DEVELOPMENT OF IN SITU HYBRIDISATION FOR CYTOKINES IN REJECTING AND TOLERANT CARDIAC ALLOGRAFTS

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Declaration

All work in this thesis was undertaken by the author with the exception of the cardiac transplants which were carried out by Mr J. Tweedle, Mr B. Jaques and Mr J. Casey in the animal facility of the University of Glasgow, and the purification of the OX38 monoclonal antibody which was done by Mr E. Campbell and Mr C. Muirhead in the Department of Surgery, Western Infirmary, Glasgow.

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Summary

An In situ Hybridisation method was developed and utilised to evaluate and pinpoint the cellular presence of IL2 and IL4 mRNA transcripts produced by Th1 and Th2 subsets respectively of activated T cells in frozen tissue sections of Lewis heart allografts from rejecting DA recipients and from recipients tolerised by Cyclosporin, Blood Transfusion and Antibody therapy regimens. A panel of monoclonal antibodies was used on the tissue sections initially and confirmed the presence of the activated T cells known to express the cytokines being studied. The Polymerase Chain Reaction was used next on tissue homogenates from the same samples to establish the presence of IL2 and IL4 mRNA transcripts and showed that IL2 mRNA sequences were present in all rejecting samples, but not in all tolerised samples, whereas with the exception of some day 2 grafts, IL4 transcripts were present in all other rejecting grafts, and also in CsA and Transfused grafts but not in all Antibody treated grafts. As the presence of cytokine transcripts had been established by PCR, the ISH method was utilised to attempt to pinpoint specific cellular production of the IL2 and IL4 transcripts in the frozen sections of the tissue. All rejecting grafts and grafts from Transfused recipients were found to be positive for IL2 transcripts whereas CsA treated recipients grafts were negative, and Antibody treated recipients grafts found to be variable. IL4 transcripts were found in 50% of the rejecting grafts only, suggesting that ISH is not sensitive enough to record low levels of mRNA expression and that the use of both the ISH and PCR methods in tandem would be more beneficial in intragraft cytokine transcription detection.

Abbreviations

APC	Antigen Presenting Cell
APES	3 Aminopropyltriethoxysilane
B cell	B Lymphocyte
BCIP	5-Bromo-4-Chromo-3-Indolyl Phosphate
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	Complimentary Deoxyribonucleic Acid
CHO	Chinese Hamster Ovary
CsA	Cyclosporin A
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dNTPs	2` Deoxynucleoside 5` Triphosphates
DSBT	Donor-Specific Blood Transfusion
DTH	Delayed-type Hypersensitivity
EDTA	Ethylenediaminetetraacetic Acid
FCS	Foetal Calf Serum
GMEM	Glasgow modification of Eagles medium
GVHD	Graft versus Host Disease
HBSS	Hanks Balanced Salt Solution
HPLC	High Pressure Liquid Chromatography
ICAM	Inter cellular Adhesion Molecule
IFNγ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL2R	Interleukin 2 Receptor
ISH	In situ Hybridisation
kg	Kilogram
kD	KiloDalton
LDA	Limiting Dilution Analysis
LFA	Lymphocyte Function Associated Antigen
mRNA	Messenger Ribonucleic Acid
MHC	Major Histocompatability Complex
mAb	Monoclonal Antibody
mg	Milligram (10^{-3} gram)
MLR	Mixed Lymphocyte Reaction
ml	Millilitre (10^{-3} litre)
MST	Mean Survival Time
NBT	Nitro Blue Tetrazolium
ng	Nanogram (10^{-9} gram)
NFAT	Nuclear Factor for Activated T cells
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction

RID	Radial ImmunoDiffusion
rIL2	Recombinant Interleukin 2
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
SEB	Staphylococcus enterotoxin B
SSC	Saline Sodium Citrate
TBE	Tris Borate EDTA
Tc	Cytotoxic T cell
T cell	T Lymphocyte
TCR	T cell Receptor
Th	Helper T cell
TNFα	Tumour Necrosis Factor-alpha
TNFβ	Tumour Necrosis Factor-beta
μg	Microgram (10^{-6} gram)
μm	Micron (10^{-6} metre)

Chapter 1

Introduction

1.1 Transplantation

Organ transplantation is becoming increasingly common as a form of treatment for end-stage failure of organs including the kidneys, liver, heart and lung. A continuing problem in clinical transplantation is the loss of transplanted organs and tissues through immunological rejection. As a consequence, a major research effort is directed towards understanding the mechanisms of allograft rejection and developing strategies for inducing specific tolerance towards the allograft.

1.2 Histocompatibility and transplantation

The molecular and cellular processes which culminate in rejection of a transplanted tissue are initiated by differences in cell surface molecules, termed histocompatibility antigens (Gorer et al, 1938), between donor and recipient. The major histocompatibility complex (MHC) antigens, which are the predominant cell surface antigens responsible for allograft rejection, include a group of antigens (MHC class I) which are expressed on virtually all nucleated cell types in the body, and provoke strong antibody responses. A related group of antigens (MHC class II), with a more limited and species-dependent cellular distribution, has the capacity to provoke massive cellular proliferation when two populations of class II-incompatible lymphocytes are cultured together (the in vitro mixed lymphocyte reaction or MLR).

1.2.1 Structure and function of MHC molecules

The overall structure of class I and class II MHC molecules is broadly similar. Both are members of the Immunoglobulin (Ig) superfamily (Like CD4, CD8 and T-cell receptor (TCR) molecules (Halloran et al, 1993), and are transmembrane

glycoprotein heterodimers. MHC class I has one heavy chain non-covalently associated with a β2 microglobulin subunit (β2m) that lacks a membrane anchor. The heavy chain comprises three structurally distinct extracellular domains designated α1, α2 and α3. The membrane distal α1 and α2 domains are associated with the presentation of peptides and the membrane proximal α3 domain has been shown to be associated with the binding of CD8 molecules on T lymphocytes (T cells)(Germain and Marguiles, 1993).

Class II MHC molecules consist of two non-identical glycoprotein chains, α and β which are non-covalently bound; they are of similar length and each has two extracellular domains classed as α1 and α2, and β1 and β2. The α1 and β1 distal domains form the peptide binding groove while the β2 proximal domain is the site of interaction with CD4 molecules on T cells.

The crystal structure of the peptide binding grooves of both class I and class II MHC molecules was resolved using x-ray diffraction analysis and showed that although the basic core structures of the molecules were similar, the length of the bound peptides in the grooves differed, optimally 9 amino acids for class I (Rotzschke and Falk, 1991) and 13-26 amino acids for class II (Chicz et al, 1992). These peptide fragments are recognised by the CD8/TCR complex and CD4/TCR complex cell surface molecules respectively on T cells thus initiating the activation of these cells.

1.3 Cellular basis of allograft rejection

Sir Peter Medawar was a pivotal figure in the development of the modern era of transplantation. He observed, from both clinical cases and experimental studies, that cellular infiltration of the graft was characteristic of tissue rejection. He described the phenomenon of accelerated, or second-set, rejection of repeated grafts from the same donor and he showed that second-set rejection could be transferred by lymphoid cells but not serum (Medawar, 1944 & Billingham et al, 1954). The role of alloantibody in graft rejection was thought to be negligible and research effort mainly focused on the cellular component of the rejection response.

It is now apparent that the presence of pre-formed alloantibody is detrimental to an allograft and causes hyperacute rejection. In clinical transplantation, hyperacute rejection is excluded by a pre-transplant cross match, and the contribution to rejection by the development of alloantibodies post-transplant has probably been underestimated. Recent experimental studies (Morton et al, 1993) have demonstrated the capacity of transferred alloantibody alone to cause rapid rejection in rat cardiac allograft recipients treated with anti-CD4 antibody. Nevertheless, it must be appreciated that the development of alloantibodies is entirely dependent on the activation of T helper cells in the rejection response.

1.3.1 Cellular components of rejecting grafts

Medawar's observations that the transfer of lymphoid cells effected second set rejection were supported by experiments using neonatally thymectomised mice

(Miller, 1962) and congenitally athymic nude rodent (Corley and Kindred, 1977) models which demonstrated the inability of the host to effect rejection in the absence of T cells. Reconstitution of lethally irradiated hosts by adoptive transfer of T cells was enough to restore cardiac allograft rejection (Hall et al, 1978) in the absence of alloantibody production and the speed of rejection was found to be dependent upon the number of transferred T cells. These experiments suggested that alloantigen reactive T cells were the mediators of graft rejection.

The use of radiolabelled lymphocytes and macrophages (Olewole et al, 1981) and the availability of monoclonal antibodies(mAbs), specific for a range of leucocyte antigens enabled the graft infiltrating cells to be studied much more closely (Bishop et al, 1986). The kinetics of leucocyte infiltration and the phenotypic analysis of the graft infiltrate were studied by several groups e.g. Armstrong et al, (1987), and M^{c}Whinnie et al, (1986), and it was shown that the infiltrate was highly heterogeneous comprising macrophages, CD8 positive($CD8^{+}$, cytotoxic) and CD4 positive ($CD4^{+}$, helper) T cells, with a preponderance of the $CD8^{+}$ phenotype in rejecting allografts compared to non-rejecting grafts (Bradley et al, 1985 & Padberg et al, 1988).

An essential role for $CD8^{+}$T cells in the mediation of allograft rejection was proposed following the isolation of cytotoxic T cells from rejecting organ allografts (von Willibrand and Hayry, 1978 & Hayry et al, 1979). It was postulated that alloantigen reactive $CD8^{+}$ cytotoxic T cells, which produced Interferon-gamma(IFNγ) (Klein et al, 1982 & Nathan et al, 1983) could therefore activate host

macrophages which could lead to a delayed type hypersensitivity(DTH) response, causing graft rejection. Sensitised $CD8^+$T cells were shown to restore rejection of kidney (Lowry et al, 1983), pancreas (Prowse et al, 1985), thyroid (Warren and Pemberley, 1985), and heart grafts (Gurley et al, 1986 & Herbert and Roser, 1987), and were the only effective subset in restoring rejection of class I MHC incompatible rat skin grafts (Lowry et al, 1985). Rosenberg et al, (1986), noted that $CD8^+$T cells mediated rejection of skin grafts that expressed a mutant class I protein. However, a central role for $CD8^+$T cells in rejection was questioned following a set of experiments which showed that T cell deficient mice could reject fully mismatched skin allografts only if they were reconstituted with purified syngeneic $CD8^-$ $CD4^+$T cells, and not $CD8^+$ $CD4^-$ T cells (Loveland et al, 1982 & Loveland and McKenzie, 1982) while Cobbold and Waldmann, (1986), showed either $CD8^+$ or $CD4^+$T cells could mediate rejection of fully disparate skin grafts in previously sensitised mice, but that the rejection episode required $CD4^+$T cell involvement. This synergy of $CD4^+$ and $CD8^+$T cells in rejection was also noted by other groups using MHC disparate heart graft models (Hall et al, 1985).

Studies using different rat strains, previously categorised as 'high responders' or 'low responders' by their ability to make cytotoxic lymphocytes and alloantibody (Butcher et al, 1982) showed that in 'low responder' strains e.g. DA($RT1^{avl}$), organ allografts bearing only class I MHC mismatches could not be rejected but class II MHC incompatible grafts could, whereas in 'high responder' strains e.g. Lewis($RT1^l$) where high titres of alloantibodies occurred, class I or class II incompatibilities could be rejected. Experiments carried out by Sapori et al, (1985)

and Salomon et al, (1984), showed that in 'low responders' the proliferation of $CD8^+$T cells was poor without $CD4^+$T cell help, but conversely, in 'high responder' strains, $CD8^+$T cells could be activated independently of $CD4^+$T cells but that their proliferation could be enhanced dramatically by the presence of $CD4^+$T cells. The vigorous alloantibody response, only seen in the 'high responder' hosts bearing class I disparate organ grafts showed that alloantibody, produced by host B lymphocytes(B cells) could have an important role in acute rejection in non-sensitised recipients, which challenged the widely held view that acute rejection was mediated by cellular effector mechanisms.

The use of congenitally athymic PVG ($RT1^c$) nude rats as T cell deficient hosts in adoptive transfer experiments helped to clarify the picture. These rats are incapable of making functional T cells (Bell et al, 1987) and are therefore unable to reject allografts from mismatched donors (Mason and Simmonds, 1988). Bolton et al, (1989), showed that nude PVG($RT1^c$) rats, which had been intravenously injected with purified syngeneic $CD4^+$ T cells and engrafted with an allogeneic DA($RT1^{avl}$) kidney, rapidly rejected the graft at a time comparable to animals given unseparated T cells in a similar system and that the transfer of purified naive $CD8^+$ T cells alone, irrespective of the numbers used could not cause the rejection of the kidney allograft in this system. This showed that naive $CD8^+$T cells needed $CD4^+$T cell help for their proliferation and activation.

1.3.2 The central role of $CD4^+$T cells

Experiments using conventional euthymic rats in transplants between various strains bearing MHC class I disparities showed that T cell deficient animals could only reject class I MHC disparate skin or heart grafts if $CD8^+$T cells were included in an adoptively transferred naive cell population, but these $CD8^+$T cells were only effective if they had $CD4^+$T cell help (Bell et al, 1990). Cobbold et al, (1984), using monoclonal antibodies(mAbs) to deplete hosts of $CD8^+$ and $CD4^+$T cells, found that the elimination of $CD8^+$T cells did not affect the survival time of fully MHC disparate skin grafts but that depletion of $CD4^+$T cells caused prolonged graft survival. These results were echoed by Morton et al, (1993), who showed that although anti-CD8 antibody was highly efficient at eliminating $CD8^+$T cells from rat PVG($RT1^u$) recipients of PVG R8 class I MHC disparate heart grafts, it didn't affect graft survival time and the grafts were consequently destroyed in the absence of a $CD8^+$T cell population. Conversely, administration of an anti-CD4 antibody cocktail only partially depleted the T cell population, but significantly prolonged graft survival time. From these and previous experiments, the general overall view was that the T cell requirements for allograft rejection depended on the type of tissue transplanted e.g. skin graft (non-vascularised) or vascularised organ graft, and the degree of genetic disparity between donor and recipient (Hao et al, 1987).

The complex and integrated immunologic response seen in allograft rejection has been shown to involve both humoral and cellular effector mechanisms, and in the latter, the $CD8^+$T cell subset plays an important but lesser role whereas the

$CD4^+$T cell subset orchestrates the complex series of signals and events that eventually lead to graft destruction.

1.4 CD4 and CD8 T cells

Like MHC class I and class II molecules, both CD4 and CD8 molecules are members of the Immunoglobulin(Ig) superfamily. All mature T cells express one or the other of these surface markers and this determines their binding specificity - to class II and class I MHC molecules respectively, thus restricting the T cells to recognition of antigens associated with these molecules (Aversa and Hall, 1991, Doyle and Strominger, 1987 & Norment et al, 1988).

The CD8 molecule is dimeric in structure, either αβ or αα chained with a cytoplasmic domain, a membrane anchor, an Ig domain and an N-terminal v-like domain containing loops forming contact points for engagement to MHC class I molecules. The CD4 molecule is a rod-like chain consisting of four Ig domains, an intracellular portion and a membrane anchor. It has two N-terminal domains whose function is to engage the β chain of MHC class II molecules (Halloran et al, 1993). The CD4 and CD8 molecules act as MHC class II and class I restriction elements respectively for the T cell receptor(TCR)-CD3 complex. The TCR consists of either αβ or γδ chain heterodimers which are associated with a group of molecules known as the CD3 complex. Interaction of the TCR with either alloantigen or a self-MHC expressing foreign antigenic peptide is facilitated by CD4 or CD8 molecules binding to regions of class II or class I MHC molecules respectively. The process of TCR binding to specific antigen transmits a signal to the cell cytosol, triggering a cascade

of intracellular events including tyrosine phosphorylation to which CD4 and CD8 molecules contribute by their association with the $p56^{lck}$ molecule leading to the activation of the T cell (Aversa and Hall, 1991 & Sundstrom and Ansari, 1995).

As the interaction of the TCR-CD3 complex and CD4 and CD8 accessory molecules with antigen and MHC complexes are of a low affinity, other accessory molecules have been found to be necessary to increase the overall avidity of the T cell for its target. These include adhesion molecules on T cells e.g. CD2 and LFA1 which interact with their ligands LFA3 and ICAM1 respectively on antigen presenting cells(APCs) and CD28 and its B cell ligand B7/BB1. These cell surface molecule interactions between T cells and target cells are not only responsible for the close approximation of these cells but the cytoplasmic domains present in these molecules also transmit co-stimulatory signals necessary to ensure activation of the T cell (Sundstrom and Ansari, 1995).

1.4.1 T cell responses to alloantigen

It has been shown that the T cell response to allogeneic molecules is much greater than that to nominal protein antigens (up to several hundred times greater) (Auchencloss, 1995). The strength of response is extremely important in transplantation immunology as the transplant is the only situation in which both donor and recipient APCs are available to stimulate the immune response. The cause of this powerful immune response has been shown to be a combination of different factors including the ability of T cells to recognise allogeneic MHC antigens as intact molecules independent of bound peptides (Shoskes and Wood, 1994). The ability of

the immune system to respond to foreign antigens is governed by recognition by responding T cells, of peptides derived from processed antigens in association with MHC molecules. APCs collect and process both endogenous and exogenous protein antigens into smaller peptides for presentation to $CD4^+$ and $CD8^+$T cells in the context of their expressed class II and class I MHC molecules respectively (Sundstrom and Ansari, 1995).

There are two distinct pathways by which protein antigens are degraded intracellularly for presentation in association with class I and class II molecules. Endogenous antigens are assembled in the endoplasmic reticulum and in the presence of transporter molecules e.g. TAP1 and TAP2, the peptides are carried to the MHC molecules bringing about the formation of MHC class I /peptide complexes on the cell surface with the peptide occupying the binding groove. In contrast, exogenous antigens which have been engulfed by the recipient MHC are partly degraded in the endocytic vesicles. These vesicles then bring the peptide into contact with MHC molecules as they emerge from the endoplasmic reticulum. The MHC class II/peptide complex then travels to the cell surface where like class I molecules, the peptide occupies the binding groove. Swain, (1983), showed that $CD8^+$T cells could recognise endogenously synthesised antigen in the context of self class I MHC molecules and could also respond directly to allogeneic class I MHC molecules whereas $CD4^+$T cells recognised exogenously derived antigen in the context of self class II MHC and could also respond directly to allogeneic MHC class II molecules. This ability of T cells to engage and respond to allogeneic molecules directly has

been shown to be responsible for the strong proliferative response to alloantigen and the subsequent acute rejection of allografts (Shoskes and Wood, 1994).

Although $CD4^+$T cells are incapable of recognising intact allogeneic class I molecules directly, it had been postulated that host APCs could process class I alloantigens that had been shed by the graft shortly after transplantation and present them as antigenic MHC class I peptide fragments in the antigen binding groove of self MHC class II molecules (Golding and Singer, 1984). Several more recent studies using rats corroborated this theory showing that recipient T cells could be primed by immunisation with peptides derived from allogeneic MHC class I molecules and bring about accelerated graft rejection (Fangman et al, 1992). This 'indirect' method of presentation of exogenously derived antigenic peptides is similar to that used by recipient B cells which process donor class I MHC molecules and present the peptides to $CD4^+$T cells in the context of self class II MHC molecules which in turn give cognate help to the B cell to produce MHC class I specific alloantibodies.

The central event that initiates allograft rejection is the host TCR/CD3 complex binding to the target MHC molecules containing allopeptides closely followed by the selective binding of the CD4 or CD8 co-receptors with their respective ligands on MHC class II or class I molecules respectively. After engagement, and the series of complex intracellular signalling events that occur, activation and proliferation of the T cell follows, accompanied by the generation of

cytokines which promote their growth and function and are central to the achievement of T cell dependent immune responses.

1.5 Cytokines and transplantation

Since it became apparent that both cellular and humoral reactions to protein antigens were dependent on the function of T helper cells, the question arose as to how the same cells could be involved in diverse immune responses. The answer lay in the realisation in the 1970s, that T lymphocytes activated in culture by mitogens, secreted soluble proteins into the culture supernatant that had the capacity to promote different cellular functions in different in vitro assay systems; a protein that led to B lymphocyte proliferation was termed B cell growth factor while another protein, T cell growth factor stimulated T cell proliferation.

These various soluble proteins which have a short half-life are collectively called Cytokines and regulate all important biological processes e.g. cell growth and activation, tissue repair, inflammation, immunity etc. They are produced,usually in low concentrations, by cells to engage protein receptors on other cells in the local environment. They interact in a self regulating network which if disrupted can have dramatic effects on the immune response. For this reason, many groups have studied the highly complex interactions between cytokines in transplantation to try and ascertain the influence of these proteins in graft rejection or tolerance (Dallman, 1993).

1.5.1 Th1 and Th2 helper cells

In 1986 Mossmann found that cloned lines of $CD4^+$T cells could be classified into two groups, termed Th1 and Th2, based on the cytokines they produced and their related functional activities. The subset model evolved to include many new cytokines, and Th1 cells are now classified by their production of Interleukin 2(IL2), Interferon-gamma(IFNγ) and Tumour necrosis factor-beta(TNFβ) whereas Th2 cells produce Interleukins 4, 5, 6, 10 and 13 (IL4, IL5, IL6, IL10 and IL13) (Mosmann et al, 1986, Mosmann and Coffman, 1989 & Mosmann and Sad, 1996). Human Th1 and Th2 clones produce similar cytokine patterns although IL10 is also secreted by Th1 cells in humans and by activated macrophages (Sornasse et al, 1991). A third phenotype, Th0 which produces a combination of the cytokines characteristic of the Th1 and Th2 subsets has also been described (Mosmann et al, 1991), and has been proposed to be a precursor of Th1 and Th2 clones as it was reported to be predominant in the earliest stages of some alloresponses.

In the rat, Th1 and Th2 clones have not yet been fully characterised, but peripheral $CD4^+$T cells have been divided into two populations based on the expression of the CD45RC molecule. $CD45RC^{high}$ $CD4^+$T cells have been shown to produce IL2 and IFNγ and have similar actions to mouse and human Th1 clones whereas $CD45RC^{low}$ $CD4^+$T cells produce IL4 and act like Th2 clones in mice and humans (Josien et al, 1995).

1.5.2 Differentiation of Th1 and Th2 subsets

Th1 and Th2 cells are thought to derive from the same T cell precursor, a mature naive $CD4^+$T cell which, upon stimulation with antigen, produces mainly IL2. The differentiation of effector Th1 and Th2 phenotypes may then proceed through a Th0 stage of unrestricted cytokine gene expression before expressing a more restricted cytokine profile (Kelso, 1995). Several factors have been shown to influence the differentiation into Th1 or Th2 lineages.

Cytokines themselves are the most potent inducing stimuli and it has been shown that IL12 chiefly, and to a lesser extent IFNγ, primarily induce Th1 development. IL12 is produced by macrophages and dendritic cells, and only Th1 cells and uncommitted recently activated cells bear functional IL12 receptors (Szabo et al, 1995). IFNγ promotes Th1 development by enhancing IL12 secretion by the macrophages and preserving IL12 Receptor(IL12R) expression on $CD4^+$T cells (Guler et al, 1996). Th2 development is induced by IL4 which is produced in small amounts by the T cell itself during its initial activation (Schmitz et al, 1994). $CD8^+$T cells have also been shown in mice and humans to secrete Th1 and Th2 - like cytokine patterns, termed Tc1 and Tc2. These subsets can be derived under similar conditions to Th1 and Th2 cells and once committed to either the Tc1 or Tc2 subset, neither subset can be converted to the other (Sad et al, 1995).

Two other factors have been shown to influence the Th1/Th2 balance. Costimulatory molecules on APCs have been shown to influence Th1 and Th2 differentiation. Antibodies against B7-1 and B7-2, two structurally related proteins

which interact with T cells via their specific receptor CD28 have been reported to selectively inhibit the development of Th1 and Th2 responses respectively (Thompson, 1995). Antigen dose or concentration has also been implicated, with low levels of antigen reported to induce Th1 development, and high levels, Th2 development (Hosken et al, 1995).

1.5.3 Functions of Th1 and Th2 cells

The functions of Th1 and Th2 cells have been shown to correlate with their distinctive cytokine repertoires. Th1 cells are involved in cell mediated reactions e.g. IL2 can activate cytotoxic cell functions and IFNγ induces DTH reactions, and these cells can also provide some B cell help whereas Th2 cells are primarily responsible for promoting humoral activity e.g. IL4-induced switching of B cells to Immunoglobulin E(IgE) production and eosinophil production and activation by IL5 (Mosmann and Sad, 1996).

The cytokine products of Th1 and Th2 cells are mutually antagonistic i.e. IFNγ, a Th1 product, inhibits the proliferation of Th2 cells while IL4 and IL10, both Th2 products can block cytokine synthesis by Th1 cells (Nickerson et al, 1994). Many naturally occurring immune responses have clearly shown either Th1 or Th2 cell dominance. Studies of the murine model of *Leishmania major* infection showed that resistance to, and recovery from, the disease, was associated with IFNγ synthesis (Th1) whereas disease progression was associated with IL4 synthesis (Th2). Furthermore, the course of the infection could be altered by treatment with

antibodies to IL4 or IFNγ or by administration of recombinant IL12(rIL12) early in the infection (Kelso, 1995).

These results, together with studies on allergies and autoimmune diseases showed that IFNγ and IL4 could exert opposing effects on a wide variety of immune responses e.g. Isotype switching in B cells and macrophage function and development of T cell cytokine profiles, and supported the idea that the dominant cytokine of the T cell response could be dependent on the cytokine environment during T cell activation.

1.5.4 Cytokines and allograft rejection

The contribution of cytokines to the pathways culminating in graft rejection has been extensively studied and, as a result, the Th1/Th2 paradigm has been extended to encompass the view that graft rejection is mediated primarily by Th1 cytokines, while the dominance of Th2 cytokines in the graft should predispose towards graft tolerance. Results of many studies do not conform to this concept; for example, while it has been clearly shown that DTH and cytotoxic T cell activity participate in graft rejection, characterised by intragraft expression of the Th1 cytokines IL2 and IFNγ (O'Connell et al, 1993), the presence of the Th2 cytokines IL4 and IL10 has also been noted (Dallman et al, 1991 & Takeuchi et al, 1992), suggesting that graft rejection may be driven by a mixture of the action of Th1 and Th2 cells or by Th0 cells which produce many different cytokines, and not by the Th1 lineage alone (Dallman, 1995). The strong association of IL2 expression with rejection has led to experiments being carried out using IL2 knockout mice whose IL2 gene has been

functionally silenced. The results of these experiments showed that despite a lack of detectable IL2, these mice still rejected their grafts. Analysis of the intragraft cytokine profile of these mice showed the presence of IFNγ, IL4 and IL7 (Nickerson et al, 1994), thus indicating that the presence of more than one T cell growth factor, albeit not necessarily from a T cell source may be needed to support proliferation of alloreactive T cells and rejection. Candidates may be IL7, which has been shown to enhance cytotoxic T cell proliferation (Alderson et al, 1990), and IL15, which is known to bind to the β and possibly γ chains of the IL2R and support the proliferation and activation of $CD4^+$ and $CD8^+$ T cells (Nickerson et al, 1994).

These results seemed to suggest that although the expression of Th1 cytokines e.g. IL2 is strongly associated with rejection, it may not necessarily be required for the rejection process. Krams et al, (1992), detected the messenger ribonucleic acid(mRNA) of the Th2 cytokines IL4 and IL5 in 80% of rejecting renal allograft biopsies and IL2 mRNA (Th1) in only 20% of these cases, and a TH2 response, categorised by the presence of IL4, has been reported to accompany delayed rejection (Hancock et al, 1993). It is likely that factors such as time scales, strain and species differences and organ-specific responses account for discrepancies in experimental results and the lack of consensus concerning the roles of particular cytokines in allograft rejection.

1.5.5 Cytokines and tolerance to alloantigen

Examination of the cytokine profiles expressed in recipients rendered tolerant to allografts by either pretransplant donor specific blood transfusion(DSBT), brief

cyclosporin A(CsA) treatment or high, but not low dose anti-CD4 mAb therapy has shown near universal decrease in expression of the Th1 type cytokine genes (IL2 and IFNγ) as well as persistent and/or enhanced expression of the Th2 type cytokine genes (IL4 and IL10) in the graft and recipient spleen, leading to the hypothesis that immune deviation from a Th1 to a Th2 type cytokine profile may play a major role in long-term graft acceptance (Takeuchi et al, 1992). Bugeon et al, (1992), noted a reduction of IL2 and IFNγ mRNA expression in recipients made tolerant by pretransplant DSBT, while Hancock et al, (1993), demonstrated reduced expression of IFNγ, IL2R and TNFα and increased IL4 mRNAs in rat heart grafts from recipients made tolerant by anti-CD4 mAb treatment. Morris et al, (1995), found reduced expression of IL2 but enhanced IL10 mRNA in heart allografts of recipients pre-treated with anti-CD4 mAb. Takeuchi et al, (1992), observed differential activation of the Th2 lineage in CsA treated allograft recipients although this may have been due to the drugs capacity to block IL2 transcription, thus muting a Th1-like response. The role of IL10 in tolerance induction has been studied only relatively recently. Its anti-inflammatory properties include the ability to block IFNγ release, inhibit the proliferation of Th1 cells stimulated by macrophages decrease MHC class II expression on monocytes, block IL12 release by macrophages and down-regulate macrophage cell-surface expression of the co-stimulation ligand B7. However, IL10 has since been shown to be selective in its effects on all APCs, thus limiting its applicability for blocking TH1 responses.

The dominant APC in the alloimmune response is the dendritic cell, and IL10 is unable to inhibit dendritic cell activation of naive T cells (Macatonia et al,

1993). Prolonged administration of rIL10 has been shown to have a detrimental effect on graft survival whereas a short peri-operative course of rIL10 produced only marginally prolonged heart allograft survival in mice (Lowry et al, 1995). Although it has been shown to have an indirect effect on Th1 regulation, IL10 seems to have either pro or anti-inflammatory effects on tolerance induction and this may be dependent on the nature of the APCs present and the local micro-environment.

IL4 has been classed as one of the two signature cytokines of Th2 cells, along with IL5 (Abbas et al, 1996), and its expression is closely associated with tolerance induction although whether its presence is necessary for, or a product of this process is still unclear. Its known that naive $CD4^+$T cells are activated by antigen and IL4 to express the Th2 phenotype which in turn can direct an allograft response away from a pro-inflammatory Th1 response. Strom et al, (1996), postulated that the expression of Th2 cytokines in graft tolerising therapies is linked to an autocrine Th2 pattern caused by a sparse T cell infiltrate or IL4 expression. This ongoing IL4 gene expression then polarises the Th2-type response and leads to long term engraftment by prevention of the Th1-type response. The administration of an anti-IL4 mAb at the time of treatment has been reported to abrogate the tolerising effects of a combined anti-CD4 mAb and DSBT regime, depending on the strain combination used. Strom et al, (1996), and Maeda et al, (1994), using a class II reactive $CD4^+$T cell clone, which produced IL4 and IL10, but not IL2 and IFNγ showed that it could inhibit antigen specific $CD8^+$T cell responses and prolong disparate skin graft survival.

Several groups, however, have shown that the presence of IL4 may not be necessary for, and indeed may be detrimental to tolerance induction in some models. Fanslow et al, (1991), reported that the administration of soluble IL4R to neutralise IL4 led to allograft prolongation instead of the predicted acute rejection. Farges et al, (1995), carrying out rat liver transplants using both DA→Lewis and Lewis→DA strain combinations found similar immune responses in both models except in the expression of IL4 mRNA. They noted that tolerance was associated with reduced levels of IL4, suggesting no shift to Th2. Morris et al, (1995), using a pig proislet xenograft transplantation model noted the activation of Th2 cells during rejection and a lack of IL4 expression during tolerance, but when tolerance was reversed, it was accompanied by an increase in IL4 mRNA expression.

Thus, although the application of various tolerising therapies in most cases switches a Th1 to a Th2 type response, the expression of IL4 , in the absence or diminution of IL2 expression does not guarantee graft tolerance, and the failure to express IL2 may be more valuable than IL4 expression in tolerance induction (Strom et al, 1996).

1.6 Induction of tolerance to allografts

The clinical goal of transplantation is to induce specific tolerance to alloantigen thus negating the requirement of the long term use of immunosuppressive drugs . Several different hypotheses have been advanced to explain the development of tolerance, e.g. Clonal deletion, clonal anergy and suppression (Dallman, 1993). For many years, it was suspected that suppressor cells were the key factor in peripheral tolerance

despite the fact that distinctive suppressor cells could not be identified. However, despite the findings of some groups that adoptively transferred cells from tolerant animals into slightly immunosuppressed naive recipients could prevent the rejection of the subsequent graft (Dallman et al, 1993), the concept of tolerance by immunosuppression has not been widely accepted.

The clonal deletion of antigen specific T cells in the thymus was also proposed as a potential tolerance induction mechanism. Webb et al, (1990), showed that deletion of $V\beta6^{+}$ cells occurred in adult $mls1^{b}$ mice made tolerant of the superantigen $mls1^{a}$ expressing cells although the recipient mice had been thymectomised prior to tolerance induction, thus ensuring that no new T cells could reach the periphery. It was also shown that some immunosuppressive drugs e.g. CsA could enhance T cell deletion 3 or 4 fold in superantigen ($mls1^{a}$ and SEB) immunised mice (Vanier and Proud'homme, 1992). However, deletion alone cannot account for the fact that in euthymic tolerant animals, antigen specific T cell thymic emigrants are unresponsive to the tolerising antigen and are not deleted (Dallman et al, 1993). Of all the methods postulated to induce transplantation tolerance to allografts, clonal anergy has emerged as the forerunner. Induction of anergy or unresponsiveness in antigen specific T cells has been shown to be accompanied by an inability of the alloreactive T cell to produce IL2 or proliferate in spite of the presence of cell surface IL2R (Alard et al, 1993), but that this state of unresponsiveness could be reversed by the addition of exogenous IL2 (Dallman et al, 1993). It is thought that T cell anergy results after antigenic stimulation via the TCR has occurred and is caused by a lack of or deficiency of costimulatory signals such as

B7 or cytokines and, consequently, the anergic T cell cannot participate in the rejection process (Anderson and Brennan, 1995).

As the induction of tolerance to allografts would seem to involve the alteration of the cytokine milieu, a study of the cytokine profile in several methods used to induce tolerance, eg. DSBT, CsA, and Antibody therapy as well as in ongoing graft rejection by methods such as In-situ Hybridisation(ISH) and the Polymerase Chain Reaction(PCR) may help to elucidate the complex picture of tolerance induction.

1.7 Cyclosporin A

Cyclosporin A(CsA) is a cyclic polypeptide consisting of 11 amino acids which can be extracted from two species of funghi, *Trichoderma polysporum* and *Cylindrocarpon lucidium booth* (Borel and Weisinger, 1979 & Wenger, 1983). Its efficacy as a powerful immunosuppressant in graft rejection was demonstrated in a variety of animal models whereby withdrawal of the drug after a short course of therapy did not inevitably lead to graft rejection of kidney allografts in rabbits (Green and Allison, 1978), or heart grafts in rats (White et al, 1980). The grafts survived long after the administrated cyclosporin had been metabolised and excreted. It was introduced into clinical human transplantation in 1978 (Calne et al, 1978), but as well as the beneficial effects reported, some serious side-effects came to light, such as nephrotoxicity, lymphomas and patient susceptibility to infection.

Studies correlating the dose of cyclosporin administered and the immunosuppression of cardiac allograft rejection across various incompatible rat

strains showed that a subtherapeutic dose (1.5mg/kg/day) was ineffectual, but that low therapeutic (3mg/kg/day), therapeutic (5mg/kg/day) and tolerogenic (10mg/kg/day) 14 day dose schedules produced prolonged heart allograft survival (mean survival time(mst) > 100 days). The amount of immunosuppression corresponded to blood cyclosporin levels, with the therapeutic dose (5mg/kg/day) showing blood levels of >300ng/ml (Miyagawa et al, 1991). Serum levels lower than 100ng/ml have been found to be associated with rejection, whereas levels greater than 250ng/ml are associated with toxicity (Kahan et al, 1986).

Attempts to overcome the problems of cyclosporin toxicity, while still retaining graft survival resulted in several groups using lower doses of drug or shorter time scale regimes combined with administration of donor blood (Perloff and Baker, 1984), purified donor T cells (Oluwole et al, 1989), or extract of donor histocompatibility antigens (Didlake et al, 1988), with varying degrees of success. Others, e.g. Stepkowski et al, (1989), compared normal systemic delivery of subtherapeutic levels of the drug to osmotic pump delivery continuous infusion over 14 days and found that animals under systemic treatment either by intra-peritoneal(I.P.) or oral gavage methods rejected their mismatched grafts in 8 days (mst) whereas direct local delivery of the cyclosporin showed increased graft survivals of 40 days (mst).

Although treatment with CsA prolongs graft survival, it doesn't prevent leucocyte infiltration into the graft. Some groups e.g. Chisholm and Bevan, (1988), when analysing the kinetics and phenotypes of the cell infiltrate, showed that

cyclosporin treated rats had substantially fewer infiltrating cells than rats undergoing acute rejection and that the specificity of these cells for alloantigen was also reduced Bradley et al, (1985), did not find such marked decrease in graft infiltrating cell numbers but did note that these cells could not effect any marked donor specific cytotoxicity. Ito et al, (1990), using limiting dilution analysis(LDA) showed a marked decrease in cytotoxic T cell reactivity to donor type target cells in CsA treated rats and that both $CD4^+$ and $CD8^+$T cell subsets specifically suppressed the proliferative response of recipient T cells against donor stimulator cells in the mixed lymphocyte reaction(MLR). They postulated that administration of CsA was characterised by either inactivation or clonal deletion of effector cells.

Despite the ability to induce immune unresponsiveness to solid organ allografts, it has also been shown that CsA can induce forms of autoimmunity. In rats, CsA can cause severe thymic medullary involution, categorised by a loss of medullary dendritic cells (Beschorner et al, 1987), causing partial thymocyte maturation arrest at the $CD4^+$ $CD8^+$ stage and the emigration of these immature thymocytes to the periphery and lymph nodes (Cherwoan and Goldschneider, 1991). This disruption of thymic mechanisms has been shown to inhibit the clonal deletion by apoptosis of self- MHC class II reactive cytolytic T cells and under certain conditions, induce a systemic autoimmune syndrome called syngeneic graft versus host disease(GVHD)(Gao et al, 1988). Studies by Zhang et al, (1995), have shown evidence that T cells bearing the TCR determinant $v\beta8.5^+$, which mediate the acute phase of GVHD seemed to be preferentially expanded in the periphery and thymus and appear in the allograft during CsA treatment, but do not precipitate graft

rejection. They hypothesised that these MHC class II autoreactive T cells may interact with alloreactive T cells causing a hyporesponsiveness to donor alloantigens and a partial peripheral clonal deletion of these cells.

1.7.1 Mode of action

The immunosuppression mediated by CsA involves the production , by activated T cells of cytokines including IFNγ (Wiskocil et al, 1985), and IL2 (Elliot et al, 1984). In the cell, the CsA forms complexes with cyclophilin which then binds to and blocks the activity of calcineurin, a Ca^{2+}/calmodulin dependent protein phosphatase. This in turn activates a cytoplasmic component of NFAT(nuclear factor for activated T cells) which migrates to the nucleus and binds to another component and forms the active nuclear factor NFAT which is known to regulate the transcription of cytokines, including IL2 (Schreiber and Crabtree, 1992 & McCaffrey et al, 1993). This cascade of events inhibits activation, proliferation and function of effector cells.

These effects of CsA may alter the balance between the mutually antagonistic Th1 (IL2 secreting) and Th2 (IL4 secreting) T cell subsets, for example, CsA has been shown to enhance DTH (Th1-dependent) type responses in mice (Behforouz et al 1986), but conversely, it sometimes promotes IgE (Th2-dependent) production (Chen et al, 1989). Th2 clones have also been reported to be more resistant to the action of CsA than Th1 clones (Gajewski et al, 1990). The overall effect of CsA on tolerance seems to be influenced by many factors including dose, time of administration of the drug and the type of antigenic stimulation.

1.8 Donor-specific blood transfusion

The importance of consanguinity in transplantation was noted in the 1930s, when skin grafts from one family member to another seemed to last longer than those from unrelated donors (Murray, 1992). Around the same time, the existence and importance of MHC antigens as transplantation barriers was being discovered in the mouse (Gorer, 1937), and was further defined by Snell, (1948). Billingham et al, (1953), experimentally went one stage further by showing that neonatal mice of one strain, that were injected with whole blood from a different strain could, when reaching adulthood, accept skin grafts from the immunising strain showing that donor specific tolerance could be achieved. Halasz et al, (1964), using a canine renal allograft model showed that subcutaneous injection of donor blood on days 10 and 5 preoperatively significantly prolonged graft survival. In 1973, two different groups, both using human renal allograft models reported the beneficial effects of blood transfusion on graft survival. Newton and Anderson, (1973), used pre-transplant transfusion of donor lymphocytes under cover of azathioprine, a potent immunosuppressant, to achieve successful acceptance of the kidney graft. Opelz et al, (1973), also reported that preoperative blood transfusions improved graft survival and went on to show that 5 or more transfusions increased the survival of allografts by 20% compared to non-transfused recipients.

One of the major drawbacks of donor specific transfusions is recipient sensitisation to the organ donor, characterised by the development of allospecific cytotoxic antibodies which can cause early graft rejection. The use of various immunosuppressive drugs, e.g. cyclosporin during the blood transfusion protocol

successfully limited the chances of sensitisation occurring, without losing the beneficial effects of the transfusion (Cheigh et al, 1991). However, the combination of possible donor sensitisation, repeated blood transfusions and the possible transmission of potentially hazardous viral infections, together with the excellent results obtained using immunosuppressive drugs, e.g. cyclosporin, prednisone and azathioprine either alone or in combination has caused most transplant centres to abandon donor-specific transfusions in living related transplants in favour of the drug regimens.

The importance of MHC antigens as transplantation barriers was investigated in experiments using congenic mice and rats. The results showed that shared class I or II MHC antigens alone, between donor and recipient, was sufficient to produce graft enhancement, but sharing of both class I or class II MHC was necessary for maximal effect on graft survival (Soulillou et al, 1984), while Madsen et al, (1988), using a mouse cardiac allograft model clearly demonstrated that by pretreating recipients with syngeneic cells that had been transfected with class I or class II MHC genes, graft survival could be prolonged.

The roles of blood cell components in allograft enhancement has been studied by several groups, and it has been shown that in a Lewis→DA renal allograft model, recipient treatment with enriched B cells, which express both class I and class II MHC antigens results in indefinite graft survival, as does pre-treatment with T cells ($CD4^+$ and $CD8^+$) and $CD4^+$T cells alone (both class II expressors) whereas

$CD8^+$T cells (class I expressors) were totally ineffective (Cranston et al, 1987 & Olewole et al, 1989).

Groups studying graft infiltrating cells have shown that, compared to grafts in non-transfused recipients, blood transfused rats have a more rapid leucocyte infiltration on day 3 post-transplant, but by day 5, both enhanced and rejecting grafts showed similar levels. Accelerated induction of donor class I and class II MHC has also been noted in enhanced grafts, thought to be caused by cytokines, e.g. IFNγ released by the activated infiltrating T cells (Wood et al, 1988). The levels of donor-specific cytotoxicity, produced by graft infiltrating cells and splenocytes in vitro are broadly similar in enhanced and rejecting graft recipients, although a decrease in $CD8^+$T cells and IL2R positive cells has been noted in enhanced grafts, whereas $CD4^+$T cell numbers remained similar (Armstrong et al, 1987). Dallman et al, (1989, 1991) found that blood transfusion-induced tolerance was accompanied by diminished IL2-dependent T cell proliferation, and that the tolerising effect of donor blood transfusion could be reversed by administration in vivo of exogenous recombinant IL2(rIL2). As there was no reduction in recipient cytotoxicity against donor cells in vitro, it seemed likely that tolerance induction was not due to clonal deletion of alloreactive T cells.

A number of groups studying the $CD4^+$ Th dichotomy in allograft rejection and enhancement found that in rejection, Th1-like cells dominate whereas in tolerance induced by DSBT, Th2-like cells producing IL4 and IL10 persisted in grafts and spleens, suggesting that tolerance to alloantigen was caused by

preferential activation of Th2 cells which could suppress Th1 cells (Takeuchi et al, 1992). Josien et al, (1995), analysing $CD4^{+}$T cells CD45RC phenotype, which in rats mimics the Th1/Th2 dichotomy seen in mice and humans, found that there were less $CD45RC^{high}$ (Th1-like) cells present in DSBT recipient grafts on day 5 post-transplant compared to untreated recipients. Interestingly, although $CD45RC^{low}$ cells predominated in DSBT grafts, and showed similar levels in untreated recipients, there was an underexpression of both Th1 and Th2-like cytokine mRNA levels, as detected by PCR analysis, in DSBT recipients compared to untreated ones. It was thus postulated that this deficiency of T cell helper function caused reduced levels of antidonor IgG and IgM antibodies noted by other groups (Downie et al, 1990).

In general, graft survival in DSBT seems to depend on various factors, e.g. time and volume of blood transfusion, the strain combinations of animals used and the type of graft, with heart and kidney grafts reportedly benefiting from the transfusion effect compared to skin and pancreas (Perloff and Barker, 1984 & Fabre and Morris, 1972). The transfusion protocol itself can be critical to graft survival, e.g. the duration of time between transfusion and transplant can cause either sensitisation if too short or diminished beneficial effects if too long (Fabre and Morris, 1972).

1.9 Anti-CD4

The CD4 antigen is expressed on mature peripheral T cells and immature thymocytes in rats, mice and humans (Reinherz and Schlossman, 1980). It is also expressed by monocytes, macrophages and Langerhans cells in man (Wood et al, 1983), and tissue

macrophages in rats, but not mice (Crocker et al, 1987). CD4 acts as an accessory molecule by binding to class II MHC molecules on APCs and contributes to class II restriction for antigen recognition by the αβ TCR of $CD4^+$T cells, leading to T cell activation (Janeway, 1989), and upregulation of IL2R expression (Johnson and Eichmann, 1990).

CD4 is a transmembrane glycoprotein which has a molecular weight of 55 kD and a cytoplasmic portion which has a 79% amino acid sequence homology between humans and rodents. The cytoplasmic tail is 40 amino acids long with 29 of the first 32 residues identical in humans and mice, and this fact led some groups to suggest that it was involved in signal transduction (Littman, 1987), while Veillette et al, (1988), demonstrated that CD4 was non-covalently associated with the protein tyrosine kinase $p56^{lck}$ which was known to be pivotal in intracellular signalling events culminating in T cell activation.

As the $CD4^+$T cell subset has been shown to be the main initiator of rejection of organ allografts, targeting immunosuppressive therapy to T cells expressing this molecule was seen to be an obvious method of abrogating graft rejection.

Monoclonal antibodies(mAbs), directed against the CD4 surface molecule were first given to cynomolgus monkey renal allograft recipients (Cosimi et al, 1981) while Cobbold et al, (1984), showed that mice conditioned with anti-CD4 mAbs could tolerate their skin allografts. They have also been shown to suppress antibody responses against soluble antigens (Benjamin and Waldmann, 1986), block the

development of autoimmune disease (Shizuru et al, 1988), and allow permanent acceptance of allogeneic islets of langerhans in diabetic mice, if given pre-transplant (Shizuru et al, 1987).

The timing of the administration of the antibody, in relation to antigenic challenge has been shown to be important. The inhibitory effect of anti-CD4 antibody was shown to be maximal if the interaction with its receptor occurred prior to stimulation of the TCR/CD3 complex (Nel et al, 1990). Sablinski et al, (1991), using BWH-4, a mouse anti-rat CD4 antibody, given to naive recipients of cardiac allografts as a low dose, 7 day pre-transplant regimen showed that the grafts were maintained indefinitely compared to the same regimen given for 7 days post-transplant which was barely effective. This was followed up with a similar set of experiments using sensitised recipients. Graft survival in the sensitised recipients was maximal when anti-CD4 mAb was administered prior to the sensitising skin graft (21 days), intermediate when given between skin graft and heart graft (c. 12 days) but minimal if given at the time of transplant (Sablinski et al, 1991). However, Bishop et al, (1994) using a mouse cardiac transplantation model gave the C57Bl/6 recipients a low dose of GK 1.5, a rat anti-mouse CD4 mAb one day before and after transplantation of DBA/2 hearts and reported a 56% survival rate 60 days post-operatively whereas RIB5/2, a mouse anti-rat CD4 mAb, when given daily from one day pre to day 3 post-transplant and then twice weekly for five weeks to BDIX($RT1^{dvl}$) rat recipients of Wistar-Furth($RT1^{u}$) donor kidneys, led to permanent graft survival in 50% of the rats (Siegling et al, 1993). The common factor in both

sets of experiments however was that the first antibody load was given prior to transplantation.

Many groups have studied whether the elimination of target cells is required to achieve anti-CD4 mAb mediated immunosuppression. Darby et al, (1992), compared the mode of action of a panel of six rat anti-mouse anti-CD4 mAbs of varying epitope specificities and different isotypes with their ability to deplete $CD4^{+}T$ cells in vivo, and found that antibodies of the IgG2b isotype caused profound depletion of $CD4^{+}T$ cells whereas IgG2a antibodies caused only partial depletion, but also noted that the use of either depleting or non-depleting antibodies could bring about indefinite graft survival.

Cellular clearance of $CD4^{+}T$ cells by anti-CD4 mAbs has been linked to different isotypes used (Waldmann, 1989) and it was postulated that cells are deleted by opsonisition caused by membrane bound C3b acting as a ligand for C3b receptors on macrophages and granulocytes (Darby et al, 1992). Bishop et al, (1991), using the depleting GK 1.5 antibody in a mouse heart transplant model studied the phenotypes of mononuclear graft infiltrating cells and showed that at day 6 post-transplant in rejecting hearts, 20% of the infiltrate was CD4 positive whereas in the GK1.5 treated animals, only 2% or less of the infiltrate were CD4 positive and this decreased to virtually nil by day 21 post-transplant, $CD8^{+}T$ cell numbers did not differ in rejecting or antibody treated animals. Mottram et al, (1995), using the same depleting GK 1.5 antibody showed that splenic $CD4^{+}T$ cells had been depleted to <1% on days 7 and 14 post-transplant but that the heart allografts were still able to

survive indefinitely. The percentage of $CD4^+T$ cells had recovered somewhat by day 21 post-transplant and the mice did not reject donor-type skin grafts at day 30 post-transplant, but rapidly rejected third party skin, thereby showing alloantigen specific tolerance.

Shizuru et al, (1990), likewise, using OX38, a mouse anti-rat CD4 antibody of isotype IgG2a, showed that its administration selectively depleted 80-95% of $CD4^+T$ cells and that 75% of the treated recipient ACI($RT1^a$) rat strain maintained their donor Lewis($RT1^l$) grafts indefinitely, accepted second donor strain hearts, but rejected third party ones. However, although long term ACI recipients were unresponsive to Lewis hearts in vivo, in vitro, their cellular responses against Lewis spleen cells in MLR experiments were similar to those of untreated control rats, while the experiments of Mottram et al, (1995), showed that cells from GK 1.5 treated animals caused a profound lack of growth and response to alloantigen in MLR. Also, long-term cell lines could not be grown from cells isolated from GK 1.5 treated hearts despite the addition of alloantigen, mitogen (concanavilin A) and growth factors to the medium.

Sablinski et al, (1991), and Shizuru et al, (1990), both postulated that the divergent effects of antibody treatment reported were caused by several factors including the dose of antibody used and the treatment regimens, epitope specificity of the antibody, whether the antibody was depleting or non-depleting or whether low or high responder animal strains are used as graft recipients. Yin and Fathman, (1995), using low responder ACI(RTI^a) and high responder Lewis($RT1^l$) rats as both

donors and recipients of heart and kidney allografts studied the role of OX38 mouse anti-rat CD4 mAb, administered on days 3, 2, 1 and 0 pre-operatively in prevention of graft rejection. They showed that in the low responder ACI rats, donor-specific tolerance of both hearts and kidneys was achieved by the anti-CD4 therapy whereas in the high responder Lewis rats, kidneys, but not hearts were tolerated. They also showed that kidney allograft tolerance in the high responders could induce tolerance to donor-matched allogeneic hearts grafted at day 100 post-transplant and found that the conflicting results between high and low responders correlated with a more profound and longer lasting depletion of $CD4^{+}$T cells in the low responders.

Evidence collated from many groups studying cytokine and receptor profiles in anti-CD4 mAb treated animals has shown a marked decrease in IL2R expression (Sablinski et al, 1991 & Mottram et al, 1995), and the virtual ablation of Th1-like cytokines IL2 and IFNγ and preservation of the Th2-like cytokine IL4, compared with rejecting models (Siegling et al, 1993, Mottram et al, 1995, Hancock et al, 1992 & Papp et al, 1992).

It has been postulated that this scenario may be brought about by the administered antibody coating the CD4 molecule, thus reducing the amount of CD4/MHC class II interaction sites and stimulating Th2-like cells which require a lower number of TCR/CD4 complexes to be triggered than Th1-like cells, in this way disturbing the Th1/Th2 cell balance in favour of Th2 whose cytokines, when released, can cause suppression of Th1 cells leading to preservation of the allograft (Siegling et al, 1993 & Kupiec-Weglinski et al, 1993).

1.10 In-Situ Hybridisation

In-situ hybridisation(ISH) techniques allow specific nucleic acid sequences to be detected by relying on base-pairing (G-C and A-T) between endogenous target RNA or DNA sequences and a labelled complimentary probe sequence in tissues whose morphology has been preserved. The technique was originally developed in 1969 for localising specific DNA sequences on chromosomes (Pardue and Gall, 1969), and since then, the methodology has been gradually modified to allow the exact cellular location of specific RNAs and DNAs in tissue sections or single cell preparations to be pinpointed with relative accuracy unlike other hybridisation methods such as Northern Blot analysis which can only confirm the presence or absence of specific RNA species in an organ or heterogeneous cell population (Thomas, 1980). This thesis explores the use of ISH in transplanted tissues with the aim of visualising the distribution and frequency, within the graft, of leucocytes expressing mRNA for certain cytokines which seem to play important roles in mediating rejection of , or maintaining tolerance to, transplanted tissues. ISH as a method is a very individual procedure, and has to be optimised and developed depending on various parameters, including the size of the target molecule, copy numbers of target molecule available for hybridisation to, types of tissue to be used and alterations to that tissue (e.g. fibrosis etc.) depending on whether the graft is rejecting or tolerant.

1.10.1 Probes

Initial ISH studies used mainly double-stranded complimentary DNA probes (Lloyd and Landefeld, 1986), which were synthesised by either nick translation or random

priming and had to be denatured before use, or, single stranded complimentary RNA probes, also known as Riboprobes (Hoeffler et al, 1986),whose synthesis involves linearisation by a restriction enzyme of a plasmid into which a probe sequence has been cloned, and transcription of sequences downstream of the appropriate initiation site by a purified RNA polymerase.

1.10.2 Probe labels

Riboprobes and DNA probes were usually labelled by the enzymatic addition of a radioisotope of choice, e.g. Tritium(^{3}H), Phosphorus(^{32}P), Iodine(^{125}I) or Sulphur(^{35}S) and detected by autoradiography. Although these methods were highly sensitive, several disadvantages in their use became obvious, including the very long exposure times required by low-energy isotopes (^{3}H), limited spatial resolution problems with high-energy isotopes (^{32}P), the potential health hazards and safety measures required and the limited shelf-life of short half-life isotopes (Larsson, 1989). However, some of the major obstacles were removed by the modification of these nucleic acid probes by the introduction of non-radioactive labels.

Two types of non-radioactive labelling exist. The Direct method, whereby the detector(reporter) molecule e.g. fluorescein is directly incorporated into the probe, and is visualised immediately after hybridisation with no need for immunocytochemistry to visualise the signal (Dirks et al, 1991), or the Indirect method, where the similarly incorporated reporter molecule is detected by affinity cytochemistry. Two of the main reporter molecules used are biotin and digoxigenin.

Biotin

This is a member of the vitamin B complex and was developed as a reporter molecule by Langer et al, (1981). It has since been very widely used and is detected by anti-biotin antibodies or more usually by streptavidin which has a higher binding capacity. However, a major drawback to the use of biotin is that certain tissues including liver and kidney contain high endogenous biotin levels which can cause serious problems with background staining.

Digoxigenin

The digoxigenin system, developed by Boehringer Mannheim Ltd, does not have this problem as its only natural source is the digitalis plant from which the steroid is extracted and, consequently, should not cause any background staining. Like biotin, it is incorporated enzymatically into nucleic acid probes and is detected with high-affinity antibodies, usually conjugated to alkaline phosphatase.

1.10.3 Oligonucleotides

The advent of synthetic oligonucleotides as probes (Oligoprobes) resulted in significant improvements in ISH methodology. These are synthesised from known nucleic acid sequences and their design and construction either in-house or commercially does not need access to cloned genes and significantly reduces probe production times.

Oligos are typically made in sizes from 15→45mers, substantially smaller than either DNA or Riboprobes. They are usually end-labelled during their

construction and have the advantage that several oligos, complimentary to different regions of the same mRNA target sequence can be manufactured and mixed in equal proportions in a hybridisation reaction, thus attaching more label onto target sequences. Their small size is also advantageous, allowing them to diffuse through the dense cellular matrix which may surround target sequences more easily than larger probes (Lewis et al, 1985).

1.10.4 Hybridisation

Each stage of the hybridisation method, from the tissue preparation at the outset, through the conditions used to obtain specific hybridisation, to the end-stage visualisation of the specific signal has to be carefully optimised.

Morphology preservation

To ensure preservation of tissue morphology, the arrestment of any potentially degrading enzymes and the preservation and immobilisation of target mRNA sequences, the tissues must be fixed as soon as possible, either pre-paraffin embedment or post-cryostat sectioning, depending on the system utilised. Different tissues require different fixatives, e.g. acetone (Farquharson et al, 1992), and paraformaldehyde and neutral buffered formalin (Farquharson et al, 1990). Many of these fixatives cross-link proteins and this can mask the target nucleic acid sequence, so permeabilisation of the tissue by acids, detergents and/or enzymes is sometimes necessary to enable probe access to the target sequence. If proteinases are used, the correct concentrations have to be titrated to avoid over-digestion of the tissue and loss of target sequence (Larsson, 1989).

Non-specific binding

Many parameters have to be considered when calculating the optimal conditions for hybridisation . Some groups use a pre-hybridisation step to block non-specific binding of probe to non-complimentary sequences or basic proteins. This usually involves an incubation with the basic hybridisation solution minus the probe, but which contains non-complimentary sequences such as yeast tRNA, salmon sperm DNA or calf thymus DNA, and denhardts solution to ensure the blockage of hydrogen bonding to basic proteins. Although blocking is necessary, it has been shown that the presence of these agents in the hybridisation solution alone is adequate thereby removing the need for a pre-hybridisation step altogether (Ogilvie et al, 1990).

Hybridisation

Various hybridisation times have been used, although most groups find it more convenient to hybridise overnight using low probe concentrations, instead of using shorter times and higher concentrations (Larsson, 1989) which may lead to increased background staining. The hybridisation temperature used depends on the melting temperature (Tm) of the hybrids formed. The Tm is the temperature at which 50% of the hybrids dissociate, therefore, any hybridisation temperature used must be below this. The Tm in turn is dependent on several factors, i.e. the higher the monovalent cation concentration and G-C base pair frequency, the higher the Tm value. Probe length is also a factor, with longer probes forming more stable hybrids. Depending

on the type of probe used, the addition of formamide is sometimes called upon to allow lower hybridisation temperatures to be used, thus avoiding any morphological deterioration that can occur to the tissues used above 50-55^{0}C (Larsson, 1989). Many groups, especially those using oligoprobes have, however, found that the addition of formamide to the hybridisation solution has led to unwanted background staining and, consequently, its use to be not beneficial to the method (Larsson, 1989 & Farquharson et al, 1992).

Post-hybridisation

During hybridisation, hybrids form between perfectly and imperfectly matched sequences. To dissociate the latter and thus reduce background staining, a series of washes of varying stringencies are usually utilised. The higher the temperature and the lower the salt concentration used, the more stringent the wash, and this is usually carried out up to a few degrees below the calculated Tm of the hybrids. Some groups however have reported that washes at the hybridisation temperature, followed by washes at room temperature are sufficient and that high stringency washes actually decreased signal detection (Bentley and Singer, 1985).

Detection

Most non-isotopic detection systems, e.g. the alkaline phosphatase conjugated anti-digoxigenin antibody system, necessitate that incubation with a substrate is necessary to form an insoluble coloured product. However, the enzymes used in such systems may themselves be endogenously present in the tissue of study, therefore they may have to be inactivated in order to avoid confusion with possible specific signal. In the

case of alkaline phosphatase, it has been shown that the addition of levamisole to the substrate solution is adequate as a precaution although generally, most endogenous enzymatic activity should be lost during the course of the different hybridisation method stages (Murray et al, 1992).

1.11 Polymerase chain reaction

The Polymerase Chain Reaction(PCR) is a technique that has been developed to amplify specific DNA sequences of interest efficiently *in vitro*, even when the starting amounts of material are extremely small. PCR was used in this thesis to determine the presence, in transplanted tissues, of leucocytes synthesising mRNA for certain cytokines. It was important to establish, by PCR, the presence of a particular cytokine in a particular tissue in order to develop the optimal reaction conditions for the detection of that cytokine by In-situ hybridisation.

The principle of PCR was first described in detail by Kleppe et al, (1971), and the methodology devised and named by Saiki, Erlich and Mullis at the Cetus corporation (Saiki et al, 1985). Early PCR procedures were tedious and laborious and usually involved moving tubes between water baths of varying temperatures and keeping a close count on the number of reaction cycles completed. Nowadays, the procedure is virtually fully automated, and involves a PCR thermal cycler in which cycle numbers, incubation times and heating block temperatures are fully programmable, allowing specific standardisation for each different DNA species being amplified.

1.11.1 Reaction requirements

There are several basic requirements for the reaction :- template, primers, buffer containing magnesium, DNA polymerase and four deoxyribonucleoside triphosphates(dNTPs).

Complimentary DNA(cDNA) is commonly used as a template due to its ease of synthesis from mRNA using an oligo d(T) primer and an RNA-dependent, DNA polymerase called Reverse Transcriptase in the presence of an excess of dNTPs at 37^0C. Two oligonucleotide primers, commonly 15→25mers, each complimentary to opposite strands of the DNA sequence of interest and oriented with their 3` ends towards each other anneal at specific temperatures to the complimentary DNA strands which have been denatured at 90-$95^0$C to separate them. Primer sequences should have similar G-C content, minimal secondary structure and low complimentarity to each other, particularly in the 3` region. The tract of DNA, lying specifically between the two primers is then preferentially amplified at a specific expansion temperature in the presence of magnesium buffer and large molar excesses of dNTPs and a DNA polymerase.

Originally, the enzyme of favour was the large fragment DNA polymerase derived from *Escherichia coli*(E coli) known as the Klenow fragment. However, this had the major drawback of having to be added after each denaturation stage due to its instability at high temperatures. The enzyme Taq polymerase extracted from the heat-loving bacterium *Thermus aquaticus* is now widely used as it is stable and

active even at the high temperatures associated with denaturation, and does not have to be added to the reaction mixture at each cycle.

1.11.2 Efficiency and specificity

The standardised cycles featuring denaturation, annealing and extension temperatures and times can be repeated ad infinitum, and specific DNA accumulates exponentially until one component of the reaction mixture becomes exhausted. 25 to 40 cycles are usually sufficient for optimum amplification although this may vary depending on the amount of starting material available and the efficiency of each amplification step. The use of 'nested primers' has been shown to be very effective at improving amplification efficiency. This involves the use of a pair of oligo primers designed to anneal to sequences internal to the original primers. Only a small amount of the original amplification product is needed, along with fresh enzyme, buffer, dNTPs etc. A further set of amplifications is then performed, providing additional specificity to the reaction and dramatic increases in the amount of amplification product (Bell, 1989).

1.11.3 Visualisation of product

The end product of this type of reaction is double-stranded DNA fragments of a defined length which can then be visualised, usually by electrophoretic migration on an agarose gel which is consequently stained with fluorescent nucleic acid binders such as ethidium bromide, and observed under ultra-violet transillumination. Appropriate molecular weight markers of known size are commonly included in each gel run and used to compare and verify product band size, which should be visible as a sharp band. Small DNA products, and sometimes primers themselves can appear as

diffuse bands near the leading front of the gel, and if extra bands appear, these may be due to the presence of single-stranded product which can occur if the primers are used at unequal concentrations.

1.12 Aims

The aims of this thesis were primarily to develop and utilise an ISH method capable of relating the potential for specific cytokine production (i.e. cytokine message) to patterns of distribution and frequency of graft infiltrating cells in both rejecting heart grafts and heart grafts from recipients rendered tolerant by antibody, blood transfusion and drug regimens. To ensure that relevant infiltrating cells were present in the allografts, morphometric analysis of the infiltrate using specific monoclonal antibodies was carried out, followed by PCR analysis to establish the presence or absence of particular cytokine mRNA transcripts in the tissue being analysed. As PCR can only detect the presence of cytokine message but cannot relate this message to pattern of cellular infiltrate (focal or diffuse), or to individual cells, an ISH method was developed to see if any pattern of association could be detected between cell infiltration and the expression of certain cytokines known to have a mediating role in organ transplantation.

Chapter 2

Materials and Methods

2.1 Animals

Inbred male Lewis($RT1^l$) and DA($RT1^{avl}$) rats were obtained from Harlan UK Ltd(Bicester, Oxon, UK), and maintained under normal temperature conditions with free access to food and water in the animal facility of the University of Glasgow. All animals used for transplantation were 8-10 weeks old.

2.2 Surgical procedures

2.2.1 Cardiac transplantation

All microsurgical procedures were carried out as described by Ono and Lindsey, (1969). Under inhalation anaesthesia (2% halothane in oxygen), Lewis donor hearts were transplanted heterotopically into the abdomens of DA recipients with the donor aorta and pulmonary artery being anastomosed to recipient abdominal aorta and vena cava respectively. Lewis hearts were also transplanted into other Lewis rats as controls. On completion of anastomoses, the clamps were removed and haemostasis achieved using Surgicel (Johnson and Johnson Pharmaceutical, UK). Cold ischaemic times were never more than 30 minutes and heartbeats were monitored daily by palpation of the abdomen. Rejection was defined as cessation of palpable myocardial contraction.

2.3 Perioperative treatments

2.3.1 Cyclosporin A

Cyclosporin A (Sandoz Pharmaceuticals, Leeds, UK), was prepared as a 10mg/ml solution in pure olive oil by heating at 70^0C for 1-2 hours with continual stirring. A dose of 15mgs/kg was administered to DA recipients by oral gavage on days 0 (transplant) and days 1,2,3 post-transplant.

2.3.2 Donor-specific blood transfusion

1ml of fresh heparinised donor Lewis blood was administered to DA recipients by injection into the dorsal penile vein, 7 days prior to transplantation.

2.3.3 Preparation and purification of MRC OX38 monoclonal antibody

Hybridoma cells, secreting OX38 (A gift from Dr. Barclay, MRC Cellular Immunology unit, Sir William Dunn school of Pathology, Oxford, UK), were injected intra-peritoneally into Balb/c mice which had been pristane-primed 2 weeks previously.

Ascitic fluid was harvested from the peritoneal cavity between 10 days and 5 weeks post injection and centrifuged at 3,000rpm(2000 x g) at 4^0C for 15 minutes. The supernatant was passed through a 0.2μm paradisc 25 disposable filter (Whatman Int., Maidstone, Kent, UK), and an equal volume of 1M glycine dilution buffer pH 8.6 (see appendix), was added before being left overnight at 4^0C. Next morning, the sample was passed through a protein-A-sepharose column (Prosep A)(Bioprocessing Ltd, Durham, UK), which had been equilibrated with 1M glycine binding buffer pH 8.6 (see appendix) at a flow rate of 10 mls/min. As Prosep A has a high affinity for the Fc portion of IgG, 0.1M citrate buffer pH 3 (see appendix), was used to elute the IgG (including OX38 which is of the IgG2a subclass).

A UV-1 single-path monitor (Pharmacia Ltd, St. Albans, UK), was used to measure the optical density of the eluate at 280nm and a Frac-100 fraction collector (Pharmacia Ltd, UK), was programmed to collect peak fractions containing IgG which were subsequently pooled and dialysed for 72 hours at room temperature against several changes of phosphate buffered saline (PBS).

The IgG2a was quantified by assay on a Radial Immunodiffusion (RID) plate (Binding site Ltd, Birmingham, UK), against a known standard (calibrator). The test sample was diluted with bovine serum albumin (BSA) (Sigma Ltd, Poole, Dorset, UK), and 5µl added to the plate which contained monospecific antiserum in agarose. When the diameter of the calibrator had reached 9mm in a minimum diffusion time of 96 hours for IgG2a, callipers were used to measure the ring diameter of the test sample, and the concentration calculated from the RID reference table supplied.

2.3.4 OX38 Monoclonal antibody therapy

DA recipients were given 10mgs/kg OX38 in PBS intra-peritoneally 3 days prior to transplantation and 3 subsequent doses of 2mgs/kg each, 2 days, 1 day and shortly before transplantation.

2.4 Excision, preparation and storage of tissue

Recipient rats were sacrificed by cervical dislocation and hearts immediately excised and bisected. One half, to be used for PCR was immediately wrapped in aluminium foil and snap-frozen in liquid nitrogen (B.O.C Cryospeed, Glasgow, UK). The remainder, to be used for Histology and ISH was placed in an aluminium foil mould, covered with Tissue-Tek O.C.T embedding medium (BDH Ltd, Lewes, Sussex, UK), and immediately snap-frozen in liqid nitrogen. All tissue was stored at -70^{0}C until use.

2.5 Histology

2.5.1 Antibodies

A panel of mouse monoclonal antibodies (Serotec Ltd, Oxford, UK), were used to detect various rat leucocyte antigens (Figure 1.1). Peroxidase conjugated rabbit anti-mouse Ig (Dako Ltd, High Wycombe,UK) was used to detect primary monoclonal antibody.

2.5.2 Gelatinisation of slides

Multispot slides (C.A Hendley, Essex, UK), were washed for 30 minutes in 2% Decon 90 (Decon Labs,Hove, UK), rinsed in running tap water and placed in a fresh solution of 0.5% gelatine, 0.125% chrome alum (BDH, UK), in deionised water for 15 minutes at room temperature before being left to air-dry overnight.

2.5.3 Cryostat sections

5μm sections were cut at -20^{0}C on a cryostat (Slee Corp., UK), and placed on the gelatinised slides which were then allowed to air-dry for 30 minutes.

2.5.4 Indirect Immunoperoxidase staining

The air-dried sections were fixed in acetone for 10 minutes at room temperature, air-dried for 45 minutes and re-hydrated in PBS. 50μl of diluted primary antibody was placed on each section which was then incubated for 45 minutes at room temperature in a humidified box before being washed three times in PBS to remove unbound antibody. The area around each section was carefully dried with tissue prior to the addition of 50μl of a 1/40 dilution of peroxidase conjugated rabbit anti-mouse Ig containing 10% normal rat serum (to avoid cross-reaction) for 30 minutes at

Designation	Specificity	References
MRC OX1	Common Leucocyte Antigen	Sunderland et al, 1979
MRC OX8	$CD8^+$T cells, some NK cells	Barclay, 1981 Dallman et al, 1982 Cantrell et al, 1982
ED1	Macrophages, Dendritic cells	Dijkstra et al, 1983
MRC OX39	IL2Rα	Patterson et al, 1987
R73	αβTCR	Hunig et al, 1989
W3/25	$CD4^+$T cells, some Macrophages	Brideau et al, 1980 Barclay, 1981

Figure 1.1 Mouse monoclonal antibodies to Rat leucocyte antigens

The panel of antibodies listed above were all purchased from Serotec Ltd UK and were used to detect various rat leucocyte antigens by the indirect immunoperoxidase method on frozen sections of tissue from rejecting and tolerised rat heart allografts as detailed in 2.5→2.5.5.

room temperature in a humidified box. After further washes in PBS, the peroxidase substrate 3, 3 diaminobenzidine tetrahydrochloride at a concentration of 0.6mgs/ml containing 0.01% hydrogen peroxide (both Sigma,UK), was added to each slide. When a noticeable colour change had occurred, the excess substrate was removed, and the slides washed in running tap water before being lightly counterstained in Harris haematoxlylin (BDH, UK), dehydrated through increasing concentrations of absolute alcohol, placed in two changes of fresh xylene and mounted in DPX mounting medium (both BDH, UK).

2.5.5 Morphometric analysis of graft infiltrate

A Leitz Dialux 22EB microscope was used to examine each section and morphometric analysis carried out by counting the number of positively labelled cells in each of ten high power fields per section. Each slide was examined at a magnification of 200 with the aid of an eyepiece graticule using a Nikon Optiphot microscope which also allowed photographs to be taken. Statistical analysis was then carried out, and the results plotted on graphs. Random sections were also examined by an independent observer to validate the results.

2.6 In-situ Hybridisation

2.6.1 Specific probes

Anti-sense oligonucleotide probes (Figure1.2), specific for rat IL2 and IL4 mRNA target sequences were computer designed, chemically synthesised, and modified by the addition of digoxigenin molecules at both 5` and 3` ends before being purified by HPLC(high pressure liquid chromatography) by R&D Systems, Abingdon, UK. Each probe consisted of a cocktail of three 30mer, equimolar(0.2μM), exon specific

IL2

	Sequence	
Probe 1	5` TGAGCATCATGGGGAGTTTCAGATTCTTGTA	3`
Probe 2	5` ATCTCCTCAGAAATTCCACCACAGTTGCTG	3`
Probe 3	5` CATTGTTGAGATGATGCTTTGACAGATGGCT	3`

A cocktail of the above 3 rat antisense IL2 oligonucleotide probes (two 31mers and one 30mer) was made by mixing equimolar amounts of each probe for use in experiments detailed in 2.6.6 and 2.6.9. Probe cocktail Tm value = 77.5^{0}C.

IL4

	Sequence	
Probe 1	5` CTGGTACAAACATCTCGGTGCATGGAGTCT	3`
Probe 2	5` CCAGACTTGTTCTTCAAGCACGGAGGTACA	3`
Probe 3	5` GGAAGTCTTTCAGTGTTGTGAGCGTGGACT	3`

A cocktail of the above 3 rat antisense IL4 oligonucleotide probes (three 30mers) was made by mixing equimolar amounts of each probe for use in experiments detailed in 2.6.6 and 2.6.9. Probe cocktail Tm value = 79.5^{0}C.

Figure 1.2 In-situ Hybridisation probes

All probes were supplied by R&D Systems Ltd UK and were categorised by the addition of digoxigenin molecules at both 5` and 3` ends. Tm values were calculated by the % G-C content method. The probes were used to hybridise with specific target mRNA sequences in tissue sections of rejecting and tolerised rat heart allografts as detailed in 2.6→2.6.11.

probes with closely matched Tm values (IL2--77.5^{0}C, IL4--79.5^{0}C)(both by %G-C content). A cocktail of six equimolar, digoxigenin labelled human Insulin 30mer oligonucleotide probes (R&D Systems, UK), was used as a negative hybridisation control and a digoxigenin labelled poly d(T) probe used as a positive control.

2.6.2 Preparation of poly d(T) probe

This oligonucleotide probe which specifically binds to poly A tails of mRNA molecules was made by mixing 4µl of 5x terminal transferase buffer, 2µl of 1mM digoxigenin-11-dUTP, 2µl of 1mg/ml $pd(T)_{20}$, 2µl of 20,000U/ml terminal deoxytransferase (all Promega Ltd, Southampton, UK), and 10µl of diethylpyrocarbonate(DEPC) (Sigma Ltd, UK), treated H_2O (see appendix) in an eppendorf tube and incubating for two hours in a 37^{0}C water bath. The labelled probe was purified by passing through a NAP-5 column (Pharmacia, UK), containing DNA-grade Sephadex G-25 which had been pre-equilibrated with 10 mls of a 0.1% sodium dodecyl sulphate(SDS), 0.1% saline sodium citrate(SSC) solution (see appendix). Probe was eluted from the column by the addition of 2mls of the SDS/SSC solution and collecting ten 200µl fractions of eluent, of which 5µl of each fraction was removed and subsequently dotted onto Hybond N$^+$ nucleic acid transfer membrane (Amersham Int., Little Chalfont, UK), and left to air-dry before being placed in a 37^{0}C incubator overnight to stabilise the probe onto the membrane.

Next day, the membrane was re-hydrated in tris-HCL pH 7.5 buffer (see appendix) for ten minutes at room temperature, placed in 1% Blocking agent (BDH, UK), in tris-HCl pH 7.5 buffer for one hour, rinsed in tris buffer again briefly before the addition of alkaline phosphatase conjugated anti-digoxigenin antibody (BDH, UK),

diluted 1/5000(150mU/ml) in tris buffer pH 7.5 containing 1% normal sheep serum (to reduce non-specific binding).

Unbound antibody was removed by several washes in tris buffer pH 7.5, and after a ten minute wash in tris-HCl buffer pH 9.5 (see appendix), black spots, characteristic of positive probe labelling were visualised by the addition of a substrate solution containing 175µl of 5-bromo-4-chloro-3-indolyl phosphate(BCIP) and 225µl nitro blue tetrazolium(NBT) (both Sigma, UK), in tris buffer pH 9.5. Fractions corresponding to the two darkest spots were pooled together and freeze dried. The resulting pellet was resuspended in 30µl of DEPC treated H_2O and was then ready for use.

2.6.3 Silane treated slides

Superfrost slides (Shandon Ltd, Runcorn, UK), were washed in 2% Decon 90 (Decon, UK), for thirty minutes, rinsed in running tap water, placed in a solution of 2% APES(3 aminopropyltriethoxysilane)(Sigma, UK), in acetone for ten minutes at room temperature and rinsed briefly in running tap water and allowed to air-dry prior to use.

2.6.4 Cell lines

Two Chinese hamster ovary(CHO) cell lines (from Dr. Barclay, MRC Cellular Immunology unit, Sir William Dunn school of Pathology, Oxford), transfected with rat IL2 and IL4 genes were used as hybridisation targets to test probe specificity.

2.6.5 Medium

Both cell lines were maintained in GMEM(Glasgow modification of Eagles medium), containing 5% heat inactivated foetal calf serum(FCS), 2mM L-glutamine, 100IU/ml

penicillin, 100μgs/ml streptomycin (all Gibco BRL, Paisley, UK), and 25μM Special salt mixture(Advanced Protein Products, Brierly Hill, UK), in 75mm^3 tissue culture flasks (Gibco, UK), in a 5% CO_2 incubator.

2.6.6 Hybridisation of cell monolayers

When cell lines were confluent, the medium was removed, the cells were washed with Mg^{2+}, Ca^{2+} -free hanks balanced salt solution(HBSS)(Gibco,UK), and 3mls of trypsin/EDTA solution (Flow Labs, Irvine, UK), added to the flask to remove adherent cells which were then transferred to 10ml sterile plastic centrifuge tubes and spun at 1200rpm for ten minutes at room temperature. The subsequent pellets were washed three times in HBSS, and cell numbers and viability checked using an improved Neubauer Haemocytometer. The cell concentration was adjusted to $2x10^6$/ml and 100μl, ≡ $2x10^5$ cells, transferred to a Shandon cytocentrifuge and spun at 800rpm for eight minutes, producing monolayers of cells spotted onto APES coated slides.

These were immediately fixed in 4% paraformaldehyde (Sigma, UK), in PBS for twenty minutes at room temperature. After subsequent washes in PBS, protein cross-linking, caused by fixation was partially digested by the addition of 1μg/ml proteinase K solution (Sigma, UK), for fifteen minutes at 37^{0}C, allowing probes access to target sequences. Further PBS washes were followed by a five minute re-fix in 4% paraformaldehyde/PBS to retain cellular integrity, and several more PBS washes to remove fixative before the tissue was dehydrated by two minute washes in increasing(50→75→95→100%) concentrations of absolute alcohol and left to air-dry for one hour.

The cells were hybridised with 40µl of hybridisation buffer (see appendix), containing various probe concentrations, (0.1→1ng/µl), or hybridisation buffer alone as control, at 37^{0}C overnight in a sealed, moist chamber, lined with tissue soaked in 4xSSC solution.

2.6.7 Detection

Next morning, the slides were washed in decreasing concentrations of SSC solution, i.e. two 30 minute washes in 2xSSC, and two 30 minute washes in 1xSSC, all at room temperature, followed by two 30 minute washes in 0.1xSSC in a shaking water bath at 45^{0}C which was slightly below the Tm of the probes calculated for that specific salt concentration, to remove unhybridised and non-specifically hybridised probe. After a brief five minute wash in tris-HCl pH 7.5 buffer (see appendix), the samples were blocked for one hour with 5% normal sheep serum in tris pH 7.5 buffer at room temperature. 100µl of 1.5mU/ml alkaline phosphatase conjugated anti-digoxigenin antibody containing 0.3% Triton X-100 (Sigma, UK), and 3% normal sheep serum was added for two hours at room temperature. Unbound antibody was then removed by washing several times in tris pH 7.5 buffer.

After a brief wash in tris pH 9.5 buffer, the slides were immersed in substrate solution containing BCIP and NBT as before (see 2.6.2) in Tris pH 9.5 buffer, but with the addition of 0.24mgs/ml levamisole (Sigma, UK), to inhibit any endogenous alkaline phosphatase activity. Colour development was checked periodically using a Nikon Optiphot microscope, and when deemed adequate, the samples were rinsed in running tap water for thirty minutes and mounted in Glycergel (Dako, UK).

2.6.8 Cryostat sections

5μm sections were cut at -20^{0}C on the Slee cryostat, placed on APES treated microscope slides and kept at -20^{0}C until fixation.

2.6.9 Hybridisation of tissue sections

The samples were removed from the cryostat and immediately fixed in a 4% paraformaldehyde/PBS solution for twenty minutes at room temperature before being washed several times in PBS, dehydrated through increasing absolute alcohol concentrations (as in 2.6.6) and air-dried for one hour at room temperature. Sections were hybridised overnight at 37^{0}C with 40μl of hybridisation buffer (see appendix) containing various concentrations of specific probe (0.1→1ng/μl), poly d(T) probe as positive control, or hybridisation buffer alone as negative control, in a sealed moist chamber as before (see 2.6.6).

2.6.10 Detection

Next morning, decreasing SSC concentration washes, blocking, antibody, and substrate stages were carried out essentially as described as before (see 2.6.7) with the exception of the positive control poly d(T) probe which, due to its nature is not as strongly bound to its target sequences (poly A tails of all mRNA sequences) as the specific probes are to theirs. It was therefore washed twice in 2xSSC for 30 minutes followed by one wash in 1xSSC for 15 minutes all at room temperature.

2.6.11 Photography

A Nikon Optiphot microscope with Nikon camera attachment was used to take photographs, using Kodak Ektachrome 64T colour reversal film (Kodak Ltd, UK), of the relevant slides.

2.7 Polymerase chain reaction analysis of cytokine cDNAs

2.7.1 RNA extraction

300mgs of frozen heart tissue was homogenised in 3mls of trizol reagent (Gibco, UK), and incubated for five minutes at room temperature to permit complete dissociation of nucleoprotein complexes. Two 1ml fractions of debris-free homogenate were placed in sterile 1.5ml eppendorf tubes, and 200µl of chloroform added. The samples were briefly vortexed, incubated at room temperature for three minutes, and centrifuged at 12,000xg at 4^0C for fifteen minutes.

600µl of the upper aqueous phase was transferred to fresh sterile tubes and 600µl of Isopropanol added to precipitate the RNA at room temperature for ten minutes. The samples were centrifuged again at 12,000xg at 4^0C for fifteen minutes and supernatants carefully removed. 1ml of 75% ethanol was added to wash the pellet, and the samples were re-centrifuged at 7,500xg at 4^0C for five minutes. The supernatants were again carefully removed, and the pellets dissolved in 40µl of DEPC/H_2O.

The purity and concentration of each sample was determined by taking optical density (OD) measurements at 260 and 280nm on a spectrophotometer. An OD of 1 at 260nm≡ 40µgs/ml RNA and a OD 260/280 ratio of 1.6→2.0 shows good RNA purity.

2.7.2 cDNA preparation

10µgs of total RNA in 24µl was primed by the addition of 2µl of 0.5µgs/ml oligo$(dT)_{12\text{-}18}$ primer (Gibco, UK), and placed in a PCR 480 Thermal Cycler (Perkin Elmer Ltd, Beaconsfield, UK), which had been pre-optimised for cDNA preparation.

The pre-optimised cycle consisted of the samples being incubated at 70^{0}C for ten minutes and cooled to 4^{0}C for five minutes, after which 10µl of 5x reverse transcriptase buffer, 4µl of 0.1M dithiothreitol and 2µl of 10mM dNTP mix (all BDH, UK), were added by careful mixing, after which the samples were incubated for a further two minutes at 37^{0}C before the addition of 2µl of 200U/µl Superscript RNAse H reverse transcriptase (BDH, UK). The mixture was re-incubated at 37^{0}C for one hour followed by five minutes at 90^{0}C, before being removed and stored at 4^{0}C short-term or -20^{0}C for longer periods.

2.7.3 Amplification of specific cDNAs

Before use, all alcohol swabbed pipettes and racks, all sterile pipette tips, tubes, and DEPC/H_2O were placed adjacent to an ultra-violet light source for thirty minutes, rendering any contaminating DNA present unamplifiable.

A Gene-Amp 9600 DNA Thermal Cycler (Perkin Elmer, UK), was used to amplify 1µl of cDNA in a 25µl reaction volume containing 0.4µl of 10mM dNTP mix (BDH, UK), 0.75µl of 50mM $MgCl_2$, 2.5µl of 10x PCR buffer, 0.1µl of 5U/µl Taq DNA polymerase (all Gibco, UK), 2.5µl of each sense (0.1µM) and anti-sense (0.1µM) primers (Cruachem Ltd, Glasgow, UK),(Figure 1.3), and sterile distilled water. For the specific amplification of IL4, 1.25µl of 50mM $MgCl_2$ was used, with a corresponding decrease in the volume of sterile water.

Pre-optimised programmes for IL2 and IL4, consisting of a five minute incubation at 94^{0}C, followed by 36 cycles for IL2 or 41 cycles for IL4 were used. Each cycle consisted of denaturation at 94^{0}C, primer annealing at 60^{0}C and polymerisation at 72^{0}C, all for thirty seconds each, and samples removed at 3 cycle

Primer	Sense	Sequence	Tm
β Actin	(+)	5` ATGCCATCCTGCGTCTGGACCTGGC 3`	82^0C
	(–)	5` AGCATTTGCGGTGCACGATGGAGGG 3`	80^0C
	Reference:- Nudel et al, 1983	Product size 607 base pairs	
IL2	(+)	5` CATGTACAGCATGCAGCTCGCATCC 3`	78^0C
	(–)	5` CCACCACAGTTGCTGGCTCATCATC 3`	78^0C
	Reference:- M^{c}Knight, et al 1989	Product size 409 base pairs	
IL4	(+)	5` TGATGGGTCTCAGCCCCCACCTTGC 3`	82^0C
	(–)	5` CTTTCAGTGTTGTGAGCGTGGACTC 3`	76^0C
	Reference:- M^{c}Knight et al, 1991	Product size 377 base pairs	

Figure 1.3 PCR primers

All the above 25mer oligonucleotide primers were purchased from Cruachem Ltd UK and were used in the amplification of specific cDNA sequences that had been extracted from rejecting and tolerised heart allografts as detailed in 2.7→2.7.4. (+) and (–) refer to sense and anti-sense sequences respectively.

intervals (at cycles 24,27,30,33 and 36 for IL2, and at cycles 29,32,35,38 and 41 for IL4), for gel electrophoresis.

Two negative controls were included with each reaction. These consisted of reaction mixtures minus primers and cDNA respectively.

A PCR reaction for β actin, to confirm RNA extraction and cDNA viability was carried out. Concentrations and volumes were similar to the IL2 amplification except that the final primer concentration was 0.1μM. Three cDNA concentrations; neat, 1/10 and 1/100 were amplified as before for 25 cycles, with the samples being removed after 15,20 and 25 cycles for gel electrophoresis.

2.7.4 Gel electrophoresis

15μl of PCR product was mixed with 4μl of loading buffer (see appendix), loaded onto a 1.5% agarose gel (see appendix), and run at 125v for one and a half hours on a Hybaid gel apparatus (Hybaid Ltd, Teddington, UK), using 5x TBE as running buffer (see appendix). 3μl of a DNA molecular weight marker (BDH, UK), 12μl of DEPC/H_2O and 4μl of loading buffer were mixed and run on every gel as a positive control and to confirm product size.

The gels were subsequently stained with 5μl of 10mgs/ml ethidium bromide (Sigma, UK), in 100mls of 0.5x TBE buffer for one hour. PCR product bands were then visualised using a UV Transilluminator and semi-quantitive analysis carried out on each gel by noting the presence or absence of specific bands, and comparing the number and intensity of the ethidium bromide stained bands where applicable.

Chapter 3

Phenotypic analysis of infiltrating cells in rejecting and tolerant rat heart allografts

3.1. Introduction

One of the hallmarks of allograft rejection is the presence of a progressive heterogeneous mononuclear cell graft infiltrate containing mostly macrophages and $CD4^+$ and $CD8^+$T cells ($M^cWhinnie$ et al, 1985).

This leads to an increase in donor class I and class II MHC antigen expression within the graft caused chiefly by cytokines, e.g. IFNγ released by activated infiltrate cells and inevitably leads to loss of graft function and its ultimate destruction (Armstrong et al, 1987). The pattern of allograft infiltration by these donor specific cells has been studied by many groups. Bishop et al, (1992) showed that the number of $CD4^+$T cells remained constant throughout the rejection episode, whereas conversely, the same group described a progressive rise of these cells peaking at the time of rejection (Bishop et al, 1991). $CD8^+$T cells on the other hand have been shown to be barely detectable at day 3 post-transplant but to increase rapidly by day 5, overtaking $CD4^+$T cell levels, and to level off, increase or diminish during the final events of graft rejection depending the animal models used (Bishop et al, 1991, Bishop et al, 1992 & Armstrong et al, 1987).

The accumulation of graft infiltrating cells in animals rendered tolerant by either CsA, DSBT or anti-CD4 monoclonal antibody therapies has also been closely studied. Bradley et al, (1985), found that cyclosporin A treated rats had similar levels of infiltrate to rejecting rats whereas Chisholm and Bevan, (1988), noted a marked decrease in graft infiltrating cell numbers in the CsA treated rats compared to those undergoing rejection, although both agreed that these cells couldn't effect any donor-specific cytotoxicity.

The use of DSBT to achieve tolerance has been shown to accelerate cellular infiltration into the graft by day 3 post-transplant as well as increasing the induction of donor class I and class II MHC antigens, while the levels of alloantigen-specific cytotoxicity have been shown to be similar in comparison with rejecting grafts (Dallman et al, 1987 & Armstrong et al, 1987).

The analysis of donor alloantigen-specific T cell presence in the allografts of recipients treated with anti-CD4 mAb has been carried out by several groups, with the added interest here being that the $CD4^+$T cell itself is being directly targeted. The administration of GK 1.5, a depleting anti-mouse CD4 mAb perioperatively has been shown to deplete intragraft $CD4^+$T cell population to as little as 1-2% by day 6 post-transplant (Bishop et al, 1991 & Mottram et al, 1995), or even to make their levels undetectable (Bishop et al, 1992). Interestingly, while there is a general consensus regarding intragraft $CD4^+$T cell depletion levels, the observed effect of this treatment on intragraft $CD8^+$T cell numbers is more controversial. A drop by > 98% in intragraft $CD8^+$T cell numbers on day 6 post-transplant has been noted (Bishop et al, 1993 & Bishop et al, 1992), although Mottram et al, (1995), using a different monoclonal antibody regimen found no diminuation of $CD8^+$T cells at all.

Taking these findings into account it was therefore of interest to compare and contrast the phenotypes and kinetics of the graft infiltrating cells at different time stages during acute rejection and during CsA, DSBT and anti-CD4 mAb tolerance induction regimens.

3.2 Analysis of graft infiltrating cells

Rejecting Lewis heart grafts from unmodified DA recipients and non-rejecting grafts from DA recipients tolerised by DSBT, CsA and anti-CD4 regimens were analysed for leucocyte infiltration.

Allografts from rejecting and anti-CD4 treated recipients were excised on days 2, 5 and 7 post-transplant whereas control syngeneic, DSBT and CsA treated grafts were taken at day 5 only. Cryostat sections were made of each sample and these were labelled with a panel of monoclonal antibodies, specific for rat leucocyte antigens (see Figure 1.1) using the indirect immunoperoxidase staining method. Morphometric analysis of each labelled section was then carried out by counting the number of positively labelled cells in each of 10 high power fields per section using a microscope. Each slide was examined at a magnification of 200 using a microscope eyepiece graticule in situ.

Means and standard deviations were calculated for each phenotype studied, and graphs of the results plotted. Day 7 rejecting grafts showed significant tissue damage and thus it was not always possible to count 10 high power fields. The morphometric analysis was carried out by the author, and random tissue sections were also analysed by an unbiased observer (Dr.S.E.Middleton) to verify the findings.

3.2.1 OX1

The MRC OX1 antibody, which binds to the common leucocyte antigen was used to measure the total leucocyte infiltration into the graft. The results are shown in Figures 2.1 and 2.2.

Infiltration by OX1 positive cells occurred more rapidly in anti-CD4 treated recipient grafts than in rejecting ones (day 2 post-transplant 102±18 versus(vs) 35±9 labelled cells per high power field). By day 5 post-transplant, both rejecting and anti-CD4 tolerised grafts showed large increases in infiltrate numbers with the tolerised grafts having nearly double the amount of OX1 positive cells than rejecting grafts (171±18 vs. 90±14). However, by day 7, the number of positive cells in rejecting grafts had increased by nearly two-fold (90±14 vs. 165±38) compared to day 5 rejecting whereas in the anti-CD4 treated animals, day 7 numbers had dropped to 131±36 from the high of 171±18 on day 5 and the rejecting grafts now had more leucocyte infiltrate than the anti-CD4 treated grafts (165±38 vs. 131±36). This was accompanied by severe myocyte damage to the heart tissue which was not evident in the antibody treated group. Day 5 OX1 positive cell levels in both DSBT and CsA treated recipients were higher than the rejecting grafts but lower than the antibody treated grafts, and all groups tested were significantly higher than the syngeneic control grafts.

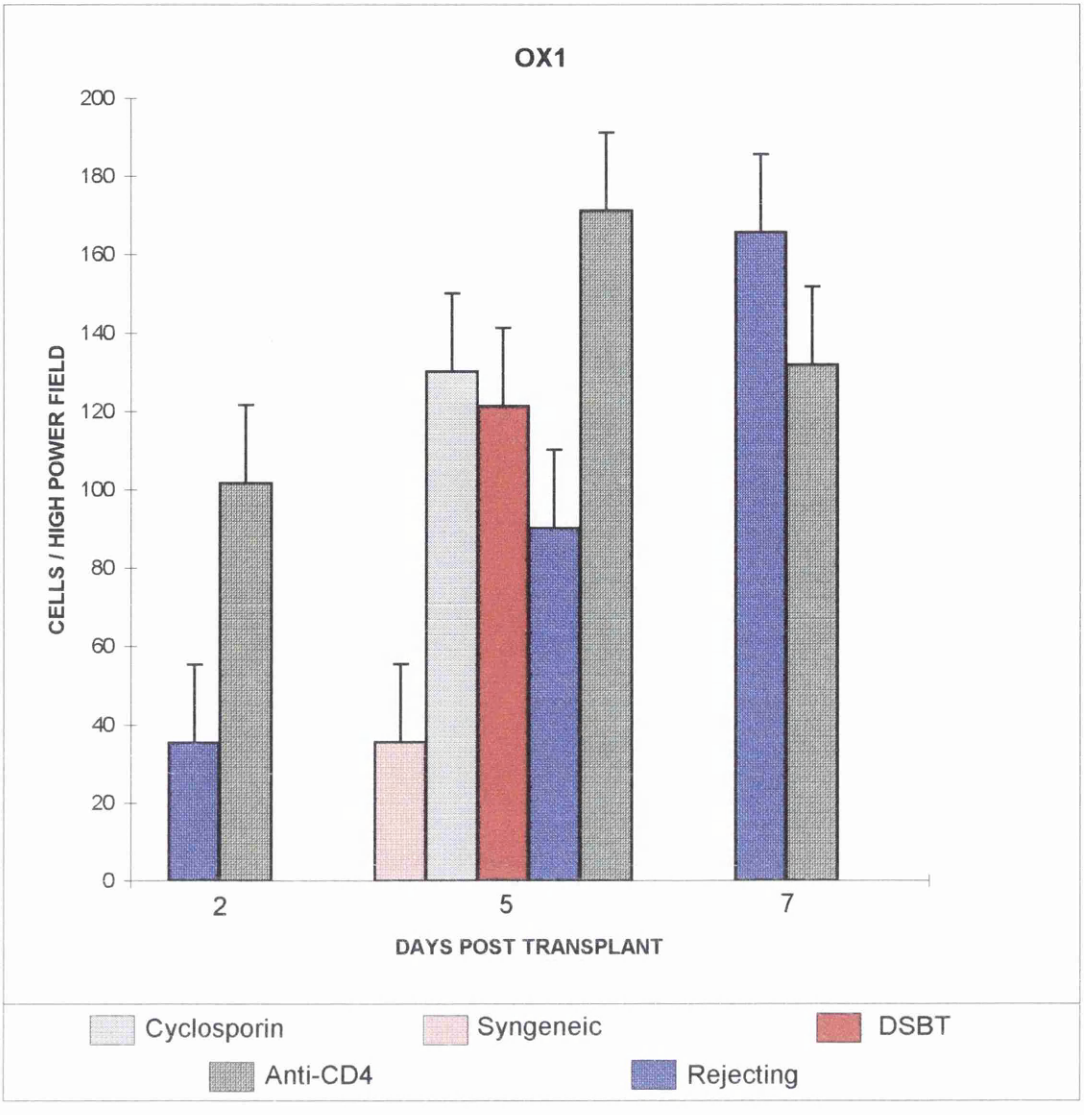


Figure 2.1 Morphometric analysis of OX1 positive cell infiltrate in Rejecting and Tolerised Lewis Heart Allografts in DA recipients.
Lewis grafts were removed on different days after transplantation from Rejecting(n=4), DSBT(n=2), Cyclosporin A(n=2) and OX38 Anti-CD4 treated recipients(n=4). Cryostat sections were labelled with OX1 antibody (against the leucocyte common antigen) using the indirect immunoperoxidase method, and the number of positive cells/ high power field was determined. Syngeneic Lewis heart grafts were stained as a control. The results are expressed as the mean and standard deviation of cells in 10 high power fields.

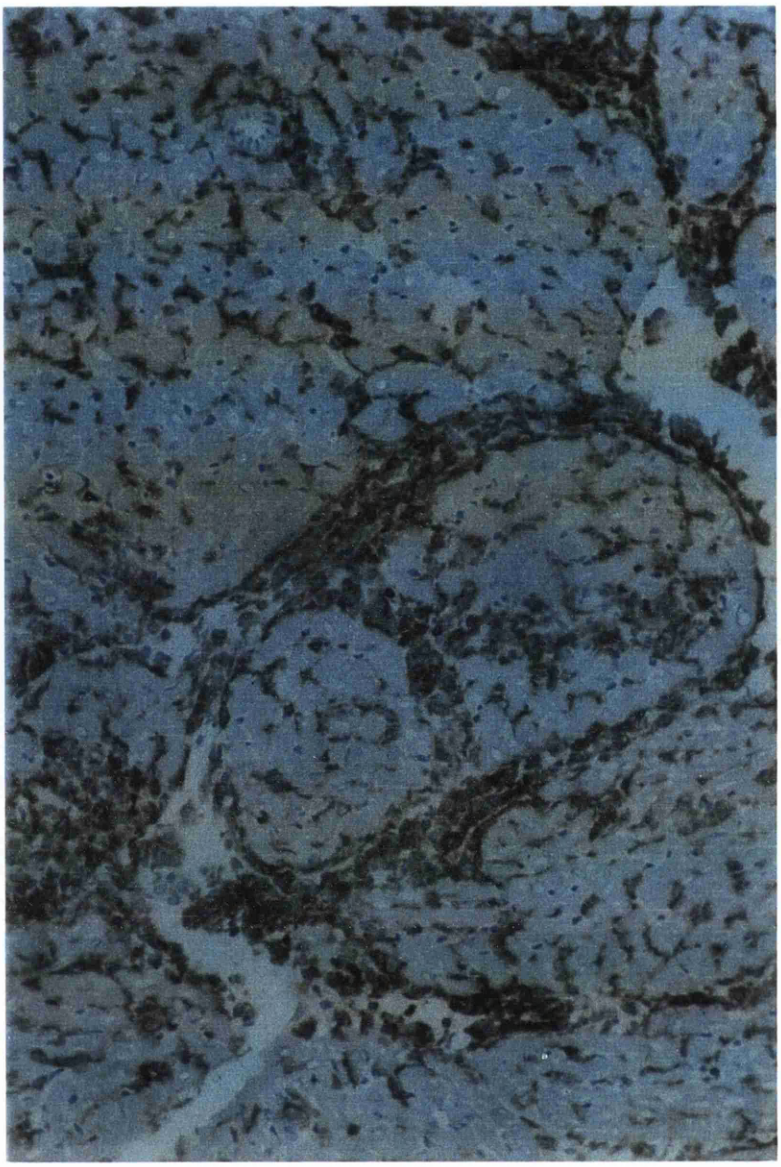


Figure 2.2 OX1 labelling of a Lewis Heart Allograft from an OX38 Anti-CD4 treated DA recipient at Day 5 post transplant. (x200)
Cryostat sections of Lewis hearts were labelled with OX1 antibody (labels Leucocyte Common Antigen) using the Indirect Immunoperoxidase method. Sections counterstained with Haematoxylin.

3.2.2 OX8

The MRC OX8 antibody was used to stain $CD8^+$T cells and some natural killer (NK) cells, and the results can be seen in Figures 2.3 and 2.4.

On day 2 post-transplant, the number of OX8 positive cells in the rejecting grafts was barely discernible compared to those in the anti-CD4 treated animals (2±2 vs. 27±3) although by day 5, there had been a large influx of $CD8^+$T cells in both rejecting and anti-CD4 treated grafts and there was still a significantly higher number in the anti-CD4 grafts. The levels of OX8 positive cells in syngeneic and CsA treated grafts on day 5 were very low, although DSBT grafts had a higher number of positive cells than the rejecting grafts (49±8 vs. 32±4). On day 7 however, in anti-CD4 treated grafts, the number of OX8 positive cells more than halved (75±10 vs. 33±4) while rejecting grafts showed only a small diminution in numbers and were now broadly comparable with the antibody treated grafts (27±6 vs. 33±4)

3.2.3 ED1

ED1 monoclonal antibody was used to label graft infiltrating cells of the monocyte/macrophage phenotype. The results are shown in Figure 2.5.

On day 2 post-transplant, ED1 positive cells were nearly four times more common in anti-CD4 treated grafts than in rejecting ones (77±17 vs. 20±7), roughly mirroring the OX1 results. By day 5, ED1 positive cells in the rejecting grafts increased dramatically from day 2 (68±18 vs. 20±7) and was now at a similar level to

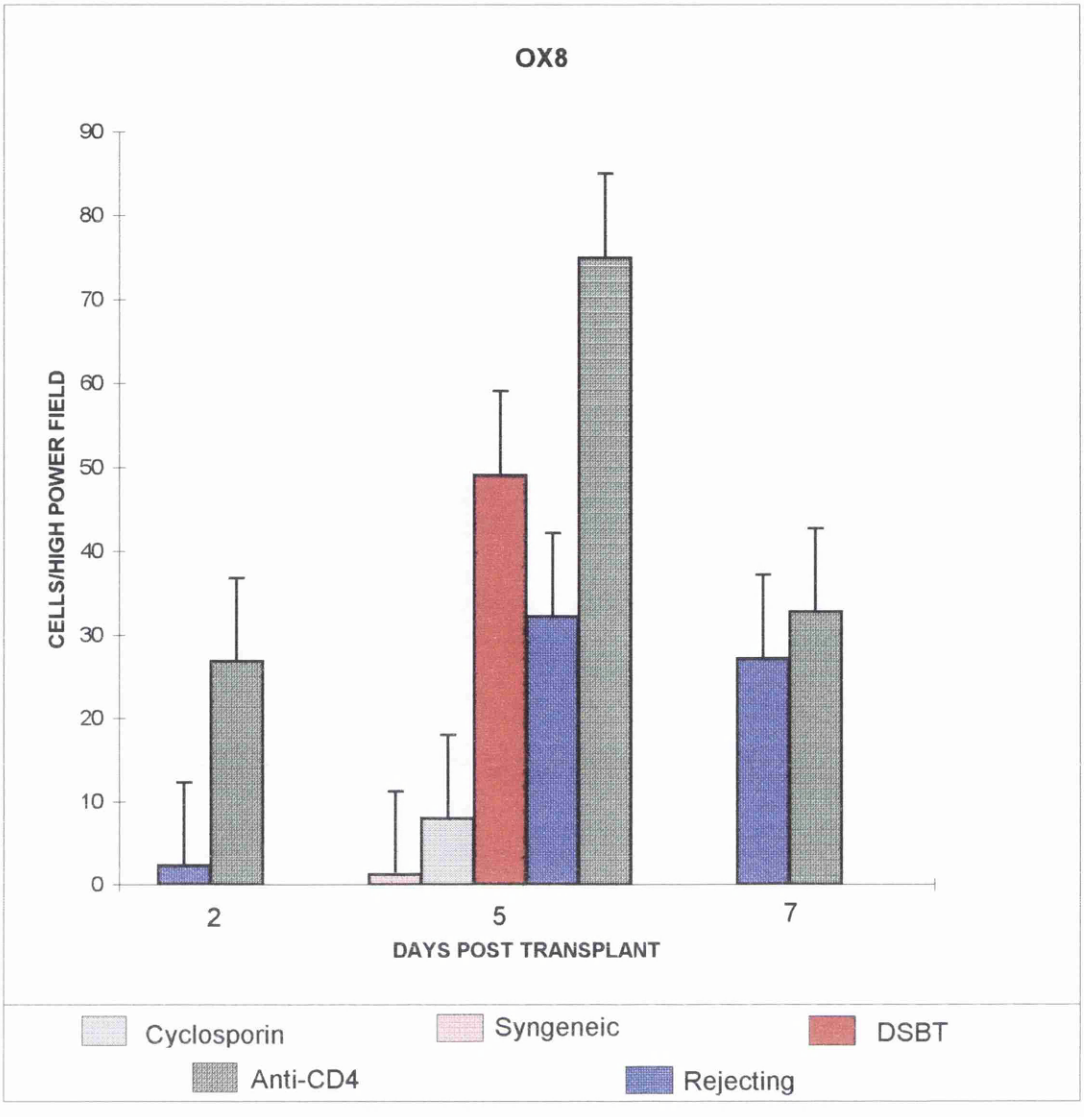


Figure 2.3 Morphometric analysis of OX8 positive cell infiltrate in Rejecting and Tolerised Lewis Heart Allografts in DA recipients.
Lewis grafts were removed on different days after transplantation from Rejecting(n=4), DSBT(n=2), Cyclosporin A(n=2) and OX38 Anti-CD4 treated recipients(n=4). Cryostat sections were labelled with OX8 antibody (against CD8 positive cytotoxic T cells and some NK cells) using the indirect immunoperoxidase method, and the number of positive cells/ high power field was determined. Syngeneic Lewis heart grafts were stained as a control. The results are expressed as the mean and standard deviation of cells in 10 high power fields.

Figure 2.4 OX8 labelling of a Lewis Heart Allograft from an OX38 Anti-CD4 treated DA recipient at Day 5 post transplant. (x200)
Cryostat sections of Lewis hearts were labelled with OX8 antibody (labels Cytotoxic T cells and some NK cells) using the Indirect Immunoperoxidase method. Sections counterstained with Haematoxylin.

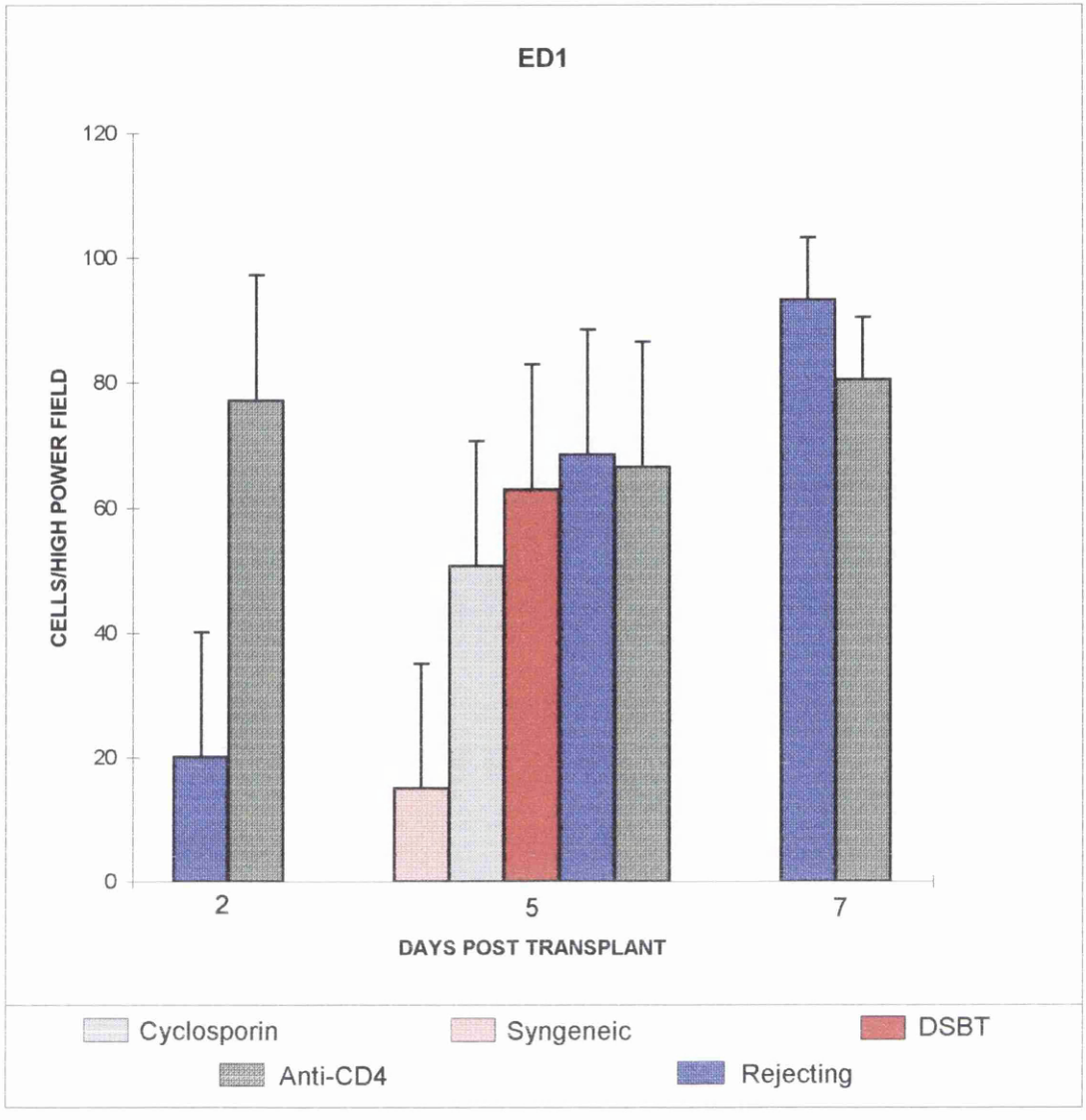


Figure 2.5 Morphometric analysis of ED1 positive cell infiltrate in Rejecting and Tolerised Lewis Heart Allografts in DA recipients.
Lewis grafts were removed on different days after transplantation from Rejecting(n=4), DSBT(n=2), Cyclosporin A(n=2) and OX38 Anti-CD4 treated recipients(n=4). Cryostat sections were labelled with ED1 antibody (against monocytes/macrophages) using the indirect immunoperoxidase method, and the number of positive cells/high power field was determined. Syngeneic Lewis heart grafts were stained as a control. The results are expressed as the mean and standard deviation of cells in 10 high power fields.

the antibody treated grafts. DSBT and CsA treated grafts also contained a large influx of cells on day 5 (63±9 vs. 50±25 respectively), but was still lower than both antibody treated and rejecting grafts which by day 7 post-transplant showed further increases in ED1 positive cells (80±8 vs. 93±19 respectively).

3.2.4 W3/25

As can be seen in Figures 2.6 and 2.7, there was a steady progressive increase in W3/25 positive infiltrating cells ($CD4^+$T helper cells and some macrophages) from day 2 to day 7 in rejecting allografts (19±6 vs. 82±19), whereas in the anti-CD4 treated grafts, although the level of positive cells was higher than in rejecting grafts on day 2, the figure was barely half that of the rejecting grafts (38±4 vs. 82±19). On day 5, all grafts except the syngeneic control grafts contained more W3/25 positive cells than in the anti-CD4 treated grafts.(It should be noted that the W3/25 antibody binds to an epitope on the CD4 molecule which is different from that bound by the OX38 anti-CD4 mAb used for tolerance induction.

3.2.5 OX39

MRC OX39 antibody was used to label the α chain of the IL2R which is upregulated during T cell activation. The results can be seen in Figures 2.8 and 2.9.

There was a massive increase from barely perceptible levels on day 2 post-transplant to day 5 (1±1 vs. 35±14) in rejecting grafts although by day 7, the numbers fell again, albeit only slightly. Anti-CD4 treated recipients had twenty times more OX39 positive cells than rejecting grafts on day 2 and although there was a slight rise on day 5 post-transplant, the numbers fell back again on day 7 and showed

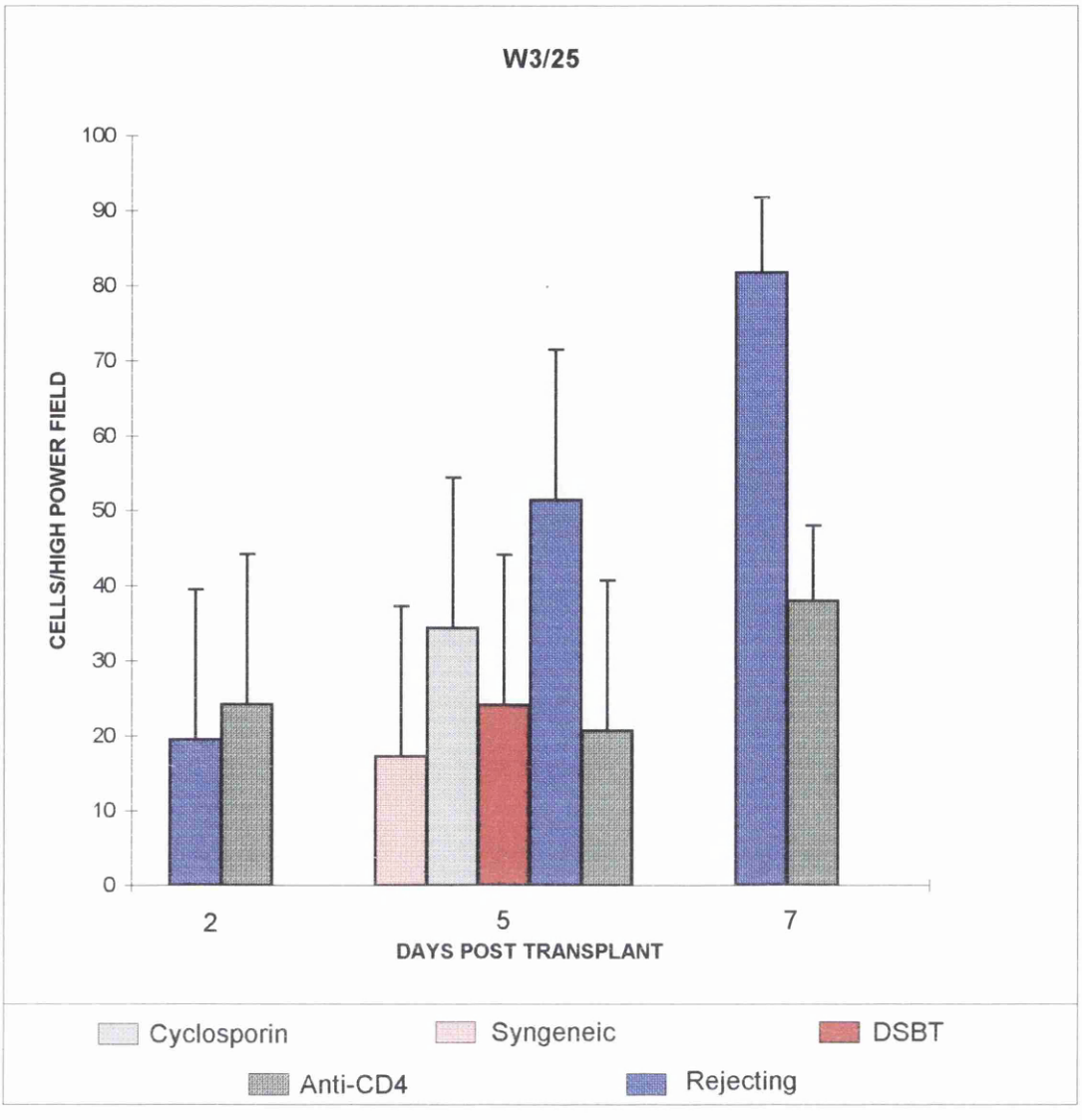


Figure 2.6 Morphometric analysis of W3/25 positive cell infiltrate in Rejecting and Tolerant Lewis Heart Allografts in DA recipients.
Lewis grafts were removed on different days after transplantation from Rejecting(n=4), DSBT(n=2), Cyclosporin A(n=2) and OX38 Anti-CD4 treated recipients(n=4). Cryostat sections were labelled with W3/25 antibody (against CD4 positive T helper cells and some macrophages) using the indirect immunoperoxidase method, and the number of positive cells/ high power field was determined. Syngeneic Lewis heart grafts were stained as a control. The results are expressed as the mean and standard deviation of cells in 10 high power fields.

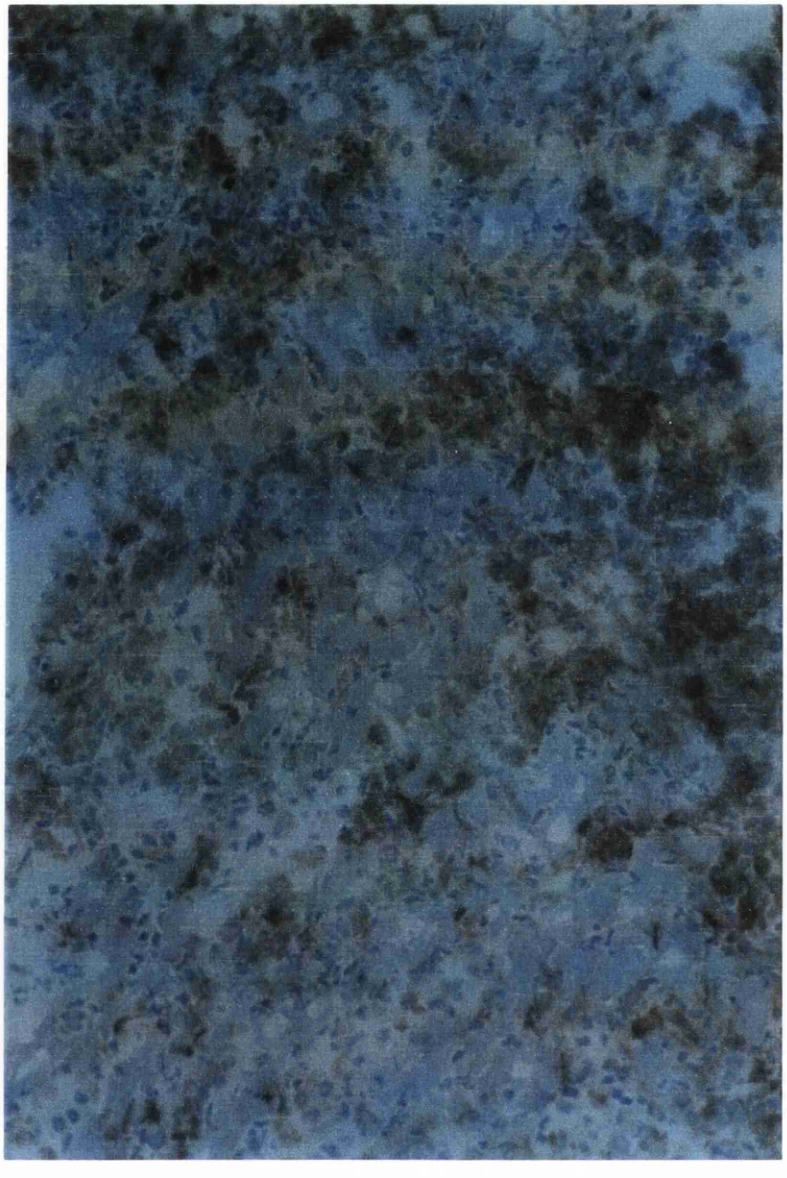


Figure 2.7 W3/25 labelling of a Rejecting Lewis Heart Allograft from a DA recipient at Day 7 post transplant. (x200)
Cryostat sections of Lewis hearts were labelled with the W3/25 antibody (labels CD4 T helper cells and some Macrophages) using the Indirect Immunoperoxidase method. Sections counterstained with Haematoxylin.

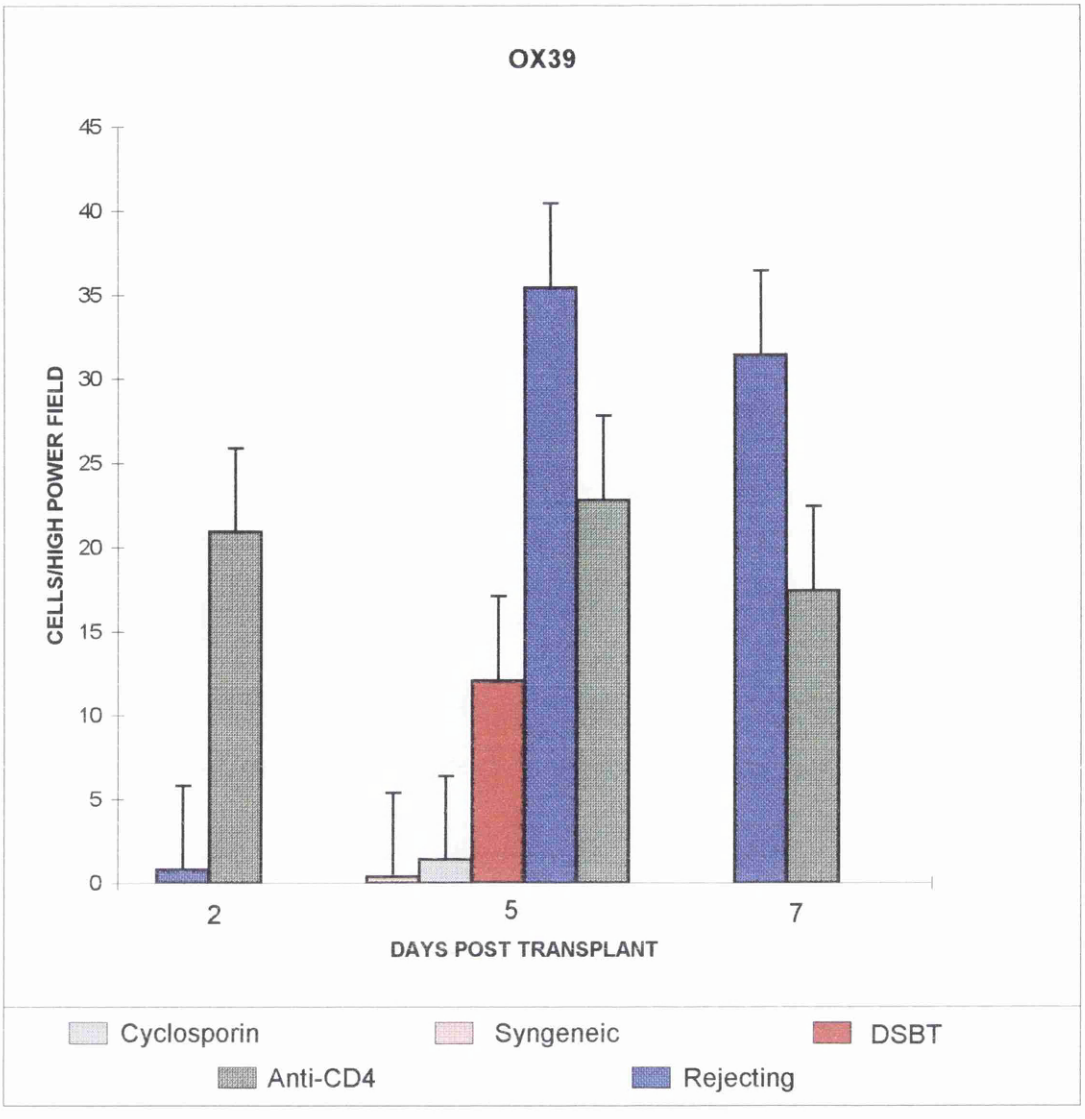


Figure 2.8 Morphometric analysis of OX39 positive cell infiltrate in Rejecting and Tolerised Lewis Heart Allografts in DA recipients.
Lewis grafts were removed on different days after transplantation from Rejecting(n=4), DSBT(n=2), Cyclosporin A(n=2) and OX38 Anti-CD4 treated recipients(n=4). Cryostat sections were labelled with OX39 antibody (against IL2 Receptor alpha chain) using the indirect immunoperoxidase method, and the number of positive cells/ high power field was determined. Syngeneic Lewis heart grafts were stained as a control. The results are expressed as the mean and standard deviation of cells in 10 high power fields.

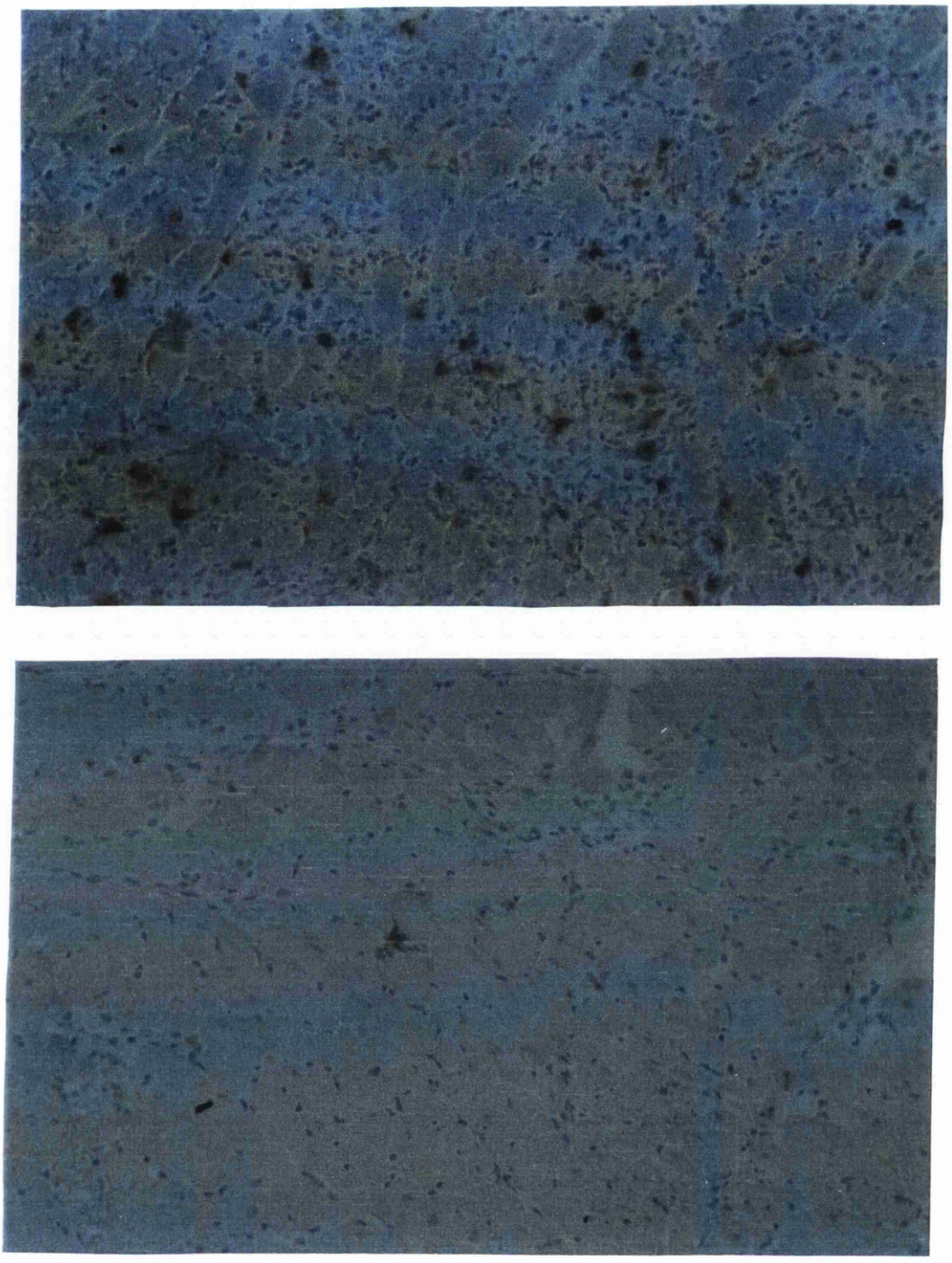


Figure 2.9 OX39 labelling of both Rejecting and Cyclosporin A induced Tolerant Lewis Heart Allografts from DA recipients at Day 5 post transplant. (x200)

Cryostat sections of Lewis hearts were labelled with OX39 antibody (labels IL2R alpha chain) using the Indirect Immunoperoxidase method. Sections counterstained with Haematoxylin.

a) Top - Rejecting Lewis heart.

b) Bottom - Cyclosporin A induced Tolerant Lewis heart.

barely half the levels of IL2R positive cells than rejecting grafts. Levels in control syngeneic and CsA treated recipients on day 5 were very low (0.4±0.5 and 1.4±1 respectively) whereas DSBT treated recipients grafts were somewhat higher, but still only half the anti-CD4 levels (12±4 vs. 23±4).

3.2.6 R73

The R73 monoclonal antibody was used to label the αβTCR positive infiltrating cells and as can be seen in Figure 2.10, from day 2 to day 7, there was an increase from virtually undetectable levels (2±1), to 70±14 in the rejecting grafts. Anti-CD4 treated recipients grafts also showed a progressive rise to higher levels by day 7 (82±4) albeit from a higher starting level on day 2 (23±3) compared to the rejecting graft (2±1). The highest level detected of all three different time points was in the DSBT treated recipient grafts (83±24) although the error margins were somewhat higher.

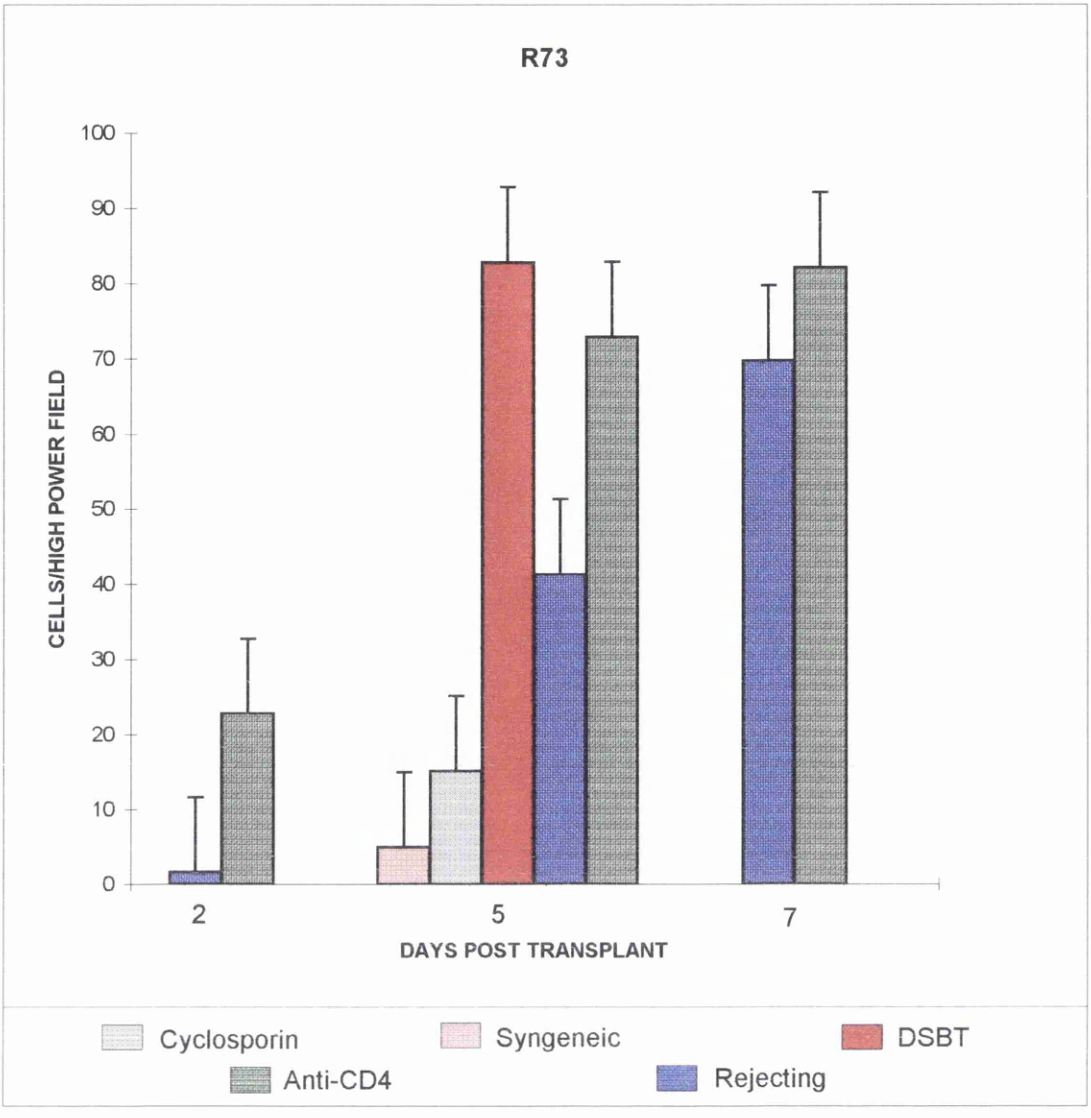


Figure 2.10 Morphometric analysis of R73 positive cell infiltrate in Rejecting and Tolerised Lewis Heart Allografts in DA recipients.
Lewis grafts were removed on different days after transplantation from Rejecting(n=4), DSBT(n=2), Cyclosporin A (n=2) and OX38 Anti-CD4 treated recipients(n=4). Cryostat sections were labelled with R73 antibody (against alpha/beta T cell Receptor) using the indirect immunoperoxidase method, and the number of positive cells/ high power field was determined. Syngeneic Lewis heart grafts were stained as a control. The results are expressed as the mean and standard deviation of cells in 10 high power fields.

3.3 Discussion

From these results, it can be noted that rejecting Lewis heart allografts from DA recipients were characterised by a progressive heterogeneous mononuclear cell infiltrate categorised by the presence of OX1 positive cells that peaked shortly before graft rejection on day 7 post-transplant. Interestingly, graft recipients treated pre-operatively with a tolerising regimen of the non-depleting anti-CD4 OX38 mAb showed a more rapid cellular infiltrate into the graft, although by day 7, infiltrate levels were not as high as in the rejecting grafts. Similar results were reported by Armstrong et al, (1987) using DSBT as the tolerising regimen. On day 5 post-transplant, both DSBT and CsA treated recipients, like anti-CD4 ones, showed elevated infiltrate levels compared to rejecting grafts and syngeneic controls.

A detailed study of the phenotypic differences of the graft infiltrate showed that in unmodified cardiac allograft recipients, the rejection process can be characterised by the intragraft presence of an increasing number of activated $CD4^+$T helper cells (W3/25+ve) and $CD8^+$ cytotoxic T cells (OX8+ve), and that this correlated with a concurrent rise in IL2R (OX39+ve) expression which peaked at the time of rejection. The presence of large numbers of ED1 positive macrophages, again peaking at the time of rejection, and by then, being the more numerous phenotype present, allied with the drop in OX8 positive cytotoxic T cells suggests that rejection in this series of experiments may be brought about by a DTH-like response caused by the large numbers of activated W3/25 positive, OX39 positive T helper cells producing Th1 type cytokines IL2 and IFNγ, which in turn can stimulate

the ED1 positive macrophages to destroy the graft using the various lytic pathways at their disposal.

Irrespective of the more rapid influx of leucocytes in the anti-CD4 treated grafts compared to rejecting ones, it can be seen that by day 5 and day 7 post-transplant, the number of $CD4^+$T cells, as characterised by the non-competing W3/25 mAb was much lower in these tolerant grafts. This was to be expected as the non-depleting OX38 mAb used as tolerogen is directed at the CD4 molecule itself. The coating of the CD4 molecule by the antibody and subsequent reduction of CD4/MHC class II interaction sites, allied with the decreasing amounts of IL2R positive cells may cause a reduction in Th1 type cytokines IL2 and IFNγ which are usually associated with rejection episodes, thus pushing the TH1/TH2 balance towards a Th2 like environment, which has been reported by some groups e.g. Siegling et al (1993) to be beneficial to graft preservation.

The day 5 results of both DSBT and CsA treated recipients show that like anti-CD4 treated recipients, leucocyte infiltration is more rapid than in rejecting grafts. Both show similar levels of macrophage infiltration to rejecting grafts but the CsA treated grafts show less $CD8^+$ cytotoxic T cell levels and IL2R positive cells than any other group other than the syngeneic controls, although the number of $CD4^+$T cells is comparable with rejecting grafts. This distinct lack of IL2R positive cells has been noted by several groups, e.g. Padberg et al, (1988) and Hancock et al, (1990), and this, accompanied by this the disruption of IL2 production, which is a hallmark of CsA administration and the decreased donor-

specific cytotoxicity that can occur (Bradley et al, 1985) may contribute to the generation of Th2-like environment which may help to maintain the tolerant state.

The DSBT maintained grafts at day 5 also showed very low IL2R expression along with lower $CD4^+$T cell levels compared to rejecting grafts. This lack of IL2R expression in DSBT treated recipient grafts has been noted by other groups. Armstrong et al, (1987) reported that although $CD8^+$T cell infiltration was as a consequence diminished, donor-specific cytotoxicity was broadly similar to rejecting grafts in vitro. Others, e.g. Josien et al, (1995), also noted that $CD4^+$T cell levels were reduced in DSBT treated grafts compared to rejecting ones and postulated that this brought about reduced alloantibody levels and donor-specific tolerance. Since equivalent levels of donor-specific cytotoxic T cell lysis are found in both rejecting and DSBT treated allografts, it is improbable that clonal deletion of alloreactive T cells is the mechanism responsible for the induction and maintenance of tolerance. The reduced number of cells expressing IL2R, in spite of an equivalent cellular infiltrate would suggest, rather, that a state of tolerance is induced and maintained by the activation and persistence of Th2 cells, as suggested by Takeuchi et al, (1992) using similar models.

Chapter 4

Application of the Polymerase Chain Reaction method to detect the presence of specific cytokines in rejecting and tolerant rat heart allograft tissue extracts

4.1 Introduction

The application of the polymerase chain reaction(PCR) technique in the detection of specific mRNA sequences in both homogenised tissues and cell suspensions has been shown to be extremely useful when limited amounts of starting material are available (Wu et al, 1992), and allows very low levels of DNA expression (in theory as little as a single molecule) to be amplified to levels easily detectable by conventional methods such as gel electrophoresis (Krams et al, 1992). PCR was used in this thesis to determine the presence or absence of specific cytokine (IL2 and IL4) mRNA sequences in rejecting and tolerised rat heart allografts prior to the use of in-situ hybridisation(ISH) techniques which, although not as sensitive as the PCR method, can pinpoint specific cells expressing the cytokine mRNA sequences, which PCR cannot.

To verify the integrity of the RNA extracted from the heart tissue and of the reverse transcription reaction itself, a preliminary PCR for β actin, which is expressed in various isoforms in nearly all eukaryotic cells was carried out on each sample to confirm the presence of PCR detectable cDNA. Negative controls in which primers and cDNA had been omitted from the reaction mixture, and positive controls comprising known PCR positive cDNA samples of the cytokines to be amplified were included in each reaction. Pre-optimisation of the technique for the cytokines under study and β Actin had been previously undertaken in our laboratories and showed that with the exception of primer concentrations, IL2 and β actin needed similar reaction mixture concentrations, whereas IL4 needed a higher

magnesium concentration for maximum efficiency as well as slightly higher cycle numbers to maximise amplification.

The resulting PCR product was then analysed by gel electrophoresis in which a fraction of the reaction product, as well as a marker DNA ladder of known molecular weight band sizes was loaded onto a 1.5% agarose gel which was then run at 125v. When the band front had reached an appropriate distance, the gel was removed from the tank and stained with ethidium bromide which enabled the product bands to be visualised by ultra-violet transillumination. The product size was then verified against the appropriate DNA ladder marker bands, as was the appearance and absence of positive and negative control bands respectively.

4.2 Results

Hearts were harvested on days 2, 5 and 7 post-transplant from both rejecting and OX38 anti-CD4 treated rats (each n = 4), whereas CsA and DSBT treated hearts were only available on day 5 (each n = 2). They were immediately wrapped in tinfoil and snap frozen in liquid nitrogen. Ten micrograms(μg) of extracted total RNA was taken from each sample and reverse transcribed into cDNA. A β actin amplification was carried out to confirm the integrity of the mRNA using three different concentrations of cDNA (neat, 1/10, 1/100). Cytokine specific primer pairs were then used to amplify neat cDNA which was derived from equivalent amounts of the extracted RNA. The products of each reaction were then analysed by gel electrophoresis and ethidium bromide staining. Photographs were then obtained under UV transillumination. Representative band profiles of PCR product are shown

in Figure 3.1. Those shown are an average representation of each group. All profiles in each group were within + or −1 band range except day 2 rejecting and OX38 anti-CD4 treated IL4 profiles in which 75% and 50% respectively showed no bands at all. The β actin product bands shown are of day 5 rejecting and OX38 anti-CD4 treated grafts and can be seen to be equivalent. The β actin band profiles of all other samples used gave similar patterns and intensities. Semi-quantitative analysis on all samples was carried out by comparing and contrasting product band intensities.

IL2

All rejecting heart grafts tested showed detectable levels of IL2 mRNA rising from day 2 to peak at day 5 post-transplant. Although day 7 grafts had similar bands to day 5 grafts, they were not as intense, suggestive of a slight diminution of IL2 production at the terminal stage of the rejection process. OX38 anti-CD4 treated grafts showed broadly similar levels of IL2 transcripts to rejecting grafts except on day 7 post-transplant where antibody treated grafts showed slightly more bands with a slight increase in their intensity. Grafts from the CsA treated rats showed either a minimal amount of detectable transcript or none at all, while DSBT treated grafts gave only a few very weak bands.

IL4

Like IL2, IL4 transcripts in rejecting grafts peaked at day 5 post-transplant and although still detectable by day 7, the band intensity had diminished considerably. Unlike IL2 however, only 25% of the day 2 grafts showed any detectable levels of

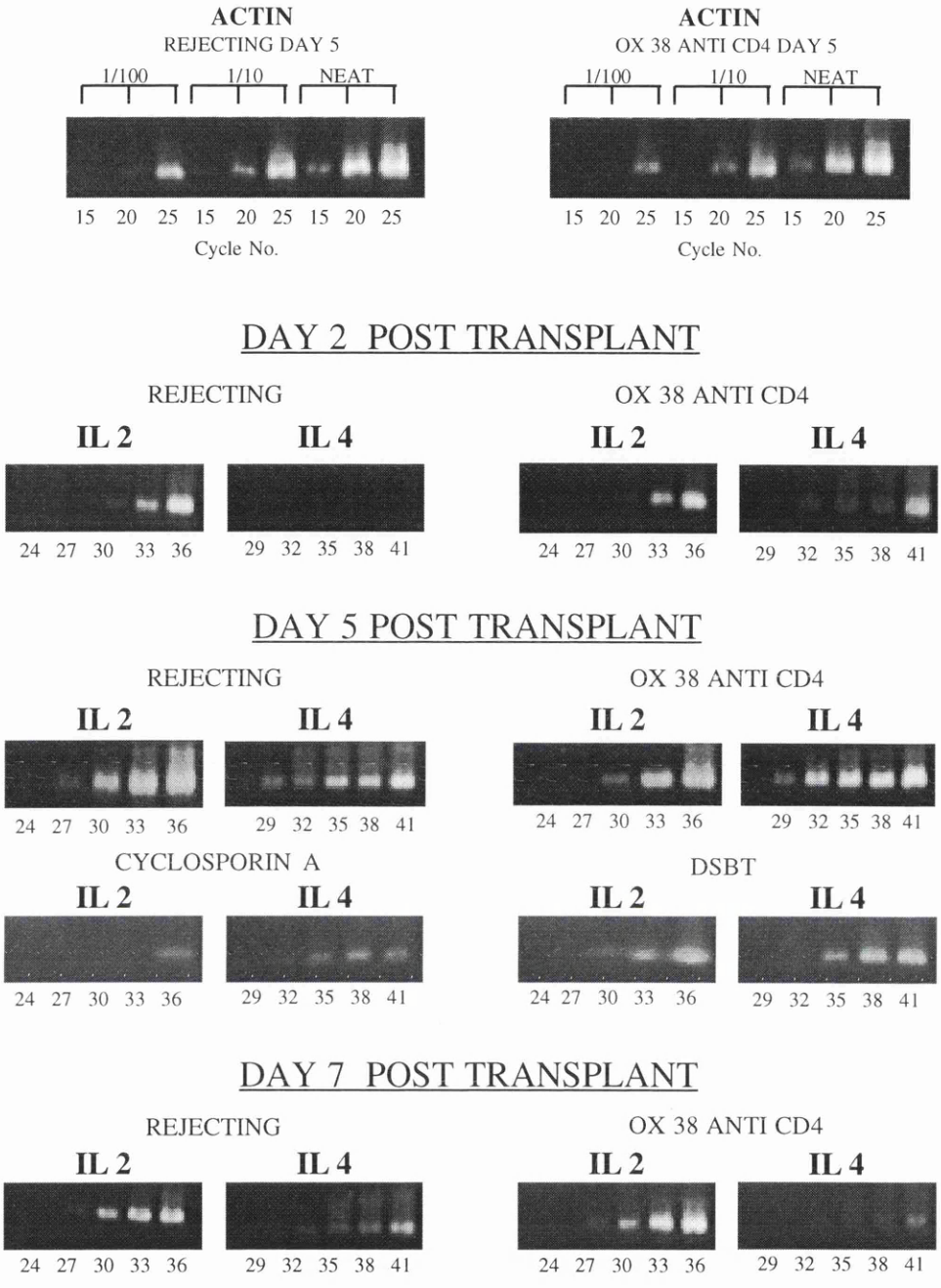


Figure 3.1 Intragraft IL2, IL4 and Actin mRNA expression of Rejecting and Tolerised Lewis heart allografts from DA recipients by PCR. Lewis hearts were excised at days 2,5 and 7 post transplant from DA recipients which were either unmodified, or had been rendered tolerant by DSBT, Cyclosporin A or OX38 anti-CD4 treatment. The tissue was homogenised, and cDNA prepared from each sample which was then amplified by PCR using appropriate primer pairs. Reaction products were analysed by gel electrophoresis and ethidium bromide staining and viewed under UV transillumination. Profiles shown are an average representation of each group. All profiles in each group were within + or - 1 band range with 2 exceptions (see 4.2).

IL4 mRNA and even then, the bands were so weak to be barely perceptible, even at the highest cycle numbers. In OX38 anti-CD4 treated rats, only 50% of the day 2 post-transplant grafts gave measurable but weak bands. By day 5, all four grafts showed bands at all cycle numbers and on day 7, although the band intensities had diminished, all grafts still had detectable levels of transcript. The grafts from CsA treated rats had very low levels of transcript whereas grafts from the DSBT treated rats had detectable cytokine levels at the highest three cycle numbers.

Summary

IL2 transcripts peaked at day 5 post-transplant in rejecting grafts compared to day 7 in OX38 treated grafts. IL4 mRNA was virtually undetectable at day 2 post-transplant in rejecting grafts, but rose dramatically to peak at day 5 before receding again, a scenario remarkably similar to that seen in the antibody treated grafts. In the CsA treated grafts, IL4 transcripts which although weak, were perceptible compared to those of IL2 which were virtually non-existent. In DSBT treated grafts, both IL4 and IL2 transcripts were relatively weak with the former, if anything, slightly the stronger.

4.3 Discussion

By using the PCR technique to detect IL2 and IL4 cytokine gene expression in the cardiac allografts, it has enabled small but equivalent amounts of RNA from each tissue to be amplified sufficiently to compare and contrast the levels of cytokine production in both the rejecting and tolerant grafts by observing both the number and the intensities of the positive bands. Direct comparisons between IL2 and IL4

levels of expression is made more difficult by the fact that higher cycle numbers were used to amplify the IL4 message compared to the IL2 to attempt to try and compensate for the expected low levels of IL4 transcripts experienced previously in our laboratory, and by others (M^{c}Knight et al, 1991).

As was to be expected by its reported mode of action, CsA treated grafts showed no perceptible levels of IL2 transcript expression. IL4 message was detected but it was not strong, suggesting that CsA treatment preferentially affects Th1 transcripts by downregulating them whilst affording a degree of protection towards the transcripts of Th2-like cells. Takeuchi et al, (1992), made similar findings while using fully mismatched mouse heart grafts. Weak positive IL2 transcripts were found only on day 4 post-transplant while IL4 transcripts were found to be more abundant on days 4 and 5, before diminishing. Some groups however have shown that Th2 cytokine production (e.g. IL4) may also be inhibited by CsA (Gajewski et al, 1990), and this may be a plausible reason for its weak expression seen in the experiments reported here.

Transcripts for both IL2 and IL4 were detected, albeit weakly in allografts from DSBT treated recipients at day 5 post-transplant. These results follow a similar pattern although at a lower order of intensity to Takeuchi et al, (1992), who reported comparable levels of both IL2 and IL4 transcripts at day 4 post-transplant with the IL2 diminishing at day 6, and IL4 increasing to peak at day 14 post-transplant. Also noted was a 10→20 fold reduction in IL2 transcripts in the transfused recipients in comparison to rejecting grafts. However, Dallman et al,

(1991), also comparing rejecting and DSBT treated grafts found that cells isolated from both sets of grafts showed broadly similar levels of IL2 transcripts, but that no IL2 was produced by the tolerised cells, concluding that tolerance was caused by alteration in the IL2 production pathway.

The finding of maximum IL2 transcript expression on day 5 post-transplant in rejecting grafts has been documented by other groups also using fully mismatched cardiac allografts models (Morgan et al, 1993 & Takeuchi et al, 1992), and these same groups noted, as we did, the appearance of IL4 transcripts on day 5, diminishing by day 7 post-transplant. These results, coupled with the findings of Krams et al, (1992), who found IL2 positive transcripts in less than 20% of rejected human kidneys, but that 46% of these same kidneys contained IL4 positive transcripts, shows that although Th1-type cytokines e.g. IL2 are necessary to promote cytotoxic cell activation and thus bring about graft destruction, the presence of Th2-type cytokines may also be necessary, e.g. to stimulate B cell production of alloantibodies.

In the experiments reported here, IL2 and IL4 transcripts both followed similar patterns of appearance in the OX38 anti-CD4 treated grafts. The appearance of IL2 transcripts in the graft showed that IL2 production did not appear to be inhibited by the antibody therapy and, indeed, the IL4 transcripts seemed to be enhanced, similar to results reported by Takeuchi et al, (1992). In comparing rejecting and antibody treated grafts, there is a marked similarity in the intragraft expression of both IL4 and IL2 transcripts, with the pattern and intensity of IL2

transcripts in rejecting grafts being mirrored in the antibody treated ones. IL4 transcripts appeared earlier post-transplant in antibody treated grafts than in rejecting ones where they were virtually undetectable. By day 5, both rejecting and antibody treated grafts expressed significant levels of IL4 mRNA, with the antibody treated grafts showing slightly more intense bands although this may be caused in part by the higher cycle numbers used in the IL4 amplification than in the IL2 one.

It would appear from the above findings that the main hallmark of tolerance induction in all three models used here is the preservation and/or enhancement of IL4 transcripts suggesting that the more 'protective' Th2-like cells play a crucial role in the maintenance of the tolerant state, while the early appearance of IL2 transcripts brings about the onset of irreversible rejection of the allograft.

Chapter 5

Development and Application of an In situ Hybridisation method to detect cytokine mRNA expression in frozen sections of rejecting and tolerant rat heart allografts

5.1 Introduction

The use of *In-situ* Hybridisation to identify specific mRNA sequences in tissue sections or single cell suspensions has allowed individual cells that express the target sequence of interest to be localised microscopically with great accuracy. Early methods using isotopically labelled DNA or RNA probes while very sensitive, also posed various problems, not least the potential hazard to health (Larsson, 1989). Important prerequisites for the success of the technique include probe specificity to and preservation of tissue target mRNA sequences. The more recent use of commercially manufactured non-isotopically labelled synthetic oligonucleotide probes has increased the reproducibility of the technique while at the same time, shortening the overall timescale involved (Larsson and Hougaard, 1990). These anti-sense oligoprobes can be specifically matched, using computer software, to any exon of the complimentary target mRNA sequence. Although they are only a fraction of the size of an average RNA probe, they may be combined in cocktails of oligoprobes specific for different areas of the target sequence to give similar levels of sensitivity.

An essential component of the ISH method is the pre-optimisation of the various stages of the technique. This involved testing different hybridisation temperatures, buffers, fixatives and post-hybridisation stringency washes to try and ensure maximum probe sensitivity and minimum background staining.

Fixatives and Pretreatments

The cross linking fixative paraformaldehyde as a 4% solution was the fixative of choice for both frozen sections and cell smears, and as the solution was treated with DEPC, a ribonuclease inhibitor, fixation was carried out at room temperature rather than at 4^0C (also inhibits endogenous ribonucleases). Twenty minutes in the fixative was enough to ensure preservation of the tissue and allow probe access to target sequences, whereas times greater than forty minutes were found to decrease signal due to possible overfixation causing dense cross-linking of tissue proteins.

Longer fixation times can be used, but this often requires the use of acids e.g. hydrochloric, or proteases e.g. proteinase K in solution to digest away some of the cross-linking, thus allowing probe access, but the amount used must be carefully titrated. Both were utilised, but the use of acid on frozen sections caused destruction of the tissue and the varying concentrations of enzymes used (1→10µgs/ml) led to overdigestion of the tissue section causing non-specific stickiness of probe when applied. Cell smears, however, did appear to benefit from the use of enzyme at the lowest concentration (1µg/ml) for fifteen minutes at 37^0C.

After the removal of fixative by several washes in DEPC treated PBS, a pre-hybridisation step using the hybridisation buffer minus probe for thirty minutes under hybridisation conditions, favoured by some groups, was found to be unnecessary as its usage did not alter the results. All sections were then dehydrated through increasing concentrations of absolute alcohol diluted with DEPC/H_2O and air dried. This step was found to increase the signal intensity, probably by ensuring that the probe was not diluted by any fluid on the tissue section.

Hybridisation buffers and Conditions

Hybridisation was carried out various temperatures :- room temperature, 37^0C, 40^0C and 42^0C using a variety of buffers containing a variety of ingredients and it was found that 37^0C was the optimum hybridisation temperature. Room temperature hybridisations led to a slight increase in background staining whereas 40^0C and 42^0C hybridisations led to less specific signal being recorded. Several buffers containing varying concentrations of deionised formamide (20→50%) were tried, but all gave rise to a distinctive background brown colouration to the tissues. Formamide is used to allow hybridisation at lower temperatures (especially good for longer RNA probes), but was found to be unbeneficial using our shorter oligoprobes so was removed from the buffer ingredients.

The use of higher concentrations of SSC in the buffer (4x) ensures binding stability of probe to target sequence, as did the addition of 5mg/ml sodium pyrophosphate. As no prehybridisation step was included, it was imperative to ensure that any non-specific binding of the probe was kept to a minimum or blocked completely during the overnight hybridisation. Denhardts reagent, containing BSA, ficoll and polyvinylpyruvate, as well as denatured salmon sperm DNA, were included in the buffer to achieve this. The inclusion of 0.5mls of 50% dextran sulphate, a volume exclusion agent, to effectively increase the probe concentration and rate of hybridisation was also found to be beneficial by slightly increasing the positive signal without causing any background staining or non-specific binding. Other additives e.g. Yeast RNA, sodium dodecyl sulphate (an RNAse destroyer) and tris-HCL pH

7.5 buffer were also tried but gave no added benefit, so were omitted from the final hybridisation buffer decided upon (see appendix 2).

Probe concentrations were very important and a series ranging from 0.1→1ng/μl were used. 0.5ng/μl was found overall to be adequate in most cases and in slides where no signal was found, the probe concentration was increased, usually to no avail except with the addition of general background staining.

Post Hybridisation and Detection

Post hybridisation washes are designed to remove any hybrids formed between imperfectly matched sequences, so washes at room temperature at a lower SSC concentration than used during hybridisation followed by a high stringency wash at 45^0C were found to be necessary to remove unwanted hybrids. Failure to use the low salt concentrations usually led to an increase in background staining.

Anti-digoxigenin antibody at 1.5mU/ml, and substrate solutions, were used as per manufacturers instructions. The antibody was used after a blocking stage designed to ensure no non-specific binding of the antibody, although the antibody solution itself also contained normal sheep serum which should have the same effect. Colour development of the signal catalysed by the substrate solution had to be checked microscopically in every experiment, and the experiment halted when an adequate colour intensity appeared. Generally, colour development was sufficient after about one hour, but samples with no specific signal were checked periodically and sometimes left in a dark cupboard overnight in case colour development was for

some reason very slow. These samples never showed any signal, and the colour was often grossly overdeveloped.

Once all these stages had been ironed out, a general method came into shape and was used for all the samples. This was used to localise cytokine mRNA production in the tissues and relate it to graft infiltrate as this gives more information about cytokine production than simply measuring secreted protein in peripheral blood. At the time of initiating this project, it still was not clear what cytokines were produced, and by which cells, in the response to the transplantation of an allograft, and it was thought that studying the pattern of certain signal cytokines in both rejecting and tolerised grafts might help elucidate the picture.

In the set of experiments carried out here, probe specificity to target mRNA sequence was confirmed by hybridising the relevant oligoprobes with cytospin preparations of transfected chinese hamster ovary(CHO) cells. These cells constitutively synthesise and secrete high levels of recombinant rat IL2 or IL4 cytokines (M^{c}Knight and Classon, 1992). Oligo d(T) probes which specifically bind to the poly A tail of mRNAs were used as positive controls in every experiment to verify the integrity of the mRNA. Negative controls were also used and these included hybridisation buffer solution minus any probe and occasionally an irrelevant human anti-sense Insulin probe, used under the same conditions as the specific IL2 and IL4 probes.

5.2 Results

Heart allografts from rejecting and OX38 anti-CD4 treated rats were sampled on days 2, 5 and 7 post-transplant, while DSBT and CsA treated rat heart allografts, as well as syngeneic control grafts were available on day 5 post-transplant only. All hearts utilised were Lewis strain hearts that had been transplanted into DA recipients(i.e. Lewis→DA).

After the rats had been sacrificed, the hearts were removed immediately and divided up. The portion for ISH was then placed in tissue-tek O.C.T embedding medium and immediately snap frozen in liquid nitrogen. 5 micron(μm) sections were cut of each tissue on a cryostat at –20°C, mounted on subbed slides and immediately fixed at room temperature in 4% paraformaldehyde to ensure the preservation of tissue morphology and the mRNA transcripts being studied. The intragraft expression of IL2 and IL4 mRNA sequences was analysed by hybridisation with specific non-isotopically labelled anti-sense oligonucleotide probes for IL2 and IL4. The specificity of the probes for their targets had been confirmed using CHO IL2 and IL4 secreting cell line smears (Figure 4.1).

The poly d(T) probe included in every experiment was consistently positive (Figure 4.2a), confirming the integrity of the mRNA sequences of the tissue under investigation and also that the conditions used were indeed favourable for hybridisation to target sequences to take place. Hybridisation buffer negative controls, also included in each experiment were always clear (Figure 4.2b), as were sections using the irrelevant insulin probe when used. This confirmed that any endogenous alkaline phosphatase present in the heart tissue had indeed been

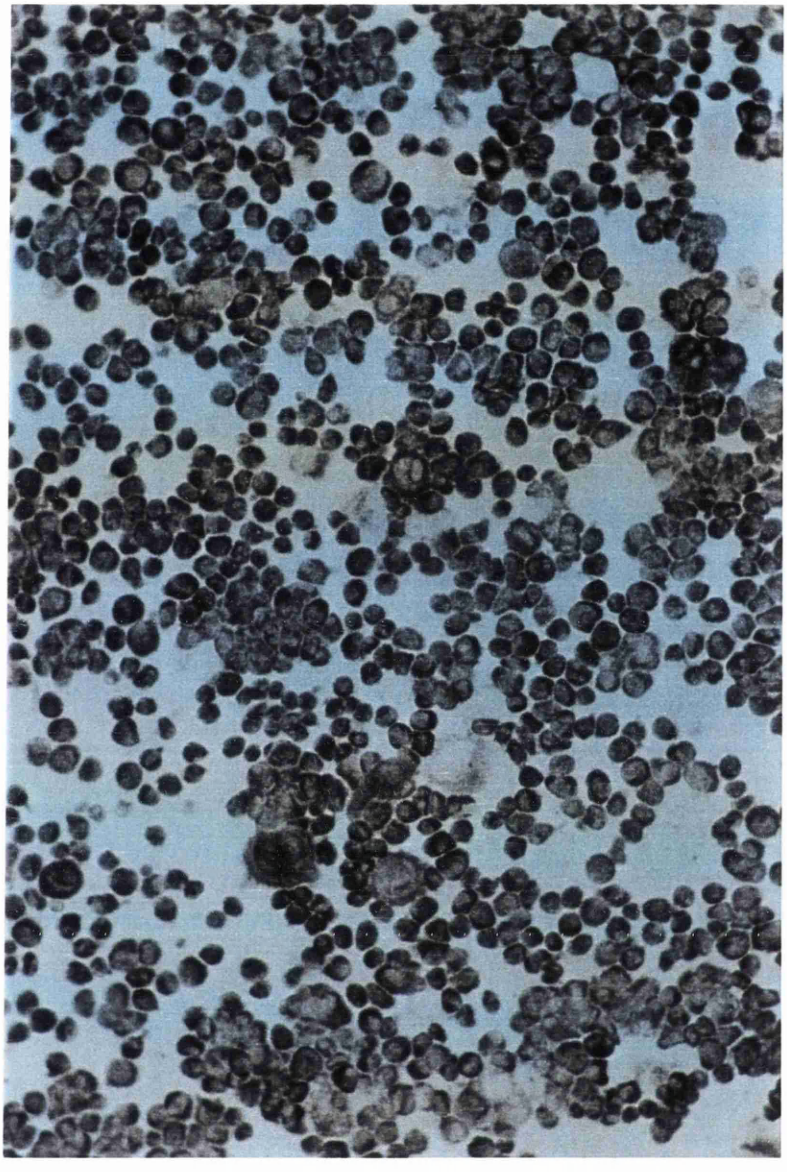


Figure 4.1 Positive labelling of an IL4 secreting CHO cell line smear using a Digoxigenin labelled anti-sense IL4 oligonucleotide probe cocktail. (x200)
Hybridisation of probe to relevant target sequences was used to confirm probe specificity. Similar results were obtained using an anti-sense IL2 oligonucleotide probe cocktail against an IL2 secreting CHO cell line. No counterstain.

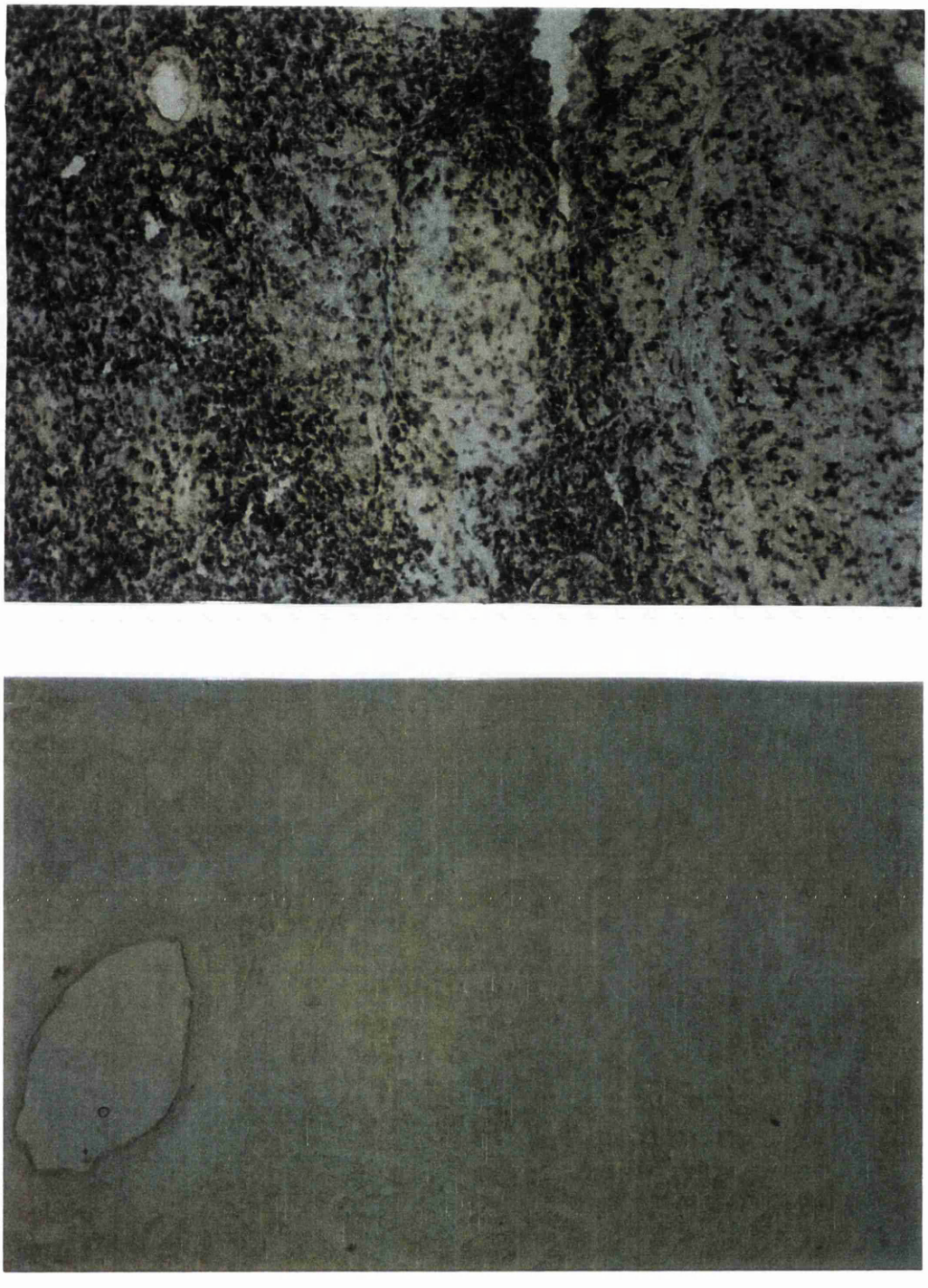
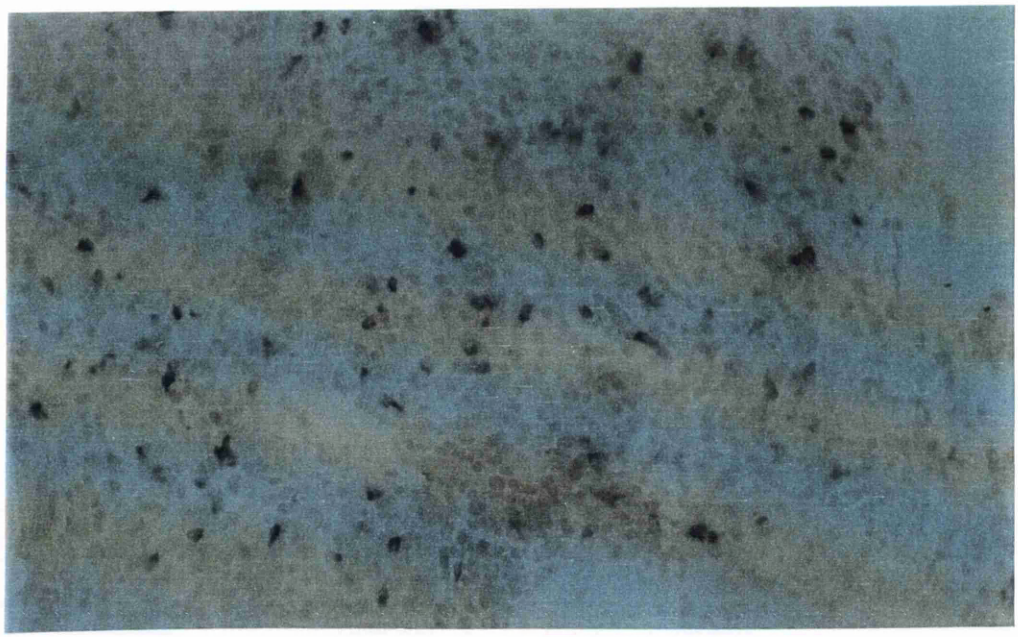


Figure 4.2 Positive and Negative ISH Controls.
Cryostat sections of Lewis heart allografts from DA recipients were hybridised with
a) Top - (x100) Poly d(T) probe as positive control.
b) Bottom - (x200) Hybridisation Buffer alone as negative control.
These controls were used in each experiment to verify mRNA integrity and confirm the absence of endogenous tissue alkaline phosphatase. No counterstain.

inhibited by the levamisole, which had been added to the NBT/BCIP solution for that purpose. After an overnight hybridisation, any non-specifically bound probe was removed by washes of increasing stringency before the administration of the NBT/BCIP/levamisole solution to enable the visualisation of the remaining specifically bound probe by optical microscopy.

IL2

IL2 positive signal occurred in all rejecting grafts (days 2, 5 and 7) post-transplant (Figures 4.3 and 4.4), increasing in frequency and intensity over the time span studied. As can be seen in Figure 4.4, day 7 rejecting grafts were characterised by gross tissue damage and irrespective of the stringency of the post-hybridisation washes, it was impossible to remove all non-specific binding due to the apparent stickiness of the tissue causing an apparent overstaining of the tissue sections. Syngeneic grafts (n=2) as well as allografts from CsA treated recipients (n=2) were both negative for IL2 irrespective of the probe concentration used, whereas grafts from DSBT treated recipients on day 5 (n=2) showed similar levels of IL2 mRNA to day 2 rejecting grafts (Figure 4.5). Positive staining for IL2 in OX38 anti-CD4 treated grafts was much more variable and was observed only in one of four grafts harvested on day 2, one of four day 5 grafts and two out of four day 7 grafts (Figure 4.6).



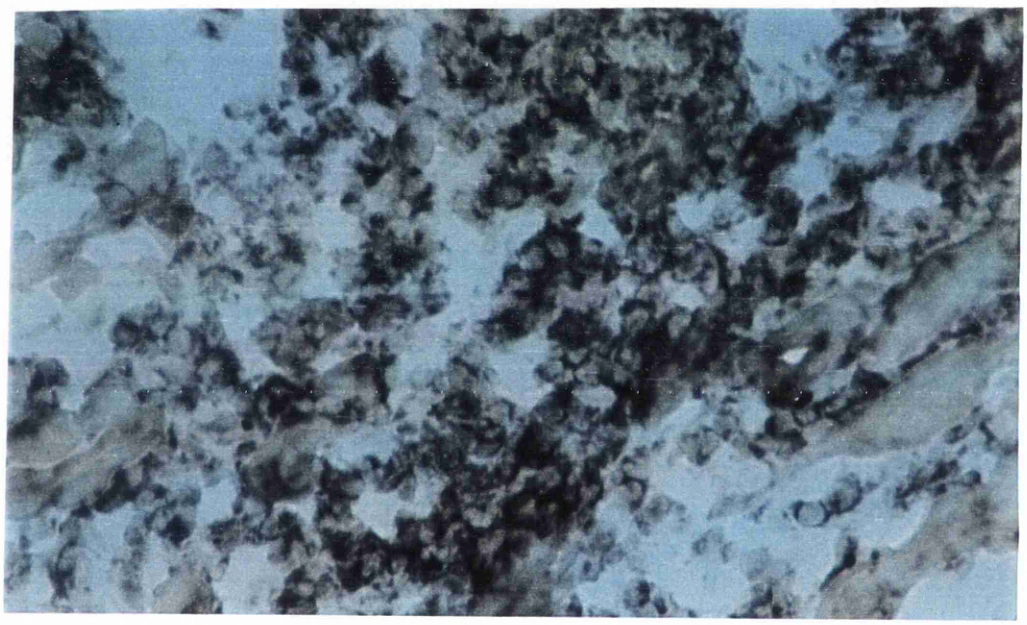


Figure 4.3 (Top) Positive IL2 labelling of a Day 5 Rejecting Lewis Heart Allograft from a DA recipient. (x200)
Cryostat sections of Lewis hearts, excised 5 days post transplant from DA recipients (n=4) were hybridised overnight with a Digoxigenin labelled anti-sense IL2 oligonucleotide probe cocktail. No counterstain.

Figure 4.4 (Bottom) Positive IL2 labelling of a Day 7 Rejecting Lewis Heart Allograft from a DA recipient. (x400)
Cryostat sections of Lewis hearts, excised 7 days post transplant from DA recipients (n=4) were hybridised overnight with a Digoxigenin labelled anti-sense IL2 oligonucleotide probe cocktail. No counterstain. Note severe tissue damage.

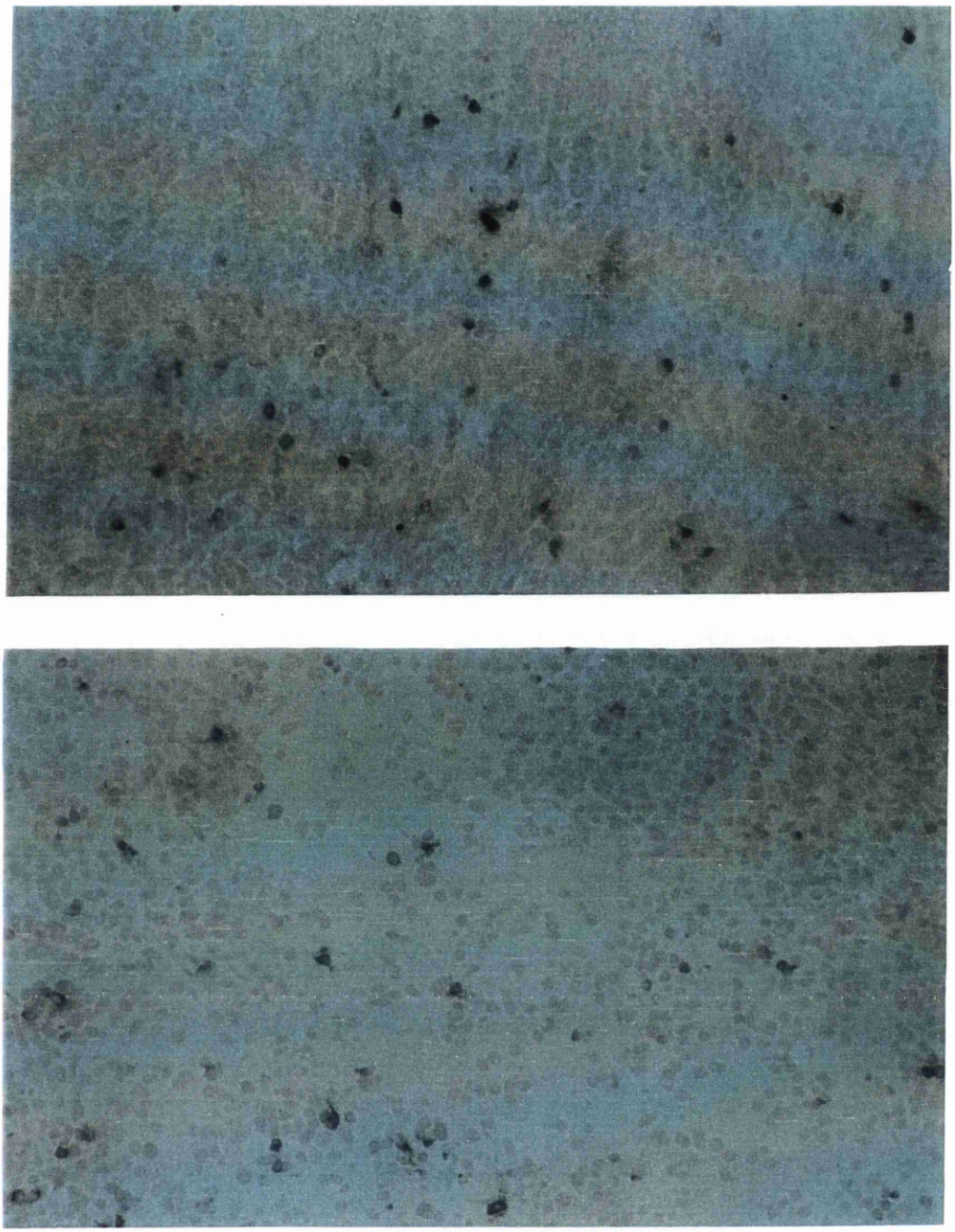


Figure 4.5 (Top) Positive IL2 labelling of a Day 5 Lewis Heart Allograft from a DA recipient Tolerised by a DSBT. (x200)
Cryostat sections of Lewis hearts, excised from donor blood transfused DA recipients 5 days post transplant (n=2) were hybridised overnight with a Digoxigenin labelled anti-sense IL2 oligonucleotide probe cocktail. No counterstain.

Figure 4.6 (Bottom) Positive IL2 labelling of a Day 7 Lewis Heart Allograft from a DA recipient Tolerised by an OX38 Anti-CD4 antibody regimen. (x200)
Cryostat sections of Lewis hearts, excised from OX38 Anti-CD4 treated DA recipients 7 days post transplant (n=4) were hybridised overnight with a Digoxigenin labelled anti-sense IL2 oligonucleotide probe cocktail . No counterstain.

IL4

Positive IL4 mRNA staining could only be found in two day 5 rejecting grafts (n=4) (Figure 4.7), and even then only weakly. No other graft, DSBT, CsA or OX38 anti-CD4 treated or syngeneic control grafts showed any positive staining.

Summary

ISH identified IL2 mRNA positive cells in all rejecting grafts but only in some OX38 anti-CD4 treated grafts (33% overall). No positive staining was found in CsA treated or syngeneic grafts whereas DSBT treated grafts were weakly positive. IL4 mRNA was only detected in 50% of day 5 rejecting grafts utilising the same hybridisation conditions as IL2. The pattern of staining was consistent with a diffuse cellular distribution.

5.3 Discussion

Both isotopically and non-isotopically labelled anti-sense probes have been used to study the cytokine mRNA repertoire of $CD4^+$ and $CD8^+$T cell subsets e.g. after mitogenic stimulation (Cardell et al, 1991, Sander et al, 1991 & M^{c}Knight et al, 1991), in anti-CD4 therapy in mice (Field et al, 1992), and in various experiments comparing allograft rejection and tolerance (Martinez et al, 1993, Yard et al, 1994 & Bugeon et al, 1992) to try and ascertain if the production of various cytokines correlated with either state.

In the set of experiments reported here using digoxigenin labelled oligonucleotide probes, graft rejection seemed to be associated with a sequential rise in intragraft IL2 mRNA expression by infiltrating cells from day 2 to day 7

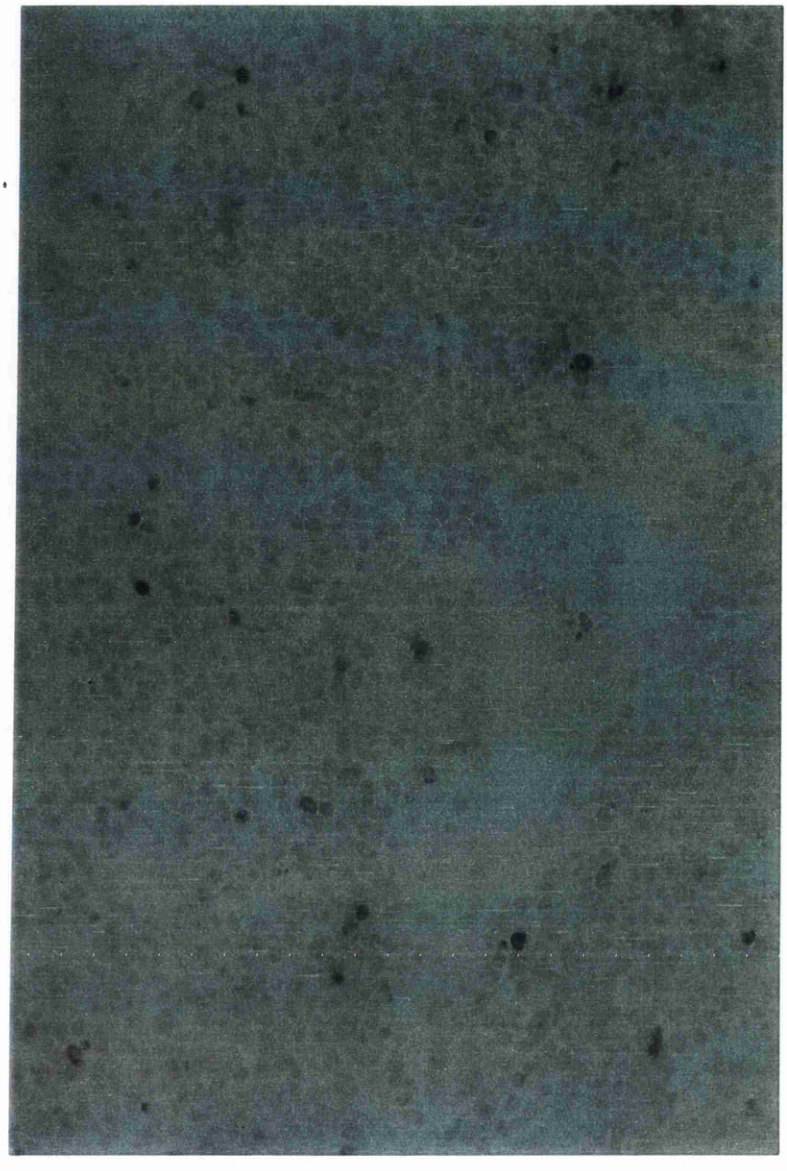


Figure 4.7 Positive IL4 labelling of a Day 5 Rejecting Lewis Heart Allograft from a DA recipient. (x200)
Cryostat sections of Lewis hearts, excised 5 days post transplant from DA recipients (n=4) were hybridised overnight with a Digoxigenin labelled anti-sense IL4 oligonucleotide probe cocktail. No counterstain.

post-transplant with rejection occurring immediately afterwards. This in itself would tend to suggest that rejection, in this model of high responder donors and low responder recipients is driven by the presence of committed Th1 cells releasing IL2 and probably IFNγ leading to the activation of macrophages and cytotoxic T cells. The presence of IL4 on day 5, released by the more protective Th2 subset in no way detracted from the ongoing process of rejection and it is possible that the rejection process was driven by a combination of the actions of both Th1 and Th2 subsets, as has been postulated elsewhere (Dallman, 1995).

The complete absence of IL2 transcripts in the grafts of CsA treated rats is not surprising as one of the main modes of action of the drug is the inhibition of IL2 production by activated T cells (Elliot et al, 1984), and has been shown to occur at the pre-transcriptional level (M^{c}Caffrey et al, 1993). The lack of detectable IL4 mRNA transcripts in these grafts is slightly puzzling as it has been reported that Th2 clones, (IL4 producing), may be more resistant to the actions of CsA than Th1 clones, and that negating the action of these Th1-like cells may alter the Th1/Th2 balance in favour of the latter (Gajewski et al, 1990). It would therefore be probable that IL4 should have been detected albeit at low levels. It is also possible that as only day 5 post-transplant samples were available here, that IL4 production had peaked earlier or later than the time sampled and would have been strong enough to be detected.

The same scenario may account for the lack of IL4 mRNA transcripts found in DSBT grafts which again were only available on day 5 post-transplant. Some expression, even at low levels, might have been expected as some studies have

shown that Th2-like cells persist in grafts from animals tolerised by transfusion causing suppression of the Th1-like cells (Takeuchi et al, 1992), which could account for the low level of IL2 mRNA transcripts recorded here in comparison to day 5 rejecting levels.

The complete absence of positive IL4 mRNA signal in the OX38 anti-CD4 treated grafts is enigmatic, as evidence from several groups has shown that Th2-like cytokine expression is preserved or even enhanced in the grafts of non-depleting antibody treated animals at the expense of IL2R positive cells and Th1-like cytokine (IL2 and IFNγ) expression, which has been shown to decrease (Siegling et al, 1993 & Mottram et al, 1995). This may account for the findings here i.e. that IL2 message was only detectable in 25% of day 2, 25% of day 5 and 50% of day 7 grafts. The lack of detection, especially of IL4 transcripts may lead to the assumption that the ISH method itself is just not sensitive enough to record low levels of transcript even after repeated attempts using different hybridisation parameters e.g. probe concentrations and hybridisation temperatures etc. Similar problems have however been reported by others. Unlike IL2 and IFNγ transcripts, IL4 mRNA expression in Con A stimulated rat $CD4^{+}$T cells could not be detected using radiolabelled probes, and it was concluded that this was due to the low level of IL4 gene expression, also noted in mice and humans (M^{c}Knight et al, 1991). IL2 transcripts, especially in rejecting grafts was obviously strong enough, with enough copy numbers/cell to be detected with relative ease, but in OX38 anti-CD4 treated grafts, differentiation between weakly positive cells and any background staining was more problematical.

Chapter 6

Final Discussion

6.1 Discussion

In the experiments described in this thesis, we sought to analyse intra-graft expression of the cytokines IL2 and IL4 produced by Th1 and Th2 subsets respectively of activated T cells using molecular biological methods to compare their appearance in rejecting and tolerised rat heart allografts at various time points post-transplantation.

Before the specific analysis of the IL2 and IL4, it was important to study allograft cellular infiltrates by light microscopy of tissue sections labelled with specific monoclonal antibodies. This not only confirmed the presence of graft infiltrating cells, including cells likely to produce the Th1 and Th2 signature cytokines, but also allowed the kinetics of graft infiltration to be studied in both the rejecting and tolerised graft models.

Studying the results of the phenotypic analysis of the infiltrate appears to show that in the Lewis→DA rejecting model used here, rejection is characterised by high levels of OX39(IL2Rα) positive T cells and ED1 positive macrophages in a Th1-type environment. In spite of the presence of a dense graft infiltrate of mononuclear cells containing large numbers of $CD8^+$ cytotoxic T cells and ED1 positive macrophages in allografts from animals tolerised by DSBT, anti-CD4 and CsA regimens, no rejection occurred. This may be associated with the much reduced levels of OX39 positive and $CD4^+$T cells seen in these grafts which may cause a shift in Th1/Th2 cytokine balance towards a more protective Th2-type environment in these models as has been reported by other groups (Siegling et al, 1993 & Takeuchi et al, 1992). As these immunohistochemistry techniques could not differentiate

between Th1-type and Th2-type cells, more sensitive methods were utilised which could detect typical cytokines from each subset e.g. IL2 from Th1-type and IL4 from Th2-type.

The *In-situ* Hybridisation technique used allowed direct cytokine mRNA expression to be identified within cells of each tissue. The method however has several reported drawbacks. It has been shown to be insensitive (Daha et al, 1994 & M^{c}Knight et al, 1991), and reproducibility to be problematical (Fealy et al, 1995).

The PCR method used is much more sensitive and can detect lower levels of message than ISH. The cytokine mRNAs were analysed in total mRNA extracted from tissue and were likely to be produced only by infiltrating cells, thus when comparing equivalent amounts of total mRNA, variations in cytokine mRNA content were likely to reflect varying proportions of cellular infiltration at different time periods post-transplant. This has several drawbacks. As the method relies on homogenised tissue samples, it can only therefore record average mRNA levels in the tissue being analysed and is incapable of detecting important differences in activation in small subsets of T cells (Bugeon et al, 1992). It is also possible, in samples taken shortly after transplantation of the allograft that the isolated RNA in the homogenised extract may contain passenger leukocytes which accompanied the transplanted organ and therefore a true picture of the allospecific immune response at its onset may be difficult to ascertain.

A major concern when analysing mRNA transcripts, especially cytokines like IL2, is that they have been shown to have a very short half-life due to the presence of AU-rich consensus sequences in the 3` untranslated region of the mRNA

and are quickly degraded in the cytoplasm following translation (Dallman et al, 1992 & Fealy et al, 1995). In order to minimise the risk of RNA degradation, samples for ISH and PCR methods were immediately encased in tinfoil following extraction and snap frozen in liquid nitrogen, thus avoiding tissue destruction prior to processing. Immediate freezing of the tissue was preferred to fixation as the time taken for paraformaldehyde fixative to diffuse into the heart tissue could not be guaranteed to be uniform, therefore, the possibility could not be excluded that transcripts at the outer areas of the subsequent tissue sections might be better preserved than those towards the centre.

The oligo d(T) probe and the β actin PCR samples, used as positive controls in the ISH and PCR methods respectively show only that mRNA sequences have been preserved in general and cannot confirm the status of the distinct cytokine sequences of interest and it is probable that some may be lost in the process. This is probably more crucial in the *In-situ* method as low levels of cytokine mRNA message can be difficult to distinguish from background staining (Krams et al, 1992) or in the case of IL4, to be detected at all (M^{c}Knight et al, 1991).

Comparing results obtained using both ISH and PCR methods on the same sample is difficult. In the ISH results reported here, only day 5 rejecting hearts showed any detectable levels of IL4, and even then in only 50% of the grafts. Using the PCR method on the same tissue, all four grafts were positive for IL4 transcripts. Day 2 rejecting graft IL4 results were similar for both methods in that none was detectable in either. Most PCR IL4 results from tolerised grafts showed that IL4 was only weakly expressed in these grafts with the exception of day 5 anti-CD4 treated

grafts which showed similar levels of transcript to day 5 rejecting grafts, some of which were positive by ISH. The lack of positive signal by ISH in these grafts is puzzling but it is possible that this may be due to large numbers of Th2 cells producing very small amounts of IL4 as opposed to a smaller number of cells making larger amounts of the cytokine, thus making it very difficult to be detected by the less sensitive ISH method as compared to the PCR. With IL2, detection rates using ISH and PCR were broadly similar in rejecting, CsA and DSBT grafts. The only discrepancy was in anti-CD4 treated grafts where only 25% of day 2, 25% of day 5 and 50% of day 7 grafts were positive for IL2 by ISH, whereas by PCR, all grafts tested were positive although some, e.g. day 2, were fairly weak. This, like the IL4 results may also be due to low levels of transcript produced by cells causing difficulties in detection especially when background staining was evident.

Combining the results obtained using both ISH and PCR methods to ascertain if a distinct Th1 or Th2-like pattern can be characterised in either the rejecting or tolerant models used, seems to suggest that rejection in this Lewis→DA strain combination is driven by cells of the Th1 (IL2 producing) lineage. These cells seem to appear much earlier in the rejection process than cells of the Th2 (IL4 producing) lineage, whose initial appearance on day 5 post-transplant cannot stop the already committed Th1 cells from producing cytokines such as IL2 and IFNγ. These cytokines can stimulate macrophages and $CD8^+$ cytotoxic T cells to bring about irreversible graft destruction in spite of the presence of the more protective Th2 cytokines like IL4, a scenario that has also been shown by other groups (Krams et al, 1992 & Hancock et al, 1993).

The virtual ablation of IL2 production seen in grafts tolerised by CsA here is in line with most other groups findings as the mode of action of the drug is the inhibition of IL2 production (Takeuchi et al, 1992). The production of IL4 also seems to have been slightly affected as has been reported previously (Gajewski et al 1990). DSBT treated grafts showed minimal effect on IL4 production but a reduction in IL2 mRNA expression, and this is in general agreement with findings by others (Bugeon et al, 1992). The use of the non-depleting OX38 anti-CD4 monoclonal antibody to induce tolerance brought about a slight decrease in IL2 production by Th1-like cells compared to levels in rejecting grafts sampled at a similar time scale while IL4 produced by the Th2-like cells was, if anything, enhanced. These findings show that total depletion of $CD4^{+}T$ cells is not necessary to obtain tolerance in this model and that the antibody seemed to preferentially target Th1-like cells whose actions seemed to be slightly suppressed, while at the same time enhancing or sparing the functions of the Th2-like cells, findings similar to those reported by Siegling et al, (1993), and Kupiec-Weglinski et al, (1993).

However, both methods, even when used in tandem, can only confirm the presence or absence of the specific transcript under investigation, and cannot prove that the specific mRNA transcript has been translated into a biologically active molecule. Dallman et al, (1991),using DSBT induced tolerance in a rat renal allograft model recorded that both rejecting and tolerant grafts contained similar levels of IL2 mRNA but that cells isolated from the tolerant rats could not make biologically active IL2, compared to the high levels of the cytokine produced in the rejecting rats, and postulated a post-translational alteration to the IL2 production pathway and

showed that tolerance in that particular model could be abrogated by the addition of recombinant IL2.

All three methods used here to tolerise recipients to fully mismatched heart allografts show that tolerance seems to be accompanied by a preserved or heightened Th2-type response at the expense of the Th1-type whereas the earlier appearance of Th1-type cytokines in rejection, compared to the Th2-type, may predispose the irreversible onset of rejection in this model, despite the presence of Th2-type cytokines.

From the results recorded here, it may be concluded that the use of both ISH and PCR methods in detecting IL2 and IL4 intragraft transcripts is beneficial. Although ISH has emerged as a less sensitive technique than PCR, it has the ability to allow individual cell expression of transcripts to be viewed microscopically in the graft whereas the use of the much more sensitive and reproducible semi-quantitative PCR technique is necessary to detect low levels of transcript which are a consequence of some tolerising regimens used, and a combination of both techniques gives a better representation of the cytokine milieu in the graft at the time of study.

Appendix

Buffers and Solutions

Glycine Dilution Buffer (see 2.3.3)

1M Glycine / 0.3M NaCl pH 8.6

Glycine :	75gms
Sodium Chloride :	17.6gms
Sterile Deionised Water :	to 1 Litre
5M Sodium Hydroxide :	sufficient to adjust pH to 8.6

Glycine Binding Buffer (see 2.3.3)

1M Glycine / 0.15M NaCl pH 8.6

Glycine :	75gms
Sodium Chloride :	8.8gms
Sterile Deionised Water :	to 1 Litre
5M Sodium Hydroxide :	sufficient to adjust pH to 8.6

Citrate Buffer (see 2.3.3)

0.1M Citrate

Citric Acid monohydrate :	21gms
Sterile Deionised Water :	to 1 Litre
5M Sodium Hydroxide :	sufficient to adjust pH to 3.0

DEPC treated H_2O. (see 2.6.2 etc.)

2mls of DEPC (Diethylpyrocarbonate) is added to 2 Litres of Deionised water, left for 24 hours and sterilised by autoclaving for 30 minutes at 136^0C and 2.2bar. DEPC inhibits RNAses and DEPC treated water is used to make solutions in the Hybridisation methods.

SSC (Saline Sodium Citrate) Solution (see 2.6.2 etc.)

Stock Solution of 20xSSC
1xSSC ≡ 0.15M NaCl / 0.015M NaCitrate pH 7.0

Sodium Chloride :	350.6gms
Sodium Citrate :	176.4gms
DEPC / H_2O :	to 2 Litres
5M Sodium Hydroxide :	sufficient to adjust pH to 7.0

Tris Hydrochloride Buffer pH 7.5 (see2.6.2 etc.)

Stock Solution of 10x (see below) -use at 1X conc.≡ 0.15M NaCl / 0.1M Tris-HCl

Tris-HCl :	157.6gms
Sodium Chloride :	87.7gms
DEPC / H_2O :	to 1 Litre
5M Sodium Hydroxide :	sufficient to adjust pH to 7.5

Tris Hydrochloride Buffer pH 9.5 (see 2.6.2 etc.)

Stock Solution of 10x Tris-HCL (see below)-use at 1X conc.≡ 0.1M Tris-HCl

Tris-HCl :	157.6gms
DEPC / H_2O :	to 1 Litre
just before use, the following are added to 1X Tris-HCl	
Sodium Chloride :	5.844gms (≡ 0.1M)
Magnesium Chloride $6H_2O$:	10.16gms (≡ 0.05M)
5M Sodium Hydroxide :	sufficient to adjust pH to 9.5

Hybridisation Buffer (see 2.6.2 , 5.1 etc.)

20xSSC :	1ml
Denhardts solution :	0.5mls
Salmon Sperm DNA :	0.05mls
Sodium Pyrophosphate :	0.25mls
50% Dextran Sulphate :	0.5mls
DEPC /H_2O :	to 5mls

Store at 4^0C. Warm up to 37^0C before use.

Loading Buffer (see 2.7.4)

DEPC/H_2O :	70mls
Glycerol :	30mls
Bromophenol Blue :	0.4gms

1.5% Agarose Gel (see 2.7.4)

1.5gms of Agarose (Sigma, UK) is dissolved in 100mls of sterile deionised water in a microwave and allowed to cool slightly before being poured onto the gel apparatus and allowed to set with lane marker combs in situ.

Running Buffer (see 2.7.4)

A Stock Solution of 5xTBE is made up as follows:

Tris Base :	54gms (≡ 0.089M)
Boric Acid :	27.5gms (≡ 0.089M)
EDTA :	1.25gms (≡ 0.00125M)
DEPC/H_2O :	to 1 Litre

solution should be pH 8.3 without adjustment.

The above solution should be diluted 1/10 with sterile deionised water shortly before use ≡ 0.5xTBE

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