MECHANISMS RESPONSIBLE FOR INDEFINITE SURVIVAL OF CARDIAC ALLOGRAFTS INDUCED BY R73 MONOCLONAL ANTIBODY IN A RAT MODEL

by

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Abstract

The principle obstacle to successful organ transplantation is the failure of modern immunosuppressive protocols to result in indefinite allograft survival and the concurrent side effects of such protocols such as infection and malignancy. As a means of overcoming these difficulties monoclonal antibodies have been used experimentally and in clinical practice to target specific molecules involved in the rejection response in the hope that operational tolerance to a graft can be induced by a short peri transplant treatment. The central role of the α/β T cell receptor in allorecognition and graft rejection makes it an ideal target for intervention.

In this thesis, experiments were carried out using a monoclonal antibody to the rat α/β T cell receptor (R73) to investigate its action in a model of rat heterotopic cardiac transplantation.

In preliminary experiments, the mouse IgG1 monoclonal antibody R73 was administered intraperitoneally at a dose of 200μg on days -2 and -1 relative to receiving a fully allogeneic heart graft (transplantation taking place on day 0). Prolongation of heart graft survival was seen in the BN-Lewis strain combination however indefinite graft survival (>100days) was only obtained in the Lewis-DA strain combination. In order to obtain the optimal treatment regime in this strain combination, antibody doses of 50μg, 200μg and 500μg were administered to separate groups. 200μg and 500μg doses produced

permanent graft survival and so the 200μg dose on days -2 and -1 was used in all further experiments. A group of animals underwent thymectomy prior to transplantation under cover of R73. The majority of animals accepted their grafts permanently, suggesting a peripheral mechanism of action for R73. This was further supported by the observation that exogenous IL-12 administered after heart transplantation to R73 treated animals resulted in reduced graft survival.

Studies were then carried out to evaluate the effect of R73 on the peripheral T cell pool. Flow cytometric analysis of lymphocytes in the peripheral blood and lymph nodes of R73 treated graft recipients revealed marked depletion of CD4 and CD8 T cells at days 4 and 7 post transplant with reconstitution of the T cell pool by day 21. Depletion was not complete, however, and 50% of T cells were present at day 7. Delaying heart grafting until this time still resulted in indefinite graft survival in the majority of recipients. This would suggest that R73 treatment not only partially depletes peripheral T cells but also renders the remaining cells unresponsive or results in the emergence of a regulatory population of cells. Flow cytometry also revealed the presence of T cell receptor modulation after R73 treatment. Immunohistochemical staining of the heart grafts themselves was also carried out at day 7 post transplant in R73 treated and rejecting control animals. This revealed a marked reduction in the graft infiltrate of R73 treated hearts compared to rejecting untreated hearts.

In further in vitro experiments, lymph node cells from R73 treated graft recipients were harvested at various time points post transplant and stimulated

in allogeneic MLR. Cells from treated animals failed to proliferate at day 4 post transplant but proliferated normally at days 7, 21 and 100 to both allogeneic and third party stimulators. Analysis of the cytokines in the supernatants of the MLR's revealed reduced IL-2 production by R73 treated LNC compared to rejecting animals and this was observed even as long as 100 days post transplant. Cytokine gene transcripts detected by RT-PCR in the grafts removed from R73 treated and rejecting animals revealed reduced IL-2 and IFNγ gene transcription in treated animals but comparable production of IL-4, IL-10 and IL-13. It would seem therefore that the cells which remain after R73 treatment are unable to produce IL-2 normally and the absence of this critical cytokine may be an important mechanism in the action of R73.

Finally, experiments were carried out to investigate whether R73 ligation of the TCR would result in alterations in apoptotic activity in graft recipients. Propidium iodide staining and flow cytometric analysis of LNC revealed increased apoptotic activity in the lymph nodes of R73 treated graft recipients at day 7 post transplant compared to rejecting and normal animals. This increased activity depended on the presence of a heart graft as DA animals given R73 alone displayed baseline apoptotic activity. Interestingly, TUNEL staining of heart grafts from R73 treated and rejecting animals revealed increased apoptotic activity in the heart grafts of rejecting animals suggesting that apoptosis occurs both as a tolerogenic mechanism perhaps by altered signalling via the TCR and as a mechanism of graft destruction by infiltrating T cells.

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Declaration

The work reported in this thesis was carried out entirely by the author Mr John Casey under the supervision of Professor JA Bradley unless otherwise specified in the text. I declare that this work has not been submitted for any previous degree. Part of the results have been presented at the Medawar Prize Session of the British Transplantation Society Meeting in Oxford, 1996.

Abbreviations

APC- antigen presenting cell

BSA- bovine serum albumin

CyA- cyclosporine A

DAB- diaminobenzidine

ER- endoplasmic reticulum

FACS- fluorochrome activated cell spectrometry

FITC- fluorescein isothiocyanate

HLA- human leukocyte antigen

IDC- interstitial dendritic cell

Ig- immunoglobulin

IL- interleukin

IVC- inferior vena cava

LNC- lymph node cell

mAb- monoclonal antibody

MHC- major histocompatibility complex

MLR- mixed lymphocyte reaction

PBL- peripheral blood lymphocytes

PBS- phosphate buffered saline

PE- phycoerythrin

PKC- protein kinase C

TCR- T cell receptor

TNF- tumour necrosis factor

TUNEL- terminal deoxynucleotidyl transferase mediated dUTP nick end labelling

CHAPTER ONE

Introduction and review of the current literature

1.1 History and current state of solid organ transplantation

The latter half of the 20th century has seen the emergence of solid organ transplantation as the treatment of choice for patients with end stage disease affecting the kidneys, liver, heart, lungs and pancreas. As a result, thousands of patients each year now have an alternative to either long term artificial organ support or death and as the experience of specialist centres increases, more and more patients each year are being considered candidates for organ transplantation.

Tissue grafting has been carried out both in the plant world and experimentally in animals for centuries, in fact the word "graft" was coined in the 16th century from the Greek word "greife", the tool used to perform plant grafting. Interestingly, it was noted even in this early age that strict conditions were required for successful grafting between plants. Boronio in 1804 described the first experimental skin grafts in sheep, however long term graft success was exceptional. Successful autologous split skin grafting was first carried out by the surgeons Reverdin, Thiersch and Ollier at the end of the 19th century and although allografting and xenografting was attempted at this time they were unsuccessful.

Solid organ transplantation was shown to be technically possible in large animal models as far back as the turn of the century, and in 1933 the first human

kidney allograft was carried out by the Ukranian surgeon Yu Yu Voronoy (Voronoy, 1936). Long term graft function was achieved in 1954 with the transplantation of a kidney between identical twin brothers in Boston and long term allograft function obtained in Paris in 1960 with the aid of immunosuppression (Hume et al, 1955, Serevelle et al 1951). With current immunosuppressive protocols one year graft function in the region of 80-90% is attainable in kidney, liver, heart, lung and pancreas transplantation.

The technical success of solid organ transplantation is mainly due to the work of the surgeons Alexis Carrel (1875-1944) and Mathieu Jaboulay (1860-1913) who developed techniques for vascular anastomoses allowing experimental transplantation to be carried out in animal models. In 1902 the first renal transplants in animals were carried out independently by Carrel and the Austrian surgeon Emerich Ullman. Carrel went on to carry out experiments using heart, lung and intestinal grafts (Carrel and Guthrie 1905). In 1906 Jaboulay attempted the first renal transplant in man, grafting the kidney of a recently killed pig onto the elbow of a woman with nephrotic syndrome. The graft had to be removed on the 3rd day and did not function. Voronoy in 1933 transplanted the kidney of a cadaver onto the thigh of a woman in acute renal failure but again the graft failed to function adequately and was removed on day 6. In 1954, however, the first successful human kidney allograft was carried out between identical twins at the Brigham hospital in Boston (Murray et al 1955). This event greatly encouraged the field of transplantation to

progress but emphasised the necessity for tissue compatibility at this time. In 1958 the first renal transplant under immunosuppression was carried out at the Brigham in Boston using total body irradiation and bone marrow transplantation. Although the graft functioned and did not show signs of rejection, the patient died of marrow suppression. 1960 saw the first successful transplant of a kidney between non twin siblings under cover of steroids. In this year, the first unrelated kidney transplants were carried out using total body irradiation and 6-mercaptopurine as immunosuppression. This event provided the justification for the continuation of clinical trials of renal transplantation and immunosuppressive protocols.

In the 1950's experimental liver transplantation began to develop a surgical protocol and in 1963 Thomas Starzl carried out the first human liver transplant. Unfortunately the first patient, a 3 year old child with biliary atresia, died due to coagulopathy. Later that year, Starzl carried out a successful human liver transplant using treatments to overcome the coagulopathy and using a combination of corticosteroids, actinomycin C and azathioprine as immunosuppression (Starzl et al, 1968). During the 1970's, liver transplantation activity plateaued out due to poor long term results and it was only with the introduction of veno-venous bypass and the advent of Cyclosporine A that liver transplantation expanded and became a recognised therapeutic modality for end stage liver disease in the 1980's.

The history of cardiac transplantation has received far wider publicity than any other organ the first successful human transplant being carried out by Christiaan Barnard in South Africa in 1967 (Barnard, 1967). The first attempt at human cardiac transplantation was carried out by James Hardy in Mississippi in 1964 using the heart of a chimpanzee and much of the work developing the technique of orthotopic cardiac transplantation was carried in out in dogs by Norman Schumway and Richard Lower in Stanford (Lower and Schumway, 1960). Cardiac transplantation, however only became a widely recognised therapeutic modality in the 1980's due to the poor results as a result of graft rejection prior to this.

In the wake of the success of these transplants, whole pancreas and lung transplantation have now become accepted methods of treating failure of these organs and are being carried out with improving results in specialist centres throughout the Western world.

1:2 The development of modern immunosuppression protocols

The increasing success of organ transplantation over the last 30 years is due principally to the evolution of modern immunosuppressive drugs. In 1959 the antimitotic drug 6-mercaptopurine was shown to induce immunological tolerance in rabbits and subsequently its metabolite azathioprine was shown to be more effective and less toxic in dog renal allografts (Calne, 1960, Calne, Alexander and Murray, 1962) and this drug was then used effectively in clinical transplantation in 1963 (Murray et al, 1963). Corticosteroids were also known to have immunosuppressive properties and the combination of prednisolone and azathioprine became the "gold standard" for immunosuppression in the 1970's (Groth, 1972). Further progress in improving graft survival was made during this time by the use of lymphocytotoxic crossmatching and the matching of major histocompatibility antigens between the donor and recipient and by the use of antilymphocyte preparations. It was also noted around this time that deliberate pre transplant blood transfusion had a beneficial effect on graft survival (Opelz et al, 1973, Opelz et al, 1992).

The next major advance in transplantation immunosuppression was made in 1976 by Jean Borel and his co-workers in the Sandoz laboratories in Basle, Switzerland with the discovery of the drug cyclosporin A (CsA) (Borel et al, 1976). CsA works by inhibiting interleukin-2 synthesis and subsequently T-cell clonal expansion and was shown experimentally to prolong graft survival in

animals (Kostakis et al, 1977, Calne and White, 1977) and subsequently in clinical transplantation (Calne and Wood, 1985, Calne, 1987). As a direct result of the introduction of cyclosporin A in the 1980's, the one year survival of solid organ allografts has increased from around 60% in the 1970's to 80-90% in the 1990's with modern immunosuppressant regimes consisting of a combination of cyclosporin, azathioprine and prednisolone. CsA treatment does, however cause generalised immunosuppression and therefore patients are at increased risk of infection and malignancy. In addition, CsA has been found to be nephrotoxic in the longterm (Thomson, 1989).

Despite the advances made with the introduction of CsA, grafts are still lost at the rate of 5% per annum and new immunosuppressive agents are being developed to combat this. These include Tacrolimus (FK506), Sirolimus (Rapamycin), Mycophenylate Mofetil and Brequinar Sodium.

Tacrolimus is a macrolide antibiotic which acts, as does CsA, by inhibiting the transcription of the IL-2 gene. It does this by binding to the cytosolic immunophilin FK-BP which then inhibits the translocation of nuclear factor of activated T cell (NF-AT) and subsequently the transcription of IL-2. Studies of Tacrolimus in clinical organ transplantation have shown it to be at least as effective as CsA, however it does share the nephrotoxicity seen in CsA treated patients (Jain et al, 2000, Preston et al, 1998).

Rapamycin (Sirolimus) acts by binding to the same cytosolic immunophilin as Tacrolimus (FK-BP), but acts by altering intracellular signalling in the T cell after IL-2 ligation. Clinical trials of rapamycin have suggested that it is a potent immunosuppressive agent which appears to act synergistically with CsA and possibly with Tacrolimus and so may allow a reduction in the dose of these drugs and therefore hopefully a reduction in the incidence of their side effects without loss of efficacy (Kahan, 2000, Latta, 2000, Khanna, 2000, Halloran, 2000).

Mycophenylate mofetil (MMF) is a new immunosuppressant drug which has recently been introduced into clinical practice in this country. It acts by inhibiting DNA synthesis in T cells through blocking the activity of the enzyme inosine monophosphate dehydrogenase (IMPDH). MMF is mow being used widely both in primary immunosuppression protocols and as an alternative to azathioprine in sensitised patients(Carl et al, 1997, Seebacher et al, 1999, Puig et al, 1999).

Brequinar sodium is a drug initially developed as an anti-neoplastic agent which inhibits the activity of the enzyme dihydroorotate dehydrogenase (DHODH) and disrupts purine biosynthesis and therefore proliferation of activated cells (Makowka et al, 1993a and b). Although its effect in transplantation is likely to be due to its antiproliferative effect on alloreactive T cells it has been suggested that it may have more complex immunosuppressive effects including inhibition

of IL-2 production (Forrest et al, 1994). Brequinar has not yet been accepted widely for clinical use but early experimental work suggests that it is a potent immunosuppressive agent (Kawamura et al, 1993).

1:3 The use of antibodies in clinical transplantation

Biological immunosuppression in the form of both polyclonal and monoclonal antibodies has been used clinically and experimentally in organ transplantation for many years. The success of these preparations has been at times disappointing, however as antibody production technology has improved and the preparations have become more refined, antibodies have become a useful adjunct to chemical immunosuppressants.

The use of polyclonal antibody preparations such as antilymphocyte serum (ALS) and antilymphocyte globulin (ALG) was pioneered by Starzl in the 1960's (Starzl, 1968). These preparations are raised by immunisation of horse, rabbits or goats with human lymphoid cells and act by depleting T and B lymphocytes from the circulation by opsonisation and by impairing the function of residual lymphocytes by modulating cell surface molecules (Powelson and Cosimi, 1994).

ALG was used during the 1970's along with azathioprine and steroids but did not improve graft survival compared with azathioprine and steroids alone

(Cosimi, 1976). ALG is currently used in some centres as induction therapy at the time of transplantation or in steroid resistant rejection as "rescue" therapy (Cosimi, 1988).

The production of monoclonal antibodies was first described by Kohler and Milstein in 1975 when they developed mouse tissue culture cell lines which produced anti sheep red blood cell antibodies (Kohler and Milstein, 1975). This hybridoma technology allowed the production of antibody preparations with consistent reactivity to particular antigens (Galfre et al, 1979). Monoclonal antibodies have been raised against many of the molecules involved in transplant rejection. Table 1.1 reviews the studies to date which have used monoclonal antibodies in a clinical setting. Despite the large number of monoclonal antibodies available, few have gained widespread acceptance as

Monoclonal antibody	Target molecule	Type of graft	Number of patients	Reference
OKT3	CD3	Kidney	123	Ortho Multicentre Trial Study Group, 1985, Delmonico et al, 1988
BMA031	α/β TCR	Kidney	24	Knight et al, 1994
T10B9.1A-31	α/β TCR	Kidney	76	Waid et al 1992
Daclizumab	CD25	Heart	55	Beniaminovitz, et al 2000
Daclizumab	CD25	Kidney	267	Ekberg et al, 2000
Daclizumab	CD25	Kidney	116	Nashan et al, 1999
Daclizumab	CD25	Islets	7	Shapiro et al, 2000
Basilximab	CD25	Kidney	174	Kahan et al, 1999
BT563	CD25	Heart	60	Gelder et al, 1996
LO-Tac	CD25	Liver	129	Reding et al, 1996
Odulimomab	LFA1	Kidney	101	Hourmant et al, 1996
Enilomab	ICAM1	Kidney	262	Salmela et al, 1999
BL-4	CD4	Kidney	12	Morel et al, 1990
MAX.16H5	CD4	Kidney	30	Reinke et al, 1995
OKT4A	CD4	Kidney	30	Matas et al, 1995
Campath 1H	CD52	Kidney	31	Calne et al, 2000

Table 1.1. Studies using monoclonal antibodies in clinical organ transplantation

effective immunosuppressants. Until recently, OKT3 was the only monoclonal antibody used widely in transplantation. This is a murine IgG2a monoclonal antibody directed against the CD3 molecule on the surface of T lymphocytes and it has been shown to be effective in reversing acute rejection by profound T cell depletion and modulation of the CD3/TCR complex (Cosimi et al, 1981, Masroor et al, 1994, Ortho Multicenter Transplant Study Group, 1985, Delmonico et al, 1988). The use of OKT3 has, however, been limited due to the attendant side effects of its use namely increased risk of infection and malignancy (Thistlethwaite et al, 1987, Monaco et al, 1987, Cosimi et al, 1987, Fung et al, 1987) and the cytokine release syndrome following the first dose (Chatenoud et al, 1990, Ambramowicz et al, 1989).

Monoclonal antibodies act in three ways. Firstly they can block a key cytokine or receptor ligand interaction in the immune system. One example of this is anti-CD25 mAb which blocks the interaction of IL-2 with its receptor. Secondly they can act by targeting and opsonising the target cell or molecule allowing recruitment of immune effector cells to destroy the cell. This mechanism appears to depend on adequate expression of the target molecule as well as the presence of the Fc region of the antibody. Campath 1H, an antibody directed against CD52, is a potent recruiter of effector cells and is thought to work in this way. Thirdly, monoclonal antibodies can achieve their cytotoxic effect by crosslinking target receptors and triggering apoptotic pathways by direct signalling mechanisms (Glennie and Johnson, 2000).

The lack of success of monoclonal antibodies in clinical transplantation is partly due to the complexity of the response in humans and also to the fact that the antibodies used have been derived from animals. They have a short half life in the serum and are not universally effective in triggering human effector functions such as the complement system and Fc receptors. In addition, these mAbs can trigger the production of human anti mouse antibodies (HAMA) resulting in hypersensitivity reactions, reduced efficacy and enhanced clearing of the antibody from the serum (Schroff, 1985). In an attempt to overcome this problem, recombinant DNA technology has been used to produce rodent antibodies with varying degrees of humanisation (Riechmann et al, 1988). This has been achieved by either forming "chimeric" mAbs where the variable region of the rodent mAb is transplanted onto the constant region of the human antibody, or by producing "humanised" antibodies where the antigen binding loops, the so called complementarity determining regions (CDR), are derived from the rodent antibody and the rest of the mAb is human. Humanised antibodies are thought to be less immunogenic than chimeric antibodies while retaining the effector activity of the human antibody, however the humanisation process often results in a reduction of antibody affinity (Clark M, 2000). Examples of these engineered antibodies are CAMPATH-1, directed against human CD52 and used in lymphomas and autoimmune disease, and anti-IL-2R mAb (anti-Tac).

Humanised monoclonal antibodies such as Daclizumab and Basiliximab have been shown to be effective as primary immunosuppression without the associated side effects of OKT3 in a variety of clinical transplant settings (Berard JL et al, 1999). Daclizumab, a genetically engineered humanised IgG1 monoclonal antibody specific for the alpha chain of the IL-2 receptor, has been used in addition to primary immunosuppression in renal transplantation with a reduction in acute rejection episodes but without any increase in side effects or lymphoproliferative disorders at 1 year post transplant (Ekberg et al, 2000, Ekberg et al, 1999, Iverson et al, 2000, Nashan et al, 1997 and 1999). Daclizumab has also been used with encouraging results in cardiac transplantation, reducing the incidence of acute rejection episodes during the induction phase (Beniaminovtz et al, 2000). Attempts at using daclizumab instead of calcineurin inhibitors such as CsA and Tacrolimus have proved disappointing (Hirose et al, 2000). Perhaps one of the most important uses of daclizumab in clinical transplantation has been its use in successful human islet transplantation, allowing a steroid free regimen to be used (Shapiro et al, 2000). Basiliximab is also a chimeric, humanised anti IL-2 receptor mAb and has been used in trials of renal transplantation with encouraging results (Kahan et al, 1999).

CAMPATH-1H is a humanised rat monoclonal antibody directed at the cell surface glycoprotein CD52, commonly called the CAMPATH-1 antigen. It

effectively lyses human T and B lymphocytes, clearing them from the blood for up to a month after administration of the antibody. A recent study has shown that renal allografts can be maintained on low dose CsA alone after CAMPATH-1H treatment allowing a state of almost or "Prope" tolerance to exist (Calne R et al, 2000).

The TCRα/β molecule has also been a target for monoclonal antibody intervention in clinical transplantation and is, in an experimental context, the subject of this thesis. BMA031 and T10B9.1A-31 are mouse anti-human α/β TCR mAbs which have been used in clinical transplant studies. BMA031 has been tested in phase II and III clinical trials compared with OKT3 in renal transplantation with comparable efficacy (Knight et al, 1994, Dendorfer et al 1990, Chatenoud et al, 1993). T10B9.1A-31 has been used in a phase II trial again compared to OKT3 and has been shown to be equally effective in reversing acute kidney graft rejection (Waid et al, 1992).

Monoclonal antibodies directed against the adhesion molecule LFA-1 have also been used in clinical studies. The antibody 25-3 was used in early studies in 1995 to test tolerability and in a follow up study by the same group the antibody Oduli-momab directed against the alpha chain of LFA-1 was used in 101 patients as induction therapy for kidney transplantation with comparable efficacy to rabbit ALG (Hourmant et al, 1996). The monoclonal antibody enilomab, directed against the adhesion molecule ICAM-1 has been tested in a

trial of 262 recipients of cadaveric renal transplants. Enilomab was given along with standard immunosuppression and while the antibody appeared safe to use, there was no significant difference in acute rejection rates or graft survival in the antibody treated group compared with those patients receiving conventional immunosuppression alone (Salmela et al, 1999).

The CD4 molecule has been the subject of much experimental work in transplantation but few monoclonal antibodies directed at this molecule have been tested in clinical setting. OKT4A has been used in a study along with standard triple therapy to assess tolerability with some success (Matas et al, 1995). Small studies have been carried out comparing the anti CD4 mAbs BL4 and MAX.16H5 with ALG and OKT3 and ALG respectively. These studies showed that anti CD4 monoclonal antibodies have similar efficacy compared to the control groups (Morel et al, 1990, Reinke et al, 1995).

1:4 The immunological basis of transplantation

The graft acceptance induced by immunosuppressant drugs is due to non specific immunosuppression and this results in a variety of side effects, principally increased risk of malignancy and infection. Recipients of organ transplants are at increased risk of developing a wide range of malignancies mainly lymphomas, skin cancer (particularly in high risk regions) genitourinary malignancy, leukaemia, alimentary tract tumours and Kaposi's sarcoma. The

overall risk is approximately threefold (Sheil et al,1993). Infection due to viral, bacterial and fungal organisms are more common and are associated with up to 85% of post transplant deaths (Drummer et al,1983, Ho et al 1983).

It is perhaps, then through a greater understanding of the immunology of the rejection process and how it can be manipulated that we will develop less toxic methods of inducing long term graft acceptance. The initial work into transplant immunology was carried out by Sir Peter Medawar who described the rejection of skin grafts in rabbits and noted that a second graft from the same donor was rejected more rapidly. This was termed the "second set" phenomenon and Medawar proposed that it was mediated by "immunocompetent cells" (Medawar, 1944). In 1954 Mitchison carried out adoptive transfer experiments on allogeneic tumour grafts in mice and demonstrated that lymphocytes can directly attack the graft. Also in 1954 Billingham, Brent and Medawar transferred immunity to skin grafts in mice using lymphocytes (Billingham et al, 1956).

The importance of humoral factors in transplant rejection was also recognised and antibodies were chemically characterised in 1936 by Tiselius. The existence of heavy and light chains was described by Porter and Edelman in 1959 and the genetic background to the diversity of antibodies described by Tonegawa in 1977. Kissmeyer-Nielsen documented the importance of antibody in the

hyperacute rejection process in 1966 but the relative importance of antibody in acute graft rejection still remains controversial.

The identification of the specific transplantation antigens responsible for rejection began with George Snell in 1948 who coined the term "histocompatibility genes" and later that year Gorer identified the H2 locus in the mouse (Gorer et al, 1948). The MHC in man was first described in 1958 by Jean Dausset and was an essential step in developing tissue typing and the HLA system.

Much of the recent research in transplantation has been directed towards inducing tolerance to an organ allograft using a short pre or peri transplant course of treatment directed against specific molecules involved in the alloimmune response. The ultimate aim of such therapy is that the adaptive immune system of the recipient would be unresponsive to donor specific molecules without non specific immunosuppression.

1:4:1The Major Histocompatibility Complex (MHC)

The MHC consists of a series of linked genes present, in man, on the short arm of chromosome 6. This is the most highly polymorphic region of the genome and was first described in mice in 1948 (Gorer et al, 1948). These genes code

for 3 classes of glycoprotein molecules called MHC class I, II and III. The first human MHC antigens were identified in 1958 by Dausset et al and the HLA A and B series documented in 1968. These discoveries were followed by the C series in 1970 and the class II antigens in 1973. In the mouse, the MHC is present on the mid portion of chromosome 17 and is called the H-2 complex and in the rat the MHC is present on chromosome 20 and called RT1 (Gunther et al, 1979, Gill et al, 1978, Klein J, 1979). In humans there are at least 17 class I loci but the loci relevant to transplantation are HLA-A, -B and -C. The class II region in humans is encoded by HLA-DR, -DP and DQ (Trowsdale et al, 1992,Campbell and Trowsdale, 1993), in the mouse by the H-2I region genes including I-A and I-E and in the rat by the RT1B and RT1D regions (Blankenhorn et al, 1983, Blankenhorn and Cramer, 1985).

Class I MHC is a heterodimeric membrane glycoprotein molecule consisting of a 45 kDa α chain which is non covalently associated with the 12kDa β-2 microglobulin. Its crystalline structure was discovered in the 1980's by a combination of affinity chromatography to outline the amino acid sequence and x-ray crystallography to determine the 3 dimensional structure (Bjorkman et al, 1987). The structure of the class I molecule is shown in figures 1.1a and b. The α chain is a transmembrane polypeptide with 5 distinct domains. α_1,α_2 and α_3 are extracellular with the α3 domain being membrane proximal and α4 and α5 comprise the transmembrane and intracellular domains respectively. The β_2-microglobulin is non covalently associated with the extracellular portion of the

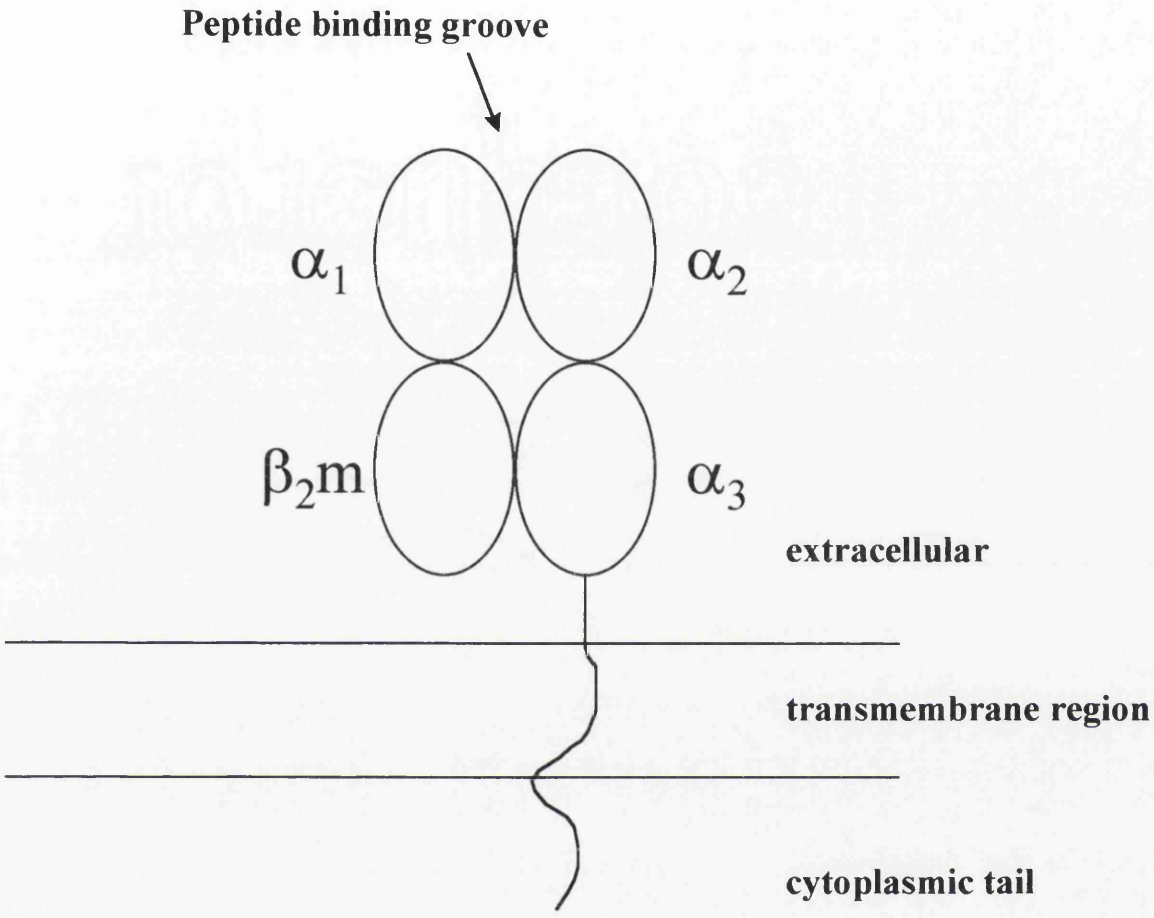


Figure 1.1a Schematic representation of the MHC class I molecule

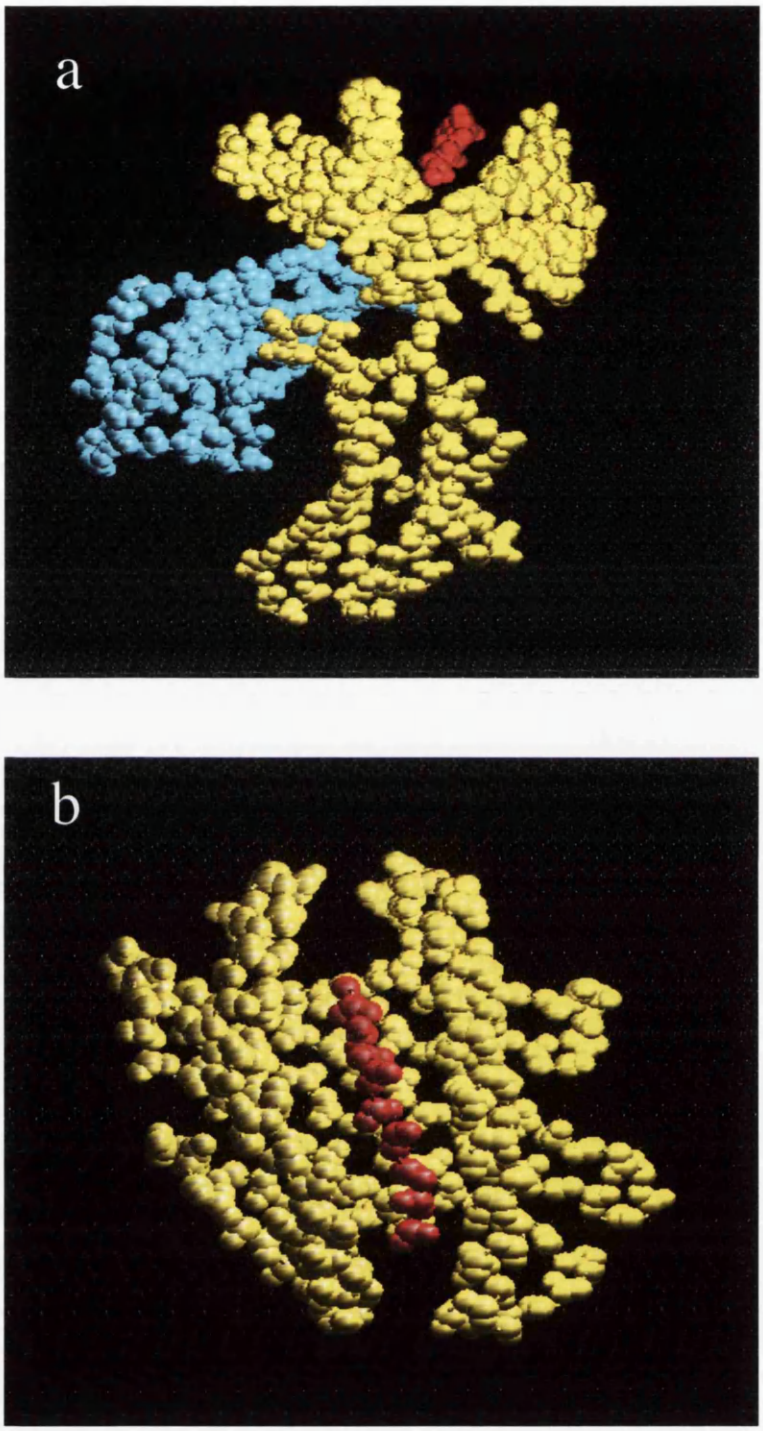


Figure 1.1b Structure of MHC Class I. The HLA-A2 molecule depicted as a space filling model viewed from the side (a) and from above (b) as seen by the TCR. (from Garboczi et al, 1996). α1, α2 and α3 domains are shown in yellow and the β2m is in blue. Antigenic peptide is in red.

α chain. The α1 and α2 domains form an eight stranded β-pleated sheet supporting two parallel strands of α-helix thus forming a peptide binding groove approximately 1x2.5 nm in size. The presence of peptide in the binding groove appears to be an essential part of the MHC complex since "empty" class I molecules are unstable and not expressed on the cell surface (Ljunggren et al, 1990, Schumacher et al 1990). Class I MHC molecules bind to and present endogenously derived peptide sequences to cytotoxic T-cells usually as a result of viral infection of the cell. Newly synthesised class I α chain associates with peptide in the endoplasmic reticulum, inducing a conformational change in the molecule and allowing binding of β2-microglobulin. The peptide-MHC class I complex is then transported to the plasma membrane via the Golgi apparatus and recognition by the CD8+ TCR takes place.

Class II MHC molecules are also transmembrane glycoproteins composed of 31-34kDa α and 26-29kDa β polypeptide chains, however both chains are transmembrane and consist of two extracellular domains each (Figures 1.2a and b). The peptide binding groove of the class II molecule is formed by the interaction of the α1 and β1 domains but unlike class I, the ends of the binding cleft are open, allowing bound peptide to extend out from the cleft (Rammensee et al, 1993, Neefjes and Ploegh, 1992). MHC class II molecules bind exogenously derived peptide ranging from 10 to 30 amino acids in length which has been endocytosed into the cell and degraded. The class II α and β

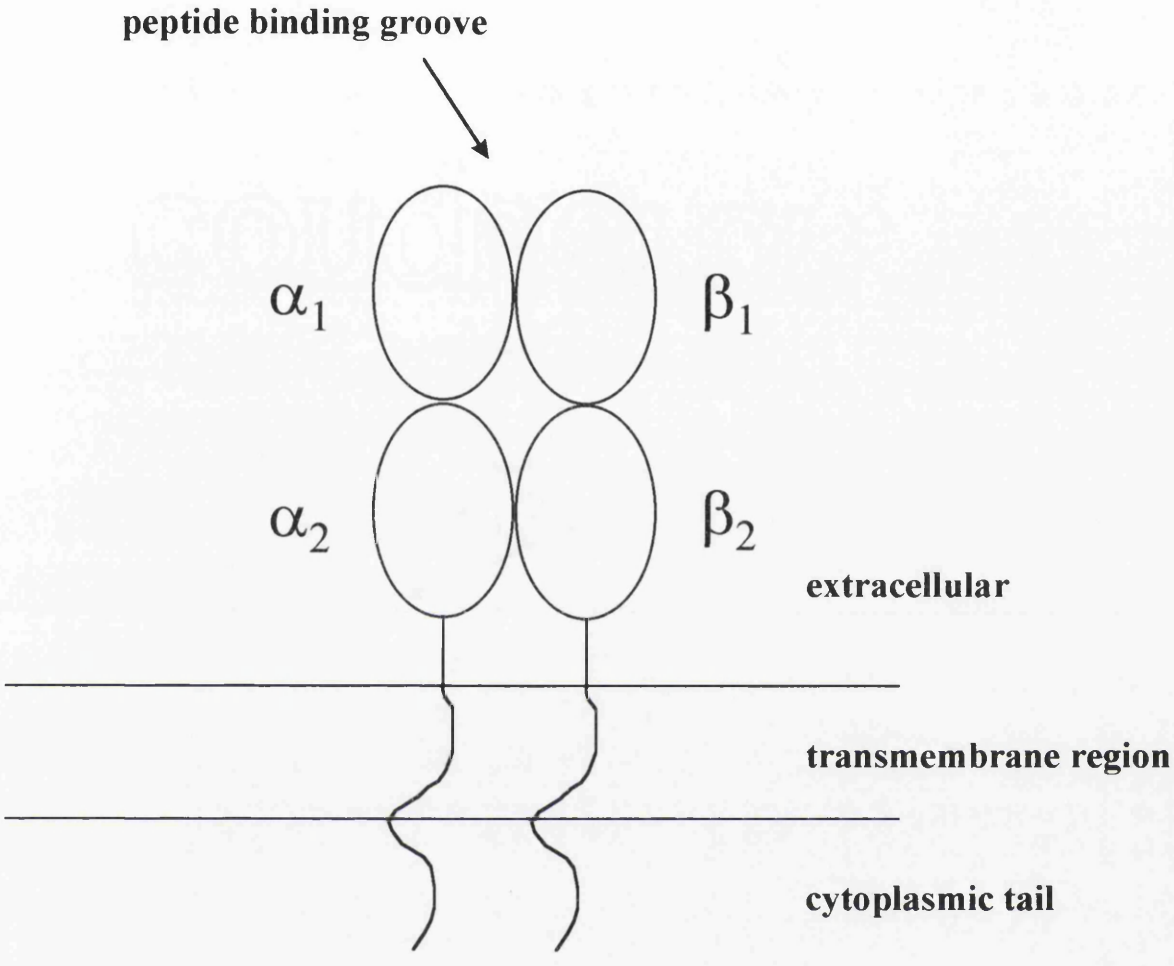


Figure 1.2a Schematic representation of the MHC class II molecule

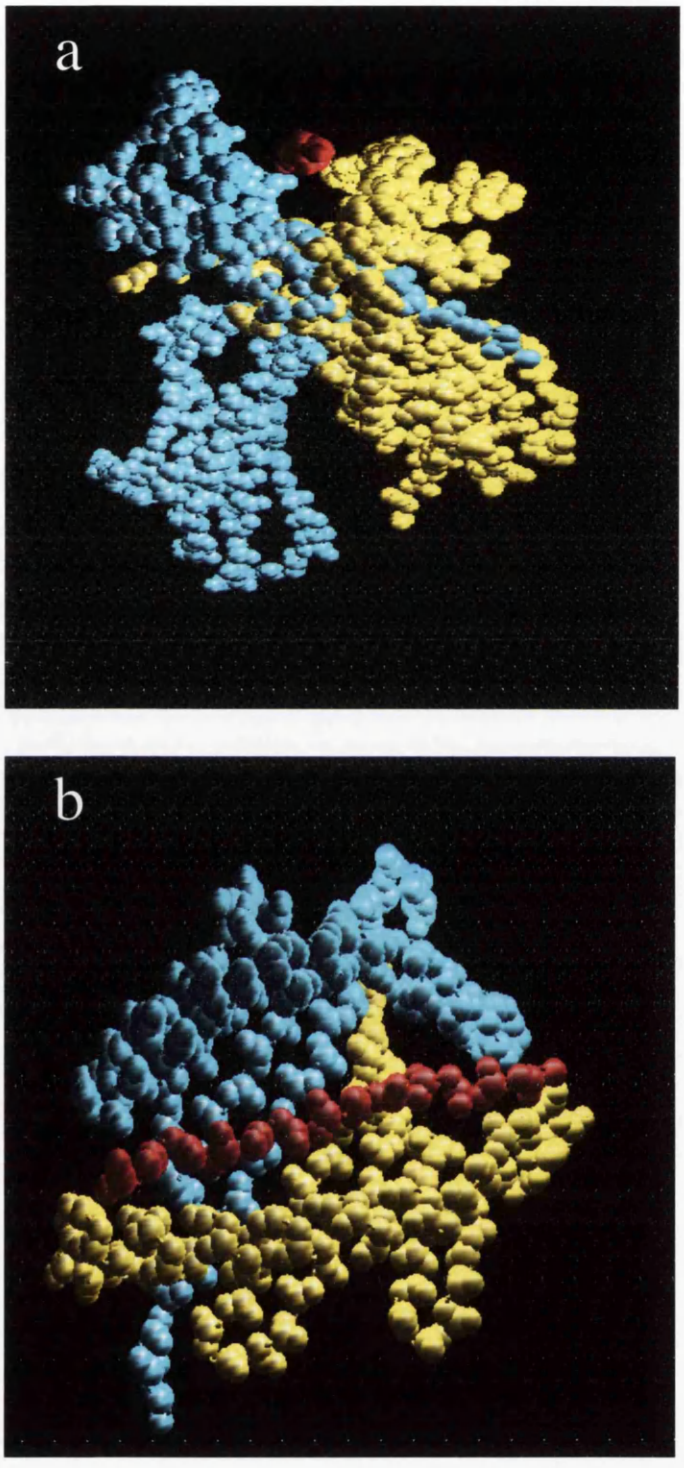


Figure 1.2b Structure of MHC Class II. The HLA-DR1 molecule depicted as a space filling model viewed from the side (a) and from above (b). Domains 1 and 2 of the α chain are shown in blue and domains 1 and 2 of the β chain in yellow. Antigenic peptide is in red (from Stern et al, 1994).

chains are synthesised in the ER where they associate with a third polypeptide called the invariant chain which is not encoded in the MHC. This association with the invariant chain is thought to be important for the transport of the class II heterodimer within the cell. This complex is then transported in the Golgi apparatus where the invariant chain cleaves, allowing peptide-MHC association in the endosomal compartment. The peptide-MHC II complex is then expressed on the plasma membrane where interaction with CD4+ TCR can take place. The class II region also encodes for proteins which deliver peptides for loading into class I MHC molecules. These are called Tap 1 and Tap 2 in humans, HAM 1 and HAM 2 in mice and mtp 1and mtp 2 in rats (Spies et al, 1992, Solheim et al, 1997, Monaco, 1993).

The class III region of the MHC encodes for components of the complement system and also includes the genes for heat shock proteins (HSP) and tumour necrosis factors α and β.

MHC class I and II molecules exhibit marked polymorphism. In the class I molecule this is mainly in the α_1 and α_2 domains with the α_3 domain highly conserved, presumably because of its association with β_2-microglobulin. In the class II molecule the β chains exhibit a much greater degree of polymorphism than the α chains, the majority of these polymorphic amino acids being grouped around the peptide binding groove. This suggests that each MHC allele only

binds a specific spectrum of peptides thus making the interaction of the peptide-MHC complex with the TCR highly specific.

1:4:2 Factors affecting MHC expression.

MHC class I antigens can be expressed on all somatic cells however studies by Daar et al (1984) using a monoclonal antibody directed against the heavy chain of HLA-ABC antigens have shown that not all nucleated cells in the body constitutively express class I. Class I antigens were not detected on corneal endothelium, some intestinal glands, central nervous system neurones, the exocrine pancreas and acinar cells in the parotid. They were only weakly detected on several tissues including islets of Langerhans, the myocardium and hepatocytes. Class I antigens are however upregulated in several immune responses including transplant rejection. In contrast, expression of MHC class II antigens is limited mainly to cells of the immune system particularly dendritic cells, B lymphocytes, Langerhans cells of the epidermis, endothelial cells and activated T lymphocytes (Daar et al, 1984). Again, expression of class II is upregulated during immune responses, particularly graft rejection, and can be induced or increased by cytokines such as IFNγ (Klareskog et al, 1986). Class II expression varies between species. Resting endothelial cells in rodents are class II negative and while in humans renal tubular cells show variable expression of class II, rat renal tubules are class II positive and mouse renal tubules class II MHC negative (Hart and Fabre, 1981, Fuggle et al, 1983, Bénson et al, 1985).

1:4:3 Minor Histocompatibility Antigens

Minor histocompatibility antigens (mH) were defined by Snell in studies in the mouse and are encoded by genes other than those of the MHC locus (Snell, 1948). Minor antigens are less immunogenic than major antigens, but studies in mice have shown that skin grafts can be rapidly rejected in some MHC identical strain combinations (Loveland and Simpson, 1986). The existence of minor histocompatibility antigens in humans was suggested by skin graft rejection between HLA identical siblings and graft versus host disease in recipients of bone marrow grafts from HLA identical siblings. Minor antigens have also been implicated in chronic organ allograft rejection and may play an important role in graft rejection via the indirect pathway and in rejection of xenografts. It has been shown that minor antigens are peptides derived from endogenous proteins and are encoded by genes located on the mitochondrial genome, the Y chromosome and the autosomes (Simpson and Roopenian., 1997)

1:4:4 The T cell receptor

T cell receptors are molecules expressed by T lymphocytes which recognise processed antigen in the form of peptides bound to MHC molecules. This MHC restricted antigen recognition is mediated by the αβ T cell receptor which is expressed on over 95% of T cells in peripheral lymphoid organs, blood

and lymph. A second type of T cell receptor, the γδ T cell receptor, is present on a small fraction of peripheral T cells which also mediates antigen recognition, but not in an MHC restricted fashion (Lawetzky et al, 1990).

Initial identification of the structure of the αβ T cell receptor was by the use of clonotypic antibodies which outlined a heterodimeric disulphide bonded molecule with a molecular weight of approximately 90kDa (Figures 1.3a and b). The heterodimer consists of an α (40-60 KD) and β chain (40-50 KD) each of which have two extracellular domains, one constant (C) and one variable (V). The junction between the V and C regions is encoded by a joining J segment gene and, in the case of the β chain, a diversity segment gene (D). The constant domain is attached to a transmembrane region by connecting peptides within which the interchain disulphide bond is formed. A short cytoplasmic tail is also present on both α and β chains. The variable domains of the α and β chains are formed into β pleated sheet structures and form the antigen binding site of the T cell receptor. Also present on all T cells is a series of polypeptides called CD3. This is tightly associated with the TCR and together they are referred to as the TCR-CD3 complex. Antibodies directed at the CD3 molecule can modulate T cell responses and are also able to co precipitate the TCR and CD3. CD3 comprises three polypeptide chains (each of 20-28 KD) on the surface of the T cell designated γ, δ and ε which are closely related to a ζ and, in rodents, a η chain. The γ, δ and ε chains are structurally related members of

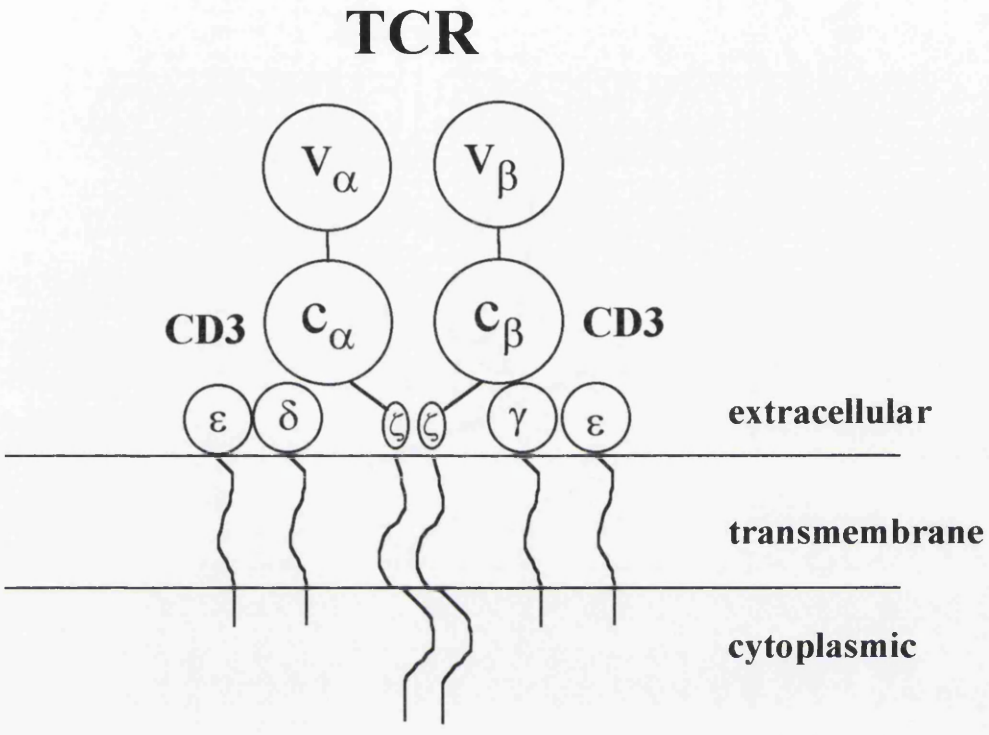


Figure 1.3a Schematic representation of the α/βTCR/CD3 complex

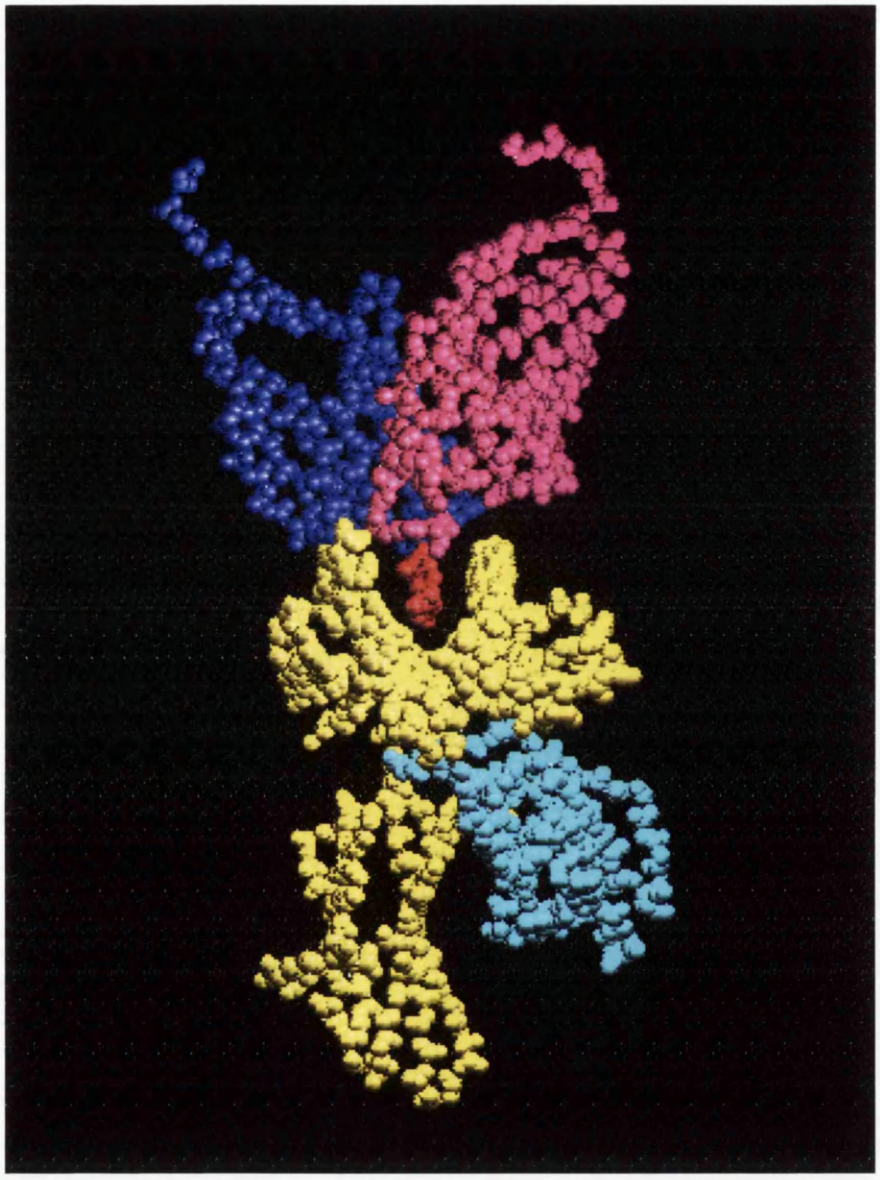


Figure 1.3b Structural basis of TCR interaction with MHC/peptide. MHC Class I molecule in yellow and peptide antigen (red) associating with the Vα (purple) and Vβ (blue) chains of the TCR (from Garboczi et al, 1996)

the immunoglobulin superfamily, whereas the ζ chain is unrelated and forms ζ-ζ dimers. The CD3 cytoplasmic domains each contain a motif called an Antigen Recognition Activation Motif or Tyrosine-based Activation Motif (TAM) (Weiss, 1991). CD3 is involved in signalling to the T cell after MHC/antigen-TCR interaction.

1:4:5 T cell development

T cells develop in the bone marrow from myeloblastic stem cells and then undergo maturation or "education" in the thymus. Briefly, thymic stromal cells present antigen to maturing thymocytes. Cells with high avidity for these self antigens are eliminated by apoptosis and cells which do not bind at all fail to proceed with maturation and die. Thus, highly autoreactive T cells and unreactive cells are eliminated by both positive and negative selection releasing only cells of medium avidity into the circulation. This process has made the thymus a potential target for immune modulation in transplant research and injection of donor antigen in the form of donor splenocytes and allogeneic peptide sequences has been carried out in animal models of graft rejection with variable success (Posselt et al, 1990, Walker et al, 1996).

1:5 Cellular Interactions in Graft Rejection

Central to the process of graft rejection is the interaction between the host T lymphocytes and the antigen presenting cells (APC) of the graft. Host T cells can be functionally divided into helper and cytotoxic subsets. These T cells express the non polymorphic membrane glycoproteins CD4 and CD8 respectively, correlating with the MHC restriction of each cell subset: CD4 +ve helper T cells are MHC class II restricted and CD8 +ve cytotoxic cells are MHC class I restricted. CD4 and CD8 were initially described simply as peripheral T cell markers, however it has been shown that they are receptors for MHC molecules and bind to structurally similar domains on their respective MHC molecules (Eichmann et al. 1987, Janeway, CA, 1989, Doyle and Strominger, 1987). Furthermore, their binding sites are distinct from the peptide binding domain allowing simultaneous binding of the TCR and CD4 or CD8 increasing the avidity of the interaction. Additionally, the cytoplasmic domains of CD4 and CD8 are associated with the intracellular protein kinase $p56^{lck}$. This enzyme is essential for tyrosine kinase activation in response to TCR ligation, and so binding of CD4 and CD8 to the same MHC molecule as the TCR results in increased tyrosine phosphorylation and amplification of the signalling pathway in response to TCR-MHC ligation (Miceli et al, 1993).

Alloantigen is presented in the form of peptide-MHC complexes by the interstitial dendritic cells (IDC) of the graft (also known as passenger leukocytes) (Roake et al, 1994). These cells are found in the parenchyma of all the commonly transplanted tissues (Hart et al, 1981, Daar et al, 1984), they are

CD45 positive and constitutively express high levels of class I and class II MHC. These are specialised immunostimulatory cells and are central to the initiation of primary cellular immune responses both in the in vitro mixed lymphocyte reaction (Steinman et al, 1978, Mason et al, 1981) and in vivo (Larsen et al, 1990). Experiments by Lechler and Bachelor demonstrated the importance of these cells in transplantation by transplanting kidney grafts into recipient rats treated with enhancing antibody to allow graft acceptance. These grafts were then transplanted into a second recipient syngeneic with the first without rejection. They concluded that this was due to replacement of graft dendritic cells with recipient IDC since injection of the second recipient at the time of grafting with donor dendritic cells restored rejection (Lechler et al, 1982). Various experimental strategies have been used to reduce the immunogenicity of organ allografts by depleting organs of IDC prior to transplantation (Goldberg et al, 1995, Brewer et al, 1989). Human vascular endothelial cells are also class II positive and can act as APC though not as efficiently as dendritic cells (Pober and Cotran, 1991, Hughes et al, 1990). Rodent resting endothelial cells are class II negative , however after in vitro culture with IFNγ they are class II positive and capable of immune stimulation (Ferry et al, 1987).

The primary immune response to an allograft is initiated by direct stimulation of T cells by the allogeneic MHC antigens on graft APC's. This direct T cell stimulation occurs because of the similarity between allogeneic and self MHC

molecules and is unique in the immune system in two respects. Firstly, MHC molecules do not require processing prior to presentation (Auchincloss and Sultan, 1996) and secondly, the precursor frequency of T cells for a direct response is one hundred times that for a direct response to other environmental peptides (Sherman and Chattopadhyay, 1993). This phenomenon of alloreactivity has been explained by the high density of foreign peptide-MHC determinants on donor APC's, allowing recipient TCR of lower affinity to respond (Bevan et al, 1984). Another explanation could be that a single allo-MHC product can stimulate multiple T cell clones by forming multiple "binary complexes" between MHC and different endogenous peptides (Matzinger et al, 1977).

In addition to direct T cell stimulation, there is growing evidence that the presentation of processed donor MHC peptide by recipient APC also plays a role in allograft rejection. Although this is the natural physiological mechanism of antigen presentation, it has been referred to as the "indirect pathway" in transplantation (Auchincloss and Sultan, 1996) . Experiments by Professor Fabre's group have shown that immunisation with donor peptide have resulted in accelerated graft rejection and an important role has been suggested for the indirect pathway in xenograft rejection (Benham et al, 1995, Fabre JW, 1996, Murphy et al, 1994, Dorling et al, 1996a and b).

The interaction between the TCR/CD3 complex and the MHC is essential for T cell stimulation, however it has been shown that full T cell activation requires co-stimulatory signals between T cell accessory molecules and their cognate APC surface proteins (Figure 1.4). The main APC-ligand receptor pairs thought to be involved in costimulation are CD40-CD40L, CD2-LFA3, CD28-B7 and ICAM1-LFA1. The TCR-MHC interaction provides signal one and the co-stimulatory molecules stimulate separate intracellular signalling pathways to provide the second signal for T cell activation. These co-stimulatory molecules are therefore potential targets for anti-rejection therapy and, as will be discussed later, experimental strategies using genetically manipulated animals and monoclonal antibodies against these molecules have been tried with variable success. Blockade of these co-stimulatory molecules may result in the T cell becoming anergic or unresponsive to further stimulation and this could be important in the induction of graft tolerance.

Following TCR/CD3 ligation, a signal is transduced from the T cell receptor complex to the nucleus of the cell to promote the transcription of genes important for T cell growth and differentiation. Antigenic engagement of the TCR/CD3 complex results in phosphorylation of the tyrosine or serine residues of the CD3 ε, γand ξ chains by $p59^{fyn}$ and ZAP 70 which are closely associated with the TCR and $p56^{lck}$. CD45 contributes to this by dephosphorylating tyrosine 505 of $p56^{lck}$. PhospholipaseC is then phosphorylated and a cascade of events is initiated. Phosphatidylinositol 4,5-biphosphate(PIP_2) is hydrolysed to

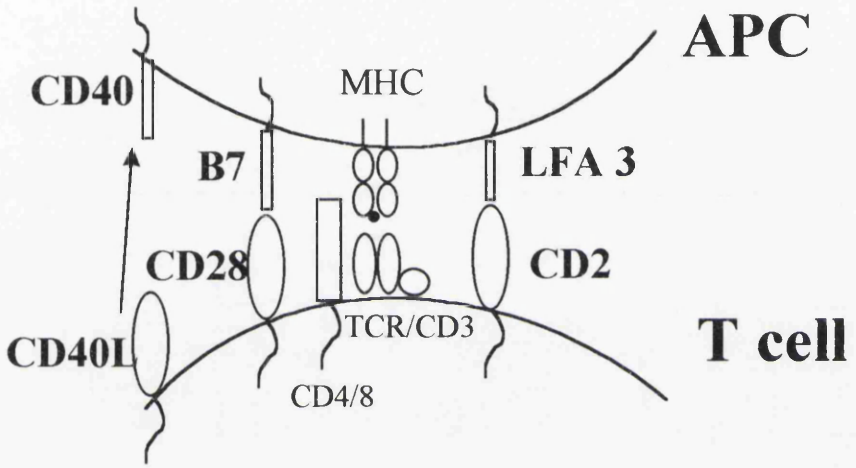


Figure 1.4 The molecular interactons involved in graft rejection

form inositol 1 4,5 triphosphate(IP_3) and diacylglycerol. This results in calcium ion mobilisation by IP_3 and the binding of diacylglycerol to protein kinase C(PKC). Increased intracellular free calcium and sustained PKC activation promotes the activation of several nuclear regulatory proteins and gene transcription.

T helper cells, usually CD 4 T cells, regulate the cellular and humoral responses by secreting a variety of soluble mediators which bind to specific receptors on the target cell surface. These mediators are called cytokines and include the Interleukins (IL-2, IL-4 etc.), the Interferons (IFN) and the Tumour necrosis factors (TNF) (Dallman et al, 1992, Nadeau et al, 1996) (Figure1.5). Particular patterns of cytokine production by T helper cells have been identified (Mosman et al, 1986). T helper cells which produce IL-2, INF-γ and TNFα are designated Th1 cells and have been shown in experimental models to be responsible for cell mediated immune responses and may be the predominant cell type involved in graft rejection. Th2 cells, which produce IL-4, IL-5, IL-6, IL-10 and IL-13 and are responsible for humoral immune responses and polarisation towards a Th2 type response, may be associated with graft tolerance in some models. Some studies have, however, found that graft rejection is associated with a Th2 type response and in some models graft acceptance is associated with suppression of both the Th1 and Th2 responses. The development of these cell types is itself regulated by cytokines. IL-12, a heterodimeric cytokine produced by monocytes, B cell, dendritic cells and NK

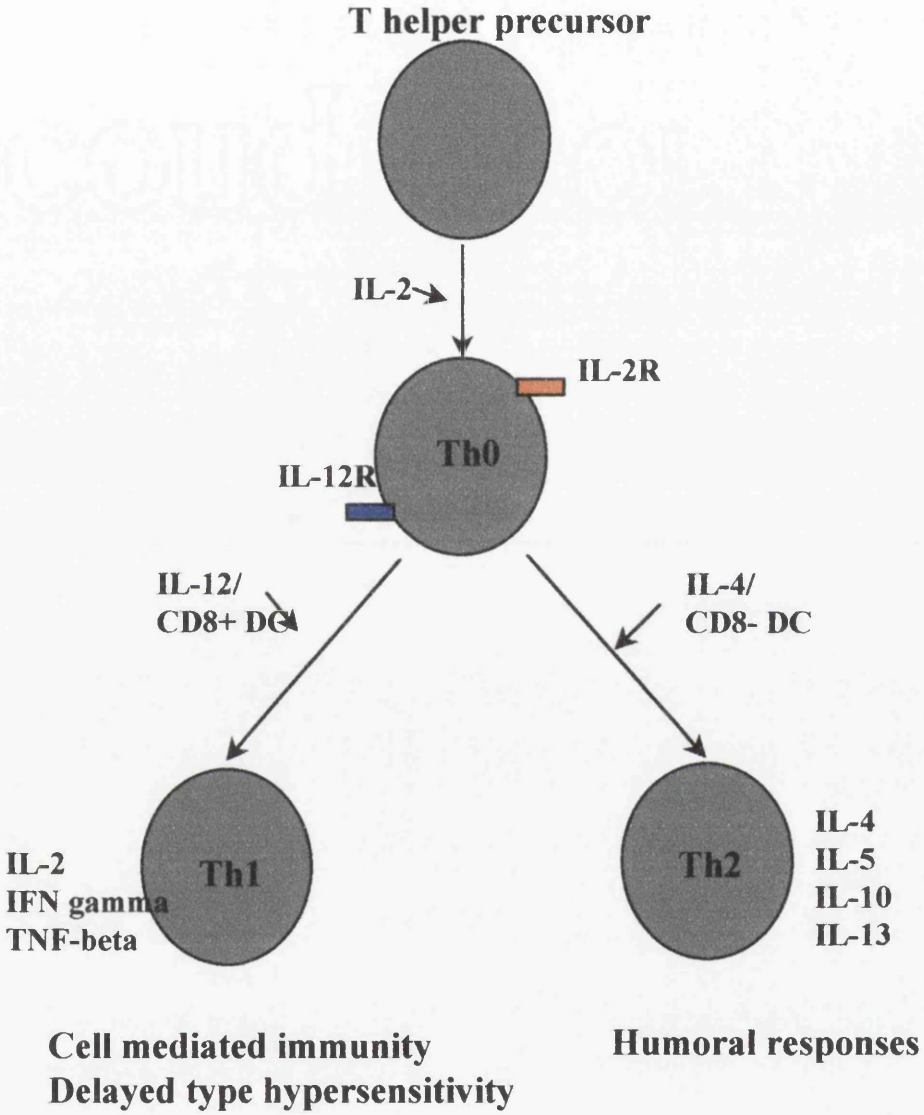


Figure 1. 5 T helper cell differentiation and cytokine production

cells, has been shown to be a key regulator of Th1/Th2 balance (Trinchieri, 1993 and 1995). Once a naïve CD4 cell is activated via the TCR it secretes IL-2 and expresses the IL-12 receptor. If it encounters IL-12 or $CD8\alpha^+$ dendritic cells then the IL-12Rβ2 chain is upregulated and differentiation along a Th1 pathway occurs. If, however, the activated Th cell encounters IL-4 and CD8α- dendritic cells the IL-12Rβ2 is downregulated and differentiation along a Th2 pathway occurs (Rengarajan et al, 2000).

1:6 The role of antibody in graft rejection

Hyperacute graft rejection as a result of preformed antibody to ABO blood group antigens and class I MHC (Baldwin et al, 1991) has been largely removed from clinical practice by the introduction of blood group matching and cytotoxic crossmatching prior to transplantation (Patel and Terasaki, 1969). Alloantibody dependant mechanisms have not been thought to be responsible for graft rejection, however it has been shown that in certain experimental circumstances alloantibody may effect graft rejection (Morton et al 1993). Alloantibody, principally IgG (Baldwin et al, 1991), may effect graft destruction either by antibody dependant cellular cytotoxicity (ADCC) or by complement mediated cell lysis.

1:7 Chronic graft rejection

Chronic rejection (or chronic transplant dysfunction (CTD) is the irreversible loss of graft function which occurs late in the post transplant period. It affects over 35% of kidney grafts and is present in 70% of lung allografts and 50 % of heart transplants at 5 years (Kouwenhoven et al, 2000). The classical pathological feature is fibroproliferative endarteritis resulting in concentric myointimal proliferation affecting the small arteries giving the characteristic "onion skin" appearance. The precise cause of chronic graft rejection is unclear and experimental models are rare, however multiple immunological factors such as histocompatibility, acute rejection, sub optimal immunosuppression, non compliance and anti-donor antibodies have been implicated. Non immunological factors such as graft ischaemia, infection, hypertension, infection and hyperlipidaemia may also play a role (Azuma and Tilney, 1995, Kouwenhoven et al, 2000,).

1.8 Mechanisms of tolerance

The traditional definition of transplantation tolerance is " specific absence of an immune response to an antigen", however, recently the concept of "operational tolerance" defined as "the survival of a functional allograft in the absence of long-term exogenous immunosuppression even though the graft recipient may

show apparent allorecognition of donor cells in vitro" has become more useful (Sachs, 1996).

Several strategies for the induction of tolerance to an allograft have been tried both experimentally and in clinical studies. The mechanisms responsible for tolerance induction can be divided into central (thymic) tolerance and peripheral tolerance. Central tolerance is due to clonal deletion of autoreactive T cells in the thymus (Burnet, 1959) and is responsible for tolerance to self antigens. The experimental evidence for this comes from work using TCR transgenic mice (Sha et al, 1988) whereby cells expressing a self reactive transgene were eliminated. TCR positive thymocytes are firstly subject to positive selection in the cortical region of the thymus where only T cells which interact with self MHC molecules survive.. Negative selection then occurs in the medullary region of the thymus where T cells expressing high affinity TCR for self antigen are eliminated by apoptosis. Thus autoreactive T cells are eliminated before they reach the periphery. Several investigators have attempted to induce tolerance to allografts by injection of donor antigen either in the form of donor cells or donor strain MHC peptide in an attempt to negatively select alloreactive T cells prior to transplantation (Posselt et al, 1990, Odorico et al, 1992, Remuzzi et al, 1991, Campos et al, 1993, Walker et al, 1996).

Work using monoclonal antibodies against T cell surface molecules such as CD4, CTLA-4 and LFA-1 has suggested that neither the thymus nor clonal deletion are required for the induction of tolerance. It would appear that, in the

transplant setting, extrathymic or peripheral mechanisms are more important for tolerance induction.

Several peripheral mechanisms have been proposed for the induction of tolerance (Figure 1.6). T cell ignorance occurs when antigen is presented in the context of MHC class I by non professional APC. The absence of costimulatory signals during T cell stimulation by either blocking co receptor interaction (in particular CD40-CD40 ligand and B7-1/2- CD28) or by antigen presentation by class II positive cells lacking co receptor (eg activated endothelial cells) can result in the inactivation of the T cell or the induction of anergy (Pleyer et al, 2000). Anergic T cells are unable to respond to further stimulation by normal APC. It has been suggested that lack of normal IL-2 production and T cell proliferation caused by incomplete stimulation is the mechanism of anergy production (DeSilva et al, 1991). It has been suggested that such tolerance is "infectious" in that a cohort of tolerant cells are able to guide or regulate other T cells towards tolerance (Cobbold et al, 1996). This may occur by anergic T cells competing with immunocompetent cells at the site of antigen recognition for costimulatory molecules and cytokines (the so called "civil service model") (Cobbald et al. 1990, Waldmann and Cobbold, 1993). In 1974 Gershon demonstrated that tolerance can be transferred by antigen specific T cells from tolerant animals (Gershon, 1975). He termed these cells suppressor cells and since then various hypotheses have been suggested to explain this observation. Most recently, suppressor or regulatory T cells have

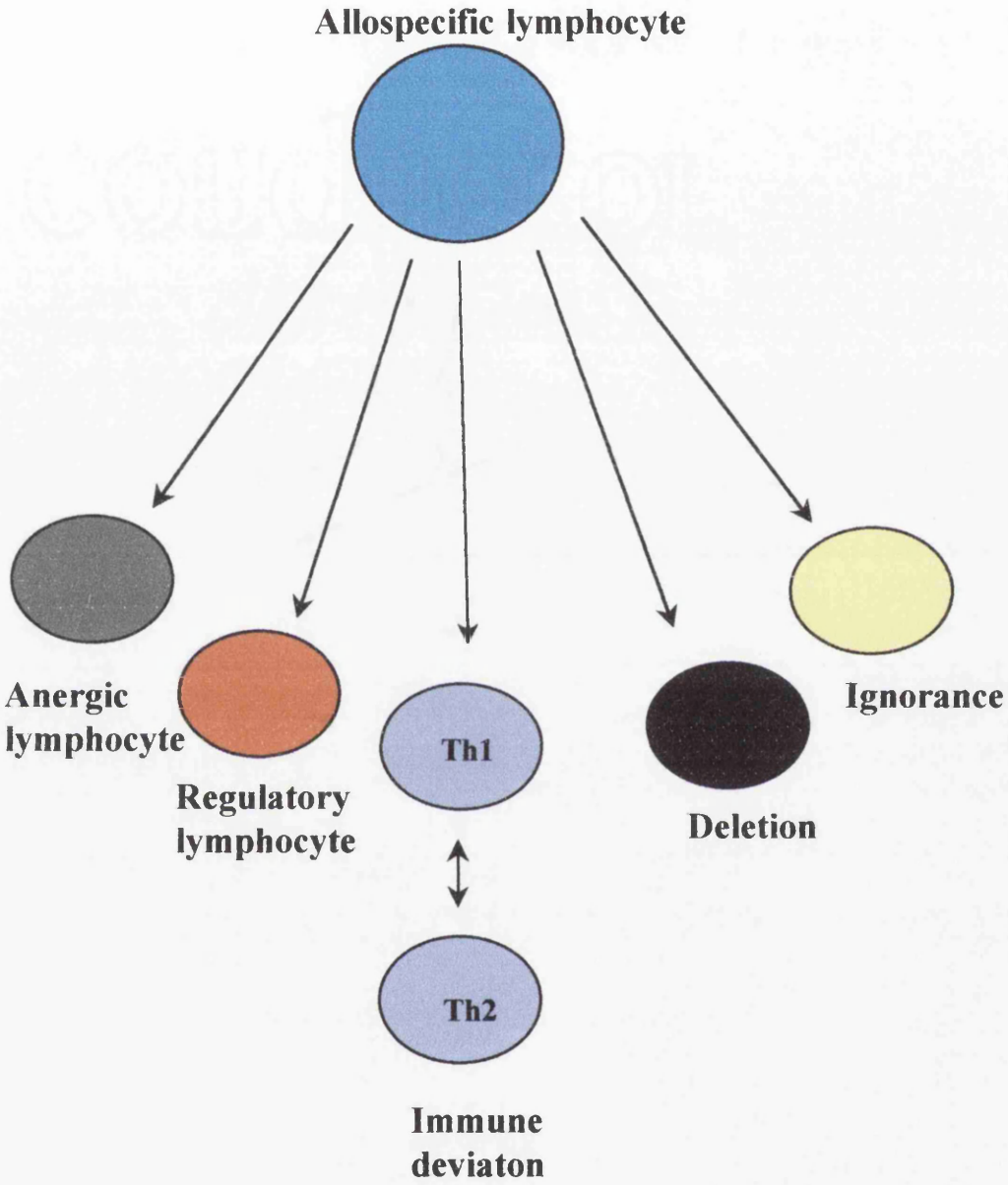


Figure 1. 6 Mechanisms of transplant tolerance induction

been found to be CD4 and CD25 +ve and a regulatory population of γ/δ CD8 T cells have been detected (Powrie et al, 1993, Harrison et al, 1996).

As previously mentioned, T helper cells can be divided into Th1 or Th2 depending on their cytokine production. It has been proposed that tolerance induction may correlate with a deviation from a Th1 to Th2 phenotype (Strom et al, 1996). An increase in IL-4 production has been seen in accepted heart grafts (Papp et al, 1992) and a reduction in IL-2 production has been noted in some models of tolerance (Dallman et al, 1991, Sayegh et al, 1995).

1.9 Monoclonal Antibodies in experimental transplantation

Monoclonal antibodies have proved useful reagents for various avenues of immunological research and their target specificity has allowed accurate definition of the cell types and molecular interactions involved in graft rejection. Monoclonal antibodies have also been used as therapeutic agents in both transplantation and autoimmune disease with variable results.

Cobbold et al described the use of monoclonal antibodies to deplete T cell subsets in thymectomised mice with resultant prolongation of skin graft survival(Nature 1984). Since then monoclonal antibodies have been raised to the major molecules involved in the rejection response in humans and rodents including CD4, CD8, CD45, MHC, CD3, CD28 TCR, LFA1, CD40, CD40L, ICAM1 and IL2 receptor(Table 1.2a-c).

Monoclonal antibodies can be used in two ways to achieve graft tolerance. They can either deplete defined cell types or they can interfere with the function or interactions of various receptors. In humans depleting anti CD3 mAb's have been used clinically for several years and depleting anti CD4, CD8 mAb's have been used in rodent models to achieve graft acceptance (Cobbold et al, 1996). Non depleting anti CD4 monoclonal antibodies have also been used successfully in rodent models to induce transplant tolerance possibly by sparing regulatory populations of cells or by inducing anergy while retaining the ability to provide host defenses to infection etc (Darby et al, 1992, Binder et al, 2000). Cytotoxic T lymphocyte A_4 immunoglobulin (CTLA4-Ig) in a similar way has been shown to block the interaction of B7 molecules with CD28 and CLTA4 on T cells resulting in prolonged allograft survival in both rat and mouse models (Turka et al, 1992, Sayegh et al, 1995, Blazar et al, 1999). In an attempt to remove donor APC, depleting anti CD45 monoclonal antibody has been infused into human kidney grafts prior to transplantation with some reduction in acute rejection episodes, however as with other monoclonal antibodies used in clinical transplantation the results have been disappointing.

Target molecule	Monoclonal antibody	Tolerogen	Model	Reference
CD3	FN18, B11	Skin	Mouse	Stevens et al, 1990
CD3	145-2C11	Islets	Mouse	Mackie et al, 1990
CD4	OKT4A	Kidney	Monkey	Wee et al, 1992
CD4	OKT4A	Skin	Monkey	Jonker et al, 1983
CD4	YTS-176.9.6	Heart	Mouse	Chen et al, 1992
CD4	YTS-191.1	Heart	Mouse	Pearson et al, 1993
CD4	OX38	Heart	Rat	Shizuru et al, 1990
CD4	OX35	Heart	Rat	Ilano et al, 1991
CD4	OX38	Heart/Kidney	Rat	Yin & Fatham, 1995
CD4	OX38	Islets	Rat	Seydel et al, 1991
CD4	OX35	Heart	Rat	Herbert&Rosser, 1988
CD4, CD8	OX38+OX8	Skin	Mouse	Cobbold et al, 1990
CD4, B7	OX38, CTLA4Ig	Small bowel	Rat	Yin et al, 1996
CD4	KT6, YTS199	Heart	Mouse	Darby et al, 1992&94

Table 1.2(a). Monoclonal antibodies used in animal models of transplantation

Target molecule	Monoclonal antibody	Tolerogen	Model	Reference
TCR	R73	Heart	Rat	Heidecke et al, 1993, 95
TCR	R73	Heart	Rat	Tsuchida et al, 1994
TCR	R73	Kidney	Rat	Heidecke et al, 1996
TCR	R73	Heart	Rat	Dufter et al, 1994
TCR	R73	Heart	Rat	Knight et al, 1994
TCR	R73	Heart	Rat	Van den Bogaerde et al, 1990
TCR	H57-597	Bone Marrow	Mouse	Drobyski et al, 1996
CD28	CTLA4Ig	Kidney	Rat	Sayegh et al, 1995
CD28	JJ319	Heart	Rat	Dengler et al, 1999
CD28, CD40	CTLA4Ig, anti-gp39	Skin/ heart	Mouse	Larsen et al, 1996
ICAM1, LFA1	Anti-ICAM1, LFA1	Heart	Mouse	Isobe et al, 1992

Table 1.2(b). Monoclonal antibodies used in animal models of transplantation

Target molecule	Monoclonal antibody	Tolerogen	Model	Reference
ICAM1, LFA1	Anti-ICAM1, LFA1	Islets	Mouse	Arai et al, 1999
IL-2R	NDS-61, OX39	Kidney	Rat	Tellides et al, 1989
IL-2R	ART-18, OX39	Kidney	Rat	Udea et al, 1990
MHC-II	HAK-75	Kidney	Dog	Yamamoto et al, 1984
MHC-II	HAK-75	Heart	Rat	Sone et al, 1987
MHC-II	BMAC4	Kidney	Rat	Priestley et al, 1992
TNFα,β	Anti-TNFα,β	Liver	Rat	Teramato et al, 1991
TNFα	Anti-TNFα	Heart	Rat	Imagawa et al, 1990
CD45RB	MB23G2, C363.16A	Islets	Mouse	Auersvald et al, 1997
CD25, CD54	NDS-61, 1A29	Liver	Rat	Gassel et al, 2000
CD2	RM2-2	Islets	Mouse	Kapur et al, 1996
IL-10	SXC.1	Heart/Liver	Mouse	Li et al, 1998
CD40L	Anti-CD40L	Skin	Mouse	Markees et al, 1997
CD2, CD28	Anti-CD2, CTLA4Ig	Hearts	Mice	Woodward et al, 1996

Table 1.2(c). Monoclonal antibodies used in animal models of transplantation

1.10 R73 monoclonal antibody

In this thesis, the experiments centre on the use of an IgG_1 mouse monoclonal antibody to the rat α/β T cell receptor. This mAb was developed and its properties described by Thomas Hunig in 1989. They found that R73 detects a pan T cell antigen expressed on 46% of splenocytes, 63% of lymph node cells and 3% of bone marrow cells. Using two colour flow cytometry, they showed that nearly all peripheral CD4+ve and 95% of CD8+ve lymphocytes react with R73. They also confirmed that it did not react with NK cells. Similar results were found in rat splenocytes. Soluble R73 was found to inhibit rat T cell activation in vitro but when the antibody is crosslinked in wells precoated with RaMIg antibodies there is marked stimulation with blast formation and IL2-R upregulation. R73 mAb was then used by the same group to characterise rat T cell subpopulations expressing α/β and γ/δ receptors (Lawetsky et al, 1990). They confirmed that peripheral T cells in the rat, as in humans and mice, can be divided into α/β TCR+ve, CD5+ve and γ/δ TCR+ve, CD5+ve and that these are phenotypically distinct from NK cells. Subsequently, Kinebuchi and colleagues have found that the determinant of this antibody exists on the constant domain of the rat TCR β chain (Kinebuchi et al, 1995). Initial investigators using R73 mAb in rats looked at its effect on experimental models of arthritis. They found that R73 suppressed established adjuvant arthritis and that this was associated with marked depletion of α/β T cells in the periphery and in the synovium of treated animals(Yoshino et al, 1992). Marked CD5 +ve

T cell depletion was also observed in these experiments suggesting that TCR modulation was not responsible for the effect on the disease process. Antibody production was not suppressed in this model.

The in vivo use of R73 monoclonal antibody was first described in a model of hamster to rat xenotransplantation. R73 was found to prolong graft survival in this model but no long term graft survival was achieved suggesting that concordant xenograft rejection is dependant on mechanisms other than T cell mediated graft destruction (Van Den Bogaerde et al, 1990).

Heidecke and colleagues observed indefinite graft survival in 20% of Lewis rats receiving a BN heart graft when a preoperative dose of 0.1mg/kg R73 was administered (Heidecke et al, 1993). This was associated with T cell depletion in the grafts and some TCR modulation. Only slightly prolonged graft survival was obtained when the monoclonal antibody was administered post transplant and in combination with sub therapeutic doses of CyA. They concluded from this study that treatment with R73 prior to transplantation results in incomplete T cell activation and anergy. Long term heart graft acceptance has been reported in the DA to Lewis strain combination when recipient animals were treated with R73 from birth to 70 days post transplant (Dufter et al, 1994). This was associated with almost complete T cell depletion during the period of mAb administration with partial recover after cessation of treatment.

A synergistic action of R73 with CyA was reported by Knight et al in 1994. In this study, a single dose of R73 administered along with CyA post transplant prolonged cardiac allograft survival in the Buffalo to Wistar Furth strain combination though indefinite graft survival was not obtained. Marked peripheral T cell depletion was again found with antibody detectable on circulating cells until day 7 after transplantation. They proposed that R73 may act by either altering T cell maturation either peripherally or in the thymus, by altering the function of mature peripheral lymphocytes or by TCR stimulation in the presence of a second signal inducing anergy. The synergism with CyA was thought to be the result of a reduction in the number of antigenic signals transduced via the TCR combined with CyA's inhibition of the calcium-dependant activation pathway downstream.

Tsuchida et al published the first report of long term cardiac allograft unresponsiveness induced by a short pre transplant course of R73 in Transplantation in 1994. 50μg of R73 mAb was administered to Lewis rats days -2 and -1 relative to receiving a fully allogeneic BN cardiac allograft. All the grafts survived indefinitely and in addition second donor specific, but not third party, grafts were accepted. In vitro experiments showed transient CD4 depletion and also TCR modulation suggesting that the mechanism of action in this model could be interference with antigen presentation by R73.

R73 has also been found to abrogate accelerated rejection in rat cardiac allograft recipients (Heidecke et al, 1995) and this was associated with marked modulation of the α/βTCR and reduced production of Th1 cytokines IL-2 and IFNγ and sparing of IL-4 as detected by immunocytochemistry.

These studies have suggested a potential role for R73 anti α/β TCR monoclonal antibody in the induction of long term graft unresponsiveness in rodent models of cardiac allografting. It appears from the results that the antibody must be administered pre transplant in order to obtain prolonged graft survival and that it may act synergistically with Cyclosporine A. The underlying mechanism of action of the antibody, however, remains unclear and mechanisms such as anergy, depletion of alloreactive T cells and polarisation towards a Th2 type response have been suggested.

1.11 Aims and experimental design

It is hoped that the use of monoclonal antibodies to specific target molecules involved in the immune response to an organ allograft will allow tolerance to an organ allograft without the need for longterm immunosuppression and the associated side effects. The α/β T cell receptor plays a central role in the molecular interactions in graft rejection and is therefore an ideal target for monoclonal antibody intervention. The main aim of this thesis was to study the ability of anti-α/βTCR therapy to promote allograft survival in a rat model of heterotopic cardiac transplantation.

The mouse anti-rat anti-α/βTCR monoclonal antibody R73 was used in an attempt to induce longterm heart graft survival in a variety of rat strain combinations. Once the conditions for promotion of prolonged graft survival were established, experiments were carried to determine some of the mechanisms by which R73 mAb can induce indefinite graft survival. Initial experiments were carried out using flow cytometric analysis to quantify the effect of R73 on peripheral CD4 and CD8 T cells and determine whether cell depletion or receptor modulation play important roles in the induction of prolonged graft survival. Further studies were carried out using cell culture and RT-PCR to determine whether immune deviation of helper T cell subsets is a potential effect of R73 therapy. Finally, the potential role of apoptosis in this model was investigated by flow cytometric analysis of lymph node cells from R73 treated animals and by TUNEL staining of explanted heart grafts.

It is hoped that the results from this thesis will provide a clearer understanding of the effects of monoclonal antibody therapy directed against the TCR and may give a clearer insight into how these affects can be obtained in human transplantation.

CHAPTER TWO

Materials and Methods

2:1 Animals

2:1:1 Rats

Inbred adult male DA ($RT1^a$), Lewis ($RT1^l$), PVG ($RT1^c$) and Brown Norway ($RT1^n$) rats were purchased from Harlan UK Ltd (Bicester). and kept under humane conditions in compliance with Home Office regulations in the animal facility at the University Department of Surgery, Western Infirmary, Glasgow and the Central Research Facility in the University of Glasgow. All animals were maintained on standard rat diet and water and used when 8- 16 weeks old.

2.1.2 Mice

Adult male Balb/c mice were purchased from Harlan UK Ltd (Bicester) and used for antibody production by intraperitoneal injection of hybrid myeloma cells. They were housed in the University Department of Surgery animal facility as above and maintained on standard diet and water.

2:2 Surgical Procedures

2:2:1 Donor cardiectomy

Animals were placed in a sealed box and anaesthetised with halothane vapourised in oxygen. Animals were then shaved and maintained under anaesthesia using halothane administered via a nose cone. Cardiac

transplantation was then carried out according to the technique described by Ono and Lindsey, 1969.

The peritoneal cavity of donor animals was entered using a long midline abdominal incision and the intestines reflected out of the abdomen and covered with a swab moistened in 0.9% saline. The inferior vena cava was then identified and the overlying peritoneum incised. 250 units of mucus heparin were then injected into the IVC. After a few minutes the animal was bled out by cutting the inferior vena cava and abdominal aorta. The chest was then rapidly opened by cutting the ribs along the mid axillary lines and reflecting the anterior chest wall cranially. The heart was then stopped and rapidly cooled by covering it with crushed ice or by administration of St Thomas' cardioplegia solution via the aortic arch. Once the heart had stopped beating the aortic arch and pulmonary trunk were dissected out with the aid of an operating microscope (Wild Heerburg, Switzerland). The inferior vena cava and right superior vena cava were then dissected out, tied with a 5/0 silk tie and divided. A single 5/0 silk ligature was subsequently passed around the junction of the left superior vena cava and the azygous vein and both vessels divided. The ascending aorta was divided 2/3 of the way to the brachiocephalic trunk and the pulmonary trunk divided just proximal to its bifurcation. A single ligature was tied around the remaining vessels at the back of the heart and the excised and placed in a petri dish containing cold saline and kept in ice until transplantation. Cold ischaemic time did not exceed 30 minutes.

2:2:2 Abdominal heart transplantation

The peritoneal cavity of recipient animals was entered through along midline abdominal incision and the intestines reflected out of the abdominal cavity and covered with a swab soaked in saline. The infra renal abdominal aorta and IVC were then dissected out and a suitable length chosen for anastomosis. Tributaries of the vein and lumbar vessels were then tied off and the vessels cross clamped proximally and distally. Occlusion clamps were then applied to the aorta and IVC of the recipient and the donor heart implanted by anastomosing the pulmonary artery then the ascending aorta end to side to the recipient IVC and abdominal aorta with a continuous 9/0 nylon suture(Ethicon Ltd, UK) under 16x magnification. The vascular clamps were then removed to re-vascularise the heart and the heart began to beat almost immediately. Any bleeding from the anastomoses was controlled with light pressure. Once the heart was beating in sinus rhythm the intestines were replaced in the abdomen and the wound closed using a continuous 3/0 catgut suture (Ethicon Ltd, UK). The animals were then allowed to recover in individual cages under a heat lamp.

2:2:2 Thymectomy

The thymus gland was removed from adult rats under halothane anaesthetia via a 2cm longitudinal incision in the neck and upper sternum and facilitated by a

short sternal split (Barbul et al, 1979). The strap muscles were split and the thymus mobilised with the aid of an operating microscope and removed with minimal bleeding. Due to the proximity of the thymus gland to the pleura of the lung, pneumothorax is a risk of thymectomy. This was avoided by the rapid closure of the neck wound after removal of the gland as the operator applied positive pressure ventilation by blowing through a syringe applied to the nose of the rat (Walker et al, 1994). This was carried out at least 2 months prior to heterotopic cardiac transplantation and subsequent analysis of peripheral blood lymphocytes by flow cytometry confirmed the depletion of Thy 1 positive lymphocytes (recent thymic emigrants).

2:3 Preparation of Cells

2:3:1 Lymph node cells (LNC)

Cervical and mesenteric lymph nodes were harvested from experimental adult male rats which were killed by cervical dislocation. Lymph nodes were stored in phosphate buffered saline containing 0.2% bovine serum albumin (PBS/BSA) and transferred to the laboratory in ice. The nodes were then cut with a scalpel and pressed through a stainless steel mesh to disrupt the node and allow removal of cells. Lymph node cells were then washed twice in PBS/BSA and resuspended in PBS/BSA and stored at 4°C until they were used

in experimental procedures. Viability was consistently greater than 95% as determined by trypan blue exclusion.

2:3:2 Peripheral blood lymphocytes (PBL)

Adult male rats were bled out by direct cardiac puncture and the whole blood thoroughly mixed with 10u/ml heparin. The heparinised blood was then mixed in equal proportions with PBS and layered on percoll (9ml percoll (Sigma Ltd)+1.3ml 10x HBSS+3ml water+130µl Hepes) and centrifuged at 400xg (2400 rpm) for 20 minutes at room temperature. Peripheral blood lymphocytes were then removed from the interface using a pipette and washed twice in PBS at 200xg for 5minutes after lysing the red blood cells (1ml water for 3 seconds followed by 1ml 2xNaCl). Cells were then resuspended in PBS/BSA and stored at 4°C until used in an experimental procedure.

2:3:3 Spleen cells

Spleens were removed from sacrificed rats and the capsule disrupted using forceps. Dead cells and debris were removed and the cells washed in PBS/BSA. Red cells were removed by hypotonic lysis and the splenocytes washed three times in PBS/BSA.

2.4 Antibodies

A variety of antibodies were used in vitro to detect rat leukocyte populations: MRC OX8 (CD8+ cytotoxic/suppressor T cells and the majority of NK cells(Gillman et al, 1982), W3/25 (CD4+ helper T cells and some macrophages (Brideau et al, 1980), ED1 (macrophages, monocytes and dendritic cells), OX19 (pan T cell marker), OX39 (IL-2Rα). All antibodies were obtained from Serotec, Oxford, UK.

2.4.1 R73 anti α/βTCR mAb production

R73 hybridoma cells (European Collection of Animal Cell Cultures, Porton Down, Wiltshire, UK) were cultured and resuspended in PBS/BSA at $2x10^5$/ml and 0.5ml injected into the peritoneum of pristane (Sigma Chemical Company Ltd, Poole, UK) primed Balb/c mice. When an adequate volume of ascitic fluid had developed the mice were killed by cervical dislocation and the ascitic collected. The ascites was then centrifuged at 400xg (2400rpm) at 4°C for 20 minutes and the supernatant removed and stored at -20°C. Antibody was then purified by Protein A affinity chromatography (Prosep-A, Bio-processing Ltd, England, UK). Antibodies were purified by adding an equal volume of diluting buffer (Glycine 75g/l, NaCl 17.6g/l, pH 8.6) for 12 h at 4°C, then centrifuging at 3000 rpm for 30 minutes at 4°C. A protein-A-sepharose column was prepared and equilibrated with binding buffer (Glycine 75 g/l, NaCl 8.8g/l, pH 7.4), then loaded with diluted ascites. The IgG was eluted with 0.1M sodium

citrate, pH 3.0, at a low rate of 2.7 ml/min into 4ml fractions and the Ig-containing fractions pooled and immediately dialysed with several changes of PBS to neutralise the pH. Purified antibodies were quantified by OD_{280}, and radial immunodiffusion (RID) using rat isotype specific RID plates and standards of known concentration (The Binding Site, Birmingham, UK).

2.4.2 rIL12 cytokine

Recombinant mouse IL-12 was a kind gift of Dr M Gately, Hoffman La Roche, Nutley, NJ.

2.4.3 In vivo administration of mAb

R73 mAb was administered to allograft recipient animals intraperitoneally at predefined doses and time points relative to transplantation in an attempt to prolong heart graft survival. Binding and activity was confirmed by flow cytometric analysis of peripheral blood lymphocytes lymph node cells from treated animals.

2.4.4 Monoclonal antibody conjugates used in flow cytometry studies

T cell populations in LNC and PBL prepared from experimental animals were identified by flow cytometric analysis. Detection was either by direct binding of fluorescein isothiocyanate (FITC) or phycoerytherin (PE) conjugated monoclonal antibodies to the relevant molecules or by detection of unconjugated mouse anti rat mAb by FITC conjugated rabbit F(ab')2 anti

mouse Ig (Dako Ltd, High Wycombe, UK)(RAM-FITC). Table 2.1 shows the antibody labeling protocol used in the flow cytometry studies.

Cell type	Labeling antibodies
CD5	OX19 + RAM-FITC
CD4	R73-FITC + W3/25-PE
CD8	R73-FITC + OX8-PE
R73 mAb +ve	RAM-FITC
R73 mAb saturation	R73 + RAM-FITC

Table 2.1 Monoclonal antibodies used in flow cytometric analyses

2.4.5 Monoclonal antibodies used in immunohistological staining of cryostat sections

Cellular infiltration of cardiac allografts was analysed by immunocytochemistry. CD4 and CD8 T cell infiltration was detected by W3/25 and MRC OX8 mAbs respectively. α/βTCR +ve cells were labeled with R73 mAb and CD5 +ve T cells detected with MRC OX1 mAb. IL-2 receptor expression was detected by MRC OX39 mAb and macrophages labeled with ED1 mAb. Antibodies were purchased from Serotec Ltd, Oxford, UK.

2.5 Flow cytometry

Depletion of CD5, CD4 and CD8 T cell populations in lymph nodes and peripheral blood of experimental animals was assessed by double labeling lymph node cells and peripheral blood lymphocytes with the relevant FITC and PE conjugates and analysed using a Coulter Epics XL flow cytometer (Coulter Ltd, Luton, UK). In addition R73 binding to T cells was analysed.

LNC and PBL were prepared as previously described. LNC were washed in PBS and resuspended in PBS/BSA at $1x10^6$ cells per tube for analysis. PBL were washed twice in PBS by centrifuging at 200xg (1200rpm) for 5 minutes and resuspended in 1ml PBS/BSA and 100µl per tube used for analysis.

Directly conjugated antibodies were added together and incubated at 4°C for 45mins-1 hour. OX19 and R73 were added as first antibodies and incubated at 4°C for 1 hour and then cells were washed twice in PBS/BSA at 200xg for 5 minutes at 4°C. RAM-FITC was diluted 1:20 in normal rat serum and added to the washed cells and incubated at 4°C for 45minutes -1hour. Cells were then washed in 3ml PBS/0.2%BSA twice at 200xg for 5 minutes at 4°C and resuspended in 0.5ml PBS for analysis.

Cell samples were then analysed using a Coulter Epics XL flow cytometer, live gates having previously been defined by forward and side scatter profiles using LNC and PBL from normal rats. 5-10000 events were counted per sample.

2.6 Immunocytochemistry

Heart grafts were excised from recipient animals at various time points, snap frozen in liquid nitrogen and stored at -70°C. 5μ cryostat sections were then cut on to triple well glass slides and fixed in ethanol and immunoperoxidase staining carried out as follows. 5μl of diluted antibody (R73, W3/25, OX8 and OX1 diluted 1:3 with PBS and ED1 and OX39 diluted 1:10 with PBS) was added (section 2.4.5) and incubated at room temperature for 45 minutes. Slides were washed three times in PBS and incubated with horseradish peroxidase conjugated rabbit anti mouse mAb (diluted 1:20 in PBS and normal rat serum) and developed using a DAB nickel substrate kit (Vector Ltd, UK). Slides were counterstained with haematoxylin, dehydrated in alcohol and a cover slip mounted and fixed with DPX. Cellular infiltrates were counted in stained sections over 10 high power fields (x40 magnification) using a cross line graticule on a Leitz Laborlux microscope.

2:7 Mixed lymphocyte culture

Unseparated lymphocytes at $2x10^5$ were prepared from mesenteric and cervical lymph nodes of experimental animals as previously described. Splenocytes from

naïve Lewis and PVG third party rats were prepared as donor specific and third party stimulators by passing spleen cells through a fine steel mesh in tissue culture medium (RPMI/FCS; RPMI 1640 medium supplemented with 2mM L-glutamine, 100U/ml penicillin, 100μg.ml streptomycin (Life Technologies), 50μM 2-mercaptoethanol (Sigma Ltd) and 5% heat inactivated FCS). Stimulator cells were washed twice and irradiated (20Gy) prior to use. Proliferation assays were set up in triplicate in 96 U-well plates (Life Technologies) using $2x10^5$ responders per well and $4x10^5$ stimulators per well. Plates were then incubated at 37°C in 5% CO_2. After 3, 4 and 5 days culture, cells were pulsed with 1μCi per well of [^{3}H]- thymidine (Amersham International PLC, Little Chalfont, Bucks, UK) for 18 hours and harvested. Thymidine incorporation by proliferating cells was carried out using an automated cell harvester and liquid scintillation counter (1205 Betaplate, LKB Pharmacia, Milton Keynes, UK).

2:8 Cytokine bioassay for IL-2

The CTLL-2 lymphocyte line was maintained by 3x weekly treatment with human recombinant IL-2 (100 Unit/ml) and subculturing in RPMI 1640 supplemented with 5% FCS, L-glutamine, penicillin/streptomycin and 2 mercaptoethanol. Serial dilutions of supernatants from day 3 mixed lymphocyte

culture of lymph node cells from R73 treated graft recipients and naïve Lewis rats were mixed with $5x10^3$ CTLL-2 cells. Twenty four hours later, the cells were pulsed with 1 μCi/well titrated thymidine (Amersham International), incubated for 18 hours, harvested onto glass filter mats and counted in a liquid scintillation counter (1205 Betaplate, LKB Pharmacia, Milton Keynes, UK). Cytokine levels (in Units/ml) were calculated from a standard curve with rIL-2.

2:9 IFNγ capture ELISA

96 well microtiter plates (Dynatech Immulon 4) were coated with 100μl/ well of anti interferon gamma antibody (rabbit polyclonal anti-mouse IFNγ, kind gift of Dr John Tite, Wellcome research laboratories, Beckenham, UK) at a dilution of 1:2000. After overnight incubation at 4°C, plates were washed three times in ELISA wash buffer (PBS containing 0.05% Tween 20) and blocked with 200μl/well of PBS containing 10% FCS for 1 hour at 37°C. Plates were then washed three times in wash buffer and 100μl/well of standard, control or sample (neat supernatant) added in triplicate and incubated at 37°C for three hours. After four further washes, the plates were incubated for 1 hour at room temperature with biotinylated ant-rat IFNγ (DB1, kind gift of Dr P Vander Meide, Rijkswik, Netherland). Plates were then washed six times and 100μl/ well of extravidin-peroxidase (Sigma Ltd) added and incubated for 1 hour at 37°C. Plates were then washed a further eight times and 100μl/well of tetramethyl benzidine (Dynatech Laboratories, Billinghurst, UK) added for 30

minutes at room temperature and plates then read at 450nm using an ELISA plate reader (Dynatech MR 5000) and Biolinx software (Dynatech Laboratories).

2:10 Semiquantitative PCR to analyse cytokine mRNA in heart grafts

One hundred milligrams of frozen tissue from explanted heart grafts (snap frozen in liquid nitrogen) were homogenized in 1ml of TRIzol (Gibco BRL, Paisley, UK) and RNA prepared according to the manufacturers instructions. The purity and quantity of mRNA was determined by OD_{260} and $OD_{260/280}$ readings. For first strand cDNA synthesis, 10μg of total RNA were primed with 1μg of oligo dT (Gibco BRL, Paisley, UK) and then mRNA was reverse transcribed in a 42μl reaction volume with 400U of Superscript RNase H reverse transcriptase (Gibco BRL) according to manufacturers instructions. For PCR, 1μl of cDNA was amplified in a 25μl reaction volume containing 0.1μl of Taq DNA polymerase, 0.4μl of 10mM dNTP mix, 2.5μl of 10x reaction buffer (Boehringer Mannheim), 2.5μl of sense and antisense primers (0.2μm final concentration except β actin which was 0.1μM), and sterile distilled water. A higher Mg Cl_2 concentration was used when amplifying IL-4 message (2.5mM). Table 2.2 shows the oligonucleotide sequences used. PCR was performed using a Perkin Elmer Geneamp PCR system 9600 with an initial cycle of 94°C for 5 minutes, followed by multiple cycles of 94°C for 30 secs,

45-65°C for 30 secs and 72°C for 30 secs with a final extension time of 5 minutes. During the exponential phase of amplification, 10 to15µl aliquots of PCR product were sampled at 3 or 5 cycle intervals and analysed by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. Positive controls included RNA from Con-A stimulated rat lymphoblasts and in the case of IL-5, a rat IL-5 transfected cell line. As negative controls, primer and cDNA were omitted independently from the reaction mixture. All PCR products were of predicted size on electrophoresis and their identity verified by Southern blot analysis using digoxygenin-dUTP labeled (Boehringer Mannheim) specific internal oligonucleotide probes selected from published gene sequences. The integrity of RNA preparation and of the reverse transcription reaction was confirmed for each tissue by including a PCR reaction for β actin using three concentrations of cDNA (neat, 1/10 and 1.100), each amplified for 15, 20 and 25 cycles.

Table 2.2 Oligonucleotide sequences used in RT PCR detection of cytokine mRNA in explanted heart grafts

Cytokine	Sense	Antisense	Internal	Reference
IL-2	CAT GTA CAG CAT GCA GCT CGC ATC C	CCA CCA CAG TTG CTG GCT CAT CAT C	TTA CAG GTG CTC CTG AGA GG	McKnight et al, 1989
IL-4	TGA TGG GTC TCA GCC CCC ACC TTG C	CTT TCA GTG TTG TGA GCG TGG ACT C	GAC TCC ATG CAC CGA GAT GT	McKnight et al, 1991
IL-5	TTC TAA CTC TCA GCT GTG TCT GGG C	AAT GCC CAC TCT GTA CTC ATC ACG C	GCA ATG AGA CGA TGA GGC TT	Uberla et al, 1991
IL-10	GTG AAG ACT TTC TTT CAA A	TGA TGA AGA TGT CAA ACT C	CTG AGG CGC TGT CAT CGA TT	Feng et al, 1993
IL-13	CAG GGA GCT TAT CGA GGA GC	AAG TTG CTT GGA GTA ATT GAG C	TCC CTG TGC AAC AGC AGC AT	Lakkis et al, 1993
IFNγ	ATG AGT GCT ACA CGC CGC GTC TTG G	GAG TTC ATT GAC AGC TTT GTG CTG G	AGC ATG GAT GCT ATG GAA GG	Dijkema et al, 1985
IL-2Rα	GTG GGG AGA TAA GGT GGA CGC AT	GAT CGA AAG GAG ACA GGC ACC C	TAT CAG GTA GCA GTG GCC AG	Page et al, 1991
IL-2Rβ	TAC TGG TCC TCG GCT GCT TCT TTG	GTG AAA GGC AGC AGA GGT GGG A	GAC CAT GCA GAT GCT CCT GT	Page et al, 1991
β-actin	ATG CCA TCC TGC GTC TGG ACC TGG C	AGC ATT TGC GGT GCA TGG AGG A	AGC AAG AGA GGT ATC CT	Nudel et al, 1983

2:11 Flow cytometric analysis of apoptotic cells

Lymph node cells were harvested from experimental animals and prepared as described earlier. The technique of propidium iodide (PI) staining of DNA to assess apoptosis has been described before (Nicoletti et al, 1991) and has been extensively used to quantify apoptosis in a heterogeneous cell population.

10^6 LNC were suspended in 100µl PBS for analysis. PI staining solutions were prepared as follows. A stock solution of 2g sodium citrate, 121mg Tris, 1044mg spermine tetrahydrochloride and 2ml Nonidet p40 was prepared in 2000ml deionised water and pH adjusted to 7.6. Trypsin was dissolved in stock solution and 450µl of this solution added to each tube containing LNC for 10 minutes at room temperature. 325µl of a solution of trypsin inhibitor and ribonuclease A was then added for a further 10 minutes at room temperature and finally 250µl of PI in stock solution was added to the samples for 10 minutes in the absence of light.

Stained cells were then immediately analysed for PI fluorescence levels on the flow cytometer (20000 events per analysis) and apoptotic cells expressed as the percentage of cells in the sub diploid region of the cell cycle profile.

2:12 In situ detection of apoptotic cells using TUNEL

Apoptosis was detected in situ using the Oncor ApoTag Peroxidase Plus In Situ apoptosis detection kit (S7101-Kit, Oncor Inc. Gaihtersburg, Maryland, USA). This technique is based on the terminal deoxynucleotidyl transferase mediated dUTP End Nick Labelling system whereby residues of digoxigenin-nucleotide are catalytically added to the 3' ends of the DNA fragments characteristic of cells undergoing apoptosis by the enzyme terminal deoxynucleotidyl transferase. This is then detected using a peroxidase detection system.

Cryostat sections of heart grafts were prepared as described above and fixed in 10% buffered formalin. The sections were then post fixed in a 2:1 solution of ethanol and acetic acid and washed in PBS twice for 5 minutes and endogenous peroxidase quenched using 3% hydrogen peroxide for 5 minutes at room temperature. An equilibration buffer was then added followed by TdT solution which was then incubated for 1 hour at 37°C. The stop/wash buffer was then added followed by the anti digoxigenin peroxidase antibody for 30 minutes. The diaminobenzidine (DAB) substrate solution was then added and the slides counterstained with methyl green. The slides were then dried in xylene and coverslips mounted using DPX.

Stained cells were counted at high power magnification (x40) over 10 high power fields per section or the whole section and results expressed as the mean

and SD of at least 3 animals. Normal rat hearts were used as negative controls along with sections where distilled water was substituted for TdT solution and slides provided commercially with the detection kit used as positive controls.

2:13 Statistical analyses

Parametric data were analysed using the Student's t test and non parametric data with the Mann Whitney U test where appropriate

CHAPTER THREE

Prolongation of cardiac allograft survival in rats treated with R73 anti alpha/beta T cell receptor monoclonal antibody

3.1 Introduction

The progress of organ transplantation is presently limited by the side effects of current immunosuppressive protocols. Much of the current research in transplantation is being directed towards the development of less toxic compounds which will reduce the need for immunosuppressive drugs such as corticosteroids, azathioprine, cyclosporin A and Tacrolimus. Ultimately, the ideal strategy is one which will result in long term graft acceptance without the need for continuous immunosuppression. One potential strategy is to target specific molecules involved in the immune response to an organ allograft using monoclonal antibodies. Monoclonal antibodies have been used in humans and experimentally in animal models against molecules present on donor APC such as CD45, LFA and B7 and molecules expressed by the T lymphocytes of the recipient CD3,CD28, CD2, CD4, CD8, CD40 ligand and IL-2R. However, the critical interaction between donor MHC-peptide and the T-cell receptor on recipient lymphocytes make these molecules ideal targets for monoclonal antibody therapy.

The direct interaction between intact MHC peptide complexes on donor APC and recipient T cells with the resultant cytokine production and clonal expansion of alloreactive T cells is seen as the principal step in the initiation of graft rejection. However, the possibility also exists that donor MHC and other polymorphic proteins in the graft can be processed and presented by recipient

APC as foreign peptide bound to recipient MHC class II to recipient CD4+ T cells via the "indirect" pathway. There has been much evidence recently to support this theory in experimental models of both allo- and xeno-transplantation and it is suggested that only a few antigenic determinants rather than the whole foreign protein are responsible for the T cell response (Dorling et al, 1996). The relative importance of the indirect pathway in the rejection of an allograft, however remains unclear.

I chose to investigate the ability of the monoclonal antibody R73 against the alpha/beta T cell receptor to prolong heterotopic cardiac allograft survival in a rat model. Heterotopic cardiac allografting in the rat is a well established model which is relevant to clinical organ transplantation since it involves the transplantation of a vascularised organ graft in a living biological system and while the transplanted heart does not contribute to the circulation of the animal, the cessation of a palpable heart beat in the abdomen is an easily detectable end point to indicate graft rejection. In addition, the immune system of the rat has been well defined and there are many reagents available for studies in this model. In this chapter, I investigated the ability of R73 monoclonal antibody to promote heart allograft survival in the rat model. R73 mAb is a mouse anti rat IgG_1 monoclonal antibody first described by Thomas Hunig in 1989. It is directed against the constant domain of the TCR β chain (Kinebuchi et al, 1995) and in soluble form this antibody inhibits T cell function in vivo and when immobilised in vitro, is a potent stimulator of T cell proliferation by

promoting cross linking of the α/β TCR (Hunig et al, 1989). R73 mAb was raised and affinity purified as described in chapter 2. R73 was administered intraperitoneally to recipient animals and hearts monitored daily by palpation. The end point was taken as the cessation of a palpable heart beat in the abdomen of the recipient animal.

3:2 Ability of R73 mAb to prolong survival of Brown Norway heart grafts in Lewis recipients.

For these initial experiments the fully allogeneic BN[Brown Norway](RT1^n) to Lewis (RT1^l)strain combination was chosen. These rat strain combinations were selected because they differ from each other at both major and minor histocompatibility antigens and BN hearts are always rejected by Lewis recipients. Initially BN hearts were transplanted into the heterotopic abdominal site of 5 Lewis animals given only an isotype matched, irrelevant control mAb, OX21 which labels human C3b inhibitor. All hearts were rejected acutely with complete cessation of palpable cardiac contraction between 5 and 8 days after transplantation (median 8 days). To determine whether R73 mAb could prolong survival of BN hearts in Lewis recipients, affinity purified R73 mAb was administered to Lewis recipients at doses of either 25μg, 50μg and 200μg IP on days -2 and -1 relative to transplantation. This treatment regime was chosen because previous reports have shown that a similar regime induces indefinite heart graft survival (Heidecke et al, 1993; Tsuchida et al, 1994) and

can abrogate accelerated kidney graft rejection in the same strain combination (Heidecke et al, 1995). As shown in figure 3.1, treatment of Lewis recipients with 50μg of R73 mAb resulted in significantly prolonged BN heart graft survival with a median survival time of 28 days (range 20-33 days, P= 0.02, Mann-Whitney test compared with control animals). Treatment with 200ug R73 mAb also resulted in significantly prolonged graft survival in this strain combination with a median graft survival of 21 days (range 17-32 days, P= 0.008, Mann-Whitney test in comparison with the control group), however a dose of 25ug was insufficient to significantly prolong graft survival (P=1, Mann- Whitney test).

3:3 Strain dependant variation in heart graft survival following R73 mAb treatment.

In contrast to the previous reports, these R73 treatment regimes failed to induce indefinite BN heart graft survival in Lewis recipients and so in further studies we investigated whether R73 mAb treatment could induce long term allograft survival in other fully allogeneic strain combinations, namely DA($RT1^a$) to PVG($RT1^c$) and Lewis($RT1^l$) to DA($RT1^a$) strain combinations. For these studies 200μg R73 were administered to recipient PVG and DA animals on days -2 and-1 prior to transplantation as before and hearts monitored by palpation. As shown in figure 3.2, 80% of Lewis hearts survived for greater than 100 days in DA recipients after this treatment regime (median

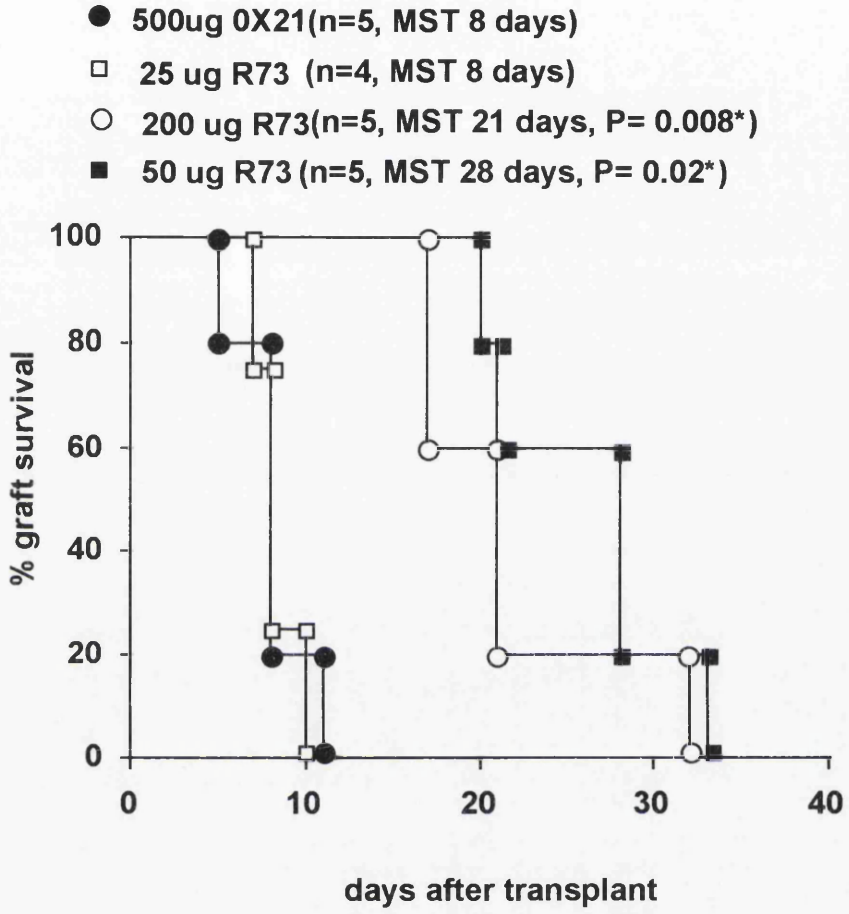


Figure 3.1 Ability of R73 mAb to prolong survival of BN heart grafts in Lewis recipients. R73 mAb was administered to Lewis recipients of a BN heart graft on days -2 and -1 relative to transplantation. Graft survival was significantly prolonged in the groups receiving 50ug and 200ug on each day, though indefinite graft survival was not seen. Animals given 25ug R73 and OX21 control mAb rejected their grafts promptly within 10 days. *Mann-Whitney test compared with control group.

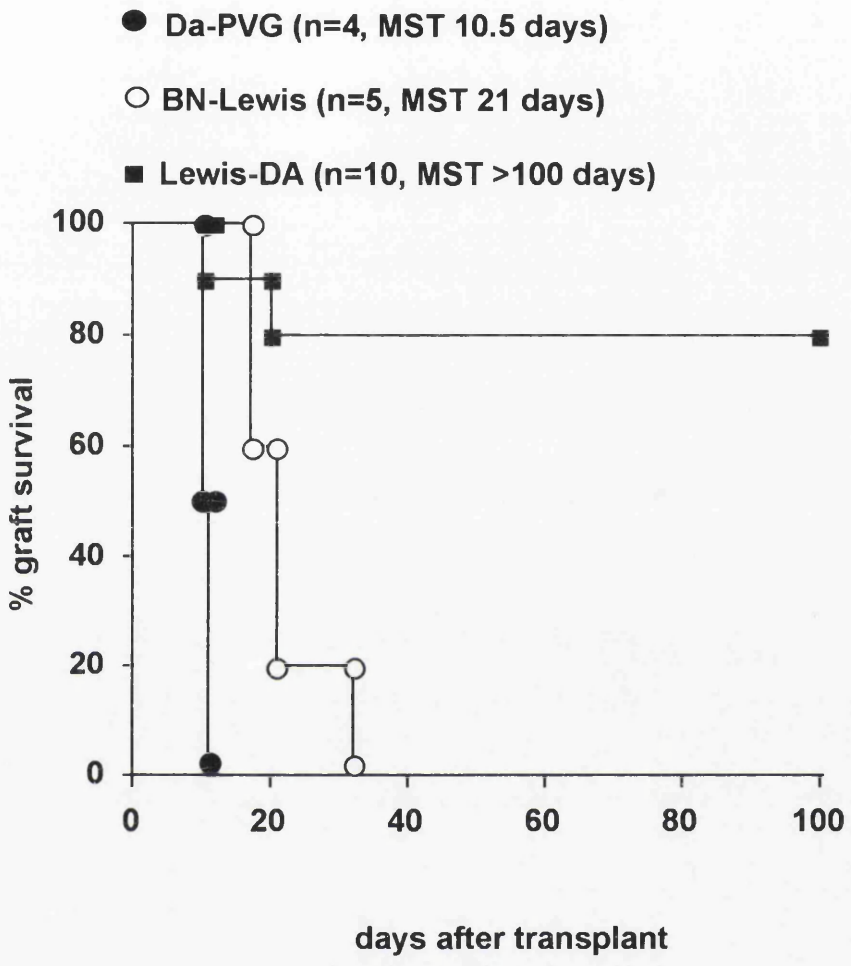


Fig 3.2 Strain dependant variation in graft survival after R73 mAb treatment. 200ug of R73 mAb was administered to PVG, Lewis and DA recipients of DA, BN and Lewis heart grafts respectively on days -2 and -1 prior to transplant. Prolonged graft survival was seen in the BN-Lewis strain combination but long term graft acceptance was only seen in the Lewis-DA low responder strain combination. Despite R73 treatment, PVG recipients promptly rejected DA donor hearts. *P=0.025, Mann-Whitney U test.

100 days, range 11 to >100, P= 0.02, Mann- Whitney test) whereas median survival in the DA-PVG strain combination was only 10.5 days (range 10-11 days). These experiments illustrate that the ability of R73 mAb to induce indefinite graft survival is highly strain dependant and although prolonged graft survival can be induced in the BN to Lewis combination, R73 mAb treatment, in our hands, only induces indefinite graft survival in DA recipients of Lewis hearts. In view of these results, the Lewis-DA strain combination was therefore used as the model in all further experiments.

3:4 Effect of different R73 doses on graft survival in the Lewis to DA strain combination

Having achieved indefinite graft survival in the fully allogeneic Lewis-DA strain combination using a 200μg dose of R73 mAb on days -2 and -1 prior to transplantation, we investigated whether altering the dose of R73 administered at these time points would influence heart allograft survival in this particular strain combination. DA recipient rats were given either 50μg, 200μg or 500μg R73 intraperitoneally on days -2 and -1 prior to receiving a Lewis heart graft and hearts again monitored by daily palpation. Figure 3.3 illustrates that 500μg doses of R73 also induce graft survival of >100 days in the majority of treated animals (P= 0.03, Mann-Whitney U test) and although a dose of 50μg induces prolonged heart allograft survival, only 25% of grafted hearts survived longer than 100 days. These experiments demonstrate that as little as 50μg of R73

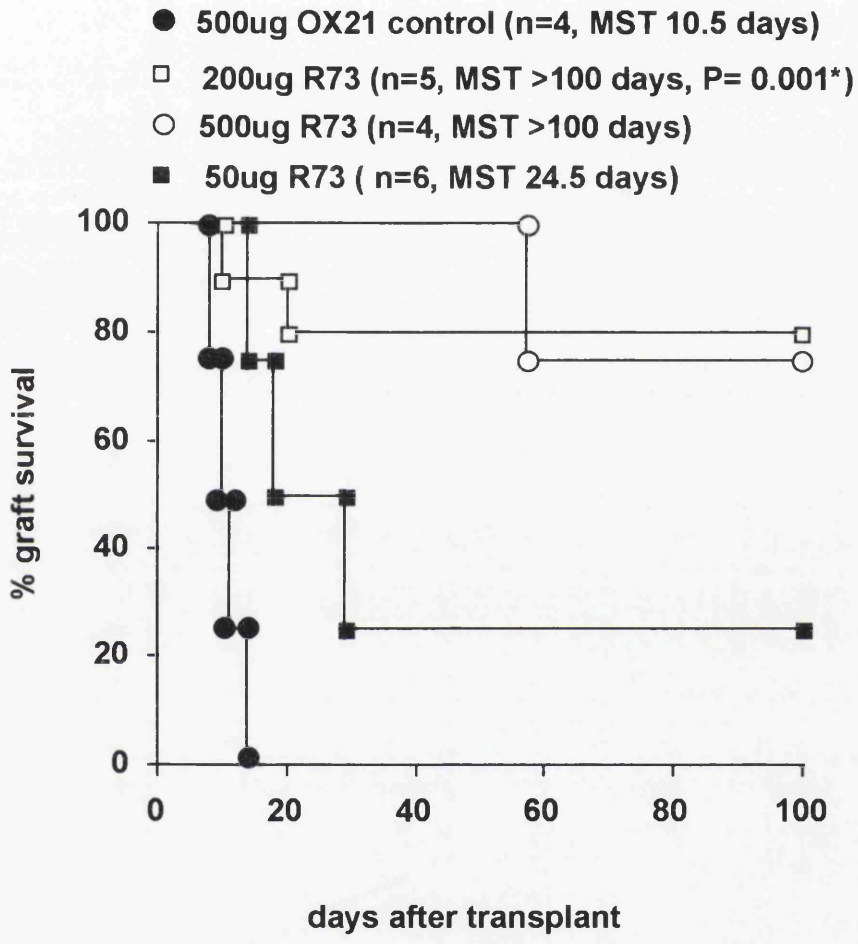


Fig 3.3 Effect of different R73 doses on graft survival in the Lewis to DA strain combination. DA recipients of Lewis heart allografts were treated with either 50ug, 200ug or 500ug of R73 mAb days -2 and -1 prior to transplant. Control animals were given an irrelevant antibody (OX21) on the same days. Hearts were monitored daily by palpation and rejection taken as the cessation of a palpable beat.The majority of animals receiving 200ug and 500ug doses of R73 accepted their grafts indefinitely whereas only 25% of those receiving 50ug mAb accepted their grafts for >100 days. *P=0.03, Mann- Whitney test.

mAb administered on days -2 and -1 relative to heterotopic heart transplantation can prolong heart allograft survival in the Lewis to DA strain combination. Indefinite heart graft survival, however, was only induced by doses of 200μg and 500μg but there was no significant difference between these two treatment regimes. It was therefore decided to use 200μg R73 mAb on day -2 and day -1 relative to heart transplantation for all further experiments.

3:5 The role of the thymus gland in R73 induced graft acceptance

The role of the thymus gland in self tolerance has been extensively investigated and based on these studies various strategies have been tried in rodent models to achieve tolerance to organ allografts by manipulation of the antigen presentation and T cell education in the thymus gland. These strategies have involved depletion of the peripheral T cell pool either by sub lethal irradiation or by administration of anti lymphocyte serum followed by the introduction of a permanent source of antigen either in the form of bone marrow transplantation along with the organ allograft (Odorico et al, 1992) or direct injection of allogeneic cells into the thymus itself prior to grafting (Posselt et al, 1990, Remuzzi et al, 1991, Campos et al, 1993) in the hope that potentially alloreactive cells in the thymus will be deleted. It has also been reported that tolerance induction by OX38 anti CD4 mAb requires the presence of an intact thymus gland in the Lewis to DA strain combination (Jaques et al, 1998). We

wished to investigate whether an intact thymus gland is necessary for long term graft survival induced by R73 mAb. We therefore carried out thymectomy in 8 DA recipients at least 10 weeks prior to transplantation. The details of the surgical technique for thymectomy are outlined in chapter 2 (materials and methods). Recent thymic emigrants in the rat express the cell surface antigen Thy-1 for several days after emerging from the thymus and then become Thy-1 negative (Mason and Williams, 1980). Complete thymectomy can therefore be confirmed by labelling Thy-1 with MRC OX7. Experiments carried out in our laboratory using flow cytometric analysis of PBLs from animals thymectomised several weeks earlier have shown complete absence of Thy-1 lymphocytes confirming complete thymectomy in this model (results not shown).

Thymectomised animals were then given 200μg R73 mAb on days -2 and -1 prior to a Lewis heart allograft as before. The results shown in fig 3.4 illustrate that despite thymectomy R73 mAb induces indefinite graft survival in the majority of DA recipients and that this was not statistically significant compared to euthymic R73 treated DA controls (Mann-Whitney test). From these experiments it can be concluded that the presence of an intact thymus gland is not required for the induction of indefinite graft survival promoted by R73 mAb in this model.

3:6 Effect of IL 12 on graft survival after R73 mAb treatment

CD4 +ve T cells play a critical role in allograft rejection and recent evidence has shown that the CD4 population can be divided in to two distinct subsets i.e.

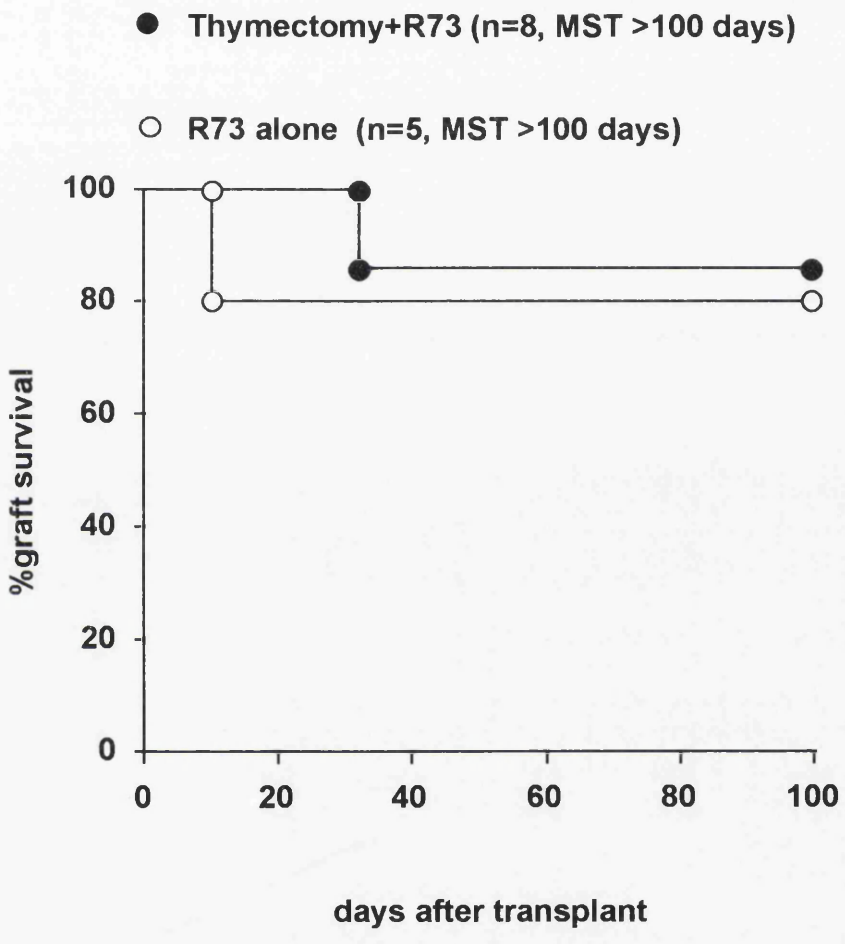


Fig 3.4 R73 induced graft acceptance does not depend on the presence of an intact thymus gland. DA rats underwent thymectomy at least one month prior to R73 treatment and Lewis heart grafting. Over 80% of rats accepted their grafts indefinitely suggesting that R73 does not achieve indefinite graft survival by acting on the thymus

Th1 and Th2 cells as defined by their cytokine repertoire. As discussed earlier, Th1 cells are broadly thought to be responsible for allograft rejection and Th2 cells for graft tolerance. IL-12 is a heterodimeric cytokine produced by phagocytic cells and B cells with a variety of effects in particular the upregulation of IFNγ production, enhancement of cytotoxic T cell and NK cell activity and promotion of Th1 responses (Trinchieri and Scott, 1994).

We investigated whether graft rejection could be induced in DA rats treated with R73 mAb by administering 1μg rIL12 (Kind gift of Dr M. Gately, Hoffman-LaRoche) to DA rats intra peritoneally on days 0-4 after receiving a Lewis heart allograft (day 0 being the day of transplantation). R73 mAb was administered as before to all recipient animals prior to grafting. Fig 3.5 shows that administration of IL12 abrogated graft survival in the majority of treated recipients with only 25% of grafts surviving greater than 100 days. This contrasts sharply with animals given R73 alone who would be expected to accept their grafts in 80% of cases.

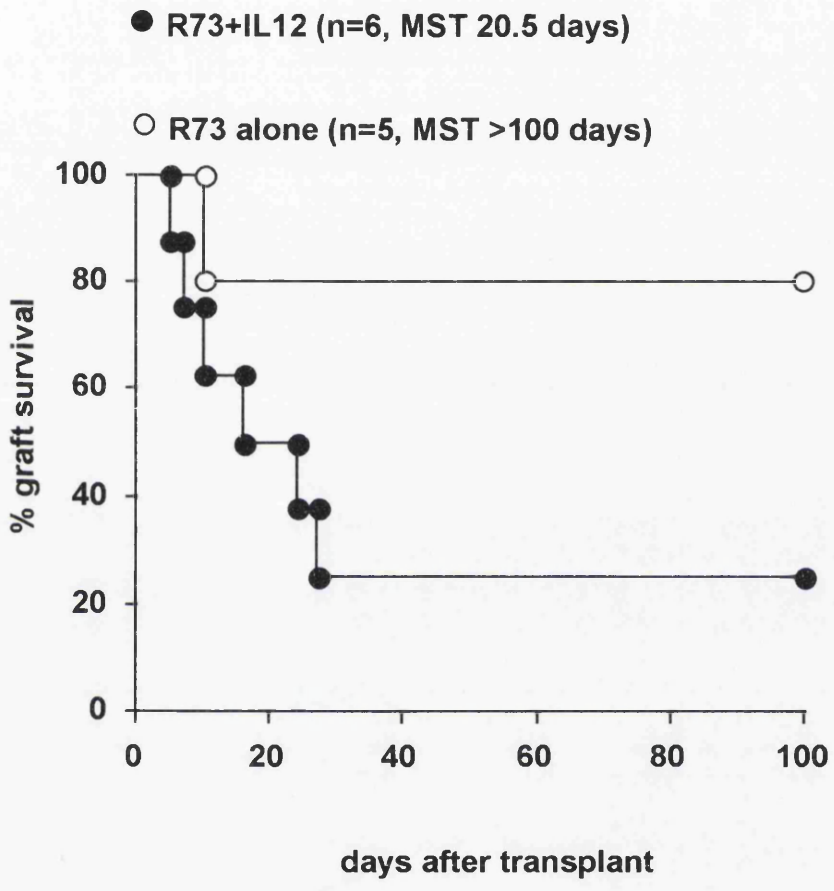


Fig 3.5 Effect of IL-12 on graft survival after R73 mAb treatment. 1ug of rIL-12 was administered to R73 treated recipient DA rats on days 0-4 relative to receiving a Lewis heart allograft. IL1-2 abrogated the indefinite graft survival induced by R73 in the majority of the R73 treated recipients.

3:7 Discussion

The goal of transplant research is the induction of long term allograft acceptance by the administration of a short course of specific therapeutic immunosuppression which does not result in long term impaired immune responses. The α/β TCR is an attractive target for immune modulation by virtue of its critical interaction with the MHC on donor cells and therefore the aim of the experiments reported in this chapter was to find out whether R73 anti-TCR mAb therapy could prolong cardiac allograft survival in a rat model and if so what factors influenced the in vivo effect of this monoclonal antibody. The results in this chapter show that a short preoperative treatment with R73 mAb can induce prolonged allograft survival in certain rat strain combinations. Only in the Lewis to DA combination, however, could indefinite graft survival be achieved using R73 mAb. Prolonged graft survival was seen in the BN to Lewis strain combination however no grafts survived longer than 35 days. This finding is consistent with previous reports (Heidecke et al, 1995). Tsuchida and colleges however, reported tolerance to rat heart allografts in this strain combination using a dose of only 50μg R73 (Tsuchida et al, 1994). In the DA-PVG strain combination R73 did not significantly prolong graft survival over untreated controls.

This strain dependant variation in response to immunomodulatory treatments has been seen in other rodent models of allograft rejection (McConnel and Hall,

1989; Ilano et al, 1989 and 1991, Walker et al, 1996). The Lewis-DA combination is regarded as a low responder strain and appears more susceptible to immunosuppressive protocols designed to induce transplant tolerance. Despite this, unmodified DA recipients acutely reject Lewis heart allografts within 10 days suggesting that the strain variation reflects a susceptibility to immune modulation rather than a weak rejection response. Prolonged, post transplant R73 mAb treatment was not used in any of the above experiments and it is interesting to note that increasing the dose of R73 mAb to 500μg did not improve long term graft survival over the 200μg dose in the Lewis to DA strain combination. This is probably due to either saturation of R73 binding sites to the α/β TCR in vivo at and above a dose of 200μg or the neutralisation of the xenogeneic mouse mAb by an anti mouse Ig response in the recipient rat. This problem has been highlighted in human transplantation using xenogeneic antibodies where repeated use of the antibody is ineffective due to such a response. Attempts have been made to overcome this by the construction of chimeric monoclonal antibodies in which the constant region of the Ig molecule is substituted with human amino acid residues. Heidecke and colleagues have observed that pre operative rather than post operative administration of R73 is essential for long term graft acceptance though this theory was not tested here (Heidecke et al, 1995).

The α/β TCR is present on the majority of peripheral T cells and those found in the thymus gland (Lawetzky et al., 1990). It is possible therefore that R73 acts

by either entering the thymus and modulating T cells centrally or depleting peripheral T cells allowing a prolonged source of alloantigen to modify T cells in the thymus resulting in central tolerance to the allograft. Removing the thymus gland prior to transplantation would abrogate graft tolerance in this model were this the case. In this model, however, thymectomy prior to transplantation had no effect on graft acceptance with over 80% of thymectomised DA rats accepting their Lewis heart allografts indefinitely after R73 treatment. It would therefore appear that R73 acts on the peripheral T cell pool to induce graft acceptance in this model and does not depend on a constant supply of immunomodulated thymic emigrants. These results contrast sharply with those reported for anti-CD4 induced transplant tolerance in the rat. Thymectomy carried out greater than 40 days prior to transplant under cover of anti-CD4 mAb abrogates tolerance in this model possibly due to the lack of immunoregulatory recent thymic emigrants which would otherwise have a suppressive effect on mature peripheral CD4 positive T cells, thereby preventing them from mediating graft rejection (Herbert and Roser, 1988, Jaques et al, 1998).

Interestingly, permanent graft survival after R73 mAb treatment in the Lewis-DA strain combination is abrogated in the majority of recipients by post transplant administration of the proinflammatory cytokine IL-12. Interleukin-12 has been shown to promote the development of CD4 +ve T cells with a Th1 phenotype and administration of IL-12 in vivo accelerates auto immune

diabetes in NOD mice (Trinchieri, 1994, Trembleau et al, 1995). Rejection of cardiac allografts in R73 treated recipients animals could result from the augmentation of cytotoxic T cell or NK cell activity in the graft induced by exogenous IL-12. IL-12 may also promote CD4 +ve Th1 cell development, a form of immune polarisation which has been shown to be associated with graft rejection in some experimental models (Takeuchi et al, 1992; Dallman et al, 1991). This observation would therefore support the hypothesis that R73 acts by altering the peripheral T cell response to a heart allograft and suggests that although T cells present in recipient animals after R73 therapy are unresponsive to alloantigen they are still responsive to exogenous cytokine.

3:8 Summary

3:2 Administration of two preoperative doses of anti-α/β TCR mAb (R73) significantly prolongs the survival of BN cardiac allografts in Lewis recipients but only when 50μg (P= 0.02) or 200μg (P= 0.008) of R73 are given at each time point. Permanent graft survival is not obtained in this strain combination.

3:3 Administration of 200μg R73 mAb prior to transplantation results in indefinite (>100days) graft survival in the Lewis to DA strain combination but does not significantly prolong graft survival in the PVG recipients of a DA heart allograft.

3:4 200μg and 500μg of R73 mAb given on days -2 and -1 are equally effective in promoting permanent graft survival in the Lewis to DA strain combination. A 50μg dose of R73 at these time points prolongs graft survival but not permanently.

3:5 The ability of R73 mAb to prolong graft survival in the Lewis to DA strain combination is not dependant on the presence of an intact thymus gland.

3:6 The effect of R73 mAb could be reversed by the administration of the proinflammatory cytokine IL-12.

CHAPTER FOUR

In vivo effects of R73 monoclonal antibody therapy on α/β TCR positive cells

4.1 Introduction

The results described in the previous chapter show that a short pre operative course of 200μg R73 anti α/β TCR mAb induces permanent survival of a Lewis heart allograft in DA recipient animals. The factors responsible for overcoming the rejection response to an allograft remain unclear despite intensive investigation by many groups. It is clear, however, that multiple effector mechanisms are involved and these can broadly be divided into central and peripheral mechanisms. Central tolerance, where alloreactive T cells are eliminated in the thymus gland, or where T cells become tolerised to alloantigen in the thymus has been suggested as a mechanism in some experimental models of transplantation (Posselt et al, 1990, Sayegh et al, 1993). Several mechanisms have also been suggested whereby monoclonal antibodies can induce permanent graft acceptance by their effects on the peripheral T cell pool. Firstly, they may cause deletion of alloreactive T cells either by clonal elimination or they may stimulate activation induced cell death in activated T lymphocytes. Secondly, anergy may be induced whereby T cells are rendered unresponsive to antigenic challenge by altered signalling events at the time of antigen recognition. This can be induced either by interruption of costimulatory signals, in particular the B7-CD28 pathway where CTLA4 Ig construct has been used experimentally with some success (Sayegh et al, 1995). It has been suggested that this partial signalling may act by altering the amplitude of TCR induced signals. Thirdly, long term allograft survival may be

induced by peripheral CD4 T cell mediated suppression. The concept of T cell suppression has been supported by adoptive transfer experiments in models of transplantation using non depleting anti CD4 mAb's and has been termed infectious tolerance (Waldmann and Cobbald, 1993).

As shown in the last chapter, the ability of DA rats to accept a fully allogeneic Lewis heart graft after R73 treatment does not depend on the presence of an intact thymus gland suggesting that in our model R73 does not act directly on the thymus gland or require the presence of recent thymic emigrants. R73 therefore would appear to act by disabling the peripheral T cell pool. This is in contrast to some reports using anti-CD4 mAb therapy in rodents which has shown that thymectomy abrogates tolerance induction suggesting a role for recent thymic emigrants in the maintenance of graft tolerance in certain models (Jaques et al, 1998, Herbert and Rosser, 1988).

In the experiments described in this chapter we sought to identify possible peripheral mechanisms of R73 induced heart allograft acceptance in the Lewis-DA rat strain combination by investigating the in vivo effects of R73 on peripheral T cell subsets and their cell surface antigens. CD4 and CD8 T cells express the α/β TCR and the interaction of the T cell receptor on these cells with allogeneic MHC on donor APC has been shown to be critical for allorecognition and rejection of an organ allograft. Depletion of CD4 and CD8 T cells has previously been shown in some experimental models to be responsible for abrogation of the rejection response (Cobbold et al, 1984). In the following experiments CD4 and CD8 T cell depletion after R73 mAb

therapy was examined using flow cytometric analysis of lymph node cells and peripheral blood lymphocytes. In addition, modulation of the α/β T cell receptor on the surface of T cells was examined by flow cytometry. In separate experiments, DA recipients of Lewis heart grafts were killed 7 days after transplantation and the heart grafts subjected to immunohistochemical analysis to determine the degree of cellular infiltration in rejecting and non rejecting grafts.

4.2 Flow cytometric analysis of T cell subsets after R73 therapy

Lymph node cells (LNC) and peripheral blood lymphocytes (PBL) were harvested from R73 treated allograft recipients sacrificed on days 4, 7, 21 and 100 after transplantation. Normal DA lymphocytes were used as controls. The cells were isolated and prepared as described in chapter 2 (materials and methods) and subjected to two colour flow cytometric analysis. The FITC and PE conjugated antibodies used in this analysis are illustrated in table 4.1.

T Cell Subset	FITC/ PE conjugate
CD4	R73-FITC + W3/25-PE
CD8	R73-FITC + OX8-PE
CD5	OX19 + Rabbit anti mouse-FITC

Table 4.1. Cell labelling protocol for flow cytometric analysis of LNC and PBL. 8µl of undiluted antibody conjugate was added to $2.5x10^5$- $1x10^6$ cells per tube for analysis. Rabbit anti mouse-FITC was diluted 1:20 with PBS and rat serum as a blocking agent.

Cells coated with R73 mAb were detected by adding rabbit anti mouse-FITC conjugate and saturation of TCR with R73 detected by incubating cells with saturating dose of R73 (8µl undiluted) and then rabbit anti mouse-FITC. Data are expressed as the mean and standard deviation of 3 or 4 animals in each group except at the day 21 time point where two treated animals were analysed. Paired comparisons were made between the treated groups and normal controls using the student's t test as were comparisons between LNC and PBL groups at each time point. Significance was taken at the 5% level in all cases.

4:2:1 T cell depletion in lymph nodes and peripheral blood of R73 treated graft recipients.

As shown in figure 4.1 and figure 4.4, the percentage of lymph node cells expressing the pan T cell marker CD5 as detected by MRC OX19 mAb was significantly reduced at days 4 (mean 25.5%, SD 2.9, P= 0.0001), and 7 (mean 28.8%, SD 2.1, P= 0.0009) in animals receiving 200μg R73 days -2 and -1 prior to a Lewis heart allograft compared with lymph node cells harvested from normal DA rats (mean 78.3%, SD 5.7). By days 21 (mean 67.2%, SD 5.4) and 100 (mean 72.6%, SD 7.4) after transplantation total T cell numbers had recovered in R73 mAb treated animals and although T cell recovery appears incomplete even at these late time points, the difference is not statistically significant.

T cell depletion in the peripheral blood of treated animals was again profound (figure 4.1b and figure 4.5). CD5 positive T cells were significantly depleted at day 4 (mean 0.3%, SD 0.4, P= 0.0009) and day 7 (mean 15%, SD 1.4, P= 0.008) compared to untreated control animals (mean 63.9%, SD 9.4). Recovery of T cells expressing this marker was again seen at 21 days (mean 63.4%, SD 18.0) and 100 days (mean 61.5%, SD 5.6) after Lewis heart grafting. Once again, although T cell recovery appears incomplete at days 21 and 100 after grafting, there was no significant difference compared to untreated animals.

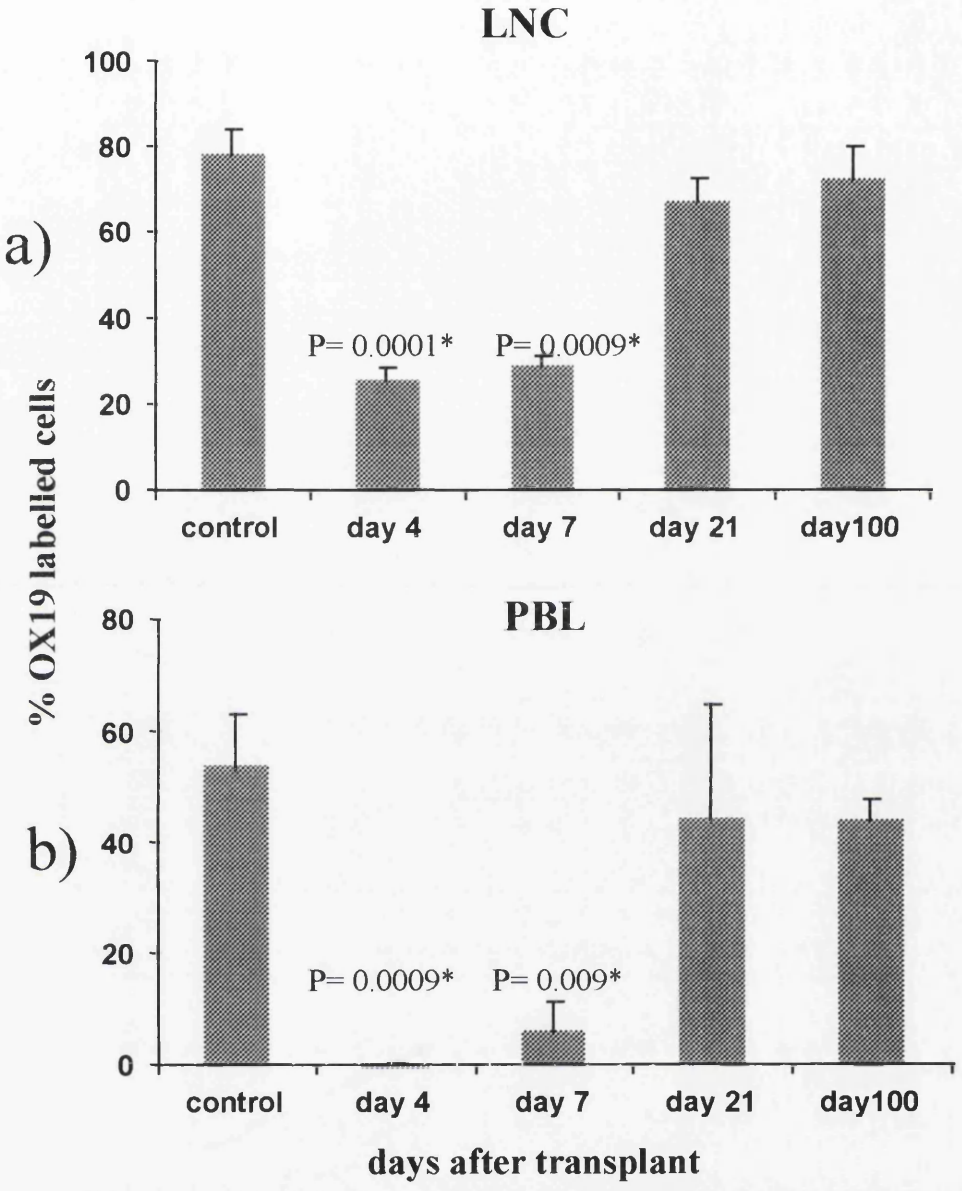


Figure 4.1. CD5 positive T cell depletion in DA recipients of a Lewis cardiac allografts.. Lymph node cells (LNC) and peripheral blood lymphocytes (PBL) were harvested on days 4, 7, 21, and 100 relative to transplantation from recipient animals treated with R73 mAb 200ug days -2,-1 prior to transplant and from normal DA control animals. Cells were labelled with MRC OX19 (CD5) mAb and then rabbit anti mouse FITC conjugate and analysed by flow cytometry. * Student's t test.

It is also notable that R73 mAb treatment depletes T cells significantly more effectively in peripheral blood than in the lymph nodes at both day 4 (P=0.0001) and day 7 (P= 0.01) post transplant.

4:2:2 CD4 T cell depletion in lymph nodes and peripheral blood after R73 mAb treatment.

Depletion of CD4 T cells in the lymph nodes harvested from R73 treated graft recipients mirrored that found for cells expressing CD5 (figures 4.2 and 4.4). Percentage of CD4 T cells was lowest at day 4 (mean 13.4%, SD 4.7, P= 0.003)) and day 7 (mean 22.4%, SD 5.9, P= 0.02) compared to normal DA animals (mean 62.8%, SD 6.3). Recovery was almost complete by day 21 (mean 57.2%, SD 12.9)and day 100 (mean 44%, SD3.7).

Depletion was again more pronounced in the peripheral blood of R73 treated animals (figure 4.2b and figure 4.5) with mean percentage of CD4 T cells 0.1% (SD 0.1, P= 0.001) at day 4 and 7.6% (SD 5.1, P= 0.01) at day 7 compared to 53.8% (SD 9.1) in control animals. Recovery of cell numbers was again complete by day 21 (mean 44.5, SD20.4).

4:2:3 CD 8 T cell depletion in lymph nodes and peripheral blood after R73 mAb treatment.

As shown in figures 4.3 and 4.4, CD8 T cells were depleted to 2.5% (SD 0.8, P= 0.001) in the lymph nodes and zero in peripheral blood (figure 4.5) of R73

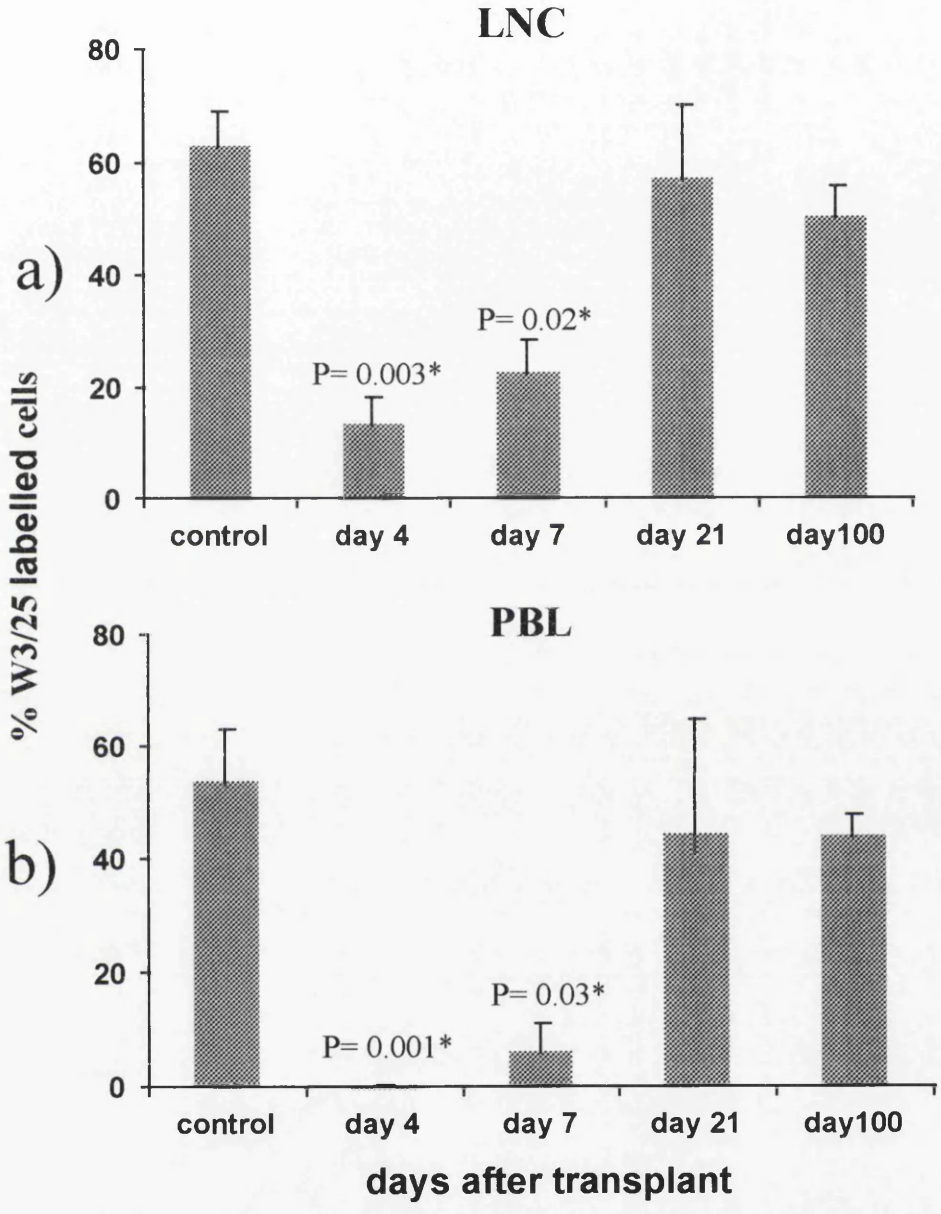


Figure 4.2. CD4 T cell depletion in DA recipients of a Lewis heart allograft.. Lymph node cells (LNC) and peripheral blood lymphocytes (PBL) were harvested on days 4, 7, 21, and 100 post transplant from recipient animals treated with R73 mAb 200ug days -2, -1 prior to transplant. The cells were then double labelled with R73 Fitc and W3/25 PE conjugates and analysed by flow cytometry. * Student's t test.

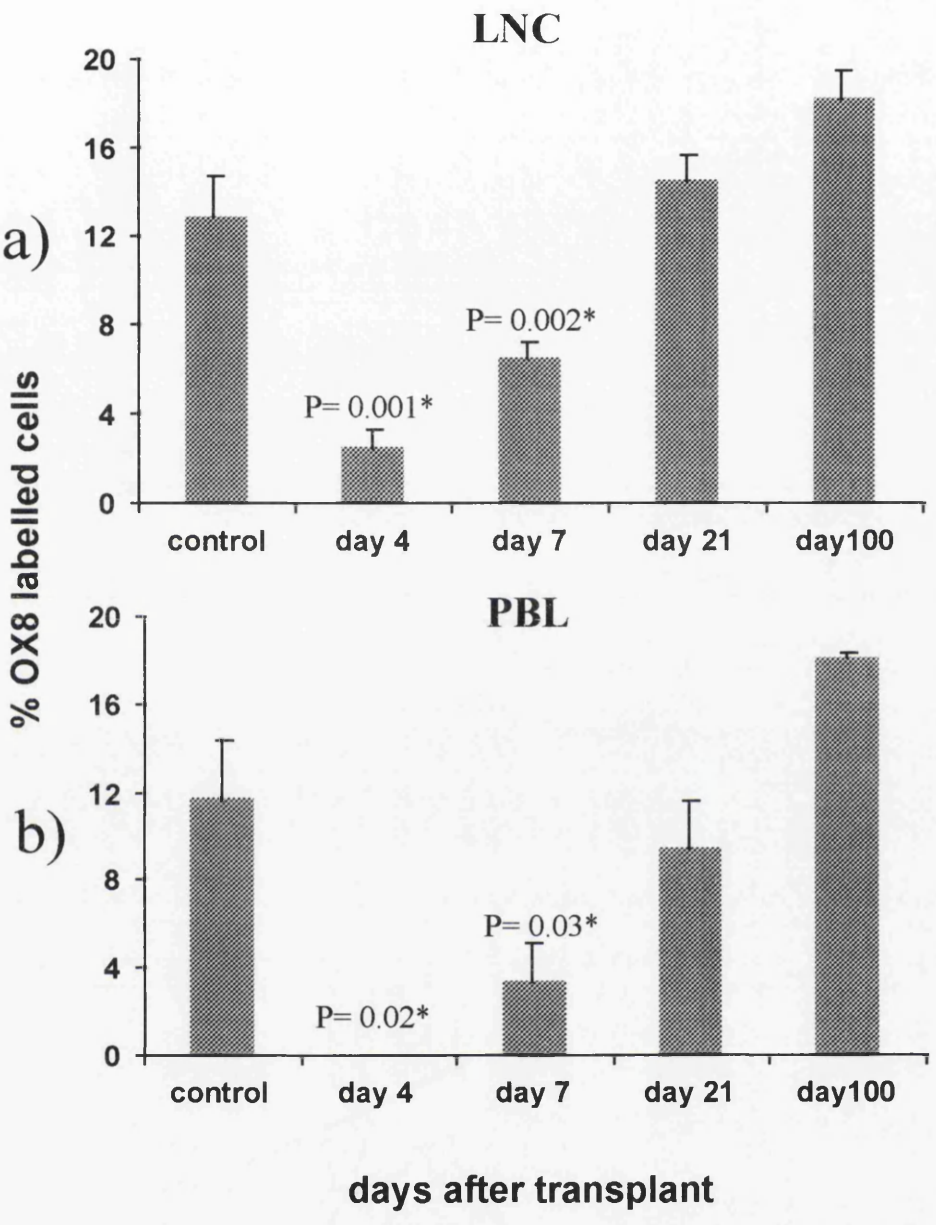


Figure 4.3. CD8 T cell depletion in DA recipients of a Lewis heart allografts. Lymph node cells (LNC) and peripheral blood lymphocytes (PBL) were harvested on days 4, 7, 21, and 100 post transplant from recipient animals treated with R73 mAb 200ug days -2, -1 prior to transplant. The cells were then double labelled with R73 Fitc and OX8 PE conjugates and analysed by flow cytometry. * Student's t test.

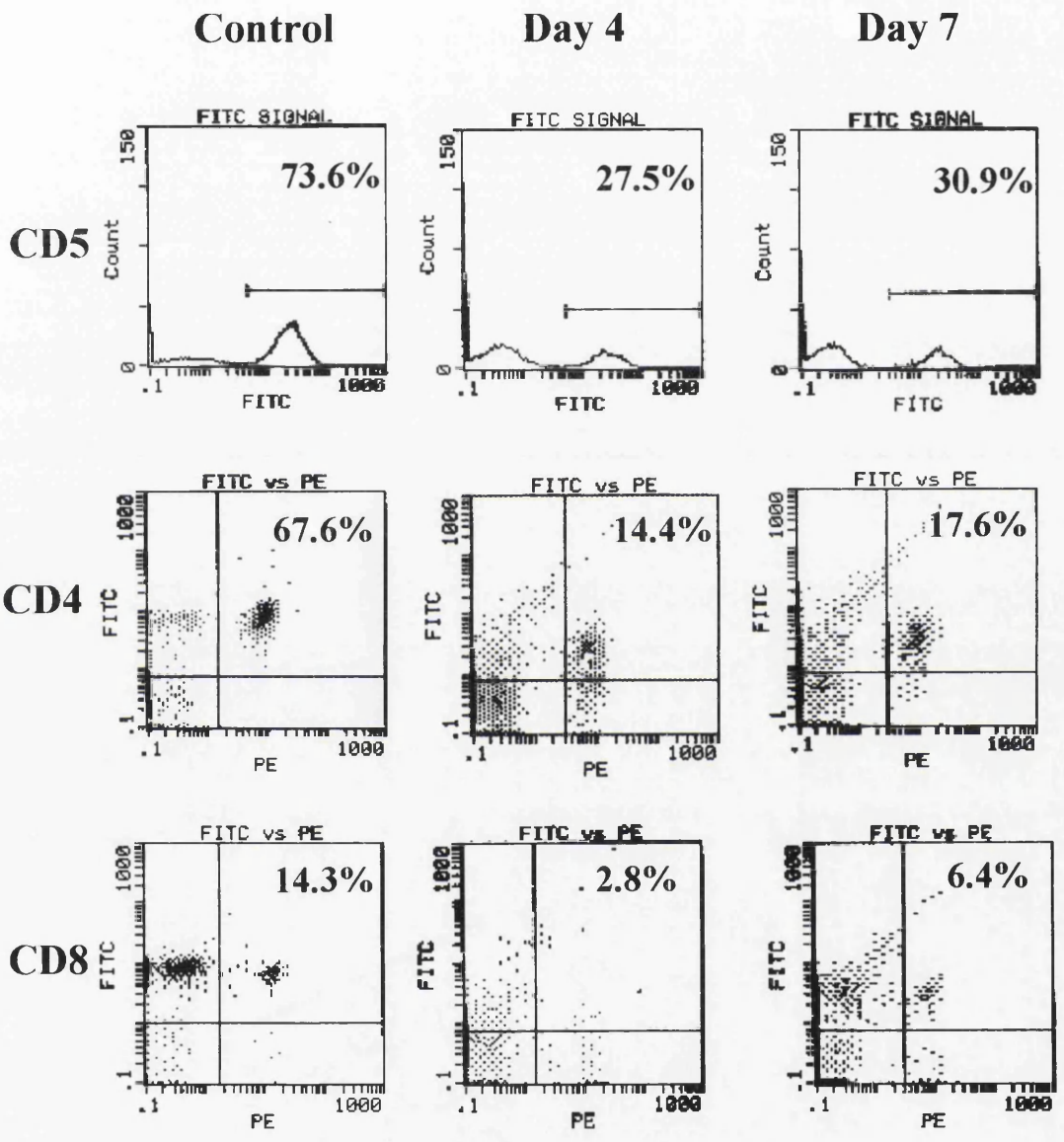


Figure 4.4 Flow cytometric analysis showing CD5,CD4 and CD8 T cell depletion in the lymph nodes of DA graft recipients after R73 mAb treatement at days 4 and 7 after grafting. Two colour fluorescence analysis using FITC onjugated R73 on Y axis (log_{10} FITC signal) and PE conjugated W3/25 (CD4) and OX8 (CD8) (log_{10} PE signals) on X axis.Single colour fluorescence analysis using OX19 (CD5) detected by rabbit anti mouse FITC conjugate on X axis (log_{10} FITC signal).

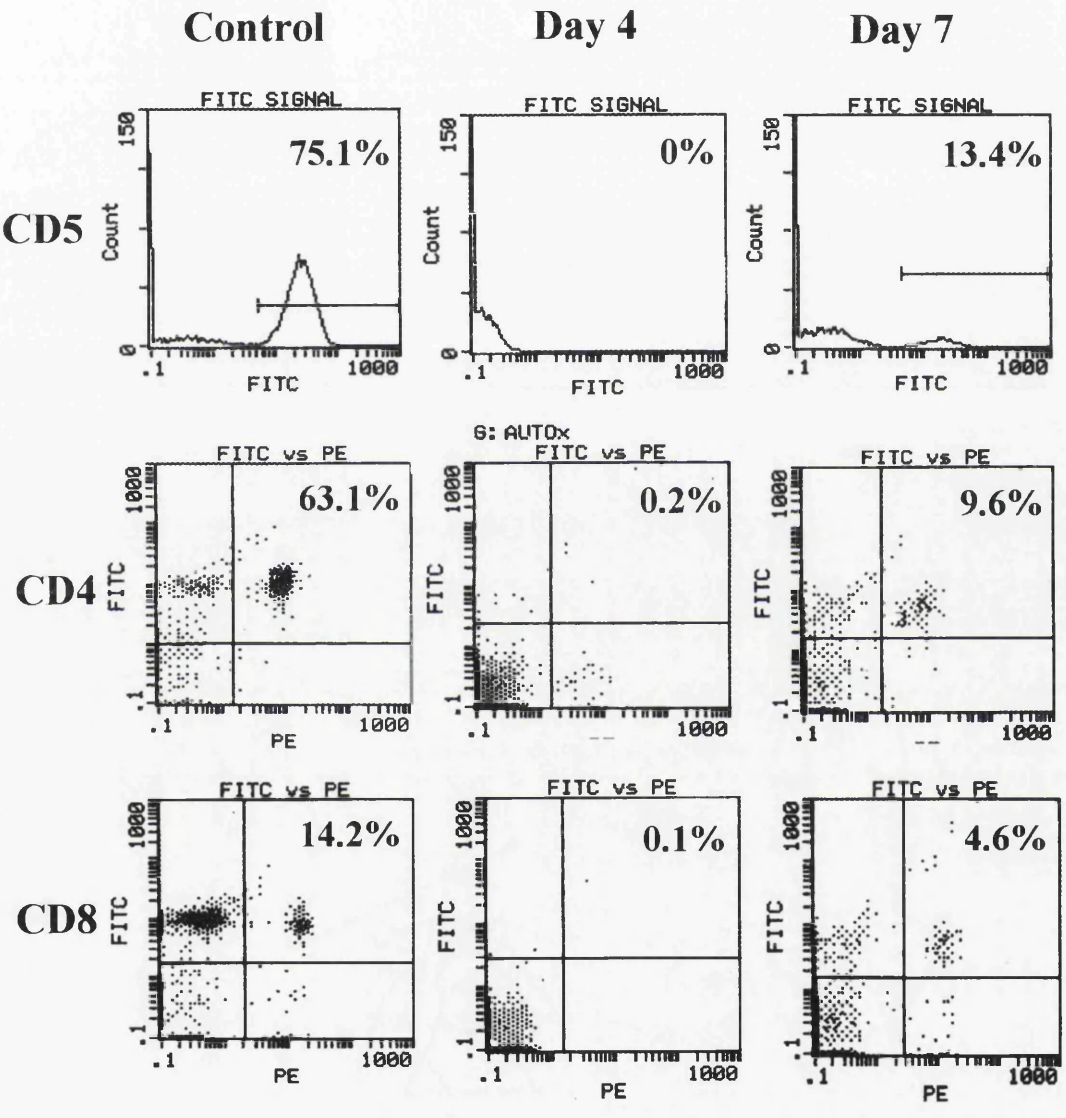


Figure 4.5 Flow cytometric analysis showing CD5,CD4 and CD8 T cell depletion in the peripheral blood of DA graft recipients after R73 mAb treatement at days 4 and 7 after grafting. Two colour fluorescence analysis using FITC onjugated R73 on Y axis (log_{10} FITC signal) and PE conjugated W3/25 (CD4) and OX8 (CD8) (log_{10} PE signals) on X axis.Single colour fluorescence analysis using OX19 (CD5) detected by rabbit anti mouse FITC conjugate on X axis (log_{10} FITC signal)

treated DA recipients of a Lewis heart graft at day 4 after transplantation, rising to 6.5%(SD 0.7, P= 0.002) in LNC and 3.4 (SD 1.8, P= 0.03) in PBL at day 7. CD 8 T cell numbers recovered to near normal at day 21 in LNC (mean 14.5% SD1.0) and PBL (mean 9.5%, SD2.2). In our experiments CD8 +ve cells accounted for 12.9% (SD 1.8) of cells in the lymph nodes and 11.8% (SD 2.6) in peripheral blood of normal DA animals.

Activated CD4 T cells are critical for allograft rejection whereas graft rejection can progress in the absence of CD8 T cells (Gracie et al, 1990). We therefore assessed whether there was a preferential depletion of CD4 T cells to account for graft tolerance in this model, however there was no significant difference in depletion of CD4 and CD8 cells at day 4 (student's t test). At day 7, there was a trend towards preferential depletion of CD4 T cells at 64% in LNC and 89% in PBL compared to 50% and 75% for CD8 T cells though this did not reach statistical significance (P= 0.1, student's t test). It appears therefore that although both CD4 and CD8 T cells are markedly depleted by R73, reconstitution of CD4 T cells is marginally slower and this may contribute to the induction of graft tolerance.

4:3 T cell receptor modulation after R73 mAb therapy

The most striking effect of R73 on the peripheral T cell pool is the depletion caused by the antibody. The depletion seen, however could be apparent if R73 was in fact modulating the T cell receptor and so blocking the binding of the

R73-FITC conjugate used for detection of these cells. Figure 4.6 shows that there is evidence of TCR modulation at day 4 in the LNC and at days 7 and 21 in peripheral blood. The graph also confirms the profound, though transient T cell depletion seen in this model.

4:4 Binding of R73 mAb to α/β TCR on T lymphocytes of R73 treated graft recipients

LNC were isolated from DA recipients of a Lewis heart allograft under cover of R73 treatment as before and incubated with rabbit anti-mouse (RAM) FITC conjugate and analysed by flow cytometry to assess R73 mAb binding at days 4, 7, 21 and 100 after transplantation. The percentage of cells labelled with RAM-FITC was compared with the percentage of total CD5 +ve T cells (figure 4.7). The results show that at day 4 post transplant 81% of T cells in R73 treated animals are bound by R73 mAb, however by day 7 R73 is detectable on only 52% of T cells. This may partly be due to CD4 and CD8 T cell recovery and partly due to internalisation of R73 by residual CD4 and CD8 lymphocytes which have not been depleted by mAb therapy.

TCR saturation of lymph node cells at day 4 and day 7 after R73 treatment and heart grafting was assessed in DA recipient animals by the comparing the peak fluorescence (PF) in LNC labelled with RAM-FITC with LNC firstly incubated with a saturating dose of R73 mAb. Figure 4.8 shows that at day 4 R73 mAb is bound to all available TCR ligands. At day 7, addition of further R73 results in

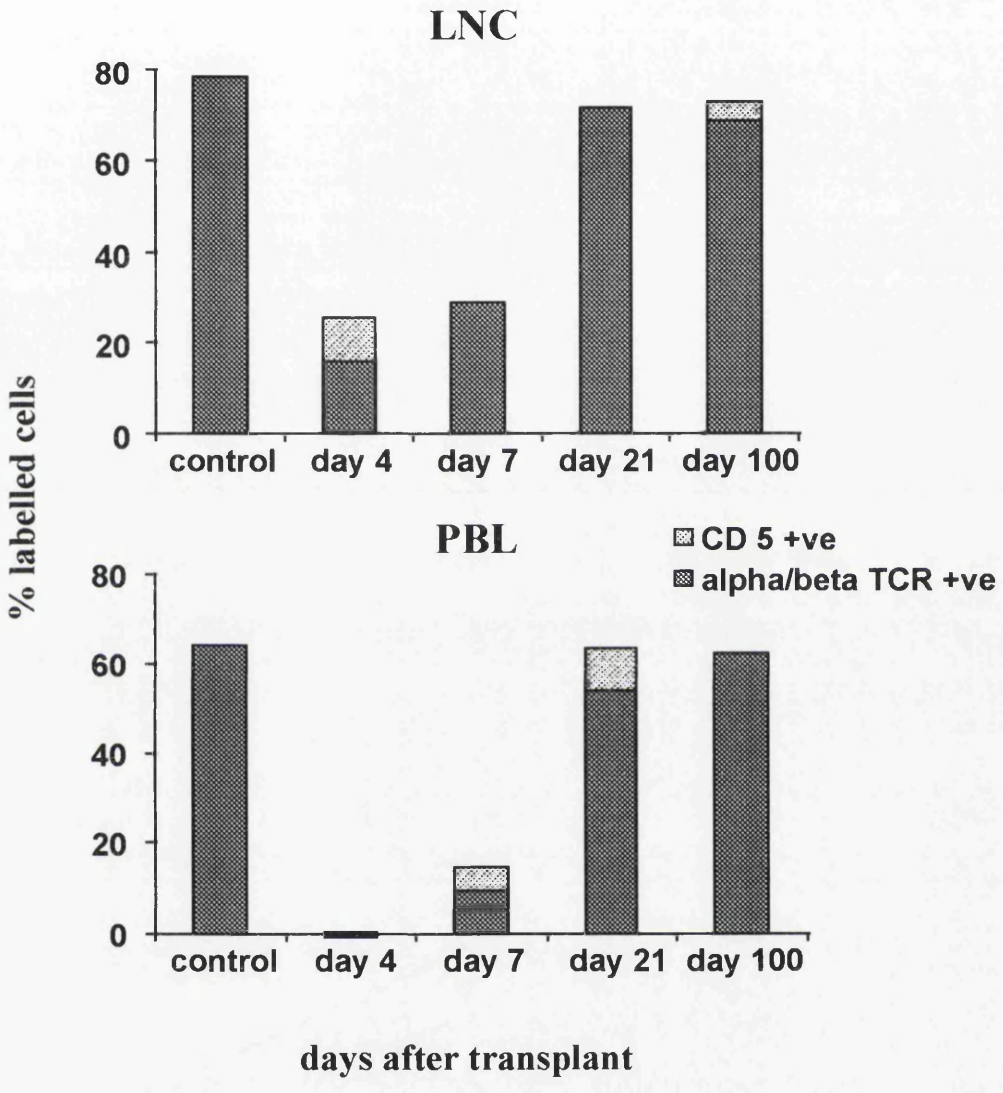


Figure 4.6 TCR modulation in R73 treated graft recipients.
These graphs illustrate the comparative depletion of alpha/beta TCR +ve cells and cells expressing the pan T cell marker CD5 in lymph nodes (LNC) and peripheral blood (PBL). There is marked depletion of cells expressing both markers at days 4 and 7 with slightly increased depletion of TCR +ve cells suggesting modulation of the T cell receptor.

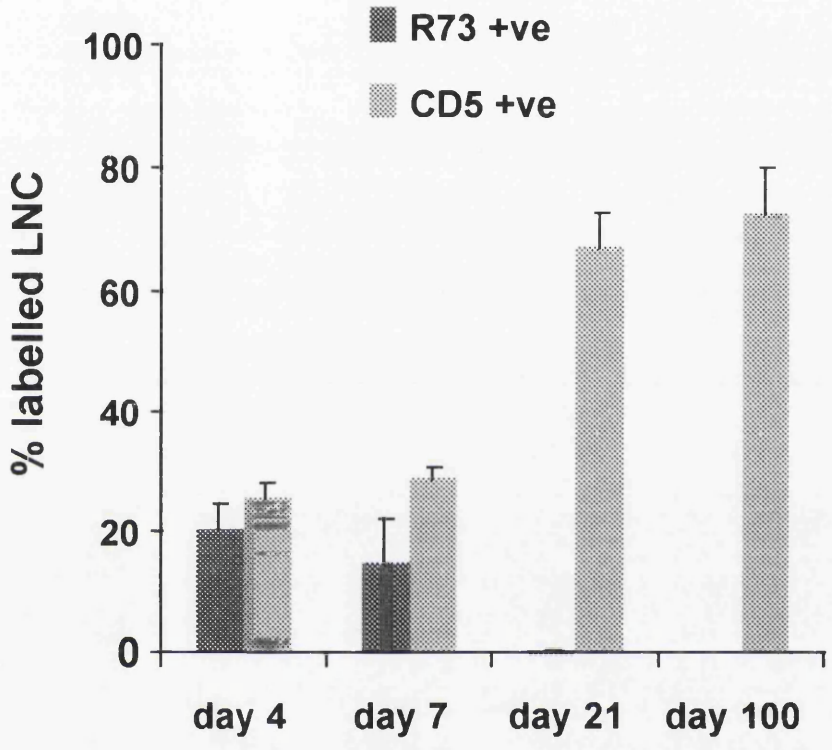


Figure 4.7 R73 mAb binding to DA rat LNC after R73 mAb treatment and Lewis heart grafting. DA rat LNC were harvested on days 4, 7, 21, and 100 relative to receiving a Lewis heart allograft and 200ug R73 day-2 and -1 prior to grafting. Cells were incubated with rabbit anti mouse FITC conjugate and analysed by flow cytometry. The results show that at day 4 81% of T cells(CD5 +ve) are labelled with R73 mAb. By day 7 R73 is bound to only 52% of T cells and by day 21 no residual R73 is present.

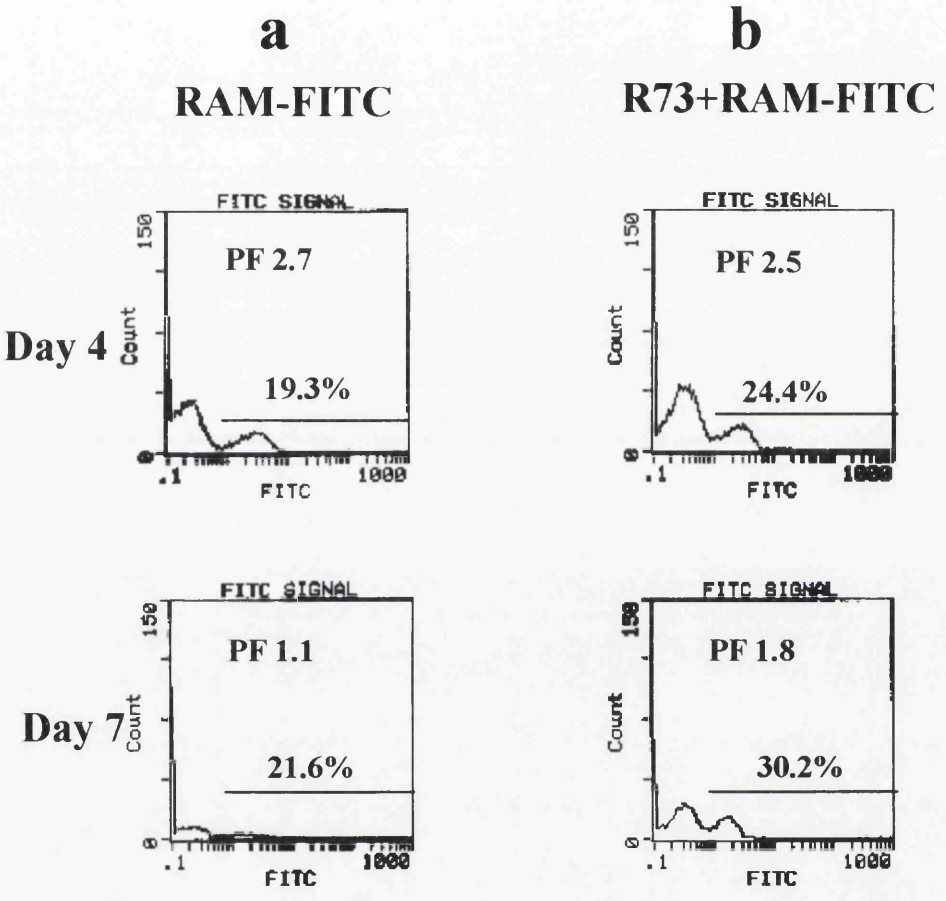


Figure 4.8 FACS histograms illustrating R73 mAb saturation in DA rat LNC at days 4 and 7 after transplant and R73 treatment. Single colour flow cytometric analysis using rabbit anti mouse (RAM) FITC conjugate (a), shown on X axis, to detect bound R73 mAb on lymph node cells (log_{10} FITC signal). Unoccupied R73 binding sites detected(b) by adding a saturating dose of R73 mAb to LNC in vitro followed by RAM FITC conjugate.

an increase in the PF and height of the peak suggesting that the majority of R73 binding sites are unoccupied.

4:5 Effect of delaying Lewis heterotopic heart transplantation by 7 days into R73 treated DA recipients.

In the light of these results, Lewis hearts were transplanted into R73 DA recipients with a delay of 7 days relative to R73 treatment. Figure 4.9 shows that the majority of grafts are accepted indefinitely in the delayed transplant group (MST >100 days). These data indicate that permanent graft acceptance can be induced in this model even at a point where only 50% of T cells are bound by R73 and when the majority of α/β TCR binding sites on the surface of the T cell are unoccupied.

4:6 Immunohistochemical analysis of graft infiltrating cells.

In order to further clarify the potential mechanisms involved in this model we carried out immunohistochemical staining of cryostat sections of heart grafts removed from R73 treated graft recipients and untreated animals rejecting their grafts.

Briefly, R73 treated Lewis heart graft recipients (n=3) and untreated control animals rejecting their grafts (n=4) were killed at day 7 after transplant and the heart graft removed and snap frozen in liquid nitrogen. 5μ sections were cut

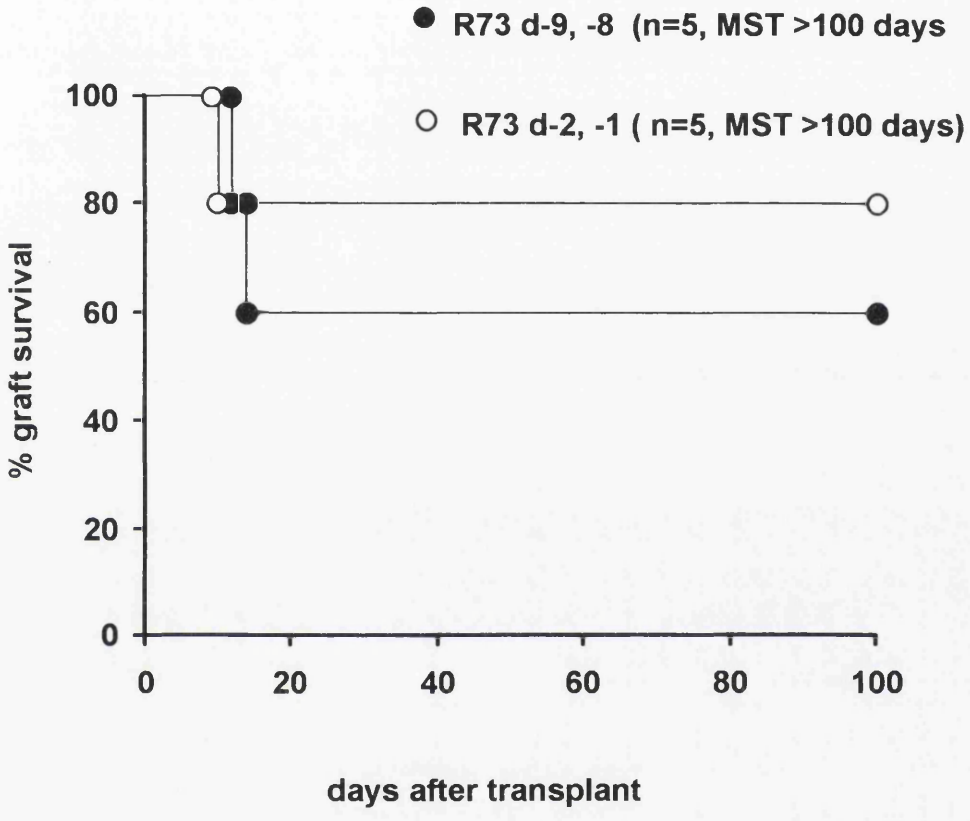


Fig 4.9 Effect of delaying heart transplant following treatment with R73 mAb. DA rats were given 200ug of R73 mAb as before and then a Lewis heart allograft either 24 hours after the last dose of antibody or 7 days later. 60% of recipient animals undergoing delayed transplant accepted their grafts indefinitely compared to 80% in the group transplanted at 24 hours.

onto triple well glass slides and fixed in ethanol. The cryostat sections were then labelled with mouse monoclonal antibodies to various cell surface markers (table 4.2) which were then detected using a biotin conjugated rabbit anti mouse mAb and stained using an HRP technique as described in chapter 2 (materials and methods).

Monoclonal antibody	cell surface marker
MRC OX8	CD8+ve cells
W3/25	CD4+ve cells
R73	α/βTCR+ve cells
MRC OX39	IL2Rα
EDI	macrophages, monocytes, dendritic cells

Table 4.2 Monoclonal antibodies used in immunohistochemical analysis of heart graft cellular infiltrates.

The results of immunohistochemical staining are shown in figure 4.10. There is a marked reduction in the degree of cellular infiltration of CD4 and CD8 T cells at day 7 in R73 treated animals which correlates with the T cell depletion noted in blood and lymph nodes. R73 (α/βTCR) expression is also markedly reduced in treated animals compared to rejecting grafts. This is probably due to combination of cell depletion and blocking of the α/βTCR in the grafts by R73 mAb. IL-2Rα expression is also reduced in R73 treated grafts. This could be

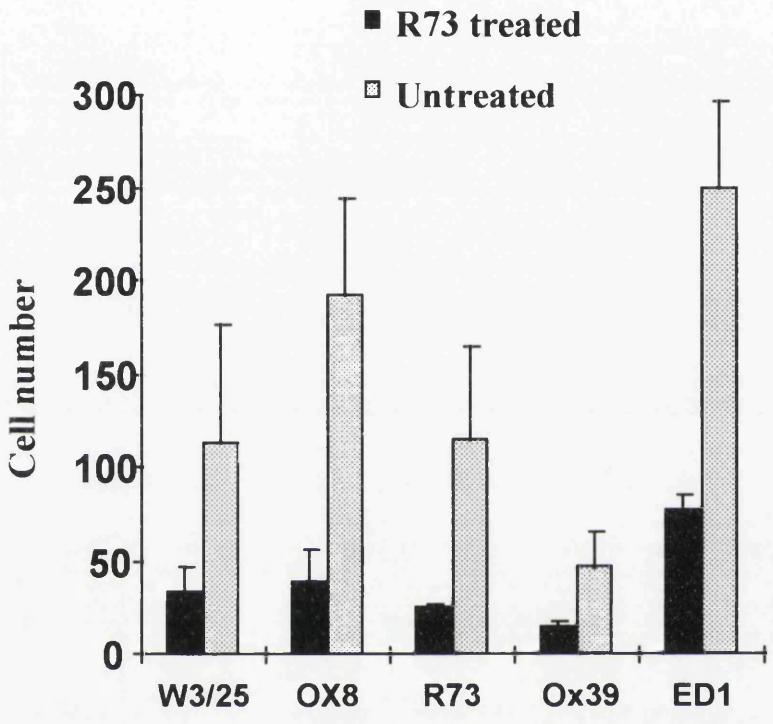


Figure 4.10 Cell infiltrates in Lewis heart grafts day 7 after transplantation into R73 treated or unmodified DA recipients. Heart grafts from R73 treated animals and rejecting controls were removed on day 7. Cryostat sections labelled with the mouse anti-rat mAb's as shown. Fewer CD4+ve (W3/25) and CD8+ve (OX8) cells were found in R73 treated hearts. IL2R (OX39) expression is also significantly lower in the treated group of animals. There were also significantly fewer macrophages (ED1) present in the R73 treated infiltrates.

due to a reduction in T cells expressing the receptor sub unit or due to the presence of anergic cells which are unable to produce IL-2. Surprisingly the monocyte infiltrate in the R73 grafts is less than half that in the rejecting grafts. R73 mAb does not directly bind to monocytes and macrophages and so this observation can be explained by lack of pro inflammatory cytokine in a graft depleted of CD4 +ve helper T cells.

4:7 Discussion

The aim of the experiments reported in this chapter was to determine the effect of R73 mAb on peripheral T cell subsets and to ascertain what proportion of peripheral T cells were coated with R73 and for how long during the early post transplant period. We also investigated whether R73 treatment resulted in TCR modulation and whether the effects on peripheral T cells were reflected by infiltration of the heart grafts themselves.

The experiments reported here demonstrate that after a brief course of R73 treatment there is marked but partial, transient depletion of CD4 and CD8 T cells in peripheral blood and lymph nodes of R73 treated graft recipients. The associated depletion of CD5 +ve cells suggests that this is at least in part, actual cell depletion rather than purely modulation of the α/β TCR by R73 binding. By day 7 post transplantation CD4 and CD8 T cell numbers are increasing, with almost complete reconstitution by day 21 post transplantation with out loss of the heart allograft. In addition R73 was detectable on T cells up to 7 days post transplant. This suggests that the treatment dose of R73 in this model is sufficient to induce peripheral T cell depletion and coating of T cells during the period when acute rejection would normally be taking place. It is probable that the lower dose of 50μg of R73 used in chapter 3 was unsuccessful in inducing permanent graft acceptance due to either insufficient T cell depletion or due to unoccupied α/βTCR binding sites in the early post transplant period. It is also not surprising that 500μg R73 did not positively

influence graft survival as depletion of T cells is profound using 200μg and also TCR saturation is obtained with the 200μg dose used in these experiments. Other groups have reported similar transient T cell depletion after pre transplant treatment with R73 without loss of the graft (Tsuchida et al, 1994, Heidecke et al, 1995) and also after R73 treatment of experimental established adjuvant arthritis (Yoshino and Cleland, 1992). Dufter and colleges, however found that in their model of R73 induced transplant tolerance that indefinite graft survival was only obtained if antibody treatment was continued for 70 days after transplantation (Dufter et al, 1994). The transient depletion and loss of circulating treatment antibody after 7 days is in stark contrast to reports using anti-CD4 monoclonal antibodies. Transplantation tolerance using MRC OX-35 mAb to deplete CD4 T cells only occurs if depletion is present for greater that 40 days after transplantation (Herbert and Rosser, 1988). Darby and colleagues demonstrated that KT6 rat anti-mouse CD4 mAb could induce indefinite graft survival in mice without depletion of peripheral CD4 T cells, however prolonged presence of circulating antibody was required (Darby et al, 1992).

There is, however evidence of TCR modulation in the residual T cells at days 4 and 7 after transplantation suggesting that alloantigen recognition by the remaining T lymphocytes may be impaired and it is possible that these cells are in fact unresponsive or that they are subject to some form of immune regulation. This theory is supported by the observation that transplanting a heart graft in to R73 treated recipients eight days after completion of antibody

treatment (day 7 in relation to normal R73 treated transplants) when less than 50% of the remaining T cells are bound by R73 mAb and when peripheral T cell numbers are returning towards normal levels still results in indefinite graft survival. It may be that R73 treatment results in the deletion of antigen specific T cells and the remaining T cells, resistant to R73 treatment, regulate or suppress the function of the new population of CD4 and CD8 T cells. There is much evidence to support the presence of regulatory T cells in mAb induced models of transplantation tolerance and in a physiological role such as mucosal immunity. Reports using anti CD4 mAbs have shown that once tolerance to a graft is established (4 weeks after stopping mAb therapy) tolerant T cells can be adoptively transferred into naïve hosts and induce specific tolerance to a second graft (Waldman and Cobbald, 1993). This has been termed infectious tolerance. There is also increasing evidence that a specialised group of regulatory T cells (T reg) may be responsible for downregulation of immune responses to both foreign and self antigens. Experiments investigating the prevention of a detrimental Th1 response to enteric foreign antigens in mice have indicated that T reg cells may be $CD4^+$ $CD45Rb^{low}$ and induced by transforming growth factor beta (TGFβ) (Powrie et al, 1993 and 1994). Regulatory CD8 T cells have also been described. Interestingly, these cells appear to be TCRγ/δ positive T cells as oral tolerance is abrogated in mice lacking γ/δ T cells (Ke et al, 1996). It has also been demonstrated that protection from diabetes induced by insulin aerosols can be transferred by adoptive transfer of γ/δ T cells from diabetes resistant NOD mice to syngeneic

hosts (Harrison et al, 1996). This observation is of particular relevance to the work of this thesis since R73 mAb only binds to α/β T cells and γ/δ T cells would therefore be spared from the depletion caused by R73 treatment. It is therefore possible, though not tested in this thesis, that the R73 resistant regulatory T cells responsible for indefinite graft survival in this model are in fact γ/δ T cells. Another potential population of regulatory cells are those CD4+ T cells which constitutively express CD25 (IL-2Rα). Congenital absence of these cells results in the development of autoimmune diseases in mice and it may be that failure to deplete these cells completely with R73 mAb results in downregulation of the alloimmune response (Hunig and Schimpl, 1997).

It has been demonstrated that CD4 T cells but not CD8 T cells are required for rejection of an allograft (Gracie et al, 1990, Bolton et al, 1989). In the experiments described above, CD4 T cells appear to be preferentially depleted at day 7 post transplant and this prolongation of CD4 depletion may contribute to prolongation of graft survival.

It is interesting to note that R73 treatment resulted in significantly increased T cell depletion in peripheral blood compared to lymph nodes. This may be due to impaired R73 access into the lymph nodes compared to peripheral blood or perhaps R73 activity is more pronounced against circulating cells compared to the more sessile LNC. It is also possible that LNC contain a population of R73 resistant T cells or indeed R73 induced regulatory cells.

Although analysis of LNC and peripheral blood allows detailed investigation of the immunological effects of R73 treatment, it is essential to determine the effects of R73 treatment on the cell populations within the heart graft. Analysis of the cellular infiltration of the grafts themselves at day 7 was therefore carried out using an immunoperoxidase technique. This revealed a reduction in CD4 and CD8 T cell infiltration compared to rejecting grafts and also reduced macrophage infiltration and IL-2R expression. These findings can be explained by the reduction in circulating T cells seen at this time point which would normally be available for recruitment in to the graft. These observations are comparable to those reported by Heidecke and colleagues in their model of cardiac transplantation in sensitised animals (Heidecke et al, 1995).

The results described in this chapter imply that indefinite graft survival induced by R73 mAb is due at least in part to peripheral T cell depletion and subsequent reduction in graft infiltration by effector cells, however other peripheral mechanisms particularly T cell suppression, depletion of alloreactive T cells and T cell anergy are also likely to play an important role in prolonging graft survival.

4:8 Summary

4:2:1 Flow cytometric analysis of T cells expressing the rat pan T cell marker CD5 (detected by MRC OX19) reveals transient depletion of T cells to one third of normal in the lymph nodes of R73 treated DA recipients of a Lewis heart allograft at days 4 and 7 post transplant. R73 mAb is significantly more effective in depleting T cells in the peripheral blood than in the lymph nodes.

4:2:2 CD4 +ve T cells are also depleted to a similar extent in the lymph nodes and peripheral blood of R73 treated animals with recovery to 50% of normal by day 7 post transplant and recovery to normal levels by day 21.

4:2:3 CD8 +ve T cells in the peripheral blood and lymph nodes are transiently depleted with reconstitution to normal numbers by day 21 post transplant. The recovery of CD8 T cells appears to be marginally quicker than CD4 T cells.

4:3 R73 mAb treatment results not only in transient T cell depletion but also in modulation of the α/β T cell receptor and so may impair TCR ligation with MHC/peptide on donor APC.

4:4 R73 mAb is bound to 81% of peripheral T cells at day 4 post transplantation, however by day 7, only 50% of cells have detectable bound

R73. In addition at day 4 all available TCR binding sites are occupied by R73 but by day 7 the majority of TCR binding sites are available for MHC ligation.

4:5 Delaying heart transplantation by 7 days after completion of R73 treatment when there is only 50% T cell depletion and the majority of TCR binding sites are unoccupied by antibody still results in indefinite graft survival in the majority of treated animals suggesting that depletion is not the only mechanism responsible for indefinite graft survival in this model.

4:6 Immunohistochemical analysis of heart grafts from R73 treated recipients and rejecting controls at day 7 after transplant shows a marked reduction in cellular infiltrate in grafts from treated animals

CHAPTER FIVE

In Vitro Characterisation of Rat Lymphocytes after R73 Treatment

5:1 Introduction

The results described in the previous chapters show that pre graft treatment of recipient DA rats with R73 mAb results in indefinite cardiac allograft survival in the majority of animals and that this is associated with marked but incomplete CD4 and CD8 T-cell depletion in the early post transplant phase. However, T cell populations begin to recover by day 7 and are almost completely restored by day 21 without the loss of the graft suggesting that the nature of the T-cell response to alloantigen has been altered. The observation that thymectomy does not abrogate the graft acceptance implies that the peripheral T-cell pool is subject to some form of immune modulation. The Th1/Th2 polarisation of CD4 helper T cells has been discussed in the introductory chapter of this thesis. T helper cells producing predominantly IL-2 and IFNγ are termed Th1 cells and are typically responsible for the promotion of cell mediated immune responses, while Th2 cells produce IL-4 and IL-10 and IL-13 and are predominantly responsible for humoral immunity. IL-2 production by graft infiltrating cells is reduced in animals tolerant to renal allografts after allogeneic blood transfusion and administration of exogenous, recombinant IL-2 abrogates tolerance in this model (Dallman et al, 1991). Other strategies for induction of transplant tolerance such as anti-CD4 treatment, and selective coreceptor blockade using CTLA4Ig have resulted in the predominance of Th2 type cytokines in the grafts of tolerant animals (Nickerson et al, 1993, Sayegh et al, 1995, Mottram et al, 1995). These

experiments have led to the hypothesis that graft tolerance is associated with polarisation towards a Th2 type response and rejection is associated with a predominance of Th1 cells. Although polarisation towards a Th1 or Th2 cytokine profile has been proposed as mechanism for peripheral tolerance or rejection respectively several studies have shown that failure to express these cytokines does not inevitably lead to rejection or long term engraftment (Larsen et al, 1996, Steiger et al, 1995).

The model of long term graft acceptance induced by R73 mAb is characterised by marked early depletion of CD4 and CD8 T cells. This depletion is transient, however, and T cell numbers are recovering by day 7 post transplant and return to near normal by day 21 without evidence of rejection of the heart graft. One possible explanation for this would be that the remaining T cells have been polarised towards a Th2 response allowing continued graft acceptance. In the following experiments, this hypothesis is tested by measuring the cytokine production by R73 treated graft recipient LNC in vitro. This was done by further stimulating the DA graft recipient LNC in vitro using irradiated Lewis stimulators and estimating the proliferative response of these cells and the cytokine production in the cell culture supernatants. Evidence for differential expression of cytokine genes in non rejecting (R73 treated) compared to rejecting (untreated) heart allografts was sought by semiquantitively measuring cytokine messenger RNA in heart grafts using RT-PCR.

5:2 In vitro proliferation of R73 treated graft recipient LNC

DA rat LNC from R73 treated graft recipients, unmodified graft recipients and normal ungrafted animals were isolated as described in Chapter 2 (materials and methods). The cells were harvested from treated animals on days 4, 7, 21 and 100 after receiving a Lewis heart allograft. These cells were then cultured in 96 well plates with irradiated spleen cells from normal Lewis rats and 3rd party PVG rats as stimulators and with the mitogen concanavalin A (conA) as a positive control. Cells were harvested 72, 96 and 120 hours after incubation and proliferation determined by (^{3}H) thymidine incorporation as previously described in Chapter 2 (materials and methods). All experiments were carried out at least three times except at the day 21 time point when they were carried out twice. Proliferation data are expressed as the means and standard deviations of peak proliferation in the multiple experiments for each group of animals. Proliferation is expressed as a proliferation index (PI) which is the ^{3}H thymidine incorporation in the lymph node cells in counts per minute (CPM) in responder LNC proliferating under experimental conditions divided by the CPM of responder LNC cultured in medium alone. Proliferation was expressed in this form to facilitate easy comparison between experimental groups. Cell culture supernatants from duplicate proliferation experiments were removed at 72 hours post stimulation for analysis of IL-2 and IFNγ production by bioassay. Data were analysed using the Mann-Whitney U test where appropriate comparing treated groups with naïve animals.

Figure 5.1 illustrates that LNC from R73 treated graft recipients harvested on days 4 post transplant showed significantly reduced proliferation to donor strain Lewis (P=0.01), 3rd party PVG stimulators (P=0.05) and conA (P= 0.001) compared to normal DA rats and unmodified rejecting controls. LNC removed on days 7, 21 and 100 post transplant from R73 treated graft recipients proliferated normally to each of the stimulators compared to normal DA and rejecting controls.

5:3 In vitro cytokine production by LNC from R73 treated graft recipients

Supernatants were harvested from allogeneic MLR at 72 hours post incubation and bioassay carried out to determine IL-2 production and capture ELISA used to measure IFN-γ production by in vivo R73 treated LNC in response to further allogeneic challenge by irradiated Lewis stimulators. The results are illustrated in Figure 5.2. IL-2 production by R73 treated responders is significantly reduced at days 4 (P= 0.008) and 7 (P= 0.002) post transplant but is once again detected at days 21 and 100 though the levels found at day 100 are still significantly reduced compared to normal untreated DA rats (P= 0.001). IFN-γ production was detected at similar levels at each time point by both R73 treated and normal untreated responders.

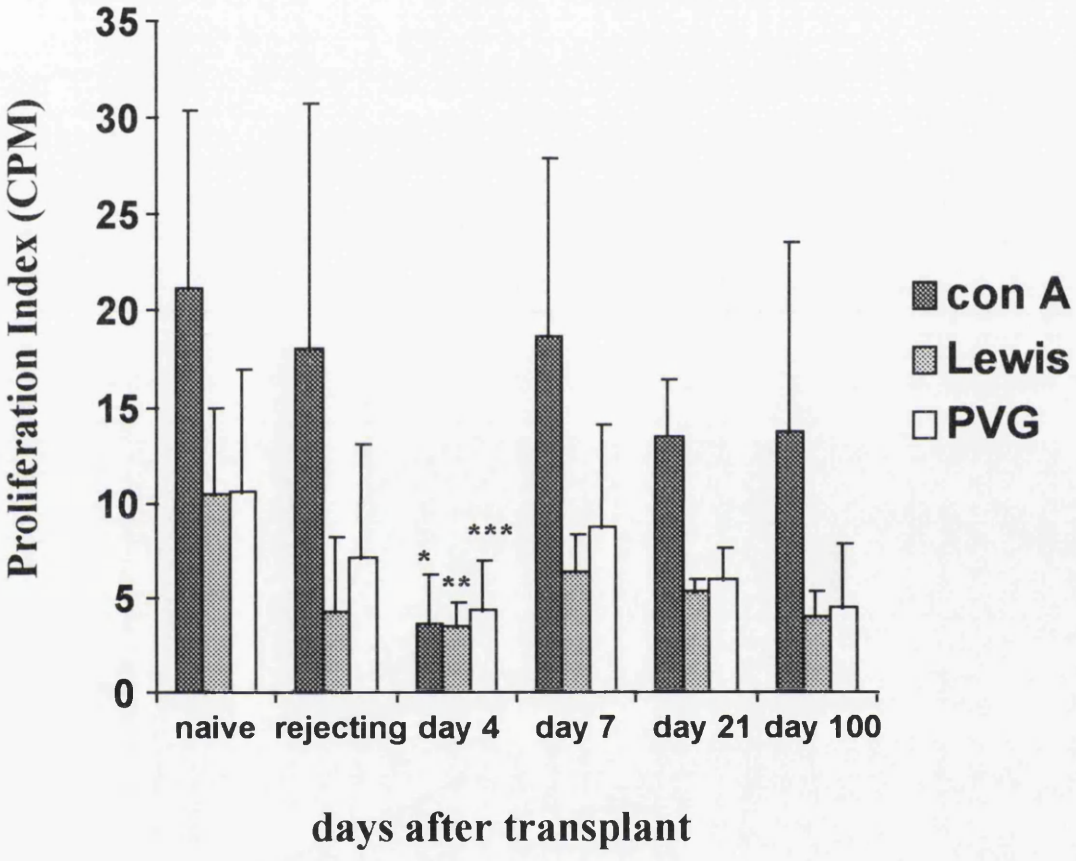


Figure 5.1 In vitro proliferation of R73 treated graft recipient LNC. DA lymph node cells from R73 treated graft recipients were isolated at various time points and MLR performed using irradiated splenocytes as stimulators from donor strain Lewis and 3rd party PVG rats and the and results expresed as an index of background proliferation (PI). R73 treated animals at day 4 show significantly reduced proliferation to both donor and 3rd party stimulators but at day 7 R73 treated cells are able to proliferate normally to donor specific, 3rd party and mitogenic stimulation. (* P= 0.001, ** P= 0.01. *** P= 0.05, Mann-Whitney U test compared to naïve animals)

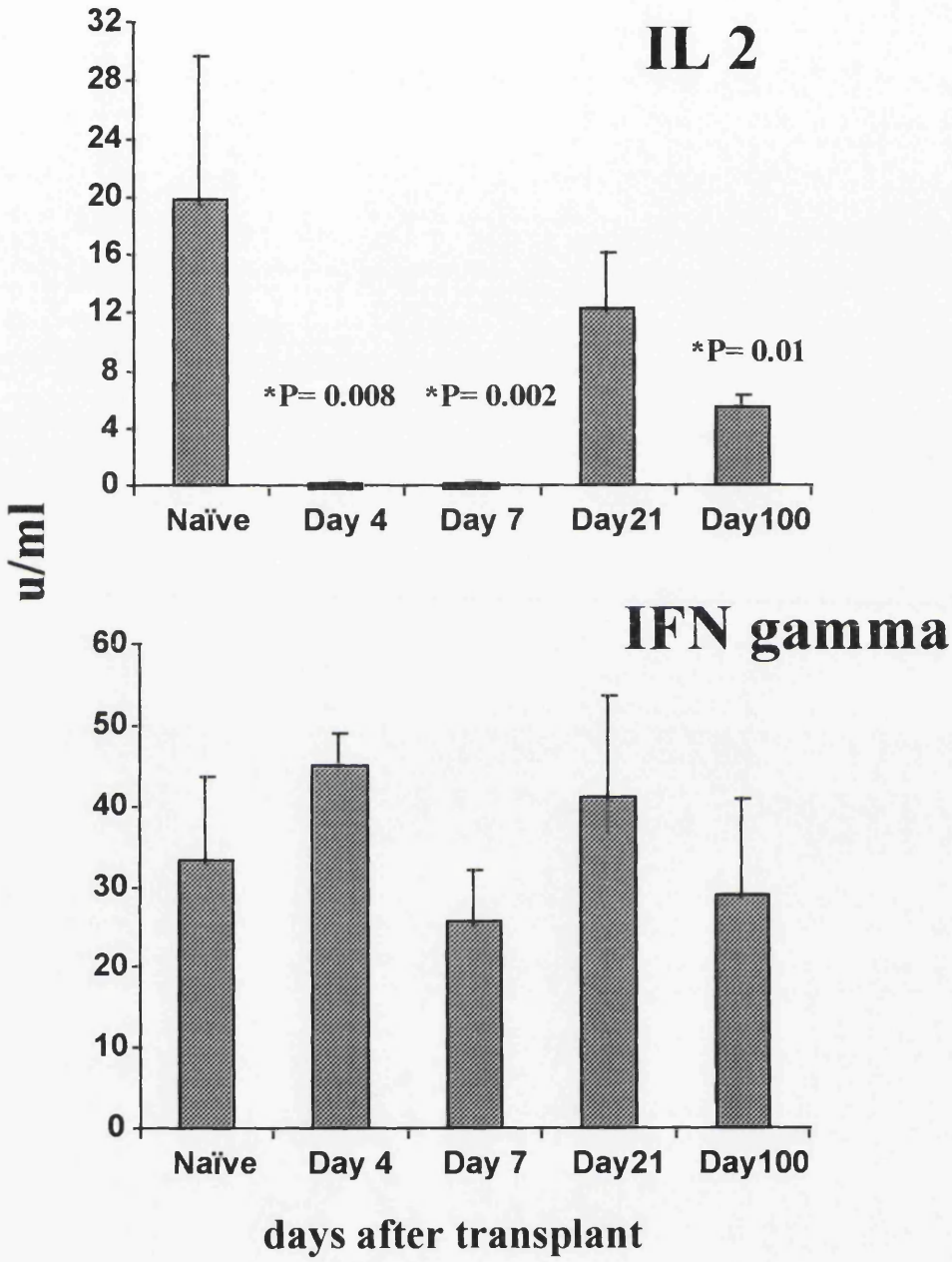


Figure 5.2 In vitro production of the Th1 cytokines IL2 and IFNgamma. Supernatents were isolated from MLR at 72 hours and IL-2 and IFN gamma production by LNC from R73 treated graft recipients and naïve DA rats detected by bioassay. LNC from day 4 and day 7 treated graft recipients appear unable to produce IL-2 in response to further allogeneic stimulation in vitro and IL-2 production remains suppressed even at day 100 after grafting. LNC retain their ability to produce IFN gamma at all time points.* Mann-Whitney U test.

These data suggest firstly that LNC from R73 treated graft recipients do not produce IL-2 in response to further antigenic challenge. This may be due to depletion of alloreactive T cells or the T cells present in the MLR are able to proliferate but incapable of producing IL-2. This theory is supported by the fact that IL-2 production is impaired even at days 21 and 100 post transplant. Another possible explanation is polarisation towards a Th2 response, however we could not assess the presence of IL-4 and IL-10 in these supernatants due to the lack of a reliable bioassay for these cytokines in the rat.

Lymph node cells from R73 treated animals produced IFNγ in quantities comparable with normal DA and rejecting LNC. There are several possible explanations for this observation. Functional NK cells capable of IFNγ production may predominate in the lymph nodes of R73 treated animals at the first 4 days after transplantation, however LNC from R73 treated graft recipients are capable of producing IFNγ at day 100 post transplant indicating that the T cells responsible for maintenance of tolerance in this model may have a Th0 phenotype.

5:4 Cytokine gene transcripts in heart grafts from R73 treated graft recipients

The analysis of cytokine production by R73 treated animals shown above reflects the characteristics of the lymph node cells isolated from these animals. It may be, however, that the cytokine production at the site of antigen

recognition, i.e. the graft itself may be more relevant. In addition bioassays can be very non specific and interpretation of the results should be cautious.

We therefore attempted to analyse cytokine production in the graft itself using semiquantitative RT-PCR. This technique lends itself well to the localised detection of the induction of cytokine genes and has been used in transplant models to outline the pattern of cytokine production in allogeneic and syngeneic heart transplantation in mice (Dallman et al, 1991). In this experiment Lewis heart grafts were removed from R73 treated DA recipients on days 4, 7, 21 and 100 after transplantation and on days 4 and 7 from rejecting control animals. Normal Lewis hearts were analysed for comparison. Hearts were divided and immediately snap frozen in liquid nitrogen until RT-PCR analysis (materials and methods). Data are expressed as the average number of PCR cycles at which mRNA was detected for each time point. Statistical comparison was carried out using the Mann-Whitney U test.

5:4:1 IL2 and IFNγ cytokine message in heart grafts from R73 treated DA recipients.

The results of the analysis for the Th1 cytokines IL-2 and IFNγ are shown in figure 5.3. There is significantly increased expression of IL-2 gene (P= 0.0005) and IFNγ gene (P= 0.004) in the grafts of rejecting animals at day 4 post transplant in comparison to normal hearts and those from R73 treated animals.

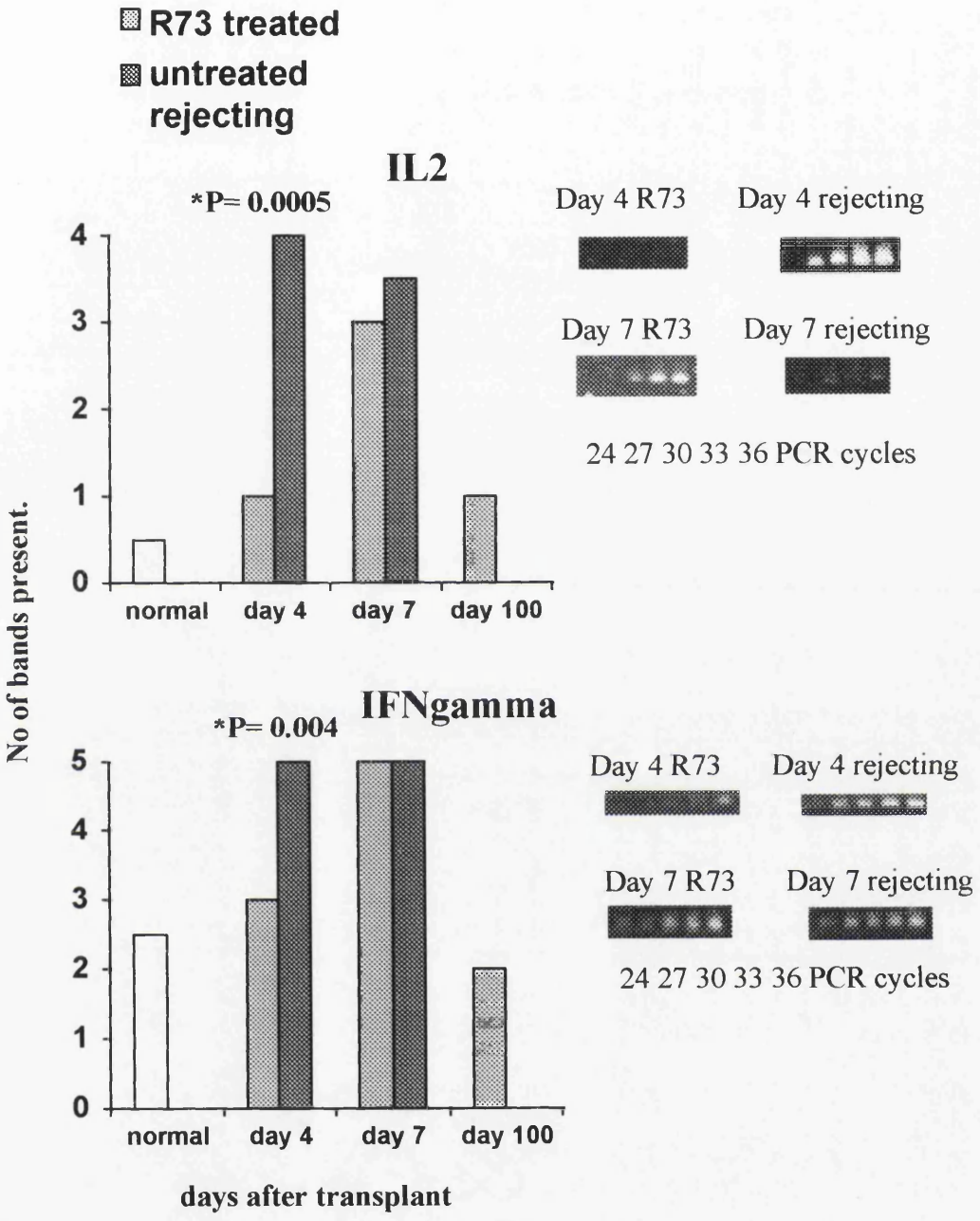


Figure 5.3 IL2 and IFN gamma cytokine mRNA in heart grafts from R73 treated DA recipients. Heart grafts were removed from R73 treated recipients and unmodified control animals rejecting their grafts at days 4, 7 and 100 after transplant and cytokine mRNA detected by semiquantitative PCR. IL2 and IFNgamma expression is significantly increased in the grafts of animals rejecting their grafts at day 4 compared to R73 treated animals. IL2 and IFNgamma production is increased in both treated and rejecting animals at day 7 and return to normal levels by day 100. * Mann-Whitney U test compared to R73 treated and normal animals.

At day 7, IL-2 and IFNγ gene expression in R73 treated and rejecting grafts is elevated compared to normal Lewis hearts. By day 100 post transplant, IL-2 and IFNγ expression have returned to baseline levels.

5:4:2 IL4, IL10 and IL13 cytokine message in heart grafts from R73 treated graft recipients.

Figure 5.4 shows the results of PCR detected mRNA for the Th2 cytokines IL-4, IL-10 and IL-13 in heart grafts from R73 treated recipients. Message for each of these cytokines was detectable at high levels in all experimental groups including normal Lewis hearts using this technique. There was no significant difference between R73 treated DA graft recipients and normal or rejecting animals.

5:5 Expression of IL-2 receptor subunits in heart grafts from R73 treated graft recipients.

The IL-2 receptor is a 15.5 kDa glycoprotein consisting of three subunits distinguishable by their affinity for ligand- IL-2Rα. IL-2Rβ and IL-2Rγ. Upregulation of the IL-2 receptor is a critical step in the activation of alloreactive T cells and impaired expression of this receptor or its subunits by T cells has been shown to result in T cell anergy and tolerance to alloantigen (Dallman et al, 1991).

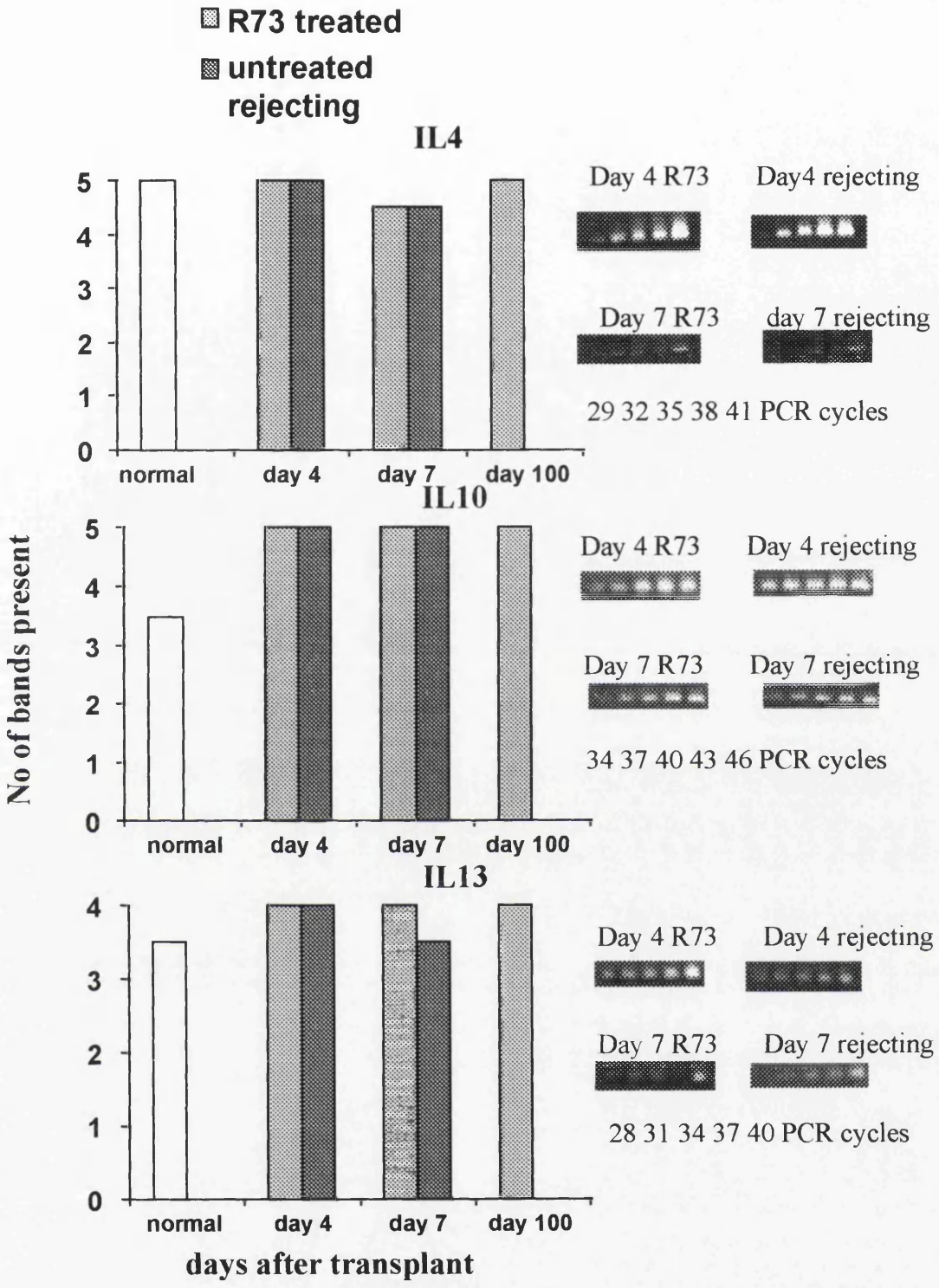


Figure 5.4 IL4, IL10 and IL13 cytokine mRNA in heart grafts from R73 treated recipients. Heart grafts were removed from R73 treated recipients and unmodified control animals rejecting their grafts at days 4, 7 and 100 after transplant and cytokine mRNA detected by semiquantitative PCR. IL4, IL10 and IL 13 message in both treated and rejecting animals was detected in all groups and was comparable to that found in normal Lewis hearts.

We assessed expression of IL 2Rα and β mRNA in R73 treated and rejecting heart grafts at days 4, 7 and 100 post transplant using RT PCR. Hearts were prepared as for PCR cytokine analysis and PCR methods were as above.

Significant upregulation of both alpha and beta IL-2 receptor subunits was seen in rejecting grafts at day 4 post transplant (P= 0.01) consistent with activation of allogeneic T cells and at day 7 (P= 0.03) in the R73 treated grafts. There was no evidence of increased IL-2R gene expression at days 4 and 100 post transplant in the R73 treated grafts compared to normal animals (figure 5.5).

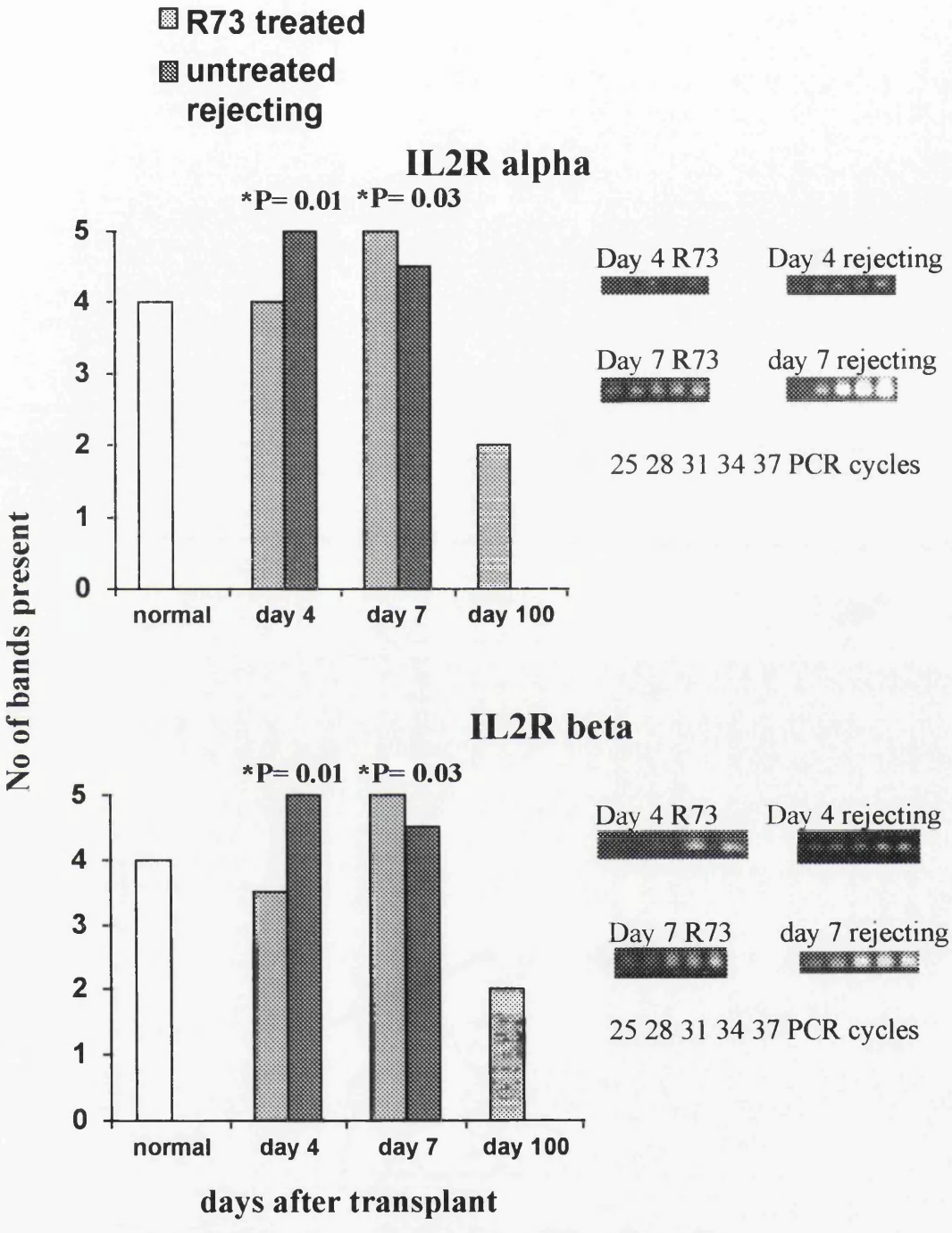


Figure 5.5 Expression of IL2 receptor subunits in heart grafts from R73 treated graft recipients . Heart grafts were removed from R73 treated recipients and unmodified control animals rejecting their grafts at days 4, 7 and 100 after transplant and IL2R mRNA detected by semiquantitative PCR. Grafts from R73 treated and unmodified rejecting rats show significant upregulation of IL2 receptor subunits compared to normal Lewis hearts at day 7 post transplant. * Mann-Whitney U test.

5:6 Discussion

The results presented in chapters three and four of this thesis demonstrate that permanent heart graft survival can be obtained using a pre transplant R73 treatment regime in the Lewis-DA rat strain combination. This is associated with transient and incomplete peripheral T cell depletion. The experiments carried out in this chapter attempt to define whether those cells which remain after R73 treatment play a role in perpetuating continued tolerance to the graft. Ligation of the T cell receptor with allogeneic MHC+peptide along with coreceptor interaction results in proliferation of CD4 and CD8 T cells. The first set of experiments described in this chapter show that LNC removed from R73 treated graft recipients at day 4 post transplant proliferate poorly in MLR when stimulated by irradiated allogeneic and 3rd party stimulators. LNC removed on days 7, 21 and 100 proliferate normally. These observations can be explained firstly on the basis of the profound T cell depletion seen at day 4. Since the MLR experiments were carried out using unseparated LNC responders, responder cells at day 4 will be mainly B cells and NK cells which proliferate poorly to allogeneic stimulation in the absence of T cell help. In addition, the smaller proportion of lymphocytes present in the MLR may result in a shift in peak proliferation outwith the time points measured. The results at days 7, 21 and 100 reflect the T cell recovery seen on flow cytometric analysis along with absence of R73 mAb at these time points. It may seem paradoxical that T cells are tolerant of a Lewis heart allograft in vivo yet proliferate to donor specific allogeneic stimulation in vitro, however the LNC have been removed from a

tolerogenic environment and perhaps there are peripheral, circulating "regulatory" or "suppressor" T cells induced by transplantation under cover of R73 that maintain the graft but fail to influence LNC in vitro. Tsuchida and colleagues have noted in their model of R73 induced graft tolerance that T cells from R73 treated graft recipients proliferate in MLR when stimulated by allogeneic heart cells but not to allogeneic or third party spleen cells. Their explanation for this finding was that expression of certain peptides is tissue specific and that tolerance may only be induced to heart specific antigens plus MHC (Tsuchida et al, 1994).

The use of tolerising therapies has been shown in various experimental models to result in immune deviation towards a Th2 type response. However, some experiments have shown that the presence of IL-4 in the absence of IL-2 and IFNγ does not necessarily lead to graft tolerance. As an explanation for these observations, it has been suggested that all T cell growth factors can, in high concentration stimulate a rejection response and as a result the cytokines IL-2 and IL-4 both present barriers to tolerance with IL-4 a weak barrier and IL-2 a strong barrier (Strom et al, 1996).

Patterns of specific cytokine production by R73 treated graft recipients were therefore investigated in this chapter by assay of IL-2 and IFNγ production by restimulated LNC in allogeneic MLR and by detection of cytokine mRNA in the heart grafts. In this R73 model, lymphocytes from animals receiving R73 therapy do not produce IL-2 after further allogeneic stimulation in vitro but are able to produce IFNγ normally. On examination of cytokine gene expression in

the allografts themselves by RT-PCR, we found that IL-2 and IFNγ message could be detected in hearts from R73 treated animals though in lower amounts than in grafts undergoing rejection at similar time points. We also detected IL-4, IL-10 and IL-13 message in both rejecting and R73 treated grafts at all time points. It must be stressed, however that the results of PCR experiments such as these are at best semiquantitative and that mRNA levels may not accurately predict levels of biologically active cytokine. This is because cytokine genes are subject to post transcriptional regulation and therefore gene expression does not necessarily correlate with protein production. It is therefore possible that IL-2 production within the graft is reduced due to post transcriptional regulation of the IL-2 gene, perhaps as a result of R73 mAb therapy. It has been suggested that this may occur due to cross linking of TCR molecules by R73 prior to encountering alloantigen and subsequent induction of an anergic state (Heidecke et al, 1996).

A critical step in T cell activation is the upregulation of the IL-2 receptor (IL-2R) and as a result this receptor has become a target for immunotherapy in transplantation (Waldmann, T, 1993, Waldmann and O'Shea, 1998). Messenger RNA for IL-2Rα and β subunits was detectable by RT-PCR in both R73 treated and rejecting grafts in our experiments with higher expression at day 4 in rejecting grafts and similar levels at day 7. Taking these results in conjunction with the above cytokine profiles and the results from immunohistochemical analysis of graft infiltrates in chapter 4, it is possible that although transcription of the IL-2R gene takes place, expression of IL-2R

protein may be prevented perhaps through altered signalling through the T cell receptor induced by R73.

Bearing these points in mind, it appears that R73 mAb treatment of heart graft recipients results in the production of T cells incapable of normal levels of IL-2 production despite the presence of IL-2 mRNA in the heart grafts. These cells are capable of IFNγ production and the graft infiltrating cells express the Th2 cytokine genes for IL4, IL10 and IL13. This suggests that the barrier to tolerance induction by IL-2 is removed either by polarisation of CD4 helper T cells towards a Th2 type response or by the induction of anergy in potentially alloreactive T cells leaving the relatively weak barrier of IL-4 to the induction of allograft tolerance. Anti CD4 treatment protocols in rodents have in some cases resulted in similar patterns of immune deviation (Binder et al, 1996, Mottram et al, 1995), however other anti-CD4 models have shown no evidence of Th1/Th2 shift (Jaques et al, 1998). Strategies which block coreceptor interaction, particularly B7-CD28 using CTLA4Ig, have resulted in inhibition of Th1 cytokines with sparing of Th2 cytokines (Sayegh et al, 1995). Interestingly, recent studies using Fc receptor non binding anti-CD3 monoclonal antibodies have reported suppression of allograft rejection associated with cytokine deviation and Th1 cell inactivation (Smith and Bluestone, 1997). The authors of this report suggest that the action of this anti-CD3 fusion protein is due to partial signalling through the T cell receptor resulting from partial phosphorylation of the CD3 ζ chain and subsequently

incomplete phosphorylation of ZAP-70. Studies using altered peptide ligands (APLs) and co-receptor blockade to provide sub optimal antigenic ligand for the TCR have also resulted in partial T cell signalling, rendering T cells unresponsive or anergic (Sloan-Lancaster, et al, 1993 and 1994, Madrenas et al, 1995). One could therefore propose that R73, anti-TCR mAb exerts its effect, at least in part, by binding to the TCR and inducing a partial signal through the T cell receptor resulting in cytokine deviation and Th1 cell inactivation.

5:7 Summary

5:2 LNC from R73 treated graft recipients harvested 4 days after transplantation proliferate poorly in vitro to both allogeneic and 3rd party stimulation. LNC's harvested R73 treated graft recipients on days 7, 21 and 100 however are able to proliferate normally in MLR when stimulated by allogeneic and 3rd party stimulators.

5:3 LNC from R73 treated graft recipients are unable to produce IL-2 when re stimulated in MLR at day 4 after transplant and IL-2 production is impaired on days 7, 21 and 100 after transplant. IFNγ production is not impaired after R73 treatment.

5:4 Semiquantitative PCR analysis of cytokine mRNA transcripts in heart grafts from R73 treated and rejecting animals shows increased mRNA for IL-2 and IFNγ in rejecting animals compared to R73 treated and normal Lewis hearts. Transcripts for IL-2Rα and β were also increased in rejecting grafts. Messenger RNA for IL-4, IL-10 and IL-13 was detected in similar amounts in all groups.

CHAPTER SIX

Apoptosis in lymph nodes and heart grafts after R73 treatment

6:1 Introduction

Since the discovery of apoptosis in 1972 a great deal of research has been carried out to elucidate its role in several biological and pathological processes including transplantation, oncology, HIV and autoimmunity. Apoptosis is a mode of cell death in which the cell undergoes a distinctive pattern of morphological and molecular changes activated by the cell itself. The cell rapidly dehydrates forming a tight sphere with a convoluted membrane. Chromatin condensation occurs at the periphery of the nucleus followed by disruption of the nuclear membrane. Endonucleases then clip chromosomes at 180 base intervals resulting in DNA fragments of oligonucleosomal length (Arends et al, 1990). The nuclear fragments and cytoplasmic contents are then packaged into apoptotic bodies surrounded by plasma membrane and phagocytosed by surrounding cells. During apoptosis there is upregulation of certain genes which appear to be required for the process to occur as well as increased protease activity (Wyllie et al, 1984). Apoptotic cells can be detected histologically and on electron microscopy by their characteristic appearance and hyperchromicity and the DNA 3' ends can be labelled with a biotinylated thymidine analogue (TUNEL). The DNA fragments stain well with fluorochromes such as propidium iodide (PI) and can be detected by flow cytometry. The products of DNA degradation are of the size of nucleosomal and oligonucleosomal fragments and so form a characteristic ladder pattern on gel electrophoresis.

Since its first description in 1972 (Kerr et al, 1972) the process of apoptosis has been extensively investigated particularly in the field of embryology where it has been found to be essential for normal development (Sulston and Horvitz, 1977). In the immune system, apoptosis plays an important physiological role in limiting the size and duration of the primary T cell immune response and in negative selection of autoreactive T cells in the thymus (Surh and Sprent, 1994). In addition it has been suggested that CD4 effector T cells kill their targets by inducing apoptosis (Hanabuchi et al, 1994, Kagi et al, 1994).

The role of apoptosis in acute allograft rejection is at best unclear. Bergese and colleges found that the pattern of apoptosis in rejecting murine allografts was inconsistent with parenchymal tissue damage as high levels were found in both rejecting and accepted grafts (Bergese et al, 1997). It has been suggested that apoptosis is associated with spontaneously accepted liver grafts in mice and may be responsible for both the elimination of alloantigen expressing donor cells during rejection and the elimination of cytotoxic T cell clones from the graft in a model of rat liver and small bowel transplantation (Meyer et al, 1998). In addition, it is thought that apoptosis conveys immunological privilege to certain sites in the body such as the testes and the eye (Bellgrau et al, 1995).

It has recently been documented that several receptor molecules may be responsible for initiating apoptosis in T cells and their targets. The TCR/CD3

complex, CD95 (Fas or Apo-1), Thy-1, CD2 and MHC class I have all been implicated in triggering programmed cell death in T cells and the specific conditions required for this to take place are being extensively investigated (Itoh, et al, 1991, Ju et al, 1994, Rouleau et al, 1997, Woodle et al, 1997, Wu et al, 1997). Immature T cells in the thymus undergo apoptosis on ligation of their TCR whereas mature, resting T cells undergo proliferation and produce IL-2 on TCR ligation. Interestingly, it has been noted that mature activated T cells are susceptible to TCR mediated apoptosis and it has been proposed that this completes a feedback loop whereby antigen stimulation induces T lymphocyte activation and growth factor production, growth factors stimulate cell cycle progression which in turn predisposes to TCR mediated cell death with strong TCR engagement activating the apoptotic process (Van Parijs et al, 1996, Cohen et al, 1996).

In our model of graft acceptance using R73 anti-TCR mAb treatment, the possibility exists that TCR ligation of activated lymphocytes by R73 mAb may play a part in the maintenance of tolerance by inducing apoptosis in alloreactive T cells. The experiments described in this chapter quantify apoptosis seen in the lymph nodes and heart grafts of R73 treated DA recipients of Lewis heart grafts.

6:2 Flow cytometric analysis of apoptotic cells in the lymph nodes of R73 treated graft recipients

Quantification of apoptotic cells in heterogeneous tissue such as lymph nodes can be difficult, however flow cytometric analysis using propidium iodide (PI) labelling of DNA strands allows us to compare the proportion of lymph node cells undergoing apoptosis in normal, R73 treated and rejecting animals (Nicoletti et al, 1991, Dive et al, 1992). Briefly, LNC were harvested as previously described and stored at 4°C. Trypsin solution was added to tubes containing 10^6 cells to remove necrotic cells, isolated nuclei and late apoptotic cells followed by trypsin inhibitor and finally the PI solution. Stained cells were then analysed for PI fluorescence on a Coulter EPICS XL flow cytometer.

Figure 6.1 shows the cell cycle histogram obtained in normal DA rats. Cells are shown in the G0/G1, S and G2 phases of the cell cycle with cells displaying hypodiploid levels of fluorescence being regarded as apoptotic. The level of apoptosis is expressed as a percentage of the total number of cells analysed with 20,000 events per analysis. At least 4 animals were used in each group and the results compared using the Mann- Whitney U test.

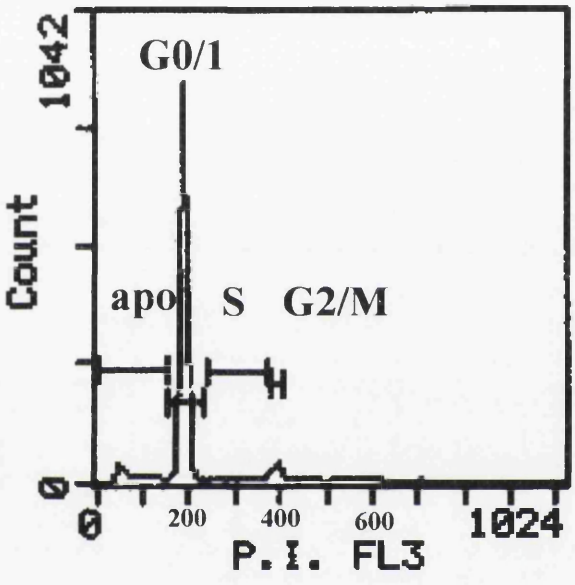


Figure 6.1 Cell cycle histogram showing phases of the cell cycle in normal DA rat LNC. G0/1 peak occurs at channel 200. The G2/M peak is set at 2x the G0/1 peak. S is between G0/1 and G2/M. Sub diploid cells are regarded as apoptotic.

6:2:1 Apoptotic lymph node cells in R73 treated graft recipients at day 4 post transplant

At day 4 after heart grafting, the number of cells undergoing apoptosis in the lymph nodes of R73 treated graft recipients (mean 16.2%, SD 8.3) is slightly higher than that found in animals rejecting their allograft (mean 7.42%, SD 5.7) but this does not reach statistical significance. In addition, similar numbers of apoptotic cells are found in the LNC extracted from normal DA rats (mean 16.6%, SD 10.3) and rats who received R73 but no allograft (mean 10.2%, SD 2.7) (Figures 6.2 and 6.3).

6:2:2 Apoptotic lymph node cells in R73 treated graft recipients at day 7 post transplant

At day 7 post transplant, however, DA rats receiving a Lewis heart allograft and R73 have a significantly higher number of cells undergoing apoptosis in their lymph nodes compared to all other groups of animals (mean 25.9%, SD 8.3, P= 0.01) including DA rats rejecting their Lewis allografts (mean 8.7%, SD 2.7) and R73 treated rats at day4. Interestingly, the apoptosis appears to depend on the presence of a heart graft in addition to R73 treatment since naïve DA rats given the same regime of R73 but no allograft show levels of apoptosis comparable to control, untreated animals. (Figures 6.4 and 6.5).

Table 6.1 compares apoptosis in LNC from R73 treated graft recipients at different time points. Apoptosis peaks at day 7 with levels comparable to

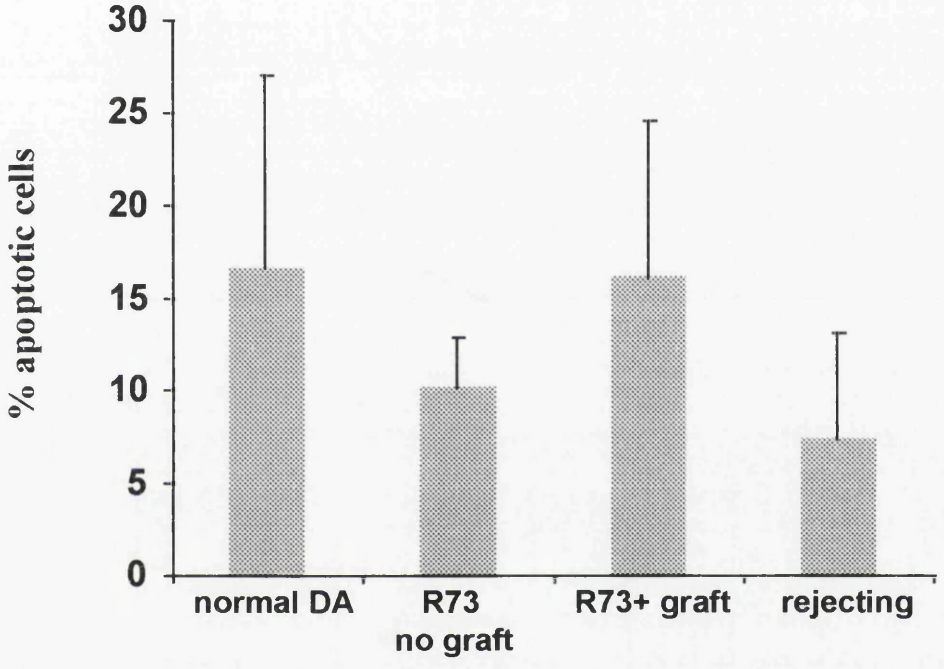


Figure 6.2 Apoptotic lymph node cells in R73 treated graft recipients at day 4 post transplant. Lymph nodes cells were harvested from R73 treated DA recipients of a Lewis heart allograft and subjected to propidium iodide staining FACS cell cycle analysis to assess the proportion of cells undergoing apoptosis. Normal DA rats, DA rats rejecting their Lewis allograft and ungrafted R73 treated animals were used as controls. No significant difference in numbers of apoptotic cells was detected in any of the groups at this time point.

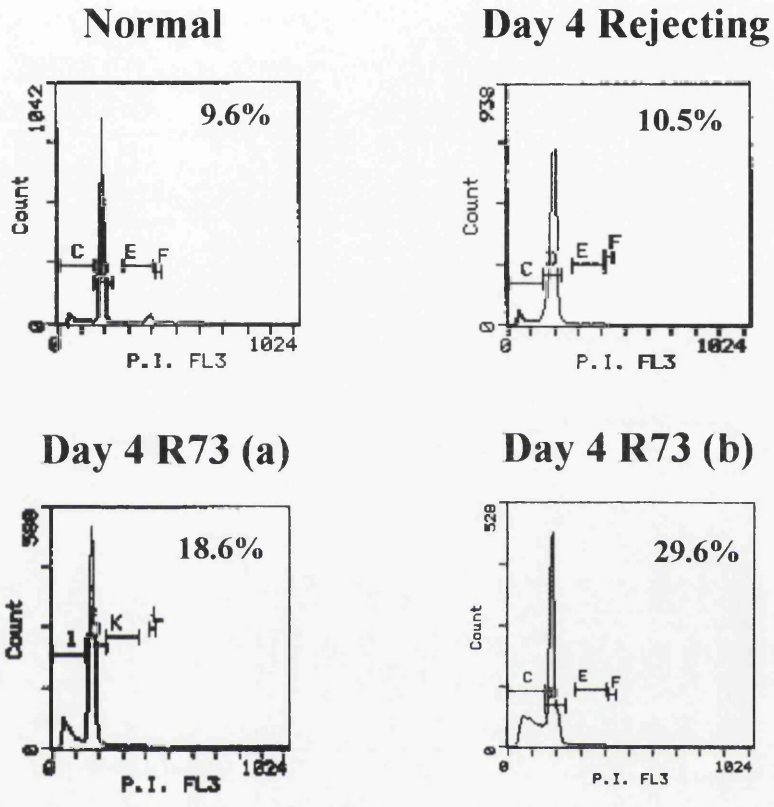


Figure 6.3 Cell cycle histograms showing apoptosis in LNC of R73 treated graft recipients on day 4 post transplant. LNC were stained with propidium iodide and analysed by flow cytometry. Histograms obtained from normal and rejecting DA animals are shown for comparison. Percentages shown are cells in the sub diploid region considered to be apoptotic.

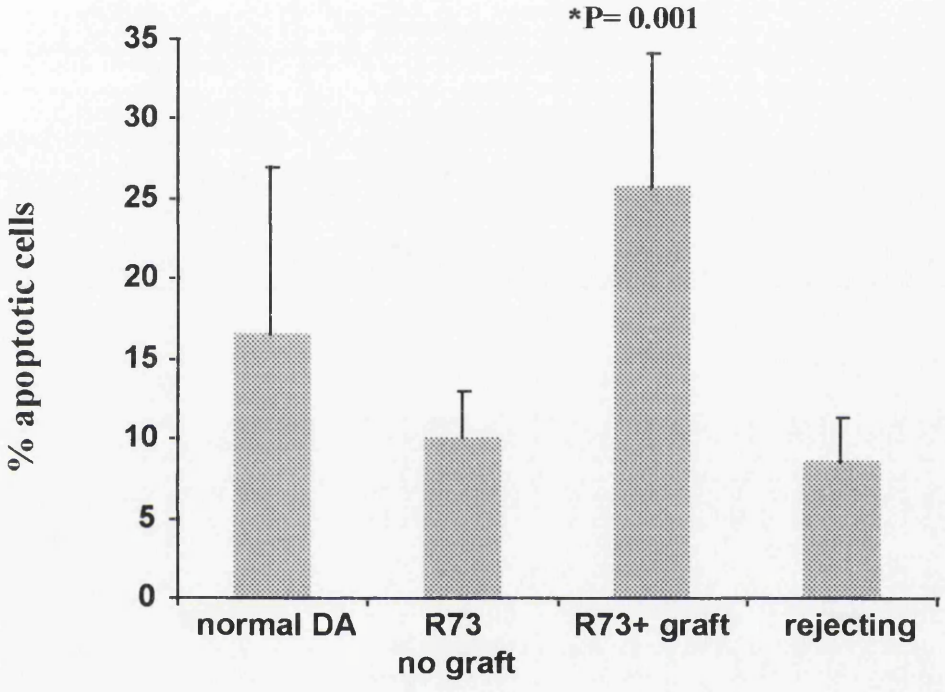


Figure 6.4 Apoptotic lymph node cells in DA rats on day 7 relative to transplant. Lymph nodes cells were harvested from R73 treated DA recipients of a Lewis heart allograft and subjected to propidium iodide staining FACS cell cycle analysis to assess the proportion of cells undergoing apoptosis. LNC from normal DA rats, R73 treated DA rats without a graft and untreated rejecting graft recipients were used as controls. At day 7, apoptosis was highest in R73 treated graft recipients and significantly higher than animals rejecting their grafts at this time point. * Mann- Whitney U test compared to rejecting controls.

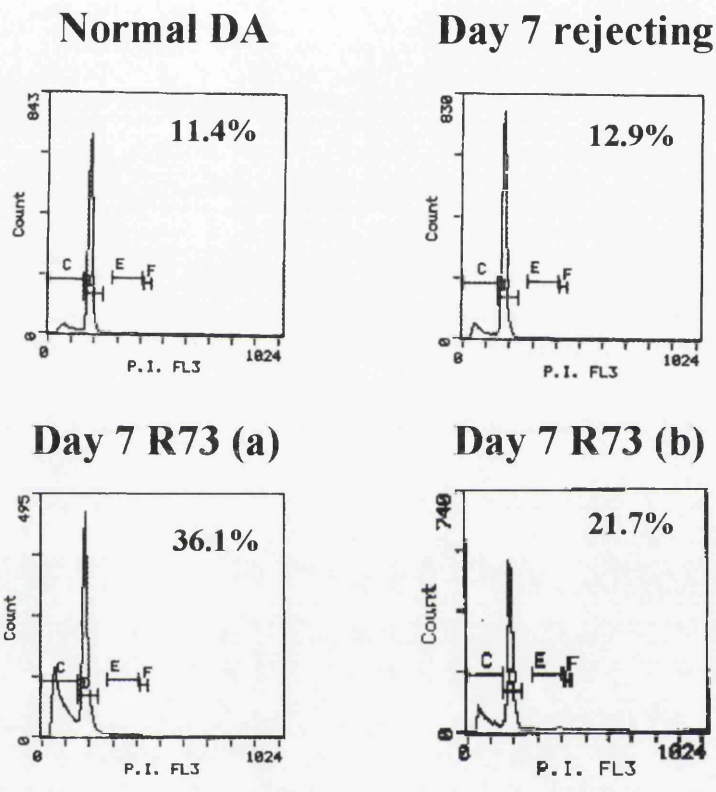


Figure 6.5 Cell cycle histograms showing apoptosis in LNC of R73 treated graft recipients on day 7 post transplant. LNC were stained with propidium iodide and analysed by flow cytometry. Histograms obtained from normal and rejecting DA animals are shown for comparison. Percentages shown are cells in the sub diploid region considered to be apoptotic.

normal DA rats at days 4 and 100 relative to transplant. This suggests that both R73 mAb and alloreactive T cells are required for apoptosis in this model since no antibody is detectable at day 100 and T cell numbers are markedly depleted at day 4.

Day after transplant	**% apoptotic cells**	
normal DA rat	16.6, SD 10.4	NS
day 4 R73 treated	16.2, SD 8.4	NS
day 7 R73 treated	25.8, SD 8.2	P= 0.01*
day 100 R73 treated	17.2, SD 7.0	NS

Table 6.1 Apoptosis in LNC from normal DA rats and R73 treated graft recipients at various time points after grafting detected by flow cytometry. * Mann- Whitney U test

These data suggest that lymphocytes in R73 treated graft recipients are subjected to apoptotic signals in excess of normal and rejecting DA rats and that the presence of alloantigen is required for this process to take place since R73 treated animals with no heart graft exhibit levels of apoptosis comparable to control DA animals.

6:3 Analysis of apoptotic cells in graft infiltrates using Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL) of cryostat sections.

As a result of the DNA fragmentation by endonucleases seen in apoptosis, there are large numbers of DNA strand breaks which can be detected by labelling their 3' ends with digoxygenin conjugated nucleotides in a reaction catalysed by exogenous TdT.

In these experiments cryostat sections of transplanted Lewis hearts from R73 treated and rejecting, untreated DA recipients were stained using TUNEL as described in chapter 2 and compared to sections from normal untransplanted Lewis hearts.

Sections were examined at x400 magnification and apoptosis quantified by counting TUNEL +ve cells in ten consecutive high power fields. The tissue distribution of TUNEL +ve cells was also noted in the sections. The data are expressed as the means and standard deviations of 3 or 4 animals in each group. Statistical analysis was carried out using the Mann- Whitney U test.

Figure 6.6 shows that apoptotic figures were more frequent in rejecting grafts at days 4 (5.8 cells per HPF, SD 2.5) and 7 (8 cells per HPF, SD 1.8) than accepted allografts at both days 4 (3 cell per HPF, SD 1.6) and 7 (4.6 cells per HPF, SD 0.7) after transplant, reaching statistical significance at day 7 (P= 0.03). This data is similar to that found by other groups studying apoptosis in liver and small bowel grafts where maximal apoptosis was found at day 7 in

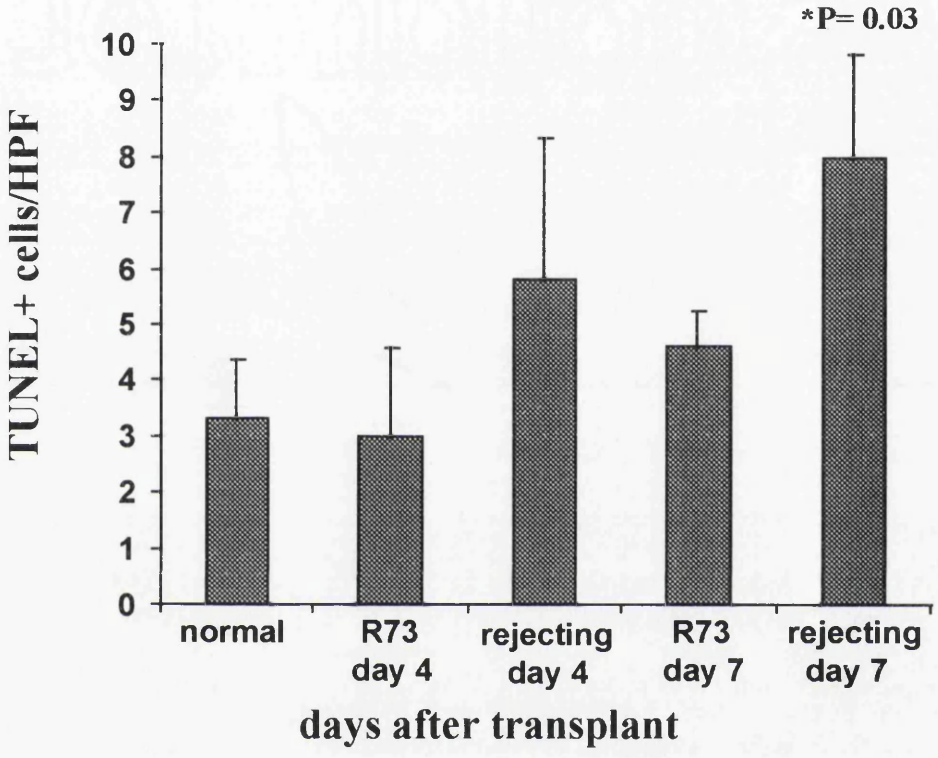
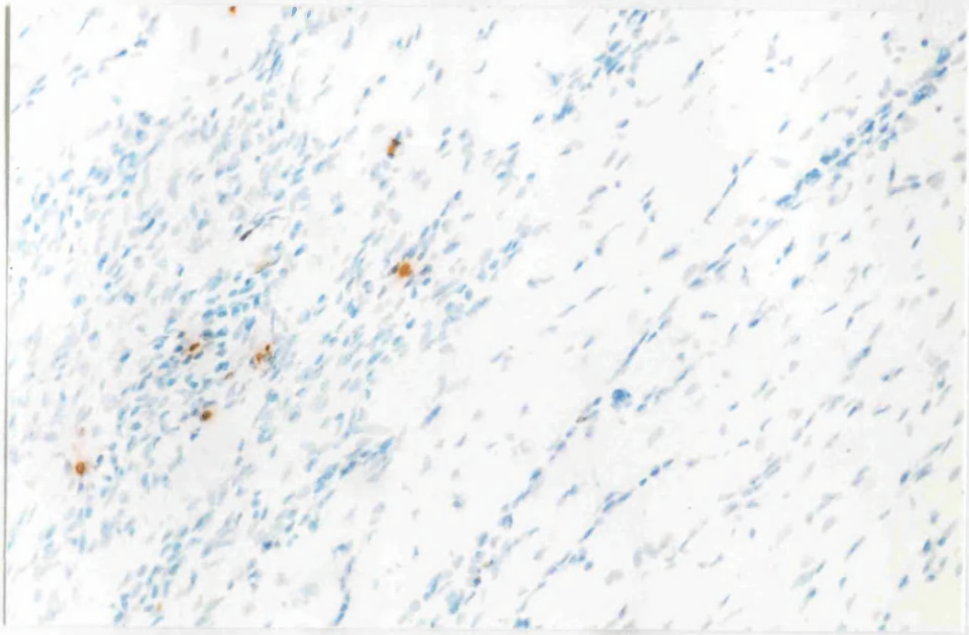


Figure 6.6 Apoptotic cells identified by TUNEL in heart grafts from R73 treated and rejecting animals. Lewis heart grafts were excised from DA recipient rats on days 4 and 7 after transplant and snap frozen in liquid nitrogen. 5u cryostat histological sections were stained using the TUNEL technique and TUNEL+ cells counted and results expressed as the mean + SD of ten consecutivehigh power fields (x400) in 3-4 animals per group. Apoptotic figures weresignificantly more frequent in the rejecting grafts compared to R73 treated at day 7 post transplant. * Mann- Whitney U test, compared to R73 treated animals.

rejecting allografts (Meyer et al, 1998). Other groups studying rejection in mouse heart allografts however, have described peak apoptotic levels at day 3 post transplant with very few figures at day 7 (Bergese et al, 1997).

The tissue distribution of apoptotic cells also differed between rejecting and R73 treated grafts with apoptosis occurring randomly throughout the graft in rejecting animals and confined to cellular infiltrates in R73 treated grafts with virtually no apoptotic figures seen within normal myocardium (Figures 6.7-6.9).

A



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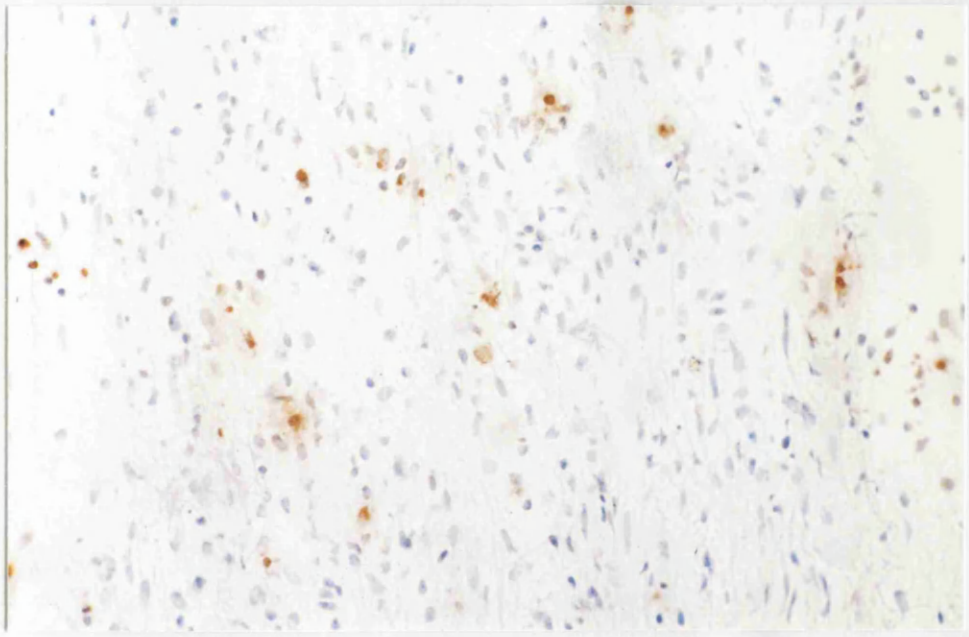

Figure 6.7 Photomicographs of heart allografts removed from (A) an R73 treated recipient at day 7 post transplant and (B) an unmodified rejecting control animal (x250). Apototic cells detected by TUNEL are stained brown. Apoptosis in R73 treated hearts is confined to cellular infiltrates but found throughout the graft in rejecting animals.

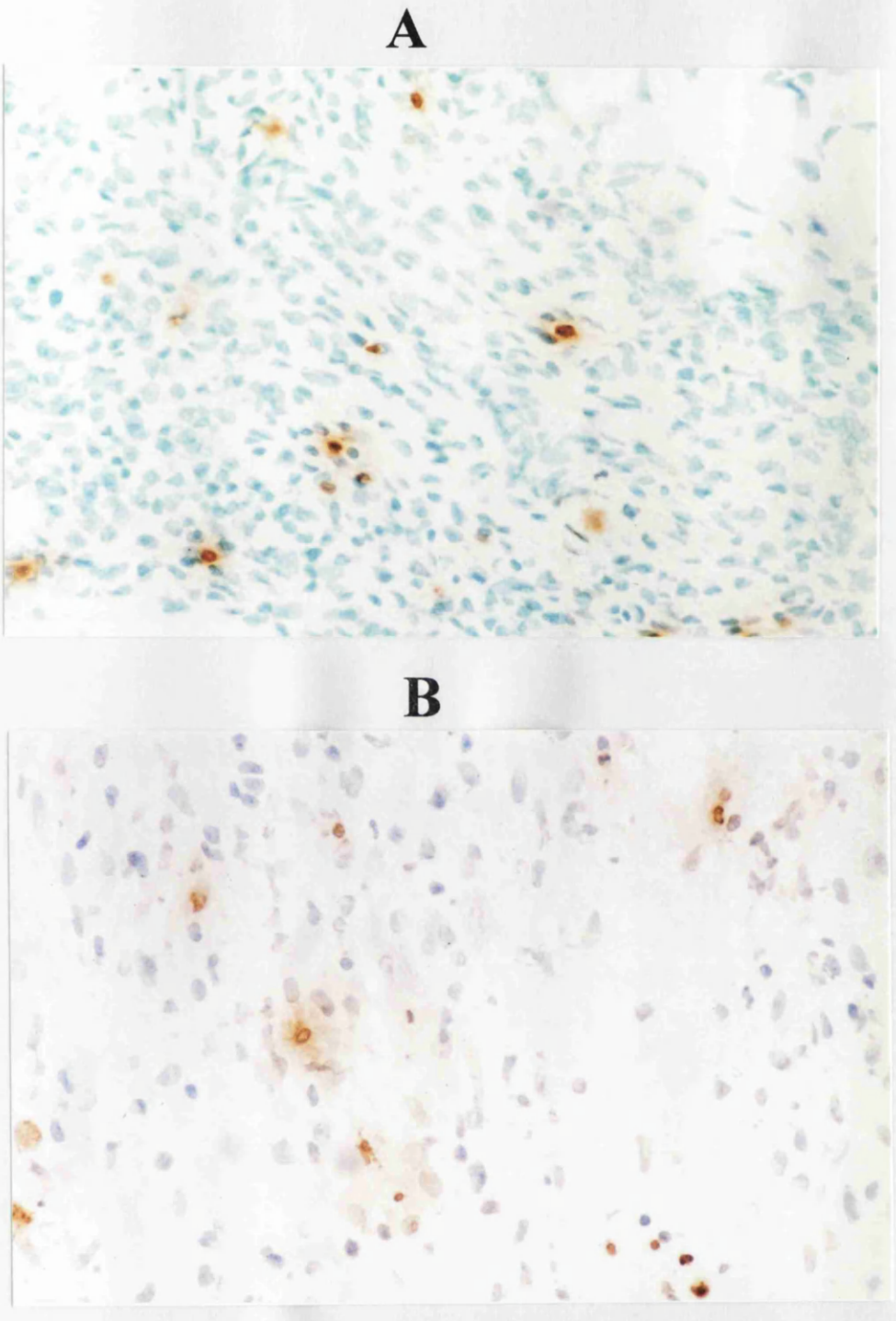

Figure 6.8 Photomicographs of heart allografts removed from (A) an R73 treated recipient at day 7 post transplant and (B) an unmodified rejecting control animal (x400). Apototic cells detected by TUNEL are stained brown.

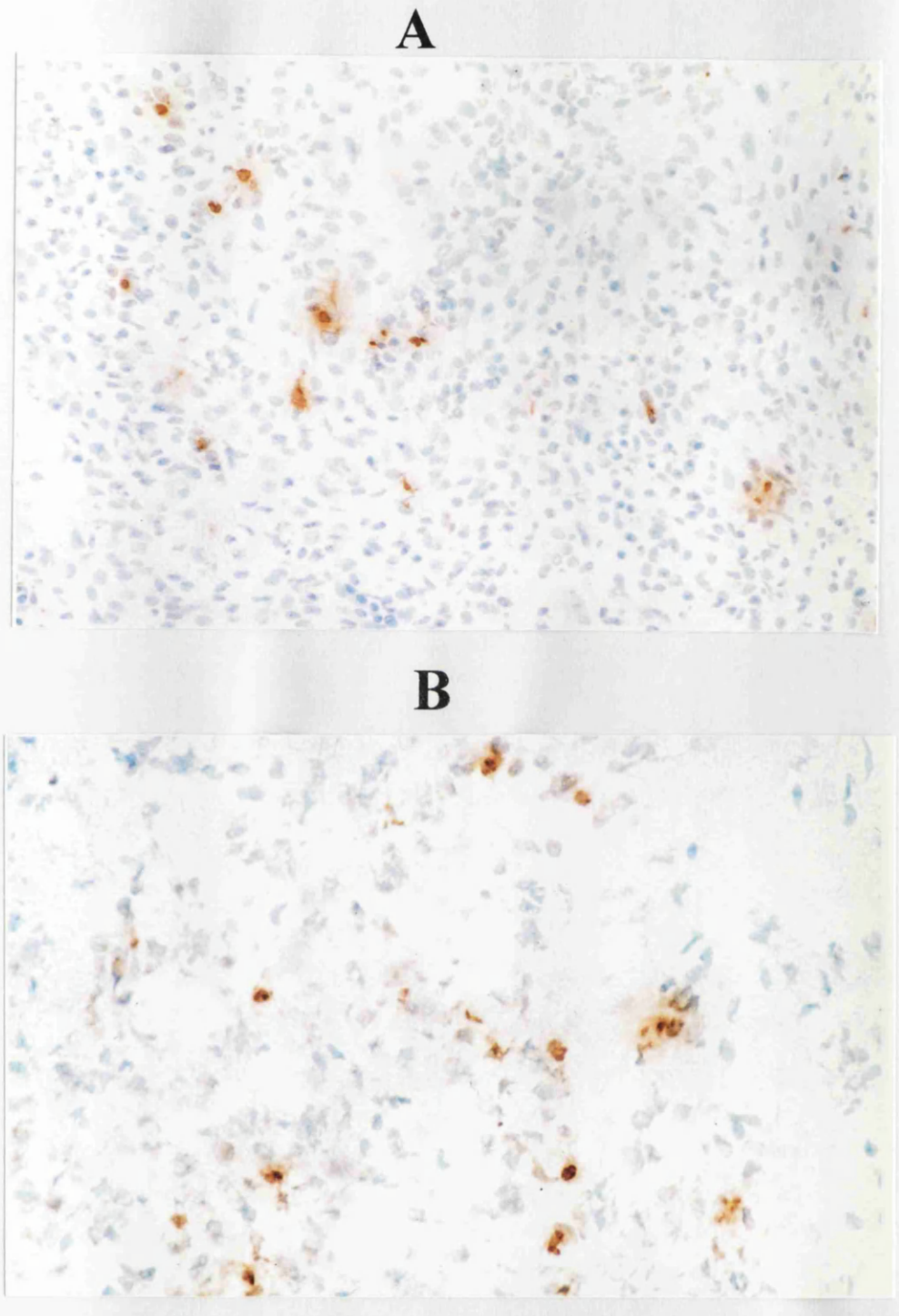

Figure 6.9 Photomicographs of heart allografts removed from (A) an R73 treated recipient at day 4 post transplant and (B) an unmodified rejecting control animal (x400). Apototic cells detected by TUNEL are stained brown.

6:4 Discussion

Alpha/beta TCR +ve T cells are principal effector cells responsible for allograft rejection (Gracie et al, 1990) and targeting these cells with R73 mAb is an effective method of abrogating the rejection process in the Lewis to DA rat strain combination. The experiments described in this chapter were carried out to determine whether apoptosis plays a role in prolonging graft survival after R73 treatment. The use of monoclonal antibodies has been shown to induce apoptosis in experimental models of tumour growth and anti-CD3 mAb has been shown to induce apoptosis in immature T cells (Trauth, et al, 1989, Smith et al, 1989). Conversely, cyclosporin A has been found to inhibit apoptosis in vitro (Anel et al, 1994).

Flow cytometric analysis was therefore carried out of PI stained lymph node cells from graft recipient animals treated with R73 mAb. Apoptotic activity was increased in the LNC of R73 treated animals compared to untreated graft recipients and normal DA rats particularly at day 7 after transplant. Interestingly, R73 treated animals who did not receive an allograft showed only baseline levels of apoptosis.

These observations suggest firstly that R73 triggers apoptosis in infiltrating lymphocytes and that this may, at least partly, be responsible for the prolongation of graft survival seen in this model. Secondly, the presence alloantigen as well as R73 mAb is required for apoptosis in this model. This can

be explained by the observation made by other groups that only activated T cells are susceptible to TCR induced apoptosis (Van Parijs et al, 1996) and is supported by the observation here that apoptosis increases at day 7 post transplant in R73 treated animals, a time point when previously depleted T cell numbers are recovering. Thus the combination of TCR ligation by MHC+alloantigen and the presence of R73 appears to result in altered signalling through the TCR.CD3 complex resulting in apoptosis of the alloreactive T cell. The continued presence of alloantigen alone, however does not result in apoptosis in lymphocytes since only baseline levels of apoptotic activity are seen at day 100 after transplant, a time point where R73 is no longer detectable.

TUNEL labelling of apoptotic cells in heart allografts gave a contrasting picture to that found in lymph node cells. Apoptotic activity was higher in rejecting grafts with only rare apoptotic figures seen in allografts from R73 treated animals. In addition, the apoptotic cells were mainly confined to groups of infiltrating cells and were absent from normal myocardium. This would suggest that apoptosis plays a different role in the graft and probably reflects destruction of allogeneic myocardial cells by cytotoxic T cells through the induction of apoptosis, a recognised mechanism of T cell killing (Hanabuchi et al, 1994). The lack of apoptotic figures in the grafts of R73 treated recipients may also be partly due to the reduced number of graft infiltrating T cells seen in this model.

The increase in TUNEL+ cells in graft infiltrates at day 7 post transplant probably reflects an increase in not only target cell killing by T cells but also the apoptosis of alloreactive T cells themselves as a physiological method of limiting the immune response itself to the destroyed allograft.

In conclusion, apoptosis plays a complicated role in both allograft rejection and prolongation of graft survival in this model. The physiological role of apoptosis as a mechanism of cytotoxic T cell killing and as a way of limiting the immune response is seen in the grafts of unmodified graft recipients. A therapeutic role of apoptosis is demonstrated in the lymph nodes of R73 treated graft recipients where activated, alloreactive lymphocytes are deleted by apoptosis, induced most likely, by the combined signalling of R73 mAb and alloantigen through the T cell receptor.

6:5 Summary

6:2 Flow cytometric analysis shows increased numbers of apoptotic cells in the lymph nodes of R73 treated recipients of a heart allograft day 7 post transplant compared to normal DA animals and recipients rejecting their grafts.

6:3 Labelling of apoptotic cells in heart grafts detected increased apoptotic activity in rejecting grafts compared to R73 treated grafts. In addition the apoptotic activity in rejecting grafts was confined to areas of cellular infiltration.

CHAPTER SEVEN

Final Discussion

Discussion

The use of monoclonal antibodies either as an adjunct to chemical immunosuppression or to induce long term graft tolerance is currently of great interest. The experiments reported in this thesis attempt to clarify the mechanisms of action, potential benefits and possible drawbacks of using a monoclonal antibody directed at the α/β T cell receptor.

In the initial experiments, R73 mAb was shown to prolong heart graft survival indefinitely in the Lewis-DA rat strain combination. An interesting new finding was that prolonged graft survival but not indefinite survival could be induced in other strain combinations. Other investigators have made similar observations in other models of transplantation, leading to the terms "high" and "low" responder strain combinations. The use of R73 mAb has been reported to induce long term heart graft acceptance in the BN-Lewis strain combination (Tsuchida et al, 1994), however permanent graft survival was not seen in this strain combination in the experiments reported here. These observation do not make the experiments in the Lewis-DA strain any less relevant, they simply imply that perhaps the immunological mechanisms responsible for graft tolerance are not different from other rat strains but are more easily activated in DA rats.

Several novel observations have been made in the subsequent experiments using R73 mAb in the Lewis-DA strain combination. The ability of DA rats to

accept indefinitely a Lewis heart allograft in the absence of an intact thymus gland confirms that the R73 acts directly on the peripheral T cell pool and that recent thymic emigrants are not responsible for extension of graft survival. The observation that exogenous IL-12 administered after R73 treatment and heart grafting could diminish the effect of R73 again suggests that R73 acts by disabling the peripheral T cell pool though not permanently. It would have been interesting, if time allowed, to investigate the effect of administering other exogenous cytokines such as IL-2 in this model and perhaps anti-IL-4 mAb in the post transplant period to further clarify whether immune deviation plays a major role in prolonging graft survival after R73 treatment.

Further insights into the mechanism of action of R73 are illustrated by the in vitro experiments outlined in this thesis. R73 treatment induces peripheral T cell depletion in the early post transplant phase with recovery to 50% of normal by day 7 and almost complete recovery by day 21. The presence of 30-50% of the peripheral T cell pool in the early post transplant period and the ability of R73 to prolong graft survival even when the transplant is delayed to a point where R73 is present in low concentration on peripheral T cells suggests that R73 does not simply act by depletion of alloreactive cells and may in fact promote the development of regulatory or suppressor T cells (which may be γ/δ +ve T cells) or may render the remaining T cells anergic. It has been suggested that soluble anti-CD3 mAb mediated immunosuppression is, in part, due to alloreactive T cell inactivation (Smith and Bluestone, 1997). Further experiments would be required to clarify these points and it would be of value

to repeat the flow cytometry to look for evidence of regulatory cells such as γ/δ T cells and CD25 +ve CD4 T cells in the early post transplant period. TCR modulation (shown by the experiments in Chapter 4) induced by R73 further supports the hypothesis that R73 acts by modifying the response of the remaining T cells to the graft.

The in vitro analysis of LNC harvested from R73 treated graft recipients outlined in Chapter 5 suggests that they proliferate poorly in the first four days after R73 treatment but proliferate normally by day 7. By contrast, however, IL-2 production by these cells is impaired even 100 days after transplantation. The presence of IL-2 has been shown to be critical for graft rejection in some models (Kirkman et al, 1985, Wood et al, 1993) and is the dominant cytokine responsible for clonal expansion of activated T cells and so impaired T cell help seems likely during the early phase post transplant in this model. Although the impaired ability of these cells to produce IL-2 and IFNγ could suggest immune deviation towards a Th2 type response, the ability of these cells to produce IL-4 and IL-10 was not investigated due to the lack of a reliable assay in the lab at the time of these experiments but would be a valuable future experiment. Intragraft transcripts for Th1 and Th2 cytokine genes were detected by RT-PCR in both R73 treated and rejecting grafts, though IL-2 and IFNγ were less readily detected in heart grafts from R73 treated animals. These findings provide some evidence of a Th1 to Th2 cytokine shift though this should be interpreted with caution. It is equally possible that it is the absence of IL-2, a

strong barrier to the induction of tolerance, rather than Th2 polarisation that is responsible for prolonged graft survival in this model (Strom et al, 1996).

It would be interesting to carry out adoptive transfer experiments using LNC from R73 treated graft recipients, transferring the cells into naïve DA hosts and then transplanting a Lewis heart graft without further treatment, to determine whether these cells could transfer immunity to the naïve animal and so provide evidence as to whether they are indeed regulatory cells or simply unresponsive. It would also be valuable to investigate the intracellular signalling events which occur in these cells in the post transplant period in the light of recent work reported by Smith and Bluestone suggesting that anti-CD3 mAb may work by altering signalling through the TCR (Smith and Bluestone, 1997). Although the results above suggest that LNC from R73 treated graft recipients are unable to produce IL-2 normally even as long as 100 days after treatment, it would be important to test whether this results in long term unresponsiveness in vivo by carrying out a second allogeneic vascularised heart graft in animals who have accepted their first grafts permanently.

An alternative and novel mechanism for the action of R73 is suggested by the results shown in Chapter 6. Activation induced cell death (AICD) of T lymphocytes is a well characterised method of T cell death and is thought to contribute to the limitation of physiological immune responses. Repeated or excessive signalling through the T cell receptor is thought to induce apoptosis in activated T cells and this can be independent of the Fas system (Van Parijs et

al, 1996). Using propidium iodide labelling of apoptotic LNC we demonstrated that cells in the lymph nodes of R73 treated graft recipients undergo apoptosis at a higher rate than that found in normal DA rats and animals rejecting their grafts. Interestingly, this was dependent not only on the presence of R73 but on a graft being present also. It would seem reasonable to propose that the combination of R73 and APC ligation results in altered intracellular signalling and activation of the apoptotic pathway.

Investigation of apoptosis in the heart grafts of R73 treated and rejecting animals provided interesting and seemingly paradoxical results. Apoptotic activity was increased in the grafts of rejecting animals compared to those transplanted under cover of R73 treatement. These results, however, illustrate the diverse role which apoptosis plays in biological systems. In the rejecting graft, it is likely that TUNEL demonstrated apoptosis of parenchymal cells mediated by infiltrating T cells in untreated animals, but FACS of PI stained lymph node cells showed apoptosis of alloreactive cells in the presence of R73 illustrating activation induced cell death in the presence of an effective antibody.

The role of the Fas/FasL system in the initiation of apoptosis has been extensively investigated (Desbarats et al, 1998, Peter and Krammer, 1998). An attempt was made using flow cytometry during the above experiments to quantify any changes in Fas expression in LNC after R73 treatment. At the time of the experiments, a satisfactory anti-rat Fas mAb could not be found and we discovered using flow cytometry that the available anti-mouse preparations

were cross reactive with other rat antigens as shown by a strongly positive signal in the negative control group of cells. It would be very interesting, however to look at Fas expression on R73 treated LNC at day 7 post transplant in the light of new mAb preparations being available.

The mechanisms underlying the action of anti-TCR monoclonal antibody are complex and diverse: depletion, anergy, immune deviation, regulation/suppression and apoptosis. Further investigations as indicated above are required to fully elucidate these. This thesis does provide, however, a novel insight into these mechanisms and provides evidence for the potential efficacy of ant-TCR directed therapy in clinical practice. Although there are many aspects of anti-TCR mAb treatment which still need to be addressed, there is no doubt that that it could provide a useful tool for investigating the complex interactions of the T cell receptor and APC and for investigating the conditions required for allograft tolerance.

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