INDUCTION OF TRANSPLANTATION TOLERANCE USING MONOCLONAL ANTIBODIES TO CD4: EXPERIMENTAL STUDIES USING A RAT HETEROTOPIC CARDIAC ALLOGRAFT MODEL

by

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Abstract

A major goal in clinical organ transplantation is to devise new strategies for the induction of specific immunological tolerance to the allograft. This would obviate the need for long-term immunosuppressive drugs and leave the immune system of the recipient otherwise intact to protect the recipient from the development of infection and malignancy. CD4 T cells play a central role in allograft rejection and the CD4 molecule has therefore attracted considerable interest as a molecular target for manipulating the allograft response. In this thesis, mouse monoclonal antibodies (mAbs) directed against the rat CD4 molecule were used in an attempt to prolong allograft survival and possibly to promote tolerance to a heterotopic cardiac allograft in the rat.

In preliminary studies, administration of the mouse IgG2a anti-CD4 mAb MRC OX38 (directed against an epitope on the distal domains of the rat CD4 molecule) for a brief period immediately prior to transplantation [10 mg/kg on day -3 and 2 mg/kg on day -2, -1 and day 0 (day of transplantation)] prolonged heart allograft survival indefinitely (>200 days) in the Lewis to DA rat strain combination. Long-standing allograft recipients displayed donor-specific tolerance by rejecting a second "third party" (PVG) heart graft but permanently accepting a second donor-specific (Lewis) heart graft.

The ability of OX38 mAb to induce transplant tolerance was highly dependent on the rat strain combination studied. Although OX38 mAb treatment consistently increased cardiac allograft survival in other fully allogeneic rat

strains tested (e.g. DA to PVG and DA to Lewis), long-term survival was not observed.

Further studies were performed with additional anti-CD4 mAbs directed against epitopes on the proximal (OX70, OX71 and OX73) or distal domains (W3/25) of the CD4 molecule. All of the mAb's tested, with the exception of W3/25, led to a marked increase in the survival of Lewis heart allografts in DA recipients and many recipients accepted their grafts permanently. The mAb W3/25 mAb depleted CD4 T cells to a lesser extent than the other anti-CD4 mAb tested, which may explain, at least in part, its inability to prolong graft survival indefinitely.

Further studies were performed to investigate some of the possible mechanisms involved in the induction and maintenance of anti-CD4 induced transplant tolerance. The ability of anti-CD4 mAbs to prolong cardiac allograft survival was significantly dependent on the presence of the thymus gland, since thymectomised DA recipients treated with OX38 or OX70 mAb did not become tolerant to Lewis strain heart grafts. Administration of exogenous recombinant interleukin-2 (rIL-2) during the induction of tolerance by anti-CD4 mAbs (OX38 and OX73) failed to abrogate induction of tolerance, suggesting that a lack of endogenous IL-2 alone does not account for allograft survival after anti-CD4 therapy. On the other hand, treatment with a neutralising anti-IL-4 mAb (MRC OX81) during the induction of tolerance with OX38 mAb reduced the efficacy of tolerance induction. Although not conclusive, this finding suggests a possible role for IL-4 during the induction phase of tolerance

following anti-CD4 mAbs therapy. The role of CD8 cells in the induction and maintenance of transplant tolerance was also examined. Administration of the depleting anti-CD8 mAb (OX8) during the induction of tolerance abrogated the ability of anti-CD4 mAb to increase cardiac allograft survival but when OX8 mAb was given to animals bearing long-term tolerant heart grafts it did not prevent such animals from accepting a second donor strain heart indefinitely. Thus CD8 cells appear to be necessary for induction but not maintenance of the tolerant state following anti-CD4 therapy. In further experiments adoptive transfer studies using splenocytes obtained from OX38 treated tolerant recipients provided evidence for the existence of immunoregulatory (or suppressor) lymphocytes. Thus, transfer of $2x10^8$ spleen cells from tolerant animals to secondary unmodified graft recipients enabled them (in 2 of 4 recipients) to maintain their graft indefinitely.

Finally, purified CD4 T cells were obtained from animals bearing long-standing Lewis heart allografts following anti-CD4 mAb treatment (OX38 or OX70) to determine their in vitro proliferative response and cytokine production. CD4 T cells from OX38 treated recipients showed near normal levels of proliferation to irradiated donor (Lewis) stimulators in the in vitro MLR. Moreover, CD4 T cells from OX38 treated tolerant animals released levels of IFNγ and IL-4 into the culture supernatants which were comparable to those produced in response to third party stimulators or released by CD4 T cells obtained from normal DA strain rats in response to Lewis stimulators cells. Interestingly, there was a reduction in IL-2 levels in culture supernatants of tolerant CD4 cells from

OX38 treated when compared with CD4 cells from normal rats. Analysis of the in vitro response of CD4 T cells from long-term tolerant animals which received OX70 mAb gave unexpected results which contrasted with those from OX38 treated recipients. Such cells showed a reduction in both proliferation and IL-2 production to both donor and third party allogeneic stimulator cells.

IFNγ levels were comparable (OX38) or marginally reduced (OX70) in culture supernatants from tolerant CD4 T cells when compared with normal CD4 T cells. Overall, these observations suggest that the cytokine pattern of CD4 T cells from animals rendered tolerant by anti-CD4 T cells is complex and variable. The findings are not consistent with the view held by some workers that anti-CD4 induced tolerance correlates with a simple polarisation of the cytokine response from Th1 (IL-2 and IFNγ) in rejection to Th2 (IL-4, IL-5, IL-6 and IL-10) in tolerance.

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Abbreviation

ADCC	antibody dependent cellular cytotoxicity.
Ag	antigen.
APC	antigen presenting cell.
ALG/S	Anti-lymphocyte globulin/serum.
B2m	β2-microglobulin.
BCGF	B-cell growth factor.
BM	bone marrow.
BSA	bovine serum albumin.
CsA	Cyclosporin A.
CDRs	complementarity determining regions.
CTL	cytotoxic T-lymphocyte.
DST	donor specific blood transfusion.
DTH	delayed type hypersensitivity.
ELISA	enzyme-linked immunosorbent assay.
ER	endoplasmic reticulum.
FACS	fluorescence-activated cell sorter.
FCS	foetal calf serum.
G-CSF	granulocyte colony stimulating factor.
GVHD	graft versus host disease.
HLA	human leukocyte antigen.
IFN-γ	interferon-γ.
ICAM	intercellular adhesion molecule

Ig	immunoglobulin.
IVC	inferior vena cava.
IL-2R	interleukin-2 receptor
Kd	kilodalton.
LFA	leukocyte functional antigen.
LNC	lymph node cells.
mAb	monoclonal antibody.
mH	minor histocompatibility antigen.
MHC	major histocompatibility complex.
MLR/C	mixed lymphocyte reaction or culture.
MMF	Mycophenolate Mofetil.
MST	mean survival time.
NK	natural killer.
PBL	peripheral blood lymphocytes.
PBS	phosphate buffered saline.
TAP	transporters in antigen processing.
TCR	T-cell receptor ($\alpha\beta$ or $\gamma\delta$).
TGFβ	transforming growth factor-β.
Th	T-helper.
TNF	tumour necrosis factor.
TCGF	T-cell growth factor.
VCAM	vascular cell adhesion molecule

Declaration

The experimental work reported in this thesis was carried out entirely by the author Dr Hamdi Ahmidat under the supervision of professor, Andrew. Bradley, unless otherwise specified in the text. I declare that this work has not been submitted for any previous application for a degree. Part of the results described in chapter 5 have been presented at the, Basic Science Symposium of Transplantation Society in September, 1995, Holland.

CHAPTER ONE

Clinical perspective of organ transplantation

1:1 The development of clinical organ transplantation

In the space of little more than 30 years, organ transplantation has progressed from an essentially unsuccessful experimental procedure conducted in a small number of pioneering medical research centres to a widespread and routine procedure performed throughout the world. Transplantation of the kidney, heart and liver are now widely practised at specialist centres across the western world and transplantation of the pancreas and small bowel are becoming more commonplace.

Despite early attempts, no real progress in organ transplantation was made until the middle of the 20th century. The first human kidney transplant was carried out in 1933 by a Russian surgeon, Vorony, who used a cadaveric kidney to treat a patient with acute renal failure caused by mercuric chloride poisoning. It is unlikely that the transplanted kidney ever functioned as it had been harvested some 6 hours after the donor's death and the graft recipient died within 48 hours of surgery (Voronoy, 1936; Hume DM, 1979).

Two important events paved the way for the development of successful kidney transplantation, namely the invention of the artificial kidney machine by Wilhelm Kolff in 1944 (Kolff et al, 1944) and the synthesis of steroid hormones and antimitotic drugs, such as nitrogen mustard (reviewed by Hamilton, 1988). In the early 1950s a small number of human cadaveric kidney transplants were performed at centres in both Paris and Boston (Servelle et al, 1951, Hume et al, 1955). However, effective immunosuppressive drugs with which to prevent rejection were not then available and despite a promising outcome in one

patient, the overall results in both centres were very disappointing . These early attempts at kidney transplantation confirmed the view that before clinical success could be expected a major immunlogical barrier would need to be overcome.

From experimental work with skin grafts it was known that exchange of tissue was possible between identical twins without encountering the problems of graft rejection and in 1954 the Boston group, headed by Joseph Murray, began a series of kidney transplants between identical twins (Murray et al, 1955). Richard Herrick, a 24 year old man with end stage renal failure received a renal transplant from his identical twin brother Ronald on December 23rd 1954. The transplanted kidney subsequently functioned well for eight years before eventually succumbing to recurrence of the primary renal disease. This important success heralded the first of many subsequent transplants between identical twins. It demonstrated the technical feasibility of kidney transplantation but the problem of immunological rejection between non-identical individuals remained.

The development of a new antimitotic drug, 6-mercaptopurine, and the discovery that it had potent immunosuppressive effects led Roy Calne in 1960 to use this agent in dogs receiving kidney allografts (Calne RY, 1960). Calne subsequently pioneered the use of azathioprine, a similar but less myelotoxic and therefore superior immunosuppressive agent, initially to prevent renal allograft rejection in dogs (Calne, Alxander and Murray, 1962) and later in clinical practice (Murray et al, 1963).

Further improvements in the results of kidney transplantation occurred over the next two decades. These were attributable to the routine use of lymphocytotoxic crossmatching prior to transplantation, the introduction of prospective matching of histocompatibility antigens, the use of antilymphocytic preparations and the adoption of deliberate blood transfusion policies (Opelz et al, 1973, Opelz et al, 1992). As a result by the 1980's renal transplantation had become the preferred treatment for most patients with end-stage renal disease, although considerable scope for further improvement in the outcome of transplantation remained.

The introduction of cardiac transplantation into clinical practice was less rapid. Following the first cardiac transplant in dogs, reported by Carrel and Guthrie in 1905 (Carrel and Guthrrie, 1905), little further interest was shown in this procedure until reliable methods of cardiopulmonary bypass were developed in the 1950s. Then it became possible to devise surgical techniques which allowed intrathoracic, orthotopic cardiac transplantation and the pioneering work in dogs reported in the 1960s by Lower and Shumway opened the way towards clinical cardiac transplantation (Lower and shumway, 1960). Advances in the treatment and control of kidney graft rejection in the late 1960s encouraged cardiac surgeons to use similar strategies for achieving success after cardiac transplantation. The first successful human orthotopic cardiac allograft was performed by Christiaan Neethling Barnard in 1967 in South Africa but unfortunately the patient died 18 days later (Barnard, 1967). Soon afterwards cardiac surgeons in North America, notably Shumway who had first developed

the surgical technique used by Barnard, established clinical programmes of cardiac transplantation and the procedure was widely adopted for the treatment of various cardiac conditions such as cardiomyopathy, ischaemic heart disease and some congenital heart diseases (Yaccoub et al, 1987).

1:2 Cyclosporin A and the modern era of transplantation

Until the late 1970s, azathioprine and steroids were the principle immunosuppressive agents used in renal transplantation (Groth CC, 1972). However, the widespread introduction of cyclosporin A (CsA) into clinical practice in the early 1980s markedly improved the early results of renal transplantation and made the results of heart and liver transplantation clinically acceptable, thereby enabling transplantation of these organs to be pursued on an almost routine basis in many centres (reviewed by Jain and Fung, 1996).

The potent immunosuppressive properties of CsA, a fungal metabolite, were first discovered by Borel while working in the microbiology department of Sandoz in Basle, Switzerland (Borel and White, 1982; Borel et al, 1976). The new agent was found to prolong survival of skin, heart and kidney allografts in experimental animals with results superior to those obtained earlier with azathioprine (Kostakis et al, 1977; Calne RY and White DJ, 1977). As with azathioprine, it was Calne in Cambridge, who showed that the new agent was also effective in clinical organ transplantation (Calne and Wood, 1985, Clane, 1987) thus heralding a new era in immunosuppressive therapy. It is now known that CsA acts predominately on helper T lymphocytes (Th), suppressing the production of a variety of lymphokines, particularly the T cell growth factor

interleukin-2 (IL-2). Although CsA selectively inhibits T cell activation and proliferation, it still leads to generalised immunosuppression and its attendant problems of increased susceptibility to infection. In addition, it may impair renal function and induce hypertension as well as causing a variety of other, usually less serious, side effects (Thomson, 1989; Schreiber and Crabtree, 1992).

1:3 FK506 and other new immunosuppressive drugs

In the late 1980s two new macrolide antibiotics, FK506 and rapamycin, were shown to be very effective in prolonging organ allograft survival in experimental animals (Kino et al, 1987). FK506 (Tacrolimus) is now licensed for use in renal transplantation and has been shown in recent clinical trials to be as efficacious as CsA in the prevention and rescue of allograft rejection as cyclosporin (reviewed by Klintmalm G, 1994; Shapiro et al, 1991).

Rapamycin is currently undergoing phase 3 clinical trials and the results of preliminary testing with this drug are promising (Sehgal et al, 1995). Both FK506 and rapamycin inhibit T cell proliferation but, despite having close structural similarity and sharing the same immunophilin, they have very distinct modes of action within the T cell. Like CsA, FK506 inhibits the very early events following T cell activation via the cell surface T cell receptor (TCR)/CD3 complex which lead to the transcription of IL-2 and other cytokine genes (reviewed by Schreiber and Crabtree, 1992). Both FK506 and CsA bind to cytosolic immunophilins (FK-binding protein and cyclophilin respectively) and the respective drug-immunophilin complex inhibits the translocation from the cytoplasm to the cell nucleus of nuclear factor of activated T cell (NF-AT),

a regulatory protein which controls transcription of IL-2 and other cytokines. Although, rapamycin binds to the same immunophilin as FK506 (FK-BP), it does not affect cytokine gene expression but acts instead at a later stage in the cell cycle blocking the response of T cells to IL-2 (Cardenas et al, 1995).

Mycophenolate mofetil is another promising immunosupressive agent recently licensed for clinical use in renal transplantation. It is an ethyl ester which is metabolised to its active ingredient mycophenolic acid after administration. Mycophenolate blocks the de-novo pathway of purine synthesis by inhibiting inosine monophosphate dehydrogenase (IMPDH), which converts inosine monophosphate to guanosine triphosphate, thus preventing DNA synthesis (Allison et al, 1993; Nagai et al, 1992). Recent multicentre trials have shown that MMF reduces the incidence of acute rejection episodes following renal transplantation but it remains to be seen whether it reduces long-term graft loss from chronic rejection (Keown PA, 1996; reviewed by Fulton and Markham, 1996).

Leflunomide, is an immunosuppressive molecule which is still at an early stage of development. It is an isoxazol derivative and is not chemically related to CsA, MMF or rapamycin. Leflunomide inhibits the proliferation of activated T and B cells (Chong et al, 1993), prevents allograft rejection in rats (Kuchle et al, 1991; Williams et al, 1993) but has not yet been fully evaluated in clinical trials (Reviewed by Brazelton and Morris, 1996, Waer M, 1996).

Finally, the immunosuppressive 15-Deoxyspergualin trihydrochloride (DSG), a synthetic form of the natural antibiotic spergualin, is produced by the bacterium,

Bacillus laterosporus. It has significant immunosuppressive activity and inhibits the activity of both B and T cells as well as macrophage and monocyte function. Its mechanism of action differs from other immunosuppressants in that it binds to the heat shock protein (hsp70). Although licensed for clinical use in Japan (Amemiya et al, 1990 & 1993), only pilot studies of DSG in organ transplantation have been conducted in Western Europe and North America (reviewed by Kaufman et al, 1996).

1:4 Biological immunosuppression (antibodies)

In addition to the "small molecule" immunosuppressive agents already described, biological immunosuppressive agents (principally antibodies) are an important addition to the immunosuppressive armamentarium.

1:4:1 Polyclonal antibodies

Polyclonal antibodies preparations directed against lymphocytes have been used in clinical transplantation for the prevention of graft rejection since the early 1960s (Starzl TE, 1968). Antilymphocyte serum (ALS) and antilymphocyte globulin (ALG) are usually raised in horses, rabbits or goats by immunisation with human lymphoid cells (lymphblasts or thymocytes). These polyclonal antibody preparations target both T and B lymphocytes and comprise a range of antibodies with specificity for different lymphocytes surface antigens which include CD45, CD3, TCR, LFA1, CD4 and CD8 (Rebellato et al, 1994, Bonnefoy-Berard et al, 1991). They deplete lymphocytes from the circulation by lysis and opsonization and they also impair the function of residual lymphocytes

by blocking or modulating cell surface molecules (reviewed by Powelson and Cosimi, 1994).

Although ALG, together with azathioprine and prednisolone, was used in renal transplant recipients as prophylactic therapy in the early 1970s, the improvement seen in graft survival failed to reach statistical significance when compared with azathioprine and prednisolone therapy alone (Cosimi AB, 1976). However, several studies have suggested that ALG may be superior to high dose steroids in reversing acute rejection episodes and also as a "rescue" therapy in steroid resistant rejection (Cosimi AB, 1988). Some transplant centres still use ALG as induction therapy in the early post-transplant period, particularly to avoid cyclosporin-induced nephrotoxicity in kidney transplant recipients (Frey et al, 1992). However, these preparations are powerful agents and there is a significant risk of lymphoproliferative disorders when ALG is combined with cyclosporin (Merion et al, 1984). Polyclonal antibody preparations such as ALS and ALG have several other limitations. These include their broad specificity, their potential for undesirable cross-reactions and the inevitable batch to batch variability which results during their manufacture. Fortunately, anaphylactic reactions or serum sickness are relatively uncommon problems (Bach and Strom, 1985).

1:4:2 Monoclonal antibodies

The advent of hybridoma technology, opened the way to development of monoclonal antibodies (mAbs) which, unlike polyclonal preparations, have predictable and consistent reactivity (Kohler et al, 1975 and Galfre et al, 1979).

However, the introduction of mAbs into clinical transplantation has been disappointingly slow. Table 1:1 summarises various mAbs which have been tried in human transplant recipients. OKT3 is the only mAb currently in wide spread use in clinical transplantation. It is an IgG2a murine monoclonal antibody directed against the CD3 molecule on the surface of T lymphocytes and was first shown by Cosimi to be an effective agent in reversing renal allograft rejection (Cosimi et al, 1981). Administration of OKT3 produces rapid and substantial T cell depletion and, in addition, residual T cells are coated with mouse antibody and display modulation of the CD3/TCR complex (reviewed by Masroor et al, 1994). OKT3 has been used successfully as a rescue therapy to treat steroid resistant renal allograft rejection (Ortho Multicenter Transplant Study Group, 1985, Delminco et al, 1988) and also has been shown in heart transplantation to reverse acute rejection episodes which are refractory to high dose steroids or ALS (Gilbert et al, 1987).

Prophylactic therapy with OKT3 is not generally used in the UK, because of the profound non-specific immunosuppression it induces and the attendant risks of infection and malignancy which may follow its use (Thistlethwaite et al, 1987, Monaco et al, 1987, Cosimi et al, 1987, Fung et al, 1987). However, some centres, particularly in the USA, use OKT3 for prophylaxis against rejection, especially when delayed graft function is encountered after renal transplantation (Kahana et al, 1990). A particular problem associated with OKT3 is that of cytokine release syndrome. This is a syndrome of fever, chills, rigors and

Table 1:1 Summary of the various mAbs which have been used in clinical organ transplantation.

Mab used	Target molecule	Type of study	No of Patients	Authors	Type of Graft
OKT3	Anti-CD3	RCT	123	(1),(2),(3)	Kidney
(BMA031)	Anti-TCR (αβ)	RCT	24	(4)	Kidney
(T10B9.1A -31)	Anti-TCR (αβ)	RCT	76	(5)	Kidney
anti-CD25	Anti- CD25	RCT	100	(6)	Kidney
Anti-Tac		RCT	40	(7)	Kidney
BT563		RCT	60	(12)	Heart
LO-Tac		RCT	129	(13)	Liver
33B3.1		RCT	40	(14)	SPK
BT563		RCT	32	(15)	Liver
Anti-LFA1	Anti-LFA1	pilot study	7	(8)	BM
25-3		pilot study	15	(16)	Kidney
BL4	Anti-CD4	Pilot study	12	(9)	kidney
MAX.16H5		Pilot study	30	(10)	Kidney
OKT4A		Pilot study	30	(11)	Kidney

Abbreviations
RCT=Randomised Clinical Trial
SPK=Synchronous Pancreas Kidney transplantation.
BM =Bone marrow.

Study Authors
(1) Ortho Multicenter Transplant Study group, 1985
(2) Thistlethwaiite et al, 1984
(3) Delmnico et al, 1988
(4) Knight et al, 1994
(5) Waid et al, 1992
(6) Soulillou et al, 1990
(7) Kirkman et al, 1991
(8) Fischer et al, 1986
(9) Morel et al, 1990
(10) Reinke et al, 1995
(11) Matas et, 1995
(12) Gelder et al, 1996
(13) Reding et al, 1996
(14) Cantrovich et al, 1994
(15) Nashan et al, 1996
(16) Le Mauff and Soulillou, 1995

generalised malaise attributable to massive release of tumour necrosis factor (TNFα,β), IFNγ and other cytokines following the first dose of OKT3 (Chatenoud et al, 1990, Ambramowicz et al, 1989).

Few other mAbs have been used to an appreciable extent in clinical transplantation (see table 1:1). The TCRαβ molecule on the T cell surface is an obvious target for mAbs and at least two different, anti-TCR mAbs have been tested in patients. BMA031 and T10B9.1A-31 are mouse anti-human αβTCR mAbs of the IgG2b and IgM class respectively. The former mAb has been tested to a limited extent for its ability to prevent renal allograft rejection and to treat graft-versus host disease following allogeneic bone marrow transplantation (Schlitt et al, 1989). BMA-031 was found to be effective as rescue treatment in steroid resistant renal allograft rejection (Land et al, 1988). More recently, the results of phase II and randomised phase III trials of BMA031 in the prevention of renal allograft rejection have been reported (Knight et al, 1994). The antibody appeared, in these limited studies, to be safe and comparable in efficacy to OKT3 (Dendorfer et al, 1990, Chatenoud et al, 1993). The mAb T10B9.1A-31 was tested in a randomised double-blind phase II trial involving 76 patients and was also found to be comparable in efficacy to OKT3 in reversing acute renal allograft rejection (Waid et al, 1992).

The IL-2 receptor (IL-2R) is another promising molecular target for mAbs in clinical transplantation. 33B3.1 and anti-Tac are both IgG2a subclass mouse anti-human mAbs directed against the IL-2R. The former mAb blocks IL-2 binding and was found to be comparable in efficacy to ALS as prophylactic

treatment to prevent early renal allograft rejection but long term follow up results have not yet been reported (Soulillou et al, 1990). In another randomised trial the anti-Tac mAb was found to reduce early acute renal graft rejection episodes but did not have a significant effect on either graft or patient survival (Kirkman et al, 1991). Gelder and colleagues, in a randomised trial involving 60 heart transplant patients, compared anti-IL-2 (BT563) with OKT3 for safety and efficacy (Gelder et al, 1996). In this study BT563 had similar efficacy to OKT3 but was devoid of the accompanying side effects (Gelder et al, 1996). BT563 was also compared to ATG as immunoprophylaxis in a randomised clinical trial involving 32 recipients of orthotopic liver transplants. The mAb was not associated with any major side effects and significantly reduced the incidence of acute rejection when compared with the standard immunosuppression regimen using ATG (Nashan et al, 1996). The anti-IL-2R 33B3.1 (a rat anti-human IgG2a monoclonal antibody directed against the α chain of IL-2) was compared with ATG as induction therapy (along with standard triple therapy) in cadaveric combined pancreas-kidney transplantation. During the follow up period, which ranged from 8 to 41 months, 33B3.1 treated patients were noted to have a higher incidence of rejection episodes than those given ATG (Cantrovich et al, 1994).

MAbs directed against the adhesion molecule LFA-1 have also been tested in preliminary clinical studies in an attempt to prevent rejection of cadaveric renal allografts. Soulillou and colleagues used anti-LFA-1 (25-3) in a pilot study involving 15 patients to test tolerability of the mAb. The 25-3 mAb was well

tolerated and the authors propose to perform further clinical trials to evaluate its role in preventing rejection (Le Mauff et al, 1995).

The CD4 molecule is an obvious and exciting target molecule for preventing graft rejection and, in an experimental context, is the principal subject of this thesis. However, anti-CD4 mAbs have, so far only been used to a limited extent in clinical transplantation. Three different mouse anti-human CD4 monoclonals, have been studied in transplant patients. Matas and colleagues used the mAb OKT4A, along with the standard triple therapy immunosuppression (CsA, Azathioprine and prednisolone), in a pilot study involving 30 recipients to assess the safety and tolerability of multiple intravenous infusions of the mAb. OKT4A was given for a total of 12 days, starting on the day of transplantation and was not associated with any obvious side effects (Matas et al, 1995).

Morel et al used the anti-CD4 mAb BL4 (IgG2a) (together with azathioprin and prednisolone) in 12 recipients of cadaveric renal allografts. Treatment with BL4 was started on day 1 after transplantation and discontinued after 2 weeks. The results using BL4 appeared comparable to those obtained with ALG although patients numbers were very small (Morel et al, 1990). In a small pilot study, Reinke and colleagues used the anti-CD4 mAb MAX.16H5 to treat acute rejection episodes in renal allograft recipients. MAX.16H5 was found to have a comparable results to OKT3 and to be better than ATG in the treatment of acute renal allografts rejection (Reinke et al, 1995). Clearly it would be premature from these limited clinical studies to draw any conclusions on the likely benefit of CD4 mAb in human organ transplantation

1:5 Current status and future goals of organ transplantation

Although the early results of organ transplantation have been improved by better immunosuppression, particularly with the introduction in the early 1980s of CsA, graft rejection and the side effects of non-specific immunosuppression (i.e. infection and malignancy) still remain the major problem in clinical transplantation. Moreover, although CsA undoubtedly improves the early graft survival after renal transplantation, it is disturbing to note that graft loss in the longer term (principally because of chronic rejection) has remained largely unchanged over the last 15 years (Thorogood et al, 1992; Calne RY, 1987).

The one year actuarial graft survival rate following cadaveric renal transplantation is around 85% but survival at 5 and 10 years is 60% and 30% respectively (Ferguson, 1995, Hayry, 1995, Azuma and Tilney, 1994, Opelz, 1989a).

In the precyclosporin era the actuarial one year survival rates for patients undergoing cardiac transplantation at Stanford University Medical Centre between 1968 and 1973 was 42% and between 1975 and 1981 it rose to 60%. CsA markedly improved the early results of heart transplantation and, in one large series of 389 patients operated on between 1981 and 1990, the one year graft survival rate was 83%. As for kidney transplantation, however, the actuarial 5 years survival for heart transplantation is around 55% and chronic rejection is a major problem (Hunt and Billingham, 1991, Wallwork, 1989).

Since the pioneering experiments of Medawar (Medawar, 1943 & 1944) a major goal in transplantation has been to devise strategies for rendering organ

allograft recipients specifically tolerant to their grafts. Tolerance induction would eliminate the problem of graft rejection and leave the recipients immune system otherwise intact for defence against opportunistic infection. Unfortunately little progress has been made in the clinic towards achieving the goal of transplantation tolerance. However, there is increasing optimism that some form of 'operational' or partial tolerance may soon be achievable and this hope has been strengthen by the results of several recent case reports.

In an interesting study, Strober reported three patients who were treated with total lymphoid x-radiation prior to cadaveric renal transplantation. Although antilymphocyte globulin and corticosteroids were given initially, all immunosuppressive therapy was subsequently withdrawn and the patients apparently displayed donor-specific tolerance and retained good function for the duration of follow up which ranged from 47 to 94 months (Strober et al, 1989). More recently, two cases of acquired immunological tolerance to renal allografts were described by Sayegh in patients who had previously undergone bone marrow transplantation from the same HLA-identical sibling (Sayegh et al, 1991). In a similar report, tolerance to an HLA-B, DR disparate renal allograft after bone marrow transplantation from the same donor was reported in a child with Wiskott-Aldrich syndrome and chronic glomerulonephritis (Jacobsen et al, 1994). Clearly the approaches used in these small case studies are not applicable to most renal or cardiac transplant recipients but never the less these interesting cases serve to indicate that some form of transplant tolerance is perhaps a realisable goal in the clinic. To achieve this goal however, requires a better

understanding of the regulation of immune system and insight how it can be manipulated to optimise tolerance induction.

CHAPTER TWO

Immunological basis of transplantation

2:1 The major histocompatibility complex (MHC)

The fate of tissue grafted from one individual to another is dependent principally upon cell-surface antigens which are encoded by that area of the genome known as Major Histocompatibility Complex (MHC). The MHC was first described by Gorer et al in 1948 (Gorer et al, 1948) as the genetic region responsible for determining the strength of skin allograft rejection in mice. It is the most highly polymorphic region of the genome and whilst this poses considerable difficulties from the point of view of the transplant clinician trying through matching to minimise the MHC disparity between organ donor and recipient, MHC polymorphism confers a survival advantage for the species because of the biological function of class I and class II MHC molecules (discussed in page 44).

The MHC is located on the short arm of chromosome six in man, the middle portion of chromosome 17 in the mouse (where it is designated H-2 complex) and on chromosome number 20 in the rat (where it is designated RT1) {Gunther et al, 1979; Gill et al, 1978; J.Klein, 1979}.

In man MHC antigens were first identified on peripheral blood leukocytes and designated human leukocytes antigens (HLA) (Dausset, 1958). The first HLA antigens identified were MHC class I antigens but the observation that individuals matched for class I HLA antigens still showed a strong in vitro mixed lymphocyte response led to the discovery of a second group of surface HLA antigens which were designated class II antigens (or D region). These

were first detected serologically in 1973 (Van Leeuwen et al, 1973; Thorsby and Piazza, 1975).

2:1:1 The class I MHC region

There are at least 17 MHC class I loci in the human, but the classical transplantation antigens are HLA-A, -B, and -C (Trowsdale et al, 1992 and Campbell and Trowsdale, 1993). In the mouse, class I molecules are encoded by the H-2D, H-2K and H-2L regions (Rammensee et al , 1993) and in the rat class I antigens are encoded by the RT1A and RT1C regions (Gunther et al, 1979 and Blankenhorn et al, 1983).

2:1:2 The class II MHC region

The human class II region is encoded by the HLA-DP, -DR and -DQ loci. In the mouse, class II antigens are encoded by the H-2I region genes, including I-A (which is equivalent to HLA-DQ) and I-E (which is analogous to HLA-DR). In the rat, the RT1B and RT1D loci encode for class II molecules which are analogous to the mouse IA and IE regions respectively (Blankenhorn et al, 1980 and 1983; Blankenhorn and Cramer, 1985).

The class II region of the MHC also contains genes which govern antigen processing (Cho et al, 1991) and are called Tap1 and Tap2 in humans (transporters in antigen processing), mtp1 and mtp2 in rats (MHC linked transporter protein) and HAM1 and HAM2 (histocompatibility antigen modifier) in mice. These genes encode for proteins which function as transmembrane pumps which deliver peptides for loading into class I MHC molecules (Spies et al, 1992; Solheim et al, 1997 and Monaco, 1993).

2:1:3 The class III region

The class III region of the MHC is telomeric to class II genes and encodes for components of the complement system including C2, C4 (C4A and C4B) and factor B. This region also includes the genes for heat shock proteins and tumour necrosis factors α and β (TNF).

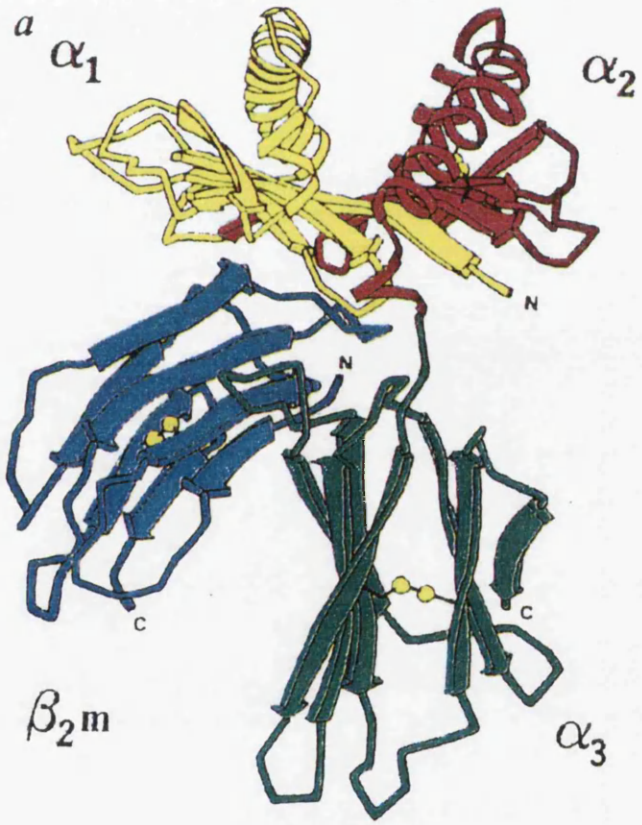
2:2 The structure of MHC molecules

MHC class I molecules are made up of a heavy (or α) chain and a light (or β) chain (β2 microglobulin) [see figure 2:1]. The α chain is approximately 44 kilodalton (KD) in humans, 47 KD in mice and 45 KD in rat and comprises approximately 350 amino acids. The non MHC encoded light chain is invariable, 12 KD in size and known as β2–microglobulin. The heavy chain consists of three extracellular domains α1, α2, α3, a transmembrane region and a cytoplasmic tail at the carboxyl terminus. β2 microglobulin is non-covalently attached to the heavy chain. The α1 and α2 domains form a platform comprising an eight stranded β-pleated sheet supporting two parallel strands of α-helix thus forming a peptide accommodating groove approximately 1 x 2.5 nm in size (figures 2:1) [Bjorkman et al, 1987]. The peptide accommodated in the binding groove appears to be an integral part of the protein complex since ''empty'' class I molecules are generally unstable and not expressed on the cell-surface (Ljunggren et al, 1990 and Schumacher et al ,1990). Class I bound peptides are typically 8 to 11 amino acids long and are tightly mounted in the groove in an extended configuration. Comparison of the amino acid sequence of a large number of human and murine class I heavy chains have indicated that

most of the polymorphic residues are located either in the α-helical sides of the peptide binding cleft or on the β–strands that form the floor of the cleft and are oriented such that the amino acid side chains point in to the cleft or toward the top of the helices. Thus polymorphism among class I MHC alleles serves to create variation in the chemical surface of the peptide binding cleft. In contrast, the α3 domain of the heavy chain and β2-microglobulin both have highly conserved amino acid sequences.

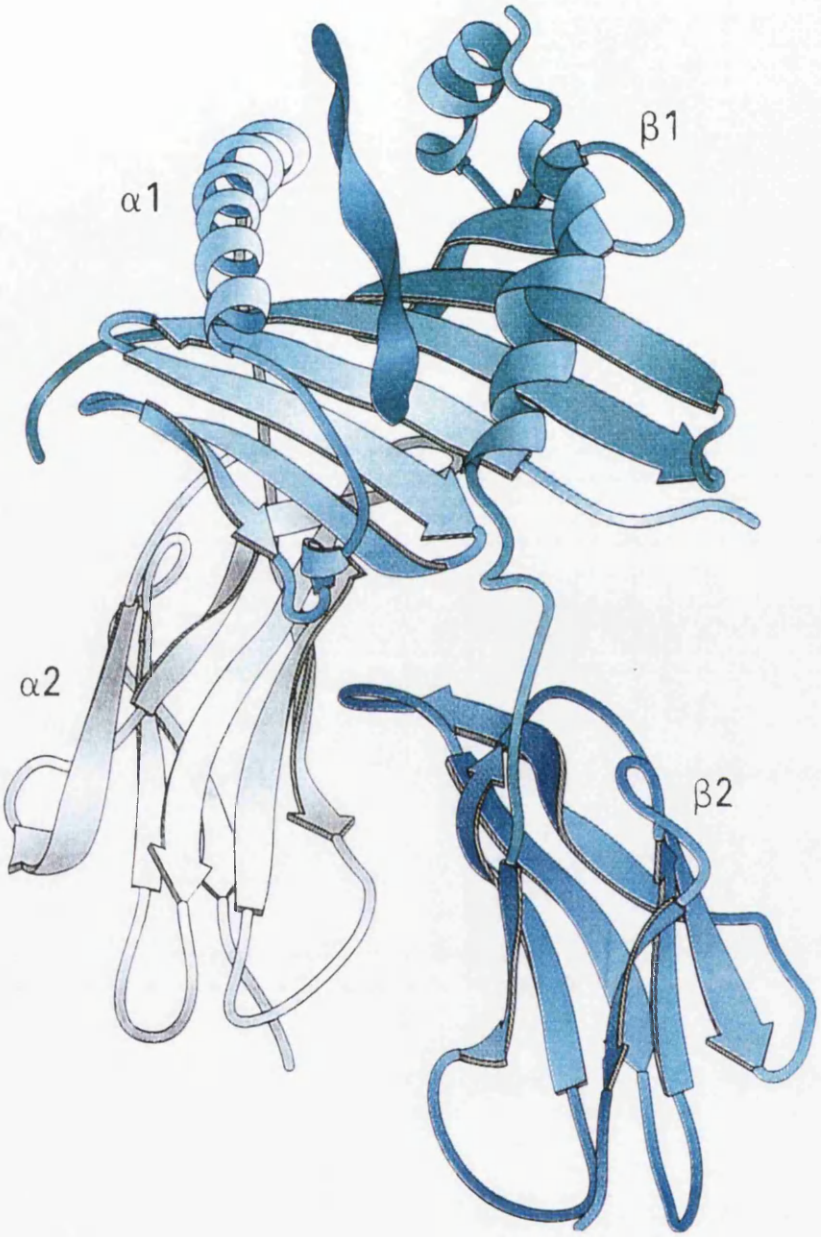
Class II MHC molecules comprise two non-covalently associated polypeptide chains (see figure 2:2). These chains (designated α and β) are similar to each other in overall structure although the α chain (32-34 KD) is slightly larger than the β chain (29-32 KD) as a result of more extensive glycosylation (Brown et al, 1993). The peptide-binding groove of the class II molecule is formed by an interaction of both chains involving the α1 and β1 domains (Brown et al, 1988a). More specifically, α1 and β1 fold to form an eight stranded β-pleated sheet platform supporting two α-helices; four strands of β-pleated sheet and one of the α-helices are formed by α1 and the other four strands of the β sheet and the other α helix are formed by β1. As in the class I structure, the α helices form the sides and the β strands form the floor of the peptide-binding cleft. Unlike class I, the ends of the class II binding cleft are open thus allowing bound peptide to extend out from the cleft. Peptides recovered from purified class II molecules range from 10 to 30 amino acids in length (mean of 14 amino acids) [Rammensee et al, 1993; Neefjes and Ploegh, 1992].

Figure 2:1 The polypeptide structure of the HLA-A2 class I molecule (Bjorkman et al, 1987)



The three dimensional structure of the extracellular region was established from the X-ray crystallographic structure of the HLA-A2 molecule.

Figure 2:2 The polypeptide structure of HLA-DR1 class II molecule (LJ Stern, 1994)



The three dimensional structure of the extracellular domains of the class II MHC molecule(HLA-DR1).

Like class I molecules, the polymorphic residues of class II molecules are concentrated in the α1 and β1 regions such that they lie in the α-helical sides or β-pleated sheet floor of the peptide-binding cleft with their side chains pointing in to the cleft or toward the top of the α helices. For class II MHC molecules, like class I MHC, the amino acids polymorphisim determines the specificity and affinity of peptide binding. A high level of polymorphism in the population ensures that some individuals are likely to bind antigenic peptides arising from any of the large number of pathogenic microbes in the environment and thereby providing an evolutionary explanation for the extensive seen in the MHC region (Lawlor et al, 1990).

2:3 Tissue distribution of MHC molecules

The distribution of class I and class II MHC antigens varies between organs and species. In general terms most nucleated cells express class I MHC whereas constitutive expression of class II MHC is much more restricted. Class I MHC is expressed on vascular endothelium cells. Renal tubule cells are weakly positive for class I antigens in both rats and humans (Hart and Fabre, 1981b ; Daar et al, 1984), but cardiac myocytes are generally class I negative. Interstitial dendritic cells are strongly class I MHC positive in both the rodents and human (Daar et al, 1984; Milton and Fabre, 1985).

Class II MHC is expressed constitutively on dendritic cells, B cells, monocytes, and macrophages, which all have the common property as antigen presenting cells for T lymphocytes. In addition, class II expression can be induced in vascular endothelium and thymic epithelium by certain cytokines (e.g. IFNγ)

(Klareskog et al, 1986; Collins et al, 1984). In rodents, resting endothelial cells are class II negative. In the rat, proximal renal tubules are class II positive (Hart and Fabre, 1981b) while in humans tubular expression of class II is variable (Fuggle et al, 1983) and in the mouse renal tubules are class II MHC negative (Benson et al, 1985).

Expression of both class I and class II MHC is dramatically upregulated by certain cytokines. Interferon γ, for example, strongly induces expression of both class I and class II MHC whereas TNFα induces expression of class I MHC (reviewed by Arai et al, 1990 and Halloran et al, 1993a). Consequently during allograft rejection rat vascular endothelial cells express increased levels of class I MHC and become class II MHC positive.

2:4 Function of the MHC molecules

MHC molecules are expressed in abundance on the surface of antigen presenting cells and their biological function is to present peptides derived from foreign proteins to the T cell and thereby stimulate a T cell dependent immune response. MHC class I molecules present endogenously synthesised peptides to CD8 lymphocytes, which are usually cytotoxic T cells (CTL) (Yewdell et al, 1990). Expression of class I molecules depends on the expression of β2-microglobulin and mice lacking a functional β2-microglobulin gene do not express class I molecules on the surface. Such animals lack mature CD8 T cells (because they are not selected for maturation within the thymus) and have defective cell-mediated cytotoxicity (Zijlstra et al, 1990).

MHC class II molecules present exogenously derived antigens to CD4 T lymphocytes (Yewdell et al, 1990). MHC class II molecules expressed on thymic stromal cells plays a key role in the positive and negative selection of CD4 T cells during thymopoiesis and genetically engineered mice that do not express MHC class II antigens lack $CD4^+$ T cells in the periphery (Cosgrove et al, 1991).

2:5 Minor histocompatibility antigens

The MHC is not the only genetic locus which influences tissue incompatibility and a large number of additional loci on different chromosomes may also play a role in graft rejection. These so called "minor" histocompatibility genes encode, minor histocompatibility antigens (mH). The ability of minor antigens to provoke graft rejection is usually, although not always, substantially less than that of MHC molecules. However, the fact that skin grafts between some MHC identical mouse strains may be rejected almost as rapidly as those between MHC incompatible strains demonstrates that minor antigens are not necessarily weak transplantation antigens (Loveland and Simpson, 1986). The precise nature of minor histocompatibilty antigens is enigmatic but over forty loci encoding allelic forms of mH antigens have been found throughout the mouse genome (Bailey, 1975). The best defined mH antigen is the H-Y antigen which is expressed by male but not female mice, and may provoke rejection of male skin grafts by syngeneic female mice (Loveland and Simpson, 1986).

Incompatibility for the mH is cumulative and multiple minor histocompatibility antigen differences can result in a significant graft rejection response. The

antigenic products of the mH loci are capable of activating either cytotoxic T-lymphocytes (CTL) or T-helper (Th) cells and those that lead to stimulation of CTL responses have been shown to be peptides derived from intracellular proteins (Wallny and Rammensee, 1990 and Griem et al, 1991).

2:6 Antigen processing and presentation

As already outlined, MHC molecules have the unique ability to bind short antigenic peptides and display them as peptide-MHC complexes in a form that can be recognised by T cells. The conversion of complex native proteins to MHC-associated peptide fragments by the antigen presenting cells is known as antigen processing.

2:6:1 Antigen processing and class I MHC transport

MHC class I molecules generally derive their bound peptide from intracellular proteins; these may be self proteins or foreign proteins (such as, those synthesised from viral RNA). Such proteins are broken down into peptides within the lysosome and class I antigen processing is inhibited by agents, such as brefeldin A, which block movement of membrane protein from the endoplasmic reticulum (ER) to the Golgi or the adenovirus E3/19K gene product, which specifically binds to class I molecules and retains them in the ER (Nuchtern et al, 1989 and Cox et al, 1990). The MHC class I associated peptides are translocated over the ER membrane by the TAP1 and TAP2 (transporter associated with antigen presentation) proteins which are located in the ER membrane (Braciale et al, 1987). Assembly of MHC class I heavy chain, β2m and peptide to form a heterotrimer complex is essential for efficient release from

ER (Ploegh et al, 1981). After release from the ER, MHC class I molecules are transported through the Golgi apparatus and the trans-Golgi reticulum network to the cell surface (Neefjes et al, 1988; Yewdel & Bennik, 1989).

As most nucleated cells express class I MHC and can therefore display peptide antigens in the class I binding groove, virally infected cells synthesising viral or mutant proteins can be marked for recognition and elimination by CD8 CTL.

2:6:2 Antigen processing and class II MHC transport

Antigens that enter an APC from the extracellular environment usually result in peptide fragments which associate with class II MHC molecules. Exogenous antigens include, for example, proteins synthesised by extracellular bacteria, fungi, and parasites as well as proteins administered during immunisation. Exogenous antigen usually enter the cell by phagocytosis or receptor mediated endocytosis. Internalised antigen localises in membrane-bound vesicles (endosomes) which facilitate degradation and intracellular transport. Class II molecules are assembled in the ER from an α-and β- chain but, in contrast to class I MHC molecules they also associate with a third molecule, the invariant chain (I), which facilitates assembly and transport of the class II MHC molecule (Anderson et al, 1992) and circumvents premature endogenous peptide binding in the ER. Class II antigen processing is sensitive to inhibition both by lysosomotropic agents, such as chloroquine and ammonium chloride, which raise the pH of these compartments and to inhibitors of endosomal proteases (Takahashi et al, 1987). After MHC class II molecules enter the endocytic pathway, they are retained there for 1-3 hours before they appear at the cell

surface (Neefjes et al, 1990). The invariant chain is degraded by proteases, thereby restoring the peptide binding capacity of class II molecules (Blum et al, 1988 and Neefjes et al, 1992).

2:7 T cell receptor structure and function

T cells recognise peptide antigens bound to MHC molecules through their T cell receptor (TCR). The TCR is a heterodimer consisting of two polypeptide chains, designated α and β, covalently linked to each other by disulphide bonds. The α chain is a 40 to 60 KD acidic glycoprotein and the β chain is a 40 to 50 KD uncharged or basic glycoprotein. X-ray crystallography has confirmed the structural similarity between the α and β chains of the TCR and immunoglobulin (Ig) polypeptides. Both α and β chains have variable (V) and (C) regions. The junction between V and C regions is encoded by a joining (J) segment gene and, in the case of β chain only, a diversity (D) segment gene.

TCRαβ receptors are present on most T cells (>95% of peripheral T cells and over 50% of thymocytes from adult mice). A small number (less than 10%) of T cells express instead the γδTCR. γδ T cells are found in higher abundance on lymphocytes located in epithelial surfaces.

The TCRαβ associates at the T cell surface with a series of polypeptides which together comprise the CD3 complex. In man CD3 consists of four polypeptide chains designated; γ , δ, ε and ζ chains (each of 20-28 KD). The γ , δ and ε chains are structurally related single domain members of the immunoglobulin supergene family whereas the ζ chain is unrelated and forms ζ-ζ dimers. In rodents, a fifth chain (η) is also present. The stoichiometry of the most common

TCRs is αβ-γδε ζζ in man or αβ-γδε ζη in rodents. The CD3 cytoplasmic domains each contain a motif called an Antigen Recognition Activation Motif (ARAM) or Tyrosine-based Activation Motif (TAM) (reviewed by Weiss, 1991).

2:7:1 CD4 and CD8 co-receptors

CD4 is a transmembrane glycoprotein which is expressed on approximately 65% of mature T cells and is a member of Ig superfamily. CD4 was originally designated T4 in humans, L3T4 in mice, and W3/25 in rats. It is an important accessory molecule and facilitates the interaction of the T cell with APCs or CTL target cells. The function of CD4 (and CD8) is intricately associated with TCR function, and these molecules are sometimes designated T cell-co-receptors. CD4 is approximately 55 KD in size, and is expressed as a monomer on the surface of peripheral T cells and thymocytes. In humans and rats, although not in the mouse, CD4 is also present on monocytes, macrophages and langerhans cells (Wood et al, 1983; Barclay AN, 1981; Steininger et al, 1984). CD4 comprises four extracellular Ig-like domains [figure 2:3] (two membrane proximal (V4 and V3) and two membrane distal (V2 and V1) domains), a hydrophobic transmembrane region and a highly basic cytoplasmic tail 38 amino acids long. The gene encoding CD4 is located on human chromosome 12 (Isobe et al, 1986; reviewed by Bierer et al, 1989 and Keegan et al, 1992).

The crucial importance of CD4 in T cell function was initially demonstrated by the ability of anti-CD4 antibodies to block class II MHC-restricted antigen stimulation of CD4 T cells both in vitro and in vivo (Reinherz et al, 1979). CD4

has two important functions in the activation of T cells. First, it serves as an adhesion molecule by virtue of its ability to bind via its two N-terminal Ig-like domains to the nonpolymorphic β2 domain of the class II MHC molecule (Eichmann et al, 1987; CA Janeway, 1989, Doyle and Strominger, 1987). Second; the CD4 molecule transduces signals and facilitates TCR complex-mediated signal transduction upon binding class II MHC , thereby promoting T-cell activation. Monoclonal antibodies directed against CD4 may have either stimulatory or inhibitory effects on MHC-independent T cell activation triggered by anti-TCR or anti-CD3 antibodies (Saizwa and Janeway, 1987). After stimulation of T cells by antigen plus MHC (or anti-TCR mAbs) there is rapid phosphorylation of serine residues in the cytoplasmic tail of the CD4 molecule. Furthermore, a lymphocyte-specific protein tyrosine kinase, called Lck (or $P56^{lck}$), is physically associated with the cytoplasmic tail of the CD4 molecule and is essential for full T cell activation (Miceli et al, 1993).

The CD8 receptor is a glycoprotein present on class I restricted T cells and contributes to activation of these cells by binding to class I MHC on antigen bearing cells. The CD8 molecule was originally designated Ly2 antigen in the mouse, T8 in man and OX8 in the rat. An important role for CD8 in T cell activation was first suggested by studies demonstrating that anti-CD8 mAbs could block target cell lysis by class I restricted cytotoxic T cells but not by the small subset of cytotoxic class II-restricted (CD4) cytotoxic cells (Braakman et al, 1987; reviewed by Bierer et al, 1989). CD8 comprises two chains (α and β), each with a single immunoglobulin-like external domain. The cytoplasmic region

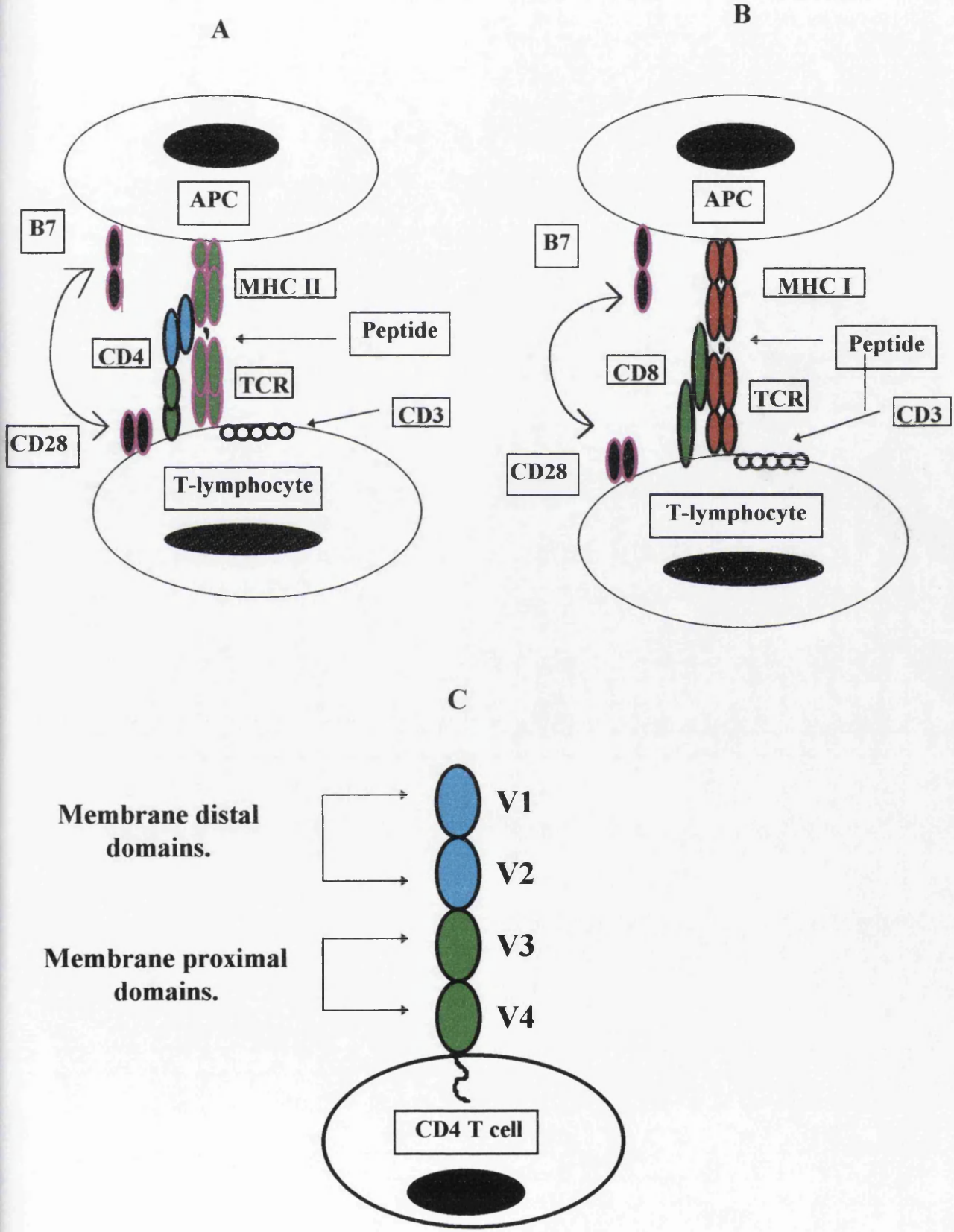
of the α chain includes a binding site for the src-related tyrosine kinase $P56^{lck}$, through which the co-signalling effects of CD8 engagement are presumably mediated. The α chain can be expressed as a full length form or alternatively a spliced α form which lacks the C-terminal cytoplasmic domain including the $P56^{lck}$ binding site. Most of the CD8 molecules are expressed as αβ heterodimers but α homodimers also exist (Bierer et al, 1989; Miceli and Parnes, 1993).

2:7:2 T cell activation

T cell activation requires two transmembrane signals. The first signal is delivered through the engagement of TCR with MHC/peptide complex (figure 2:4) and the second signal is provided through interaction of additional T cell-APC ligand-receptor pairs on T-cell and APC, including ICAM1-LFA1, CD2-LFA3 and CD28-B7 (figure 2:3).

TCR-mediated activation results in the phosphorylation of a number of intracellular proteins including the Src-family of protein kinases (e.g. $P59^{fyn}$) and the CD3 ARAM motifs. Thereafter, the tyrosine-phosphorylated ARAMs recruit additional effector molecules by serving as interaction sites for ZAP-70 kinase which contains a specialised binding domains (SH2) for phosphotyrosine residues. TCR engagement is not usually sufficient to trigger T activation and engagement of CD4 or CD8 co-receptors also necessary (reviewed by Fields and Roy, 1996).

Figure 2:3 CD4 and CD8 T lymphocyte after engagement with T cell receptor (A &B). CD4 molecule extracellular domains (C).



2:8 Allorecognition

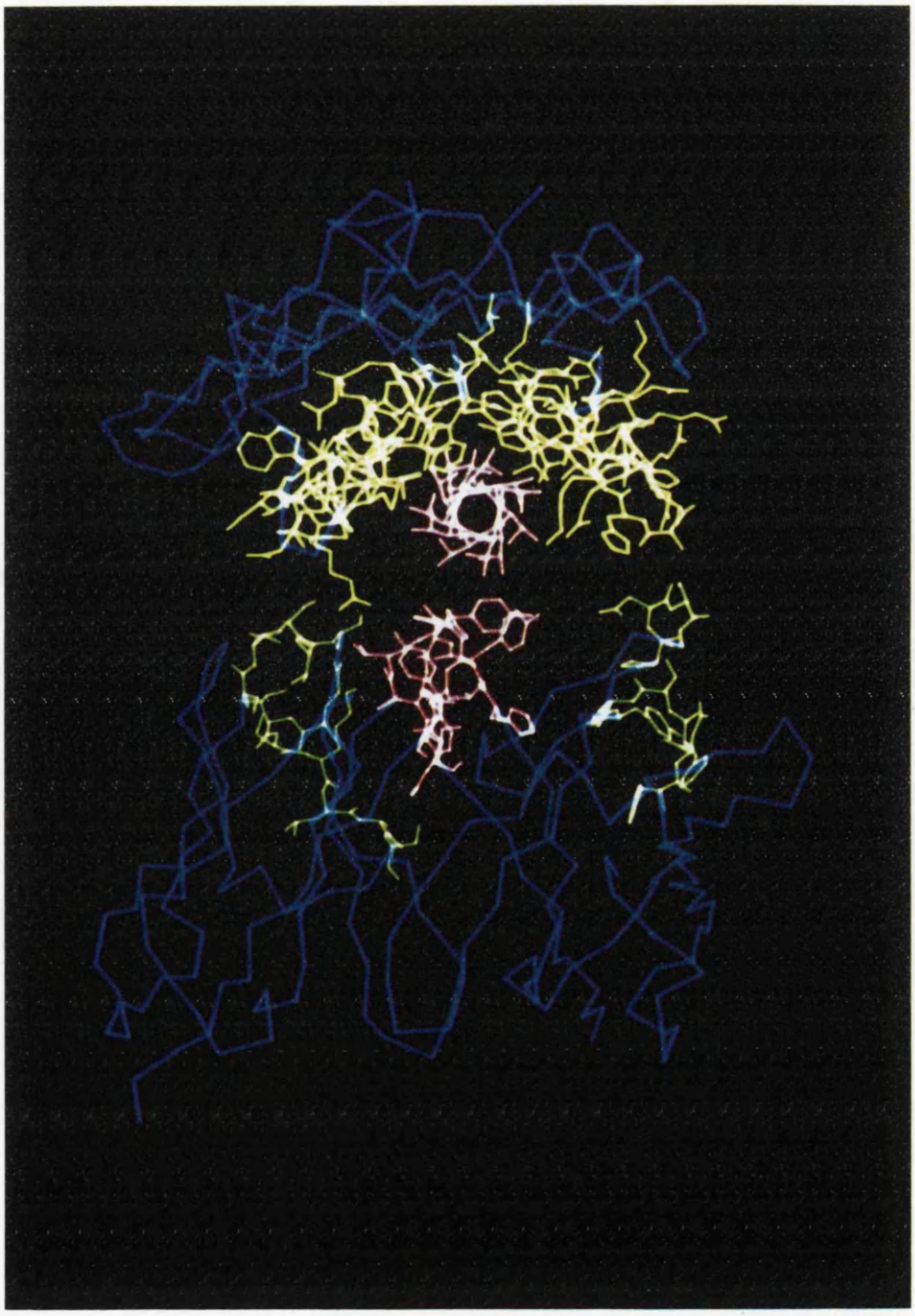
It has long been recognised that the normal T cell repertoire contains a very high frequency (1-10%) of T cells capable of recognising and responding to alloantigens (Sherman and Chattopadhyay, 1993). Two principal hypothesis have been proposed to explain this high frequency of alloreactive T cells.

Matzinger and Bevan (Matzinger et al, 1977) proposed that a single allo-MHC product can stimulate multiple T cell clones by formation of multiple "binary complexes" between MHC and different endogenous peptides. In the light of current understanding of TCR-MHC interactions, this hypothesis would imply that alloreactive T cells recognise allogeneic MHC molecules together with a wide range of different endogenous peptides displayed by the MHC molecules thereby stimulating multiple T cells clones each with specificity for different peptides presented by donor MHC.

Bevan (Bevan et al, 1984) subsequently proposed an alternative, but not mutually exclusive, possibility suggesting that the high frequency of responding T cells results from the very large number of MHC molecules displayed by APC ("high determinant density hypothesis").

Further clarification of the molecular basis of allorecognition was provided by Lechler and his colleagues (Lombardi et al, 1989) in studies using T cell clones specific for HLA-DR. On the basis of their experimental findings they proposed that an allo-MHC molecule can be envisaged as having two important functional sites. The first site comprises amino acid residues on the upper face of the α helices which make direct contact with TCR. The second site is

Figure 2:4 The tri-molecular complex of T-cell receptor, MHC molecule & peptide.(Robert Lechler, 1994)



formed by amino acids which form the antigen binding groove i.e. those residues on the inner surface of the α-helices and the exposed area of the β strands forming the floor of peptide binding groove. These sites determine the nature of the bound peptide but do not contact the TCR directly. Lombardi et al showed that, depending on the nature of the MHC disparity, the TCR may recognise polymorphic residues on either the α helices or the bound peptide itself (Lombardi et al, 1989).

Allogeneic MHC molecules are unique as antigens because they are able to directly stimulate T cells without the need for processing and presentation by recipient APC. This is known as direct allorecognition (reviewed by Auchincloss and Sultan, 1996). In addition, allogeneic MHC molecules may also be treated like nominal protein antigens and processed and presented as short antigenic peptides in the context of self-MHC by recipient APC, so called indirect allorecognition (reviewed by Auchincloss and Sultan, 1996).

2:8:1 The indirect allorecognition pathway

Although the direct pathway of T cell allorecognition is generally thought to be the principal pathway responsible for acute rejection, there is now increasing evidence that the indirect pathway may also plays a significant role (Shoskes and Wood, 1994). A role of the indirect pathway in allorecognition was first postulated by Butcher and Howard in 1982 (Butcher GW and Howard JC, 1982) and by Lechler and Batchelor in the same year (Lechler et, 1982a). The latter authors argued that the indirect pathway accounted for the rejection of rat kidney allografts which lacked donor strain APC (Hart et al, 1980; Lechler and

Batchelor, 1982a & 1982b). Later, Fangmann and colleagues (Fangmann et al, 1992a) provided convincing evidence for the indirect pathway by demonstrating that Lewis rats primed with synthetic peptides corresponding to the A^a class I molecule displayed accelerated rejection of A^a disparate DA skin grafts. Similarly, Benichou and co-workers showed that spleen cells from BALB/c and SJL mice given AK strain skin allografts proliferated strongly when stimulated with AK class I MHC derived peptides in vitro (Benichou et al, 1992). Confirmatory data on the role of the indirect pathway was subsequently provided by several other groups (Shirwan et al, 1995, Gallon et al, 1995).

The potential contribution to graft rejection of T cells activated by indirect pathway is not limited to rejection of skin grafts. Benham et al, reported that semi-allogeneic (DA X Lewis) F1 kidney allografts which had been depleted of donor interstitial dendritic cells (to minimise the possibility of direct T cell allorecognition) were rejected more rapidly by Lewis recipients which had been primed with DA class I peptides prior to transplantation (Benham et al, 1995). Despite such experimental data, information regarding the indirect pathway in clinical transplantation is still very limited.

The relative roles of the indirect and direct pathway in the rejection process is not clear, although it is generally held that the direct pathway is the major route responsible for acute graft rejection in non-sensitised recipients whereas the indirect pathway may play a contributory role in accelerated allograft rejection in previously sensitised recipients as well as in chronic graft rejection (Benham et al, 1995; Auchincloss and Sultan, 1996). The activation of recipient CD4 T

cells by polymorphic donor peptides presented on various recipient APC might enhance various effector mechanisms of rejection. Fabre's group, have proposed that T cells activated by the indirect pathway may provide help for conventional effector mechanisms, notably antibody production, cytotoxic T cell generation and intragraft DTH reactions (Fangmann et al, 1992b, Parker et al, 1992).

2:9 Mechanisms of graft rejection

Current understanding of the mechanisms responsible for graft rejection is largely based on the immunological response of laboratory animals to tissue or organ grafts from genetically dissimilar donors. The rejection process can, for the ease of description, be divided in to three different stages, namely the afferent stage, the central stage and the efferent stage.

2:9:1 Afferent phase of allograft rejection

During the **afferent phase** of allograft rejection, the immune system of the recipient recognises and becomes primed to graft alloantigens. Bone marrow derived dendritic cells within the transplant, the so called passenger leukocytes, play a key role in host sensitisation (reviewed by Roake et al, 1994). Dendritic leukocytes are found in the parenchyma of all commonly transplanted tissues (Hart et al, 1981a & Daar et al, 1984). These cells express high level of MHC class I and II antigens on their surface and are potent stimulators of the in vitro mixed lymphocyte reaction (Klinkert et al, 1980, Steinman et al, 1978, Mason et al, 1981). After organ transplantation, donor DC can be identified in the recipient spleen between days 1 and 4, where they form clusters with alloreactive lymphocytes and stimulate alloimmunity (Larsen et al, 1990, Austyn

and Larsen, 1990). The importance of the DC in initiating rejection of allografts in vivo was first demonstrated by Lechler and Batchelor (Lechler et al, 1982a). They transplanted rat kidneys into intermediate hosts which had been pre-treated with anti-donor alloantibody (enhancing antibody) to prevent rejection of the kidney (so-called passive enhancement). After several weeks, to allow donor bone marrow derived dendritic cells to disperse and be replaced by those of recipient origin, the kidneys were re-transplanted into a second unmodified hosts syngeneic with the intermediate host. In certain rat strain combinations, kidneys devoid of donor DC were not rejected by the second host. However, if the secondary recipient was injected with donor strain dendritic cells at the time of retransplantation, graft rejection was fully restored, thus demonstrating the importance of DC in initiating allograft rejection.

Certain other cell types, notably endothelial cells, are also capable of acting as antigen presenting cells, although they are generally much less efficient in this respect than DC and monocytes which are usually designated "professional" APCs (Hirschberg et al, 1981). Purified vascular endothelial cells are able to stimulate proliferation of allogenic lymphocytes in vitro (Pober and Cotran, 1991 and Hughes et al, 1990). As already noted, human endothelial cells as well as rodent endothelial cells do not constitutively express class II antigens, and in the resting state would not, therefore, be able to directly activate CD4 T cells. However, rat endothelial cells which have been cultured in vitro in the presence of IFNγ to induce class II MHC are able, when injected intravenously, to sensitise recipients and accelerate allograft rejection thereby confirming the

ability of rodent endothelial cells to initiate an alloimmune response (Ferry et al, 1987).

2:9:2 Central phase of allograft rejection

The central phase of graft rejection encompasses the complex cellular interactions between APC and lymphocytes, and between lymphocyte subsets (e.g. T-B cell collaboration) and takes place mainly in the lymph nodes and spleen of the recipient. The T helper cell, usually a CD4 T cell, plays a central role in regulating and orchestrating the various cellular and humoral effector mechanisms responsible for graft rejection and does so by elaboration of a variety of cytokines (reviewed by Dallman , 1992 and Nadeau et al, 1996).

Cytokines are soluble proteins which act through binding to specific receptors, in either an autocrine or a paracrine fashion and thus serve to mediate bi-directional cell-cell communication. They are important not only for activation of the immune response but also for regulating of both the magnitude and nature of the resulting immune response. Fig 2:5 depicts the role of different cytokines in the generation of the alloimmune response. A detailed discussion of the role of individual cytokines in the development of the immune response is beyond the scope of this thesis but some important points are worthwhile highlighting (see later page 67).

2:9:3 T cell subsets and the efferent phase of allograft rejection

T lymphocytes are essential for allograft rejection (Dallman et al, 1982, Dallman and Mason, 1982, Bradley and Bolton, 1992). In T cell deficient rodents, reconstitution with CD4 T cells alone is usually sufficient to restore allograft

rejection (Bolton et al, 1989; Gracie et al, 1990; Bell et al, 1990). However, in certain experimental situations (some mutant class I MHC disparate mouse strain combination), adoptive transfer of CD8 T cells alone is sufficient to restore rejection of skin allografts (Rosenberg et al, 1992).

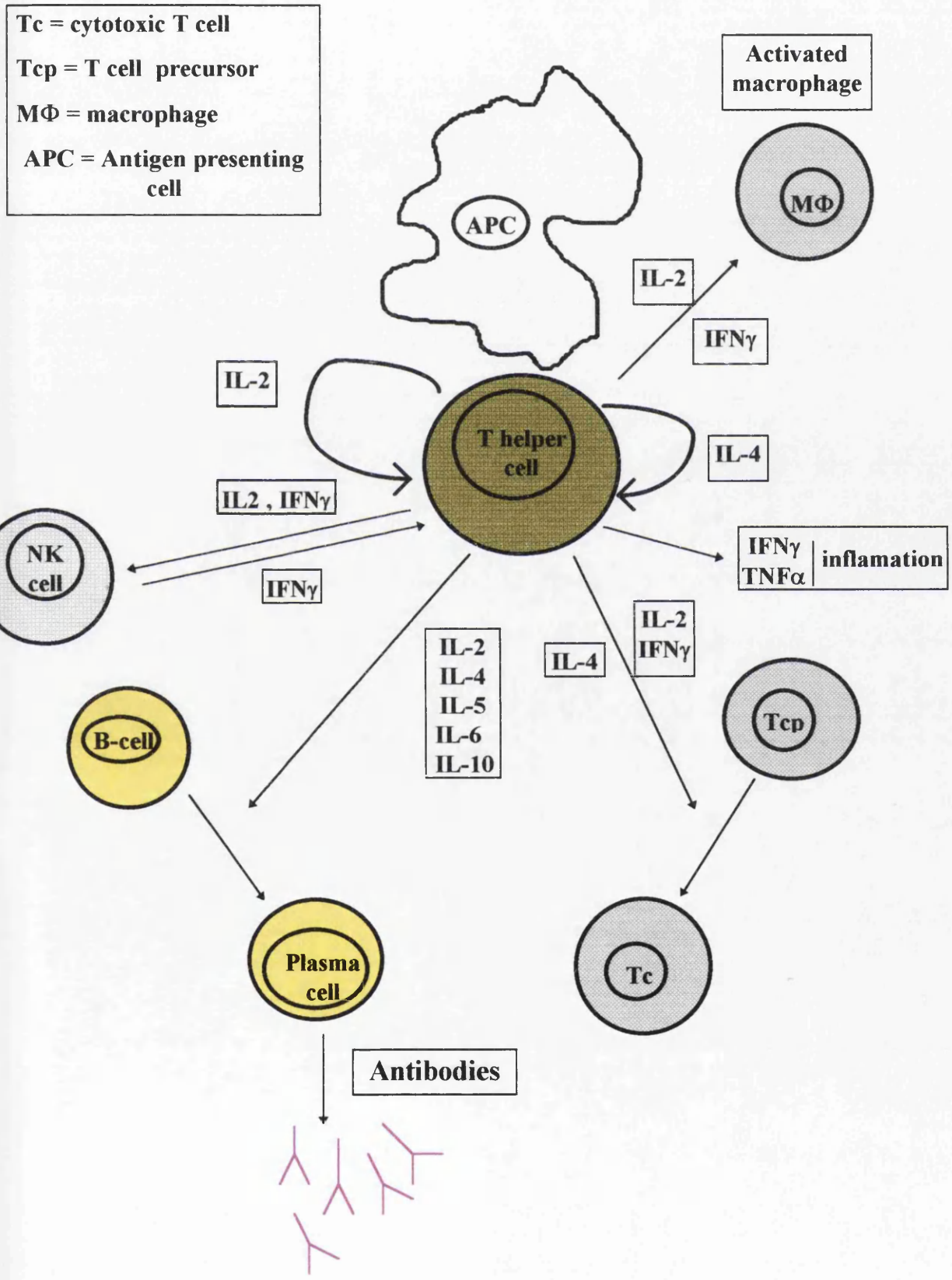
The T cell-dependent cellular and humoral effector mechanisms responsible for mediating allograft rejection are those which the immune system normally employs to deal with infectious agents i.e. there is no unique effector mechanisms responsible for the non-physiological phenomenon of allograft rejection. Specific cytotoxic T cells, activated macrophages and natural killer (NK) cells, and antibody dependent mechanisms may all, in principle, result in graft rejection. The relative role of each of these effector mechanisms in graft rejection has not been clearly defined and is probably dependant on factors such as the type of graft, the MHC disparity and whether the recipient has been previously sensitised (Bradley, 1996).

Overall, cell-mediated immune mechanisms are generally thought to be more important than antibody dependent responses in mediating acute allograft rejection in non-sensitised recipients and there has been much debate on whether specific cytotoxic T cells or DTH mechanisms are the most important (Bradley, 1996).

2:9:3:1 Cytokines and the generation of immune response

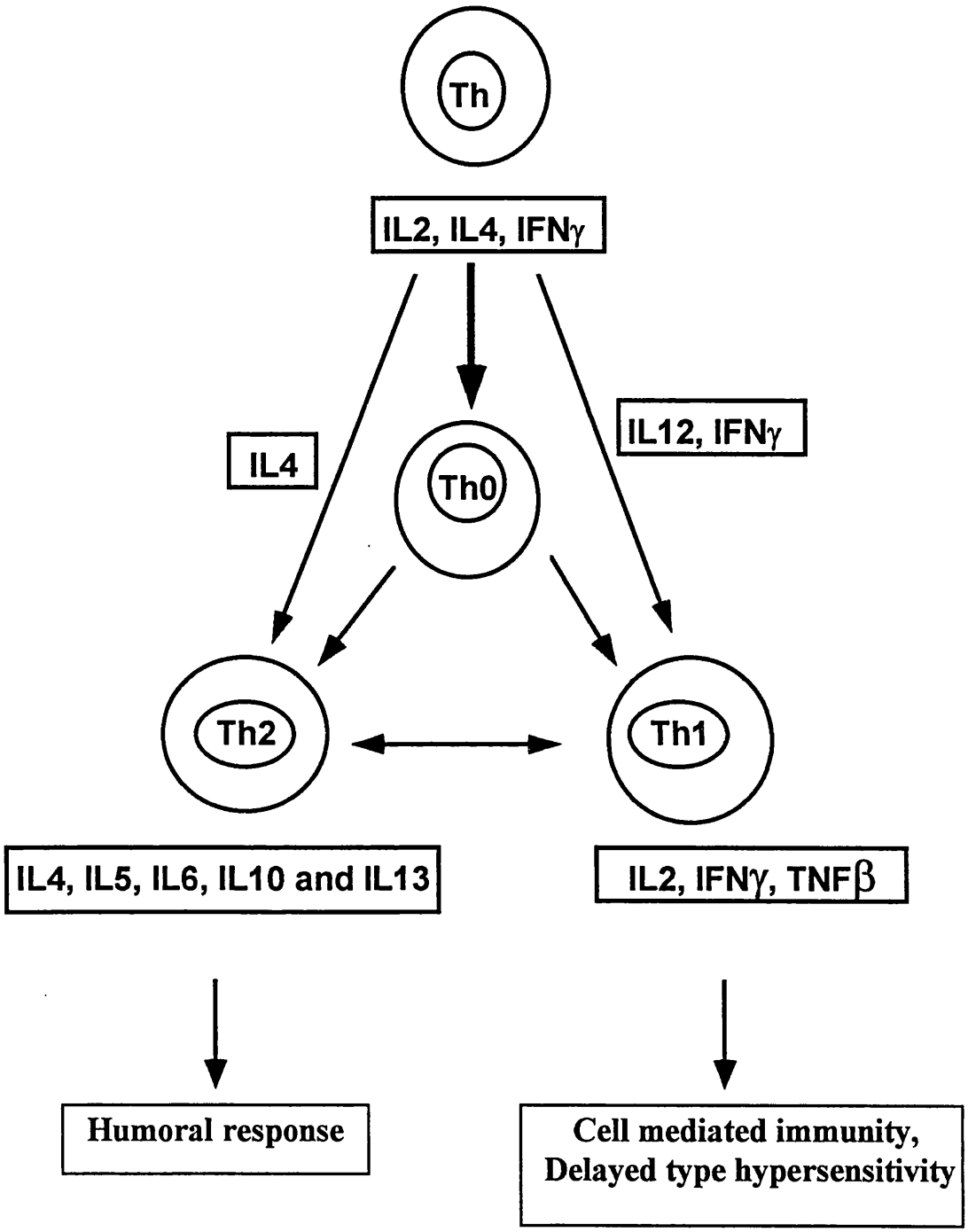
In 1986, Mosman and colleagues reported that most cloned lines of murine CD4 T cells could be classified into two groups, designated Th1 and Th2, based on

Figure 2:5 The central role of the T helper cell and cytokines in the generation of allo-immune response



the type of cytokines they produced and their related functional activities as shown in figure 2:6 (Mosman et al, 1986). This work led to the development of the Th1\Th2 paradigm which encompasses the concept that CD4 T cells (Th0) differentiate into one of two T cells subsets, Th1 and Th2, and these each produce a distinctive pattern of cytokines. Th1 cells generally produce IL-2, IFNγ, TNFα and are responsible for producing cell-mediated immune responses. Th2 cells generally produce the cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 and are important for humoral immune responses. Both T helper types produce IL-3, TNFα and granulocyte colony-stimulating factor (GM-CSF). Interestingly, the development of Th1 and Th2 cells is itself closely regulated by cytokines. IL-12 and IFNγ promote the development of Th1 cells whereas IL-4 promotes the Th2 cells. There is also strong cross-regulation between the two T cell subsets (reviewed by Smith, 1989). IL-4, inhibits the development of Th1 responses whereas IL-12 and IFNγ, inhibit the development of Th2 cells. IL-12, in particular, has recently emerged as a key immunoregulatory cytokine controlling the balance of Th1 and Th2 responses. It is a heterodimer consisting of two covalently linked glycosylated chains (P40 and P35), and is produced mainly by monocytes, B cells and also dendritic and NK cells (Trinchieri,1993 and 1995).

Figure 2:6 Cytokine production by Th1 and Th2 T cell subsets.



Th2 and Th1 cells exert strong cross-regulation, IFNγ from Th1 cells inhibits Th2 cells whereas IL-4 from Th2 cells inhibits Th1 cells. Th0 cells may produce a mixed (Th1 and TH2) cytokine pattern.

2:9:3:2 The role of antibody in graft rejection

It has long been recognised that preformed cytotoxic alloantibodies may cause hyperacute rejection of kidney allografts in sensitised recipients. Such antibodies usually arise from a previous kidney transplant, pregnancy or blood transfusion (Kissmeyer-Nelsen et al, 1966; Williams et al, 1968; Patel and Tersaki, 1969). The binding of antibody to vascular endothelium leads to a sequence of events including complement fixation, platelet aggregation, fibrin deposition and graft destruction (Busch et al, 1971).

In the clinical setting preformed antibodies to class I MHC and to ABO blood group antigens are responsible for hyperacute rejection (Baldwin et al, 1991) but fortunately the clinical incidence of this problem has been greatly reduced by blood group matching, and screening recipient serum for the presence of cytotoxic alloantibodies against donor spleen cells and then avoiding transplantation in the presence of a positive cytotoxic crossmatch (Patel & Terasaki, 1969). Although IgM is the most efficient class of antibody for activating complement in vitro, IgG is more harmful to the transplanted organ (Baldwin et al, 1991). Alloantibody dependent effector mechanisms have not usually been thought to be responsible for acute rejection of organ allograft but Bradley and colleagues recently showed in the rat that alloantibody alone is able, in some circumstances, to effect acute allograft rejection (Morton et al, 1993). Alloantibody may, in principle, mediate acute rejection by either antibody-dependent cellular cytotoxicity (ADCC) or by complement dependent lysis. In clinical practice evidence is also emerging which suggests that anti-class

I alloantibodies may be associated with a vascular pattern of acute rejection during the first 6 months of transplantation (Halloran et al, 1993b).

2:10 Chronic graft rejection

Chronic rejection is responsible for the majority of late graft failures in clinical practice and is seen in all types of organ transplant i.e. kidney, heart and liver. Neither the aetiology nor the pathophysiology of the process is particularly well understood (Hayry et al, 1993, Paul and Benediktsson, 1993). However, several potential risk factors have been implicated in its aetiology. HLA matching is important and there is undoubtedly a lower rate of kidney allograft attrition in grafts from living related donors compared to kidneys from cadavers (Isoniemi et al, 1994). HLA-DR mismatching in particular increases the incidence of both acute rejection episodes and long term graft loss. Other important recipient factors contributing to chronic rejection are a high serum cholesterol and high LDL and triglyceride levels, as well as infection with cytomegalovirus (Almond et al, 1993).

The principle histopathological findings during chronic rejection, irrespective of the type of organ transplanted, are arteriosclerosis and interstitial fibrosis (reviewed by Hayry, 1995). Although both antigen-dependent and antigen-independent factors appear to contribute to the vascular endothelial injury which characterises chronic graft rejection, immunological factors are probably most important. Circulating anti-donor antibodies and antigen-antibody complexes may play an important role in triggering endothelial injury (Hayry, 1995). Much

effort is currently being devoted to dissecting further the complex events underlying chronic allograft rejection.

CHAPTER THREE

Transplant tolerance: experimental strategies and possible mechanisms.

3:1 Transplantation tolerance

The simple definition of transplantation tolerance as 'specific absence of an immune response to an antigen' does not adequately explain many of the more recent observations emerging from experimental studies of transplantation tolerance and there is wide agreement that the concept of 'operational tolerance' may be more useful. Operational tolerance is 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 (Sachs, 1996). Operational tolerance can be achieved or induced by a variety of mechanisms.

3:2 Strategies for inducing experimental transplantation tolerance

The strategies for inducing experimental transplantation tolerance can be broadly divided into those protocols in which some form of antigen pre-treatment is given (e.g. donor-specific blood transfusion, donor bone marrow or donor spleen cells) and experimental models in which non-specific immunosuppression is given around the time of transplant (either chemical immunosuppressant such as CsA or biological agents, notably monoclonal antibodies, against cell surface molecules or immunoregulatory cytokines).

3:2:1 Antigen pre-treatment

Its has been known for many years that donor specific blood transfusions (DSTs) may have a beneficial effect on the survival of subsequent renal transplant in rodents and humans (Marquet et al, 1971, Fabre and Morris, 1972, Opelz et al, 1973, Sollinger et al, 1986). In humans, pre-renal transplant DST

was used at Washington University School of Medicine in 1971 (Newton et al, 1973; Anderson et al, 1982). In the late 1970s and early 1980s pretransplant blood transfusion was widely accepted as having a significant beneficial effect on the survival of renal allografts from both living related and cadavers donors. Potential recipients were typically pre-treated with three aliquots (200-ml each) of whole blood DST at bi-weekly intervals under azathioprine coverage. Similar results have been obtained with a single transfusion performed immediately preoperatively (Davies et al, 1992; Alexander et al, 1992; Alexander et al, 1993). It has been argued that blood transfusion only improves human kidney allograft survival when the recipient shares at least one HLA-DR antigen in common with blood donor (Lagaaij et al, 1989).

Recently, however, the improved results of transplantation together with the risk of viral disease transmission from transfusion led many centres to abandon deliberate blood transfusion as a method to improve kidney allograft survival. However, the Collaborative Transplant Study concluded that the potential benefit of blood transfusion was less than the risks of infection and presensitisation (Opelz, 1987) and interest continues in refining DST protocols. The potential of inducing transplantation tolerance by donor bone-marrow infusion has also been studied by several investigators. It is known that donor derived haemopoetic cells may be transferred to the recipient at the time of solid organ transplantation and detected as residents cells within recipient tissues many years afterwards (Larsen et al, 1990, Starzl et al, 1992). This observation, combined with evidence from experimental models that tolerance can be

achieved if allogeneic haemopoetic cells are given to conditioned recipients, has led to the hypothesis that spontaneous haemopoetic microchimerism (the presence of cells of two genetically different tissue types in one individual) may be essential for the development and maintenance of immunological unresponsiveness to organ allografts (reviewed by Sykes, 1996). Barber and colleagues, in a protocol of DBM infusion for human cadaveric renal transplant recipients, used a 10-14 days induction course of Minnesota antilymphocyte globulin in 57 patients, beginning the day after transplantation and followed 7 days later by transfusion of approximately 2-3 x 10^8 viable nucleated DBM cells/Kg body weight. All recipients were also treated with conventional triple therapy. Matched control patients did not receive DBM. While there was no difference in the number of rejection episodes, graft survival was significantly better in the DBM group than in the control group. Renal allograft survival in the DBM group at 12 and 18 months was 90% and 85%, respectively, versus 71% and 67% , respectively, for the control group. In addition it had been possible in more than half of the patients in the DBM group to taper the amount of prednisone required (Barber et al, 1991).

The induction of a state of a mixed chimerism has been observed following human solid organ transplantation, and was noted to be present consistently in a group of successful long-term renal transplant recipients, in whom donor-specific reduction or diminution in mixed leukocyte reaction was observed (Starzl et al, 1993a). Although clinical strategies have been implemented to augment the chimeric state, no definitive relationship between the development

of chimerism and immunological responsiveness has yet been identified (Schiltt, 1997). It has been suggested that the capacity of allogeneic haemopoetic cells to induce tolerance results largely from their ability to induce intrathymic clonal deletion of thymocytes with TCRs that recognise antigens expressed by the haemopoetic cells (reviewed by Sykes, 1996). In essence, bone marrow transfusion attempts to harness nature's clonal deletion mechanism. The potential mechanisms by which DST and DBM modulate the immune system are likely to be different in degree rather than in kind. These mechanisms include induction of T cell anergy, development of anti-idiotypic antibodies to HLA or TCRs, suppressor cell or veto cell activity, alteration of T cells subsets (Th1 to Th2 shift), stimulation of regulatory CD4 T cells, clonal deletion by promotion of microchimerism or a combination of these various mechanisms.

Interestingly, the finding that freedom from immunosuppression was sporadically possible in long surviving recipients of liver allografts and the concurrent evidence that chimerism was uniformly demonstrable in such tolerant patients have led to prospective trials of complete weaning of immunosuppressive drugs in selective groups of transplant patients. The first report on weaning of immunosuppression was in 1993 by Thomas Starzl. He observed that 5 of 20 randomly selected long-surviving liver transplant recipients had stopped their medication for between 7 to 13 years (Starzl et al, 1993b). In these 20 randomly selected patients along with additional 2 long-term recipients, nine were female patients who had been given livers from male donors. In all nine the Y chromosome could be demonstrated in the skin or

lymph nodes by in situ hybridisation and by PCR. Similarly, chimerism was also noted in the other 13 patients who were studied with HLA detection techniques to identify donor haplotypes (Starzl et al, 1993b).

In a small preliminary study, Starzl and colleagues weaned eight long-term recipients of renal allograft from immunosuppression therapy (Mazariegos et al, 1995). These patients were recipients of living related kidneys of more than five years duration and had at least 2 years of stable graft function. Only one patient developed a rejection episode during weaning of immunosuppression and at the time of publication, the time off immunosuppressive drugs ranged from 3 months to 29 years. Four of the patients were off immunosuppression for less than 2 years and are currently under follow up, whereas the other three patients were off immunosuppression for more than 14 years. This limited study of 8 patients used only serum creatinine as an assessment of renal function. Ramos et al in a prospective randomised trial, evaluated seventy two long-surviving liver transplant recipients for weaning off of immunosuppressive drugs. Complete weaning was accomplished in 27.1%, with a 3-19 months drug-free follow-up period. Weaning is also progressing in 47.4% and has failed in 25.4% without graft losses. This prospective trial confirmed that many long-term survivors after liver transplantation can be successfully weaned off immunosuppression (Ramos et al, 1995). However the authors cautioned that this should only be contemplated 5-10 years posttransplantation and then only with careful case selection, and monitoring with a low threshold for reinstitution of immunosuppression. Recently, Starzl and colleagues reported the weaning of

immunosuppressive therapy in 95 long-term recipients of liver transplants. Complete weaning was achieved in 18 of the 95 patients and 37 patients were, at the time of writing, in the uninterrupted process of drug weaning. In only 18 patients was weaning interrupted because of a confirmed diagnosis of rejection. Twelve patients were withdrawn from the weaning protocol due to non-compliance (Mazariegos et al, 1997). One of the conclusions from this study was that neither the presence nor the quantity of microchimerism can be used as a guide to whether immunosuppression weaning should be considered in stable, long-surviving liver transplant recipients. In long-term liver allograft recipients, about 30% of recipients apparently can come off all immunosuppression without penalty of rejection. Stopping immunosuppression after other types of solid organ transplantation usually result in their rejection (Calne, 1997).

Numerous reports have shown that injection of donor haemopoetic cells promotes allograft survival, for example, Salom et al achieved significant prolongation of rat cardiac allograft survival by perioperative intravenous injection of donor spleen cells and a single dose of CsA at day 2 after transplantation (Salom et al, 1993, Westra et al, 1991). Similarly, Wood et al have obtained indefinite survival of mouse cardiac allografts by pretreating recipients with DST and anti-CD4 mAb (Bushell et al, 1994). In a preclinical large animal model, injection of donor bone-marrow induced tolerance in bilaterally nephrectomised Rhesus monkeys who had received renal allografts. The animals received a 5-day treatment with rabbit anti-human thymocyte globulin (RATG) followed by donor BM infusion 7-8 days after the last dose of

RATG treatment. No additional immunosuppression was given and, graft survival was more than 70 days in all animals. 30% of recipients lived for more than 100 days in comparison with control animals which received RATG only and rejected their grafts within 37 days. The addition of cyclosporin and prednisone in this experimental model led to graft survival of greater than 100 days in 67% of animals (Thomas et al, 1989). Recently, Kawai et al induced mixed allogeneic chimerism and renal allograft tolerance in Cynomolgus monkeys by a regimen in which recipients were first conditioned by total body irradiation followed by donor BM and a short course of cyclosporin. This regimen led to graft survival of more than 100 days in all experimental animals, whereas control animals rejected their renal graft within 15 days. In addition, donor specific non-reactivity was confirmed by both MLR and skin transplantation. (Kawai et al, 1995). More recently, Thomas et al have induced tolerance to kidney allografts in Rhesus monkeys using perioperative anti-CD3 mAb and donor BM without the need for continuous immunosuppression. The above protocol led to graft survival of more than 120 days in about 70% of experimental animals whereas the control animals rejected their renal graft within 10 days (Thomas et al, 1997).

3:2:2 Monoclonal antibodies in experimental organ transplantation

Monoclonal antibodies that deplete or block cells carrying their target antigen offer an exciting prospect for modifying graft rejection and have been studied extensively. With this in mind antibodies to a variety of T cell surface antigens [CD4, CD8, TCR, CD3, IL2-R, LFA1, CD28, CD40 and ICAM1] and

cytokines [e.g. TNFα and IL-12] (Gorczynski et al, 1996) have been used (for summary see table 3:1). The mouse anti-rat TCRαβ mAb (R73) has been shown to prolong cardiac allograft survival in both naive and sensitised recipients (Heideck et al, 1995 and 1996). Similarly, the soluble recombinant ligand, cytotoxic T lymphocyte A_4 immunoglobulin (CTLA4-Ig), has been shown to block co-stimulatory signals delivered through the binding of members of the B7 family of molecules to their ligands (CD28 and CTLA4) on T cells. Therapy with CTLA4-Ig at the time of transplantation prolongs cardiac allograft survival in the mouse (Turka et al, 1992). In the rat, the blockade of CD28-B7 T-cell costimulatory pathway by administration of a single dose of CTLA4-Ig has been shown to result in tolerance to renal and cardiac allografts (Sayegh et al, 1995, Bolling et al, 1993).

In mice, a combination of anti-LFA1 (leukocyte function antigen) and anti-ICAM-1 (intracellular adhesion molecule) mAbs have been shown to prolong survival of cardiac allograft indefinitely (Isobe et al, 1992). Recently, Larsen and colleagues used mAbs to block CD40 and CD28 simultaneously and achieved prolonged survival of cardiac allograft and, more impressively, skin allografts in the mouse (Larsen et al, 1996). In contrast, anti-CD8 mAbs when used alone, have been found to be ineffective in prolonging cardiac or pancreatic allograft survival in rodents (Roza et al et al, 1989a+b). However, Anti-CD8 used in combination with anti-CD4 may induce tolerance to allogeneic heart and skin allografts in the mouse (reviewed by Cobbold et al, 1996).

Table 3:1 Prolongation of organ allografts survival by monoclonal antibodies in experimental animals.

Target molecule	Inducing mAb	MAb Type	Toleroge n	GS	Animal model	Reference
CD3	FN18, B11	D	Skin	PS	Mouse	Stevens et al, 1990
CD3	145-2C11	D	IL	PS	Mouse	Mackie et al, 1990
CD4	OKT4A	D	Kidney	PS	Monkey	Wee et al, 1992
CD4	OKT4A	D	Skin	PS	Monkey	Jonker et al, 1983
CD4	YTS-176.9.6	N	Heart	T	Mouse	Chen et al, 1992
CD4	YTS -191.1	D	Heart	T	Mouse	Pearson et al, 1993a+b
CD4	(OX38)	D	Heart	T	Rat	Shizuru et al, 1990
CD4	(OX35)	D	Heart	T	Rat	Ilano et al, 1991
CD4	(OX38)	D	Heart/Kid	T	Rat	Yin & Fathman, 1995
CD4	(OX38)	D	IL	T	Rat	Seydel et al, 1991
CD4	(OX35)	D	Heart	T	Rat	Herbert & Roser, 1988
CD4, CD8	OX38+OX8	D+N	Skin	T	Mouse	Cobbold et al, 1990
CD4, B7	OX38, CTLA4Ig	N	SBT	T	Rat	Yin et al, 1996
CD4	KT6,YTS199	N	Heart	T	Mouse	Darby et al, 1992 & 94
TCR	αβ TCR mAb	D	Heart	T	Rat	Heidecke et al,1995, 96
CD28	CTLA4Ig	*	Kidney	T	Rat	Sayegh et al, 1995
CD4,CD28	OX38+CTLA4	D+ *	heart	T	Rat	Yin & Fathman 1995
CD28,CD40	CTLA4-Ig,anti-gp39	*	Skin/heart	T	Mouse	Larsen et al, 1996
ICAM1,LFA1	Anti-ICAM1,LFA1	*	Heart	T	Mouse	Isobe et al, 1992
IL2-R	NDS-61, OX39	*	Kidney	T	Rat	Tellides et al, 1989
IL2-R	ART-18, OX39	*	Kidney	PS	Rat	Udea et al, 1990
MHC-II	HAK-75	*	Kidney	PS	Dog	Yamamoto et al, 1984
MHC-II	HAK-75	*	Heart	PS	Rat	Sone et al, 1987
TNFα,β	Anti-TNFα, β	*	Liver	PS	Rat	Teramato et al, 1991
TNF-α	Anti-TNFα	*	Heart	PS	rat	Imagawa et al, 1990

D = Depleting mAb **IL** = Islets of Langerhans **PS** = Prolonged survival
T = tolerance **SBT** = Small Bowel Transplantation **GS** = Graft Survival
* = Blocking mAb **N** = Non-depleting mAb

3:2:3 Targeting CD4 T cells

The CD4 molecule is an obvious target for mAb therapy because CD4 T cells play a central role in inducing and orchestrating the graft rejection responses (Mason and Morris, 1986; Hao et al, 1987; Hall, 1991 and Bradley and Bolton, 1992). In rodents, **depleting** regimens of anti-CD4 mAbs have been shown to induce tolerance to cardiac (Shizuru et al, 1990) and pancreatic islets allografts (Alters et al, 1991). **Non-depleting** anti-CD4 mAb regimens have also been reported to prolong survival of pancreas (Burkhardt et al, 1989), skin (Qin et al, 1990) and cardiac allografts (Darby et al, 1994) in the mouse. Preclinical studies in Rhesus and Cynomolgus monkeys using the anti-CD4 mAb OKT4 and OKT4a have demonstrated prolongation of both skin and renal allografts (Jonker et al, 1985 and Wee et al, 1992).

3:3 Mechanisms of transplantation tolerance

Since the pioneering studies of Medawar, a major goal in transplantation has been to develop clinically applicable strategies for tolerance induction in humans (Billingham and Medawar, 1953). A better understanding of the mechanisms responsible for inducing and maintaining tolerance is a necessary prerequisite to optimising clinically applicable protocols for inducing transplant tolerance.

The mechanisms underlying tolerance can be divided into two main categories: first thymic (or central) mechanisms and secondly extra-thymic (or peripheral) mechanisms. Although central mechanisms play the major role in maintaining tolerance to self antigens, it is likely that extra-thymic mechanisms are more important in maintaining transplant tolerance. This is undoubtedly an

oversimplification, since continuous interaction between peripheral events and those occurring in the thymus may occur to maintain a steady state of immunological tolerance. There is, however, little evidence in adult models of transplantation tolerance for clonal deletion, although this may be important in neonatally induced tolerance to alloantigen (Streilein et al, 1993). Because it is difficult in the tolerant animals to break tolerance by adoptive transfer of naive or even alloantigen sensitised syngeneic lymphocytes the idea that tolerance is maintained by an active form of suppression has gained popularity (Fabre & Morris, 1972 and Shizuru et al, 1988). A considerable body of evidence has now accumulated which suggests that tolerance is both induced and maintained by regulatory or suppressor T cells. However, the failure to identify and clone a separate population of suppressor cells in models of transplant tolerance has led to a good deal of scepticism about the existence of a separate population of suppressor cells whose only function is of immune suppression.

3:4 Central mechanisms of tolerance

3:4:1 Self tolerance

T cell tolerance to self antigens is due predominantly to clonal deletion within the thymus as the result of contact between developing T cells and self antigen expressed on thymic epithelial cells. Burnet was the first to describe in his clonal selection theory that tolerance to self antigens is achieved by clonal deletion of autoreactive lymphocytes. Evidence supporting this theory was presented by Kappler and colleagues in 1987, who used antibodies recognising Vβ 17^{+} TCR to trace the elimination of such T cell in I-E^{+} mice (Burnet, 1959; Kappler and Marrack, 1987). More recently, TCR transgenic mice were used and found to

eliminate most of the cells expressing a self-reactive transgene (Sha et al, 1988; Von Behmer and Kisielow, 1989 and 1990). Once thymocytes express a surface TCR, they are subjected to two selective pressures in the thymus. First, is the positive selection which is imposed on cortical thymocytes and leads to the survival of only those thymocytes whose TCRs interact productively with thymically expressed (self) MHC molecules. Second, is the negative selection which occurs when the thymocytes have reached the thymic medulla and involves the elimination of thymocytes with autoreactive receptors. Thus, maturing thymocytes expressing high affinity TCR receptors for self-antigens are eliminated by apoptosis before they reach the periphery. Within the thymus, bone marrow derived dendritic cells (APC), thymic epithelial cells and thymocytes themselves all have been shown to contribute to self tolerance induction. The probable mechanism underlying these selective pressures is based on the different affinities for self MHC molecule/peptide complexes displayed by thymic epithelial and dendritic cells and this process can be summed up as the thymus ignores the useless, deletes the dangerous and selects the useful (Von Boehmer et al, 1989). Along with the concept of clonal deletion, clonal anergy (which is functional inactivation of a cell, without elimination) also emerged as a mechanism to explain how the immune system develops self-tolerance and avoids autoreactivity (Quill and Schwartz, 1987).

However, a small number of T cells may escape deletion within the thymus and must be rendered tolerant by peripheral mechanisms (Jenkinson et al, 1985; Kappler et al, 1988; reviewed by Matzinger, 1993 and 1994).

3:5 Peripheral mechanisms of tolerance

The intrathymic events outlined above are clearly of great importance in the development and maintenance of self tolerance. The thymus is not able, however, to present the full range of either MHC class II or MHC class I associated autoantigens and it seems mandatory that additional systems of control for potentially autoreactive T cells must exist outside the thymus. Such mechanisms are likely to be of particular importance in the context of acquired transplantation tolerance. Four, non-mutually exclusive, hypotheses have been proposed to explain the induction of peripheral tolerance. These are, in broad terms, T cell ignorance, T cell anergy, immune deviation and suppression or active T cell regulation. Evidence that peripheral mechanisms of self tolerance exist, without clarifying how these mechanisms operate, comes from animal models which include those with spontaneous autoimmune disease and those expressing extrathymic transgene products (Bohme et al, 1990, reviewed by Kruisbeek and Amsen, 1996).

3:5:1 T cell ignorance

The concept of T-cell ignorance was originally proposed by Ohashi (Ohashi et al, 1991) following experiments in which expression of lymphocytic choriomeningitis virus glycoprotein as a transgene in pancreatic β cells was simply ignored by the immune system, evoking neither activation nor tolerance in T cells. Normally T cells are activated by the cross linking of their antigen receptor following the recognition of target antigen/MHC complexes presented on appropriate antigen presenting cells. There is evidence, mainly from studies

in transgeneic mice, that peptides may be presented in the context of MHC class I on cells which are unable to trigger a response from T cells with appropriate TCRs (Gotz et al 1990; and Miller et al, 1990). T cells apparently ignore the presentation of antigen by these non-professional APCs and are not stimulated to undergo the metabolic or functional changes characteristic of either activation or inactivation. Similarly if an allograft is devoid of professional APCs (i.e. passenger leukocytes) then it may result in development of T cell ignorance within the recipient and thereby in graft acceptance (Lafferty et al, 1986).

3:5:2 Anergy

The induction of anergy or functional inactivation of alloreactive T cells is one of the hypotheses currently receiving strong support as a mechanism of transplantation tolerance. T cells require two signals for full activation. The first signal is provided by engagement of the TCR with antigenic peptide plus MHC molecule on APCs (the tri-molecular complex figure 2:4). The second or "costimulatory" signal is provided by binding of specific receptors on T cells with their ligand or ligands on APC. It has been suggested that T cell anergy results from antigen receptor stimulation in the absence of costimulatory signals. MHC class II expression can be upregulated on various tissues including endothelial cells, kidney messengial cells and proximal tubule cells by certain cytokines, such as interferon γ. This upregulation of class II may allow these cells to function as "non-professional" APCs which are lacking the costimulatory molecules for imparting the second signal. The best characterised

costimulatory pathway is that provided by CD28 on T cells binding B7-1 and/ or B7-2 on APC (Linsley and Ledbetter, 1993 and Bluestone et al, 1995). B7 is an activation molecule found on B cells, monocytes and dendritic cells (Freeman et al, 1989; Larsen et al, 1992) whereas CD28 is found on all peripheral T cells in the mouse and up-regulated upon activation (Gross et al, 1992). Lamb and colleagues first described T cell anergy, when they showed that T-cell clones exposed to antigen on other T-cells in vitro responded in such a way that subsequent exposure to antigen presented by professional APC did not result in T cell proliferation (Lamb et al, 1983). Anergy has also been induced by stimulation of T cells with "non-professional" APCs such as islet cells (Markmann et al, 1988), keratinocytes (Gaspari et al, 1988). In these situations it seems likely that absence of costimulatory signals during stimulation is responsible for anergy induction. DeSilva et al showed that murine Th1 clones stimulated with antigen presented by normal APC in the presence of anti-IL-2 and anti-IL-2 receptor (IL-2R) antibodies also became anergic (DeSilva et al, 1991). From their study, they suggested that the lack of accessory molecules for costimulation is not the only limiting step in induction of anergy. Instead, they argued that the lack of normal IL-2 production and cellular proliferation resulting from incomplete stimulatory activity was the critical event. In vivo data coincide with evidence that prevention of CD28-B7 interaction using cytotoxic leukocyte antigen (CTLA)-4 immunoglobulin fusion protein can induce tolerance to mismatched cardiac allografts in the mouse and rat model (Lin et al, 1993; Sayegh et al, 1995). Recently Cobbold et al have introduced a

new term "infectious anergy", which describes tolerance as being both inhibition and anergy working together (Cobbold et al, 1996). They have suggested that anergic T cells might compete with immunocompetent cells and, by depriving them of important costimulatory molecules and paracrine proliferative cytokines, cause them to default to an anergic state (Waldmann et al, 1990; Waldmann & Cobbold, 1993). Once tolerance has been established it could be maintained by infectiousness, where a cohort of tolerant T-cells are able to guide naive T cells specific for the same antigen to become tolerant themselves. Waldmann et al have proposed that prolonged blockade of important functional T cell surface molecules favours non-professional presentation to T cells and this is permissive for the emergence of regulatory T cells rather than those capable of destruction. This is because professional APCs have only limited time in which they are capable of sensitising T cells, or because the regulatory T cells are functionally less susceptible to blockade of their surface CD4 adhesion receptors. Either way the result would be dominance of the regulatory T cells and once these cells became established as dominant they would be able to guide other T cells in to the same role (reviewed by Waldmann et al, 1993; Cobbold et al, 1996).

3:5:3 Immune deviation

Recently, a number of authors observed that induction of transplantation tolerance correlated with an increase in IL-4 production within accepted heart allografts (Papp et al, 1992 and Hancock et al, 1993), and reduction or absence of IL-2 expression (Dallman et al, 1991; Mottram et al 1995 and Sayegh et al,

1995). One interpretation of these experiments is the hypothesis that immunological tolerance is due to immune deviation from a Th1 to Th2 cytokine pattern. It has been postulated that a Th2 cytokine pattern is associated with transplant tolerance whereas a Th1 pattern is associated with allograft rejection (reviewed by Strom et al, 1996). Several groups have shown preferential expression of IL-4 and/or IL-10 during the induction of tolerance to renal and cardiac allografts in rodents using tolerogenic strategies which include using low dose anti-CD4 antibody treatment (Takeeuchi et al, 1992; Nickerson et al, 1994). Similarly, some investigators have been able to prevent class II disparate skin graft rejection in mice by the transfer of thymocytes expressing Th2 cytokine repertoires (Maeda et al, 1994). However, there are some exceptions to the idea that transplantation correlates strictly, with a Th1 to Th2 cytokine shift (Bugeon et al, 1992; Dallman, 1995; Strom et al, 1996; Krieger et al, 1996; Sad and Mosmann, 1995). Interestingly, Thai et al found up regulation of both Th1 (IL-2 & IFNγ) and Th2 (IL-4 & IL-10) cytokine mRNAs in mouse liver allografts that were spontaneously accepted without immunosuppression (Thai et al, 1995). More recently, studies using IL-4 Knockout mice (Garside et al, 1995) or neutralising anti-IL-10 antibody (Aroeira et al, 1995), have shown that mice can be made tolerant to ovalbumin in apparent absence of any IL-4 and/or any detectable Th2 cytokine response. From these studies, it is clear that although Th1 to Th2 immune deviation is an attractive explanation for peripheral tolerance in some experimental models, it can not be uniformly applied to all transplantation models.

3:5:4 Suppression

In many transplant models, evidence for T cell-mediated suppression has been derived from adoptive transfer systems in which T cells from an immunologically tolerant animals are able to mediate tolerance in naive recipient animals (Gershon, 1974; Kilshaw et al, 1975; Chen et al, 1993; Batchelor et al, 1984 and Lombardi et al, 1994). The first report on suppressor cells and immunosuppression was by Gershon on a seminal paper published in 1974, where he described that T cells from tolerant animals could transfer tolerance that was antigen specifc. He outlined the case for these regulatory cells which he termed suppressor cells (Gershon, 1974). Various hypotheses have been proposed to explain the mechanism of this T cell mediated suppression. It has been suggested that suppression is due to the effects of T cell-derived cytokines, such as interleukin-4 (IL-4), IL-10, or transforming growth factor-β (TGF-β), which inhibit the activation of IL-2 producing T cells (Fiorentino et al, 1989; De Waal et al, 1993; Miller et al, 1992). This is analogous to Th1/Th2 paradigm proposed by Mossmann et al, as already described. This basic model (i.e. Th1/Th2 CD4 T cells subsets) has recently been extended by Bloom et al to explain the phenomenon of suppression and includes CD8 T cells. In this model CD8 T cells are divided into two populations, type 1 which suppress B cells and have CTL function and type 2 which provides B-cell help and suppress DTH. These CD8 T cells produce lymphokines as do CD4 T cells in order to carry out these function. Bloom et al used this model in attempt to explain the T cell mediated suppression phenomenon and place it within the context of current

thinking of cytokine regulation (Bloom et al, 1992a & 1992b). A series of CD8 T cell suppressor (Ts) clones were studied and shown to have no cytolytic or helper activity and to produce no IL-2 or IL-4. These Ts clones blocked the production of IL-2 by helper CD4 T cells (reviewed by Webb et al, 1994). Sehon et al have generated clones from mice tolerated by polyethylene glycol-conjugated antigens. These clones block production of immunoglobulin in a MHC class I-restricted and antigen specific fashion. All clones from this study were Thy-1.2+, CD4-, CD8+, CD3+, TCR$\alpha\beta$+, and were not cytolytic (Mokashi et al, 1989; Takata et al, 1991; Chen et al, 1992). Another hypothesis proposes that antigen-specific T cells that have been rendered non-responsive suppress other T cells that have the same specificity in a passive manner through competition for ligands and for cytokines such as IL-2 (Cobbold et al, 1992; Lombardi et al, 1994).

An in vivo veto phenomenon resulting in clonal deletion of CD4 T helper precursors that recognise class II MHC along with suppressor cell activity has been suggested to play a role in bone marrow acceptance (Nakamura and Gress, 1990; Thomas et al, 1991) and in tolerance induced by donor-specific transfusions (Heeg and Wagner, 1990). Although it remains possible to demonstrate the phenomenon of T cell suppression, it has proved very difficult to isolate and characterise cells with suppressor activity and to explain the phenomenon of T cell mediated suppression by in vitro analysis.

Aim of the thesis

A major goal in organ transplantation is to develop strategies for inducing transplantation tolerance or partial tolerance and thereby eliminate or reduce the problem of non-specific immunosuppression and the attendant risks of infection and malignancy associated with life-long treatment with the current immunosuppresive drugs. Because CD4 T cells are central to the mechanisms of allograft rejection, there has been much recent interest in the use of mAbs directed against the CD4 molecule with a view to the possibility that this may promote allograft survival and perhaps transplant tolerance. The principle aim of this thesis was to study aspects of anti-CD4 mAb therapy as a strategy for promoting transplant survival or tolerance in a rat cardiac allograft transplant model.

Initial studies aimed, using a mouse anti-rat anti-CD4 mAb (MRC OX38), to characterise various aspects of the use of anti-CD4 mAb to prolong heart allograft survival and, in particular, determine to what extent rat strain dependant differences in the ability of the anti-CD4 mAbs to induce transplant tolerance exist. A second major aim of the work was to evaluate the ability of different anti-CD4 mAbs, particularly those mAbs directed against membrane proximal domains of the CD4 molecule, to prolong cardiac allograft survival. The third major aim of the thesis was to gain further insight into some of the possible mechanisms whereby anti-CD4 mAb are able to induce and maintain transplant tolerance in the rat. In this respect, particular emphasis was given to the question of whether anti-CD4 mAbs promote tolerance through immune

deviation of functionally distinct CD4 T cells subsets and preferential suppression of Th1 T cells (producing IL-2 and IFNγ) and/or the dominance of Th2 T cells (producing IL-4, IL-5, IL-6 and IL-10). Notwithstanding the possible limitation of extrapolating the results from such studies to clinical human transplantation, it was anticipated that the experiments performed would allow better understanding of anti-CD4 mAb therapy which might eventually be of relevance to clinical transplantation.

CHAPTER FOUR

MATERIAL AND METHODS

4:1 Animals

Inbred male rats were obtained from Harlan UK Ltd (Bicester) or were bred in the University of Glasgow Central Research Facility. They were housed in the Central Research Facility, maintained on standard rat diet and used when 8-16 weeks old. The following strains were used: PVG ($RT1^c$), DA($RT1^a$), Lewis ($RT1^l$) and Brown Norway ($RT1^n$).

4:2 Surgical procedures

4:2:1 Donor heart retrieval

The donor rat was anaesthetised by continuous halothane inhalation, opened with a midline abdominal incision and heparinised with 250 units into the inferior vena cava (IVC). The aorta and IVC were transsected to exsanguinate the animal. The thorax was opened and cold saline was applied immediately to the heart to achieve cardioplegia. Using an operating microscope (Wild, Heerbrug, Switzerland) the IVC was dissected and ligated with a 6/0 braided tie close to the atrium. The thymus was then removed and the right superior vena cava (SVC) dissected away from the thymus and connective tissue and tied close to the atrium. These two vessels were left undivided to keep the heart anchored. The heart was displaced to the right to reveal the azygous vein and left SVC which were ligated just proximal to their union by a single tie passed through the fascial plane behind the vein. The veins were divided, exposing the pulmonary trunk, which was then carefully dissected from its connective tissue (figure 4:1A). The ascending aorta was then dissected from its adherent connective tissue and transsected at two thirds of the distance from its origin to

its arch for abdominal transplantation or distal to the innominate artery for cervical transplantation. The pulmonary trunk was dissected free of the aorta and transsected close to its bifurcation and the venae cavae were then divided. Lastly, the remaining vessels forming a pedicle at the back of the heart were included in a single ligature and the heart was dissected free. It was placed in cold saline on ice and gentle external pressure applied to flush the blood from the chambers.

4:2:2 Abdominal heart transplantation

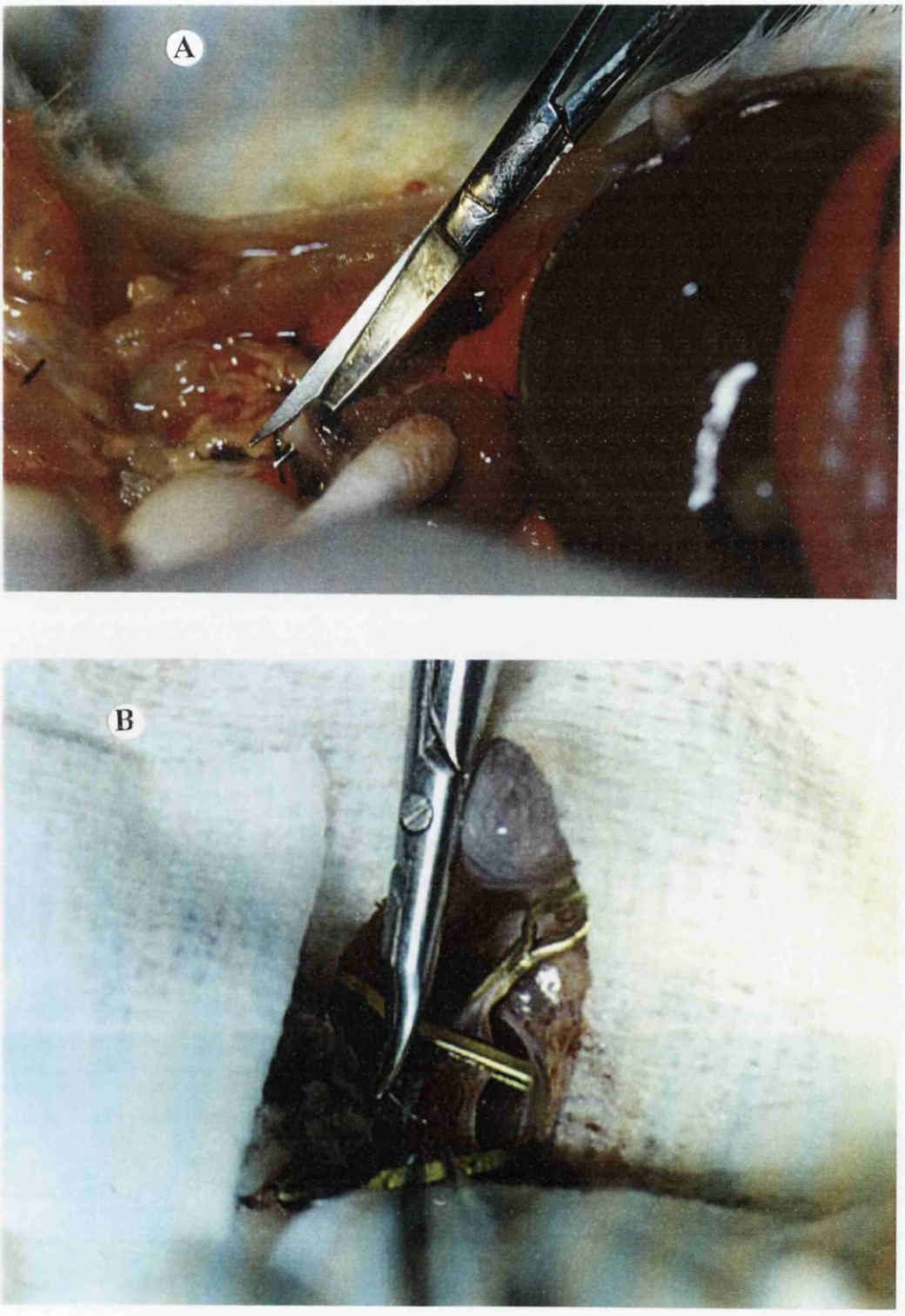
Abdominal heart transplantation was performed according to the technique of Ono and Lindsey (Ono and Lindsey, 1969). The recipient rat was anaesthetised by continuous halothane inhalation, shaved, and secured to a heated board (switched off) with tape. The recipient was swabbed with alcohol then opened with a midline abdominal incision and the intestines were displaced and enclosed in a gauze swab moistened with sterile saline. Using an operating microscope at about 10x magnification, the aorta and IVC were exposed, the overlying peritoneum teased open, and the vessels cleaned between the renal vessels and the bifurcation (figure 4:1B). A suitable length of aorta and IVC was chosen (1-1.5 cm) and all the branches and tributaries of this section were ligated with 6/0 braided ligature. Occlusion clamps were applied across the two vessels, either side of the intended anastomosis site, in such a way as to leave the vessels exposed side by side. Next an arteriotomy was made in the aorta equivalent in length to the diameter of the donor aorta. A venotomy was made similarly in the IVC and blood was aspirated from the lumina. End-to-side anastomosis of

donor aorta to recipient aorta was performed using a continuous 9/0 monofilament nylon suture (Ethicon Ltd, UK), under 16x magnification, followed by end-to-side anastomosis of the donor pulmonary artery to the recipient IVC (figure 4:2B). During anastomosis the heart was covered with a gauze swab and cold saline was continually applied. The inferior clamp was released briefly at first to allow haemostasis to begin, then the superior clamp was released. Once anastomosis was achieved the heated plate was switched on again. Ischaemic times of the heart during transplantation were around 30 minutes. The intestines were replaced and the abdomen was closed in two layers with a continuous 3/0 absorbable suture (Ethicon Ltd).

4:2:3 Cervical heart transplantation

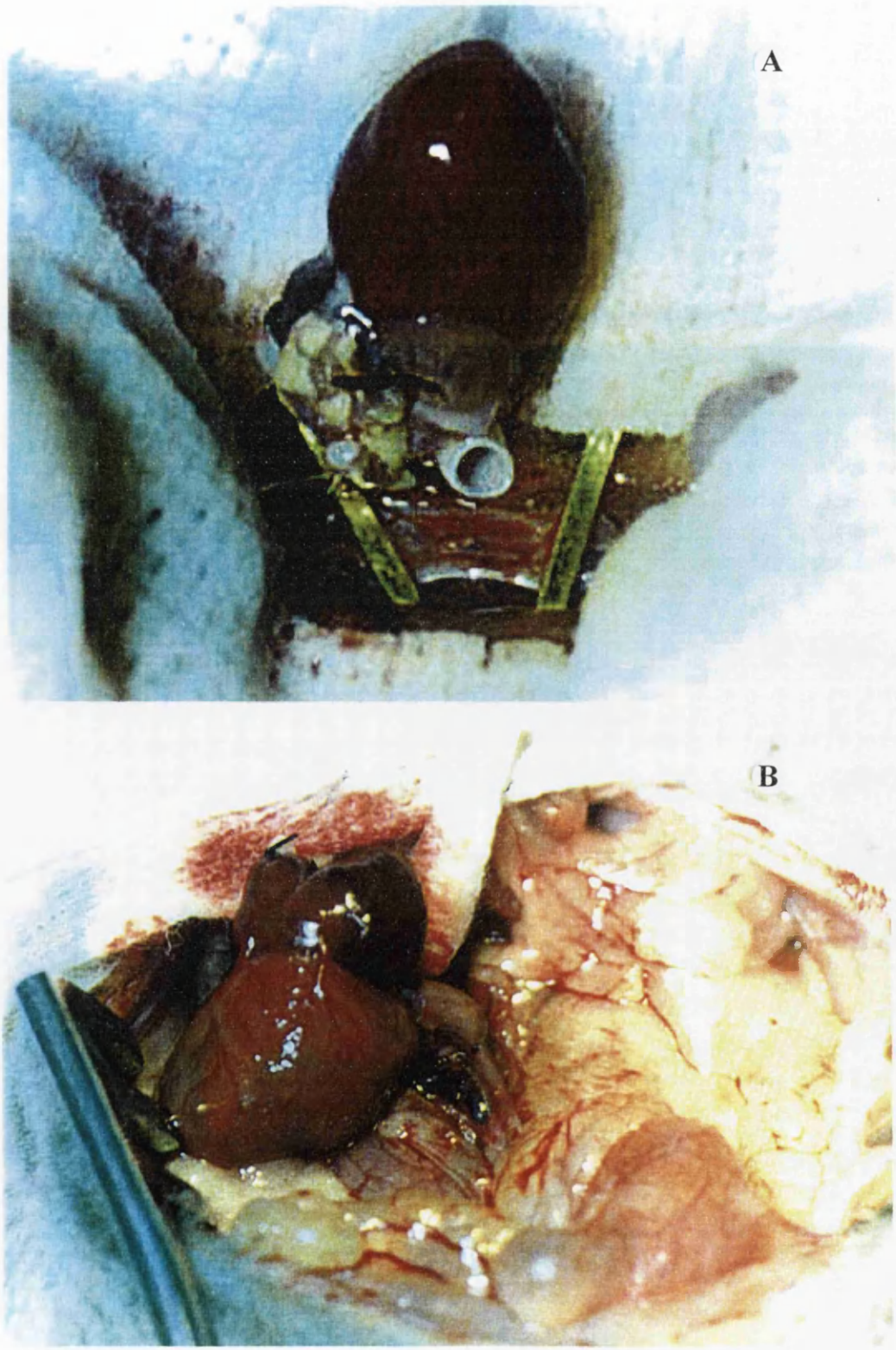
Cervical heart transplantation was performed using a modification of the method described by Heron (Ivor Heron, 1971). A right sided paramedian neck incision was made extending vertically from the upper border of the manubrium sterni to beneath the mandible. The external jugular vein was exposed and mobilised by blunt dissection. The sternocleidomastoid and strap muscles were separated by blunt dissection to expose the carotid sheet which was dissected in order to mobilise the internal carotid artery. Both vessels were individually clamped and ligated distally to leave stumps for anastomosis. End-to-end anastomosis of the donor innominate artery to the recipient internal carotid artery was performed using an interrupted 9/0 monofilament, followed by end-to-end anastomosis of the donor pulmonary artery to the recipient external

Figure 4:1 Heterotopic cardiac transplantation in the rat.



A. Donor aorta and pulmonary artery were dissected free and ready to be divided.
B. Recipient abdominal aorta and inferior vena cava were prepared below renal vessels and clamped below and above the anastomotic site.

Figure 4:2 Heterotopic cardiac transplantation in the rat.



A. Donor heart lying across the anastomotic site ready for anastomosis.
B. End-to-side anastomosis was performed between donor aorta and recipient aorta, then donor pulmonary artery in similar fashion to the recipient IVC.

jugular vein, again with an interrupted 9/0 suture. The arterial clamp was released followed by the venous clamp, and prior to closure both edges of the wound were subcutaneously dissected to prevent compression of the transplanted heart. The skin was closed in a single layer.

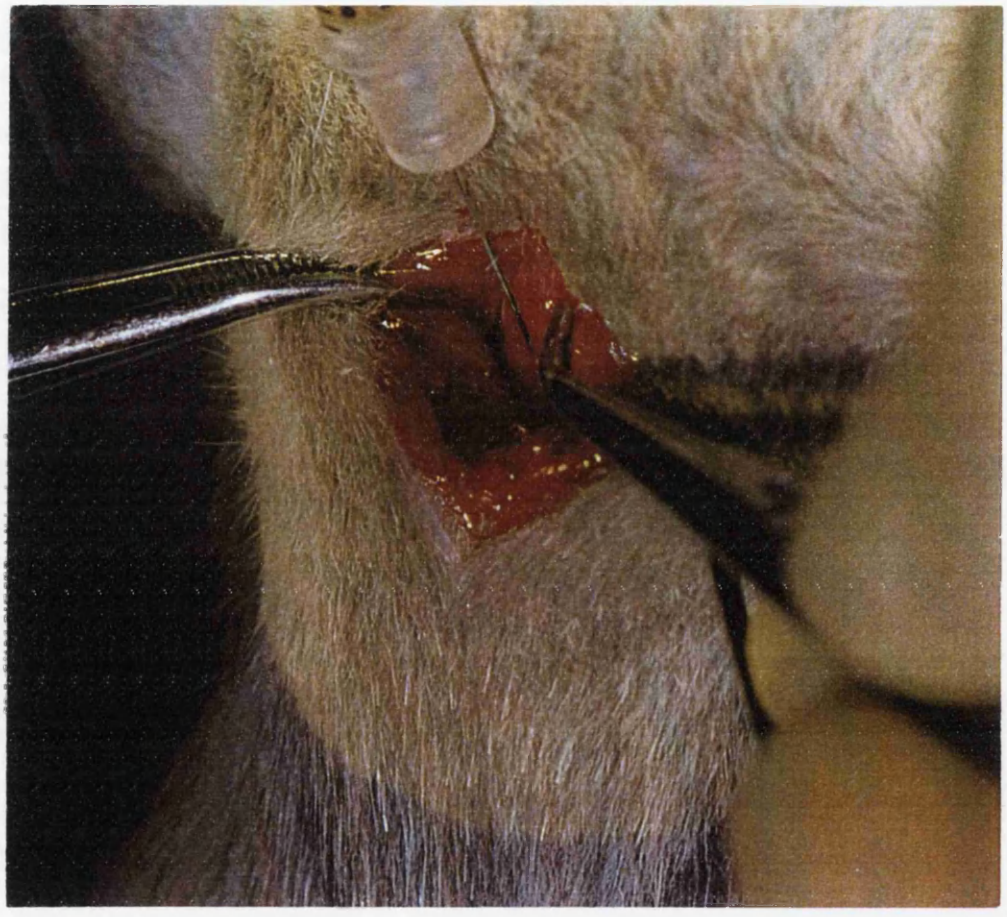
4:2:4 Assessment of graft survival

Graft survival was assessed by daily palpation of the graft in the abdomen or neck and rejection was defined as complete cessation of myocardial contraction.

4:2:5 Thymectomy

Under halothane inhalation anaesthesia a 2 cm midline skin incision was made in the neck, followed by division of the manubrium sterni of about 7-10 mm in length. The strap muscles were separated and the thymus gland was mobilised by blunt and sharp dissection using an operating microscope (figure 4:3). The blood vessels entering the gland were divided and the thymus gland was then easily removed. The skin was closed quickly with a continuous suture and positive pressure ventilation was administered (by the operator blowing through the barrel of 2 ml syringe fitted over the nose of the rat) to prevent pneumothorax. The total duration of the procedure was 5 min (Barbul et al, 1979 and Walker et al, 1994). Subsequent analysis of peripheral blood lymphocytes by flow cytometry confirmed the depletion of T lymphocytes positive for Thy1 which is expressed on recent thymic emigrant. Post-mortem examination further confirmed complete thymectomy.

Figure 4:3 Thymectomy



The thymus was exposed by midline neck incision followed by division of manubrium sterni (the needle is pointing to the thymus gland).

4:3 Antibodies

Monoclonal mouse anti-rat antibodies (mAbs) used for identifying rat leukocyte populations are described in Table 4:1. All antibodies were obtained from Serotec Ltd, Oxford, UK. Secondary polyclonal antisera were used to detect the presence of the primary mAbs by a range of techniques, for which the antiserum had been appropriately conjugated.

4:3:1 Preparation and purification of monoclonal antibodies

The anti-CD4 mAbs, OX38, OX70, OX71 and OX73 were produced as ascites from hybridomas (kindly provided by Dr Neil Barclay, Sir William Dunn School of Pathology, University of Oxford, Oxford) injected into BALB/c mice primed intraperitoneally with 0.5 ml pristane (Sigma Chemical Company Ltd, Poole, UK) 7 days previously. Ascitic fluid harvested from the peritoneal cavity was centrifuged at 3000 rpm for 15 m at 4°C and separated from fat, fibrin clot and red cells, then filtered and stored at -20 °C until purification by protein-A affinity chromatography (Prosep-A, Bio-processing Ltd, England, UK). Antibodies were purified from ascites by adding an equal volume of diluting buffer (Glycine 75 g/l, NaCl 17.6 g/l, pH 8.6) for 12 h at 4 °C, then centrifuging at 3,000 rpm for 30 mins at 4 °C. A protein-A-sepharose column was prepared and equilibrated with binding buffer (Glycine 75 g/l, NaCl 8.8 g/l, pH 7.4), then loaded with diluted ascites.

The IgG was eluted with 0.1M sodium citrate, pH 3.0, at a flow rate of 2.7 ml/min into 4 ml fractions, and the Ig-containing fractions (identified by

Table 4:1 Monoclonal mouse antibodies detecting rat leukocytes.

Antibody	Specificity	Reference
MRC OX8	CD8+ cytotoxic/suppressor T cells, majority of natural killer cells	Gilman et al, 1982
W3/25	CD4+ helper T lymphocytes, some macrophages	Brideau et al, 1980
R73	Constant determinant of the rat TCR-αβ.	Hunig et al, 1989
MRC OX12	Ig Kappa chain on B lymphocytes	Hunt & Fowler, 1981
MRC OX22	Leukocyte common antigen (CD45R) (B lymphocytes, MRC OX8+ and 67 % of W3/25 peripheral T cells, NK cells)	Spickett et al, 1983
MRC OX21	Human C3b inactivator (used as negative control)	Hsiung et al, 1982
MRC OX81	Rat IL-4	Ramirez et al, 1996

screening their optical density [OD] at 280 nm) were pooled and immediately dialysed with several changes of PBS to neutralise the pH. Purified antibodies were quantified by OD_{280} and by radial immunodiffusion (RID) using rat isotype-specific RID plates and standards of known concentration (The Binding Site, Birmingham, UK).

4:3:2 In vivo treatment with monoclonal antibodies and cytokines

A panel of anti-CD4 (W3/25, OX38, OX70, OX71 and OX73) monoclonal antibodies (mAbs) were used to treat organ allograft recipients in vivo in an attempt to induce tolerance as described in Table 4:2. MRC OX8 (anti-CD8) and OX81 (anti-IL-4) mAbs (were used also as described in Table 4:2. Human recombinant interleukin 2 (rIL-2), a generous gift from Glaxo (Glaxo IMB, UK) was used at a dose of 400 or 20,000 U/day for five days (day 0, 1, 2, 3 and day 4).

4:4 Preparation of cells

4:4:1 Peripheral blood lymphocytes (PBL).

Two to three mls of peripheral blood was collected in heparinised tubes (20 U/ml) and diluted 1:1 with phosphate buffered saline (PBS). Peripheral blood lymphocytes (PBL) were separated by centrifugation of the heparinised blood at 2000 rpm for 20 min over 75% iso-osmolar percoll (Sigma Ltd). Lymphocytes harvested from the interface were washed three times in PBS containing 0.2% bovine serum albumin (PBS-BSA).

Table 4:2 The mouse monoclonal antibodies used in vivo[a] .

Anti-CD4[b]	Isotype	Epitope/receptor
OX38	IgG2a	Domain 1 & 2 of CD4
W3/25	IgG1	Domain 1 & 2 of CD4
OX70	IgG2a	Domain 3 and 4 of CD4
OX71	IgG1	Domain 3 and 4 of CD4
OX73	IgG1	Domain 3 and 4 of CD4
OX21	IgG1	Human C3b inactivator
OX8	IgG1	CD8
OX81	IgG1	IL-4

(a) All these mAbs (except OX8 and OX81) were used at a dose of 10 mg/kg on day -3 and 2 mg/kg on day -2, -1 and day 0 (day of transplantation). OX8 was given at a dose of 5 mg/kg on day -1, 0 (day of transplantation) and day 3 after transplantation whereas OX81 was given at a dose of 5 mg/kg on day 0, 1, 3 and 5 post transplantation.

(b) These antibodies or hybridomas were a kind gift from Dr Neil Barclay (Sir William Dunn School of Pathology, University of Oxford, Oxford).

4:4:2 Lymph node cells (LNC)

Cervical and mesenteric lymph nodes were removed from rats that had been sacrified by cervical dislocation. The excised lymph nodes were pooled, cut with a scalpel blade and then pushed through a fine stainless steel mesh with a 5 ml syringe plunger, in PBS-BSA. Lymph node cells were washed two or three times and aggregates of dead cells removed with a Pasteur pipette. Viability was consistently greater than 95% as determined by trypan blue exclusion.

4:4:3 Spleen cells

Spleens were removed from sacrificed rats, and teased apart with sterile disposable plastic forceps in PBS-BSA. Cells were washed once and aggregated dead cells were removed. The erythrocytes were removed by hypotonic lysis using 5 mls distilled water followed by 5 mls 1.8% saline. Splenocytes were washed three times before use.

4:4:4 Preparation of T lymphocytes

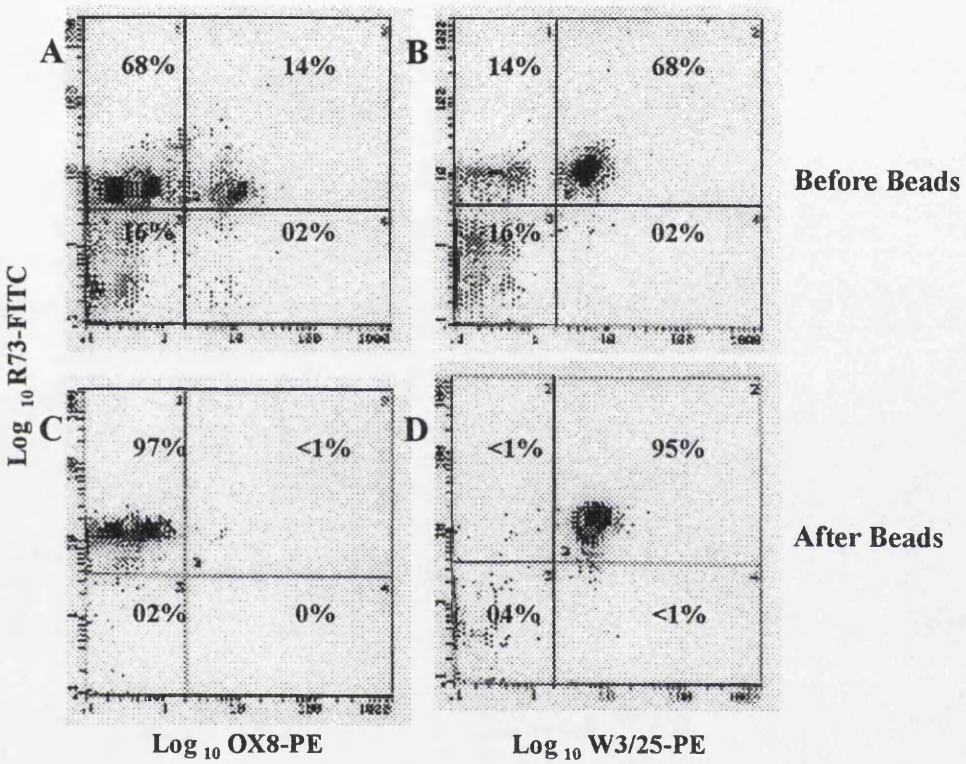
Spleen cells from OX38 treated tolerant DA ($RT1^a$) rats were prepared as described in section 4:4:3. To isolate purified T-lymphocytes (i.e. both CD4 and CD8 T cells), a saturating amount of MRC OX12 (Anti-Ig Kappa light chain, Serotec Ltd, Oxford, UK) was added to the cell suspension for 1 hour at 4 $^{\circ}C$ to coat B cells. The cells were washed three times with HBSS (Hanks Buffered Salt Solution)/2% FCS (Foetal Calf Serum) [Sigma Ltd], resuspended at $1x10^8$ cells/ml and goat anti-mouse IgG-coupled magnetic beads (BioMag, Northampton, UK) were added at a ratio of 1 ml of beads per $1x10^8$ cells. This was incubated at 4 $^{\circ}C$ for 60 min with frequent agitation, and the beads were

then removed using a magnetic particle concentrator (Dynal MPC-1, Norway). The cells were B cells depleted a second time with fresh Biomag beads, then washed three times in PBS and resuspended in PBS at $5x10^8$ or $5x10^7$ cells/ml before use in adoptive transfer experiments. Viability of cells was verified by trypan blue exclusion and were consistently > 95%. Purity of T cells was checked using flow cytometry with directly fluoresceinated R73 mAb (Phycoerythrin conjugated anti-rat α/β TCR mAb, Serotec Ltd) and was always > 90 %.

4:4:5 Preparation of purified CD4 T cells

Lymph node (LNC) or spleen cells from OX38 and OX70 treated tolerant DA ($RT1^a$) rats were prepared as described in sections 4:4:2 and 4:4:3. Saturating amounts of MRC OX8 and MRC OX12 monoclonal antibodies (Serotec Ltd) were added to the cell suspension of either the LNC or splenocytes for 1 hour at 4 °C to coat CD8+ T cells and B cells respectively. The cells were washed three times with HBSS/2% FCS, resuspended at $1x10^8$ cells/ml and goat anti-mouse IgG-coupled magnetic beads were added at a ratio of 1 ml of magnetic beads per $1x10^8$ cells, as described above. The purity of CD4 T cells was checked using flow cytometry with directly fluoresceinated W3/25 and OX8 antibodies, and was always > 95 % as depicted in figure 4:4. Here LNCs were stained directly using anti-TCR-FITC (Y-axis) and either anti-CD8-PE (left panels, X-axis) or anti-CD4-PE (right panels, X-axis). Cells were stained both before (upper panels) and after (lower panels) negative selection of CD4 T cells.

Figure 4:4 Flow cytometric analysis of LNCs before and after purification of CD4 T cells.



Double labeling flowcytometric analysis of LNCs showing (A) OX8-PE versus R73-FITC before beads. (B) W3/25-PE Vs R73-FITC before beads. (C) OX8-PE Vs R73-FITC after bead. (D) W3/25-PE Vs R73-FITC after beads.

4:5 Flow cytometric analysis of lymphocyte subpopulation

The influence of anti-CD4 mAb treatment on lymphocyte subpopulation was determined by flow cytometric analysis of peripheral blood lymphocytes (PBL) and lymph node cells prepared from treated rats. $1x10^6$ lymphocytes were incubated (in PBS-BSA on ice, for 45 min) either with fluoroscein-conjugated mAbs against rat leukocyte subpopulations, or with non-conjugated anti-rat CD4 mAbs followed, after 2 washes, by incubation with FITC-(fluorescein isothiocyanate) or PE-(phycoerythrin) conjugated rabbit F(ab`)2 anti-mouse Ig (Dako Ltd, High Wycombe, UK) diluted to 1/20 in PBS containing 10% normal rat serum to block cross reaction with rat cell surface Ig. After 2 final washes, cells were resuspended in PBS and analysed using a Coulter EPICS- XL flow cytometer (Coulter Ltd, Luton, UK).

4:6 Mixed lymphocyte culture

Single cell suspensions of purified CD4 T cells at $2x10^5$ cells/ml were prepared from mesenteric and cervical lymph nodes of OX38 and OX70 tolerant rats (responder cells) as described in section 4:4:5. Splenocytes from naive Lewis and PVG rats as donor specific and third party stimulator cells respectively were prepared by passing spleen cells through a fine steel mesh in tissue culture medium (RPMI/FCS; RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all Life Technologies), 50 μM 2-mercaptoethanol (Sigma Ltd) and 5% heat inactivated FCS. The 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 incubated at 37°C in 5% CO_2. After 3 and 4 days culture, cells were pulsed with 1 μCi/well of [^{3}H]-thymidine (Amersham International PLC, little Chalfont, Bucks, UK) for 18 hours and harvested, to determine thymidine-incorporation by proliferating cells, using an automated cell harvester and liquid scintillation counter (1205 Betaplate, LKB Pharmacia, Milton Keynes, UK).

4:7 Capture ELISA for quantification of IFNγ

100 μl/well of coating anti-interferon gamma (rabbit polyclonal anti-rat IFNγ, kind gift of Dr. John Tite, Wellcome Research Laboratories, Beckenham, UK) antibody used at dilution of 1:2000 (i.e. 10 μl aliquot in to 20 ml of 0.1M $NaHCO_3$ buffer, PH 8.2) was added to 96 well microtiter plates (Dynatech Immulon 4). After overnight incubation at 4 °C, plates were washed three times in ELISA wash buffer (PBS containing 0.05% Tween 20) and blocked with 200 μl/well of PBS containing 10% FCS for 1 hour at 37°C. Plates were washed three times with wash buffer, and 100 μl/well of each standard, control or sample (neat supernatant) was added in triplicate with 3 hours incubation at 37°C. The plates were washed four times and incubated for 1 hour at room temperature with biotinylated anti-rat IFNγ (DB1 , a kind gift of Dr. P.Vander Meide, Rijkswik, Netherland). After six washes, 100 μl/well of extravidin-peroxidase (Sigma Ltd) was added and incubated for 1 hour at 37 °C. After eight washes, 100 μl/well of TMB [tetramethyl benzidine] (Dynatech Laboratories, Billingshurst, UK) substrate was added for 30 minutes at room

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

4:8 Bioassay of IL-2 and IL-4

4:8:1 IL-2 bioassay

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 dilution of supernatants for day 3 mixed lymphocyte culture of purified CD4 T cells from OX38 and OX70 treated rats was mixed with $5x10^3$ CTLL2 cells. Twenty four hour later, the cells were pulsed with 1 μCi/well tritiated 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 per millilitre) were calculated from a standard curve with rIL-2.

4:8:2 IL-4 bioassay

Detection of IL-4 was achieved by bioassay to determine upregulation of expression of MHC class II molecules on B cells. Rat B cells were purified from lymph node cells by direct rosetting using W3/13 mAb-labelled sheep red blood cells (SAPU, Carluke, Scotland) to remove T cells, remaining erythrocytes were removed by hypotonic lysis. Purity of cell subset was checked using flow cytometry with directly fluoresceinated OX12 and was >85%. 100 μl of tissue culture supernatant was added to 100 μl /well of B cells (at $5x10^6$ cells/ml) in 96 well tissue culture plates. After incubating for 18 h at 37 °C, plates were

washed twice with 0.02% PBS-BSA. Saturating amount of FITC-labelled OX6 mAb (Serotec Ltd) was added and plates incubated for 1 hour at 4 °C. After washing three times with 0.2% PBS-BSA, the increase in class II molecules expression was measured by the Coulter EPICS-XL flow cytometer (Coulter Ltd). Assay specificity was determined by means of neutralising mAb to rat IL-4 (OX81) added at the beginning of the 18 hour culture period (at 50 µl/well of OX81 supernatant). Values are expressed as U/ml IL-4 derived from a standard curve constructed using serial dilutions of recombinant rat IL-4 obtained as tissue culture supernatant (10^4 U/ml) from a transfected CHO cell line. One unit was defined as that concentration of IL-4 that gave 50 percent of maximal induction of MHC class II on B cells as assessed by flow cytometry.

4:9 Statistical analyses

Except as specified below, survival data were analysed using Fisher's exact test, in which the proportion of animals surviving to 100 days was compared between two groups. The number of animals in each group was in general too small to permit the valid use of the chi-squared test, and hence in experiments containing more than two groups, they were compared on a pairwise basis using Fisher's exact test. Bonferroni's method was used to correct for the performance of multiple comparisons within a given experiment. For a given comparison to be considered statistically significant, a p-value of less than 0.05/n was required, where n is the number of simultaneous comparisons in that experiment. This procedure ensures that the probability of erroneously declaring any difference to be significant is less than 5% per experiment. Only comparisons of primary

interest, in most cases those of treated groups with a control group, were included in n, and this number is specified in the results for each multi-group experiment.

In some experiments in which all animals, or all but one or two, failed to survive for 100 days, the median survival times were compared with the Mann-Whitney test. Bonferroni's method was used to correct for the performance of multiple comparisons, as described above.

Flow cytometric data were analysed by performing a one-way analysis of variance (ANOVA) to determine whether there were significant overall differences between the groups within a given experiment. If the results of the ANOVA showed there to be significant differences, a pairwise comparison was made between each anti-CD4 treated group and the naive control group using Dunnett's test (Dunnett, 1964). This test corrects for multiple comparisons by controlling the error rate for the family of simultaneous comparisons, in a similar manner to Bonferroni's method. Hence, the probability of erroneously declaring the difference between any treated group and the control group to be statistically significant is less than 5% per experiment. Dunnett's test was performed only at a 5% significance level, and hence all significant differences are quoted as $p<0.05$. When further comparisons were made between different anti-CD4 treated groups within an experiment, Tukey's test was used. This is similar to Dunnett's test, but is more conservative in that it controls the error rate for the family of all possible simultaneous pairwise comparisons, rather than only those between treated and control groups.

To compare the effect of a given anti-CD4 antibody in depleting CD4 T cells in peripheral blood lymphocytes and lymph node cells, given that the percentage of CD4-positivity varied with cell type in naive animals, results were first expressed as a fraction of the mean CD4 percentage for cells of the same type in naive animals. Comparisons were then performed using Student's t test. A similar procedure was used to compare the depletion of OX22-high and OX22-low CD4-positive T cells. In chapter eight part of the results were not statistically analysed because the experiments were performed on one occasion only.

Statistical analysis was performed using Minitab for Windows 9.2 (Minitab Inc.) and Statgraphics 2.6 (Statistical Graphics Corporation) software.

CHAPTER FIVE

The ability of anti-CD4 (OX38) mAb to promote cardiac allograft survival in the rat.

5:1 Introduction

As already outlined in chapter 2, CD4 T cells play a critical role in initiating and amplifying the immunological response to an allograft. The CD4 molecule has attracted, therefore, considerable interest as a target for mAb therapy to prevent graft rejection. In this thesis, I chose to examine the ability of anti-CD4 mAb's to promote cardiac allograft survival in a rat heterotopic heart transplantation model. This experimental model is of more relevance to clinical organ transplantation than, for example, skin allograft models since it involves transplantation of a vascularised organ graft. Moreover, the model provides an easily detectable end-point (i.e. cessation of palpable cardiac contraction) with which to assess graft rejection and thereby allows an objective determination of the effect of anti-CD4 mAb treatment on heart graft survival.

By virtue of its greater size, relative to the mouse, the vascular anastomosis in the rat is more easily performed. Like the mouse the immune system of the rat has been well studied, although, the range of immunological reagents available for rat studies is not so extensive as that available for use in the mouse. Interestingly, in the rat, but not in the mouse, the CD4 molecule is found both on CD4 T cells and also on some activated macrophages (Wood et al, 1983; Barclay AN, 1981; Steininger et al, 1984). From an immunological perspective, it is also notable that rat CD4 molecule was one of the first lymphocyte cell surface molecules to be identified by monoclonal antibodies (Brideau et al, 1980). In this particular chapter I examined the ability of the mouse anti-rat CD4 mAb, MRC OX38 (OX38), to promote heart allograft survival. OX38 has

been shown in previous studies to be a partially depleting mouse anti-rat CD4 mAb of the IgG2a isotype which labels a determinant on the distal domains of CD4 molecule and this mAb has been reported, in some rat strains combinations, to promote permanent heart allograft survival (Claesson et al, 1987; Herbert and Roser, 1988; Shizuru et al, 1990; Seydel et al, 1991). After a series of preliminary experiments to establish, for myself, the rat cardiac transplant model I went on to use the model to examine the experimental circumstances and, in particular, the rat strain combinations in which treatment with OX38 was able to prolong heart allograft survival in the rat.

5:2 Preliminary experiments to establish and validate the rat heterotopic cardiac transplantation model.

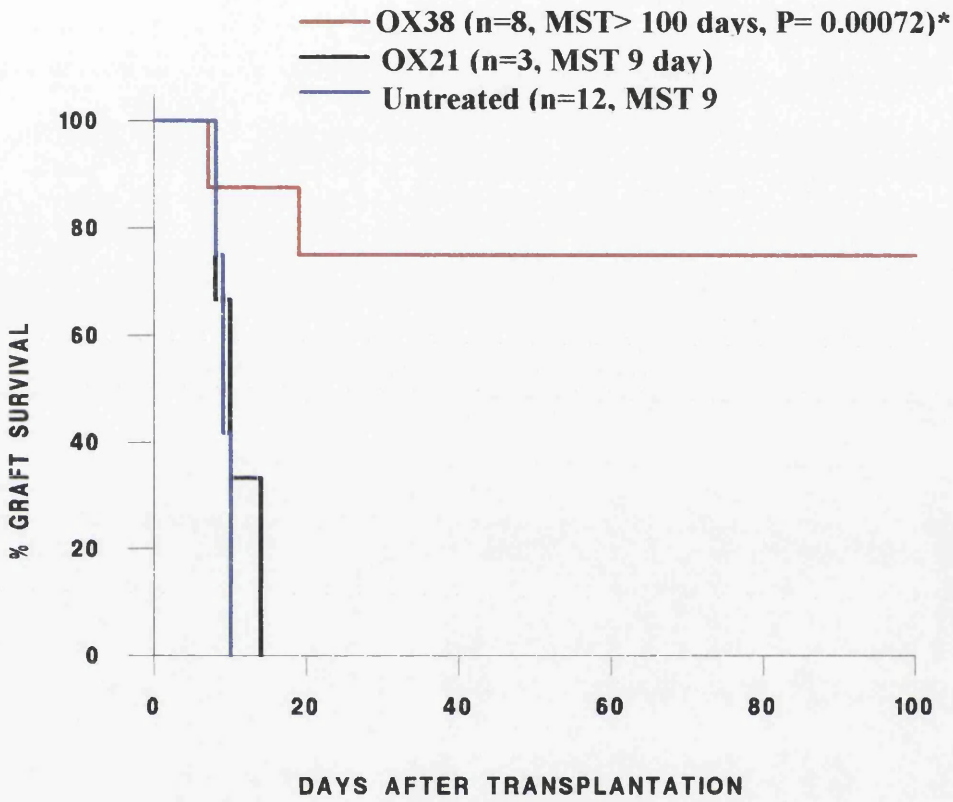
For initial transplant studies the fully allogeneic Lewis ($RT1^l$) to DA ($RT1^a$) rat strain combination was examined. The Lewis and DA rat strains differ from each other at both major and minor histocompatibility antigens and Lewis heart grafts are always rejected rapidly by unmodified DA recipients (Fabre et al, 1972a and b and extensive unpublished studies from my supervisors laboratory). In an initial experiment, Lewis hearts allografts were transplanted to the heterotopic intrabdominal site of 12 DA recipients. All of the heart grafts underwent acute rejection with complete cessation of palpable cardiac contraction between days 8 and 10 after transplantation, with a median survival time (MST) of 9 days. To confirm that cessation of graft contractility in this fully allogeneic rat strain combination was attributable to immunological rejection rather than technical failure due to my relative inexperience at this

early stage of the project with microsurgical techniques, 4 DA ($RT1^a$) to DA ($RT1^a$) syngeneic cardiac transplants were also performed. All 4 syngeneic heart grafts survived for greater than 100 days (when the experiment was terminated) with easily palpable cardiac contractility throughout the period of study, thereby excluding technical failure as a likely cause for graft destruction in the series of unmodified allogeneic heart transplants described above.

5:3 Ability of OX38 mAb to prolong survival of Lewis heart grafts in DA recipients.

To determine the ability of OX38 mAb to promote survival of Lewis heart allografts in DA recipients, DA animals were treated with affinity purified OX38 mAb at a dose of 10 mgs/kg on day -3 and 2 mg/kg on days -2, -1, and 0 (the day of transplantation). This antibody treatment regimen was chosen because it has been reported previously that a similar OX38 antibody treatment protocol induces indefinite survival of Lewis cardiac allograft in ACI ($RT1^a$) recipients (Herbert and Roser, 1988; Roza et al, 1989; Shizuru et al, 1990; Seydel et al, 1991; Yin and Fathman, 1995; Binder et al, 1996). Eight DA recipients were treated with OX38 mAb using this regimen and then transplanted with a heterotopic Lewis heart graft. As shown in figure 5:1, OX38 treatment significantly increased survival of Lewis heart grafts (MST>100 days, P= 0.00072, Fisher's exact test in comparison with the control untreated animals) and in 6 out of the 8 treated recipients permanent survival (>100 days) occurred. Two of the recipients rejected their heart grafts on days 11 and 18 respectively. An additional control group of 3 DA recipients was treated with a control mAb, OX21 (which labels human C3b inhibitor).

Figure 5:1 The ability of OX38 mAb to prolong survival of Lewis heart grafts in DA recipients.



DA ($RT1^a$) rats were treated with the mouse anti-rat CD4 mAb OX38 (or control OX21 mAb) on day -3 (10 mg/kg) and on days -2, -1 and on day 0 (2 mg/kg). On day 0, treated animals received a heterotopic Lewis ($RT1^l$) heart allograft. Graft rejection was defined as complete cessation of cardiac contractility.

*** P value refers to comparisons with unmodified control (P= 0.00072, Fisher's exact test).**

As shown in figure 5:1, all 3 of these animals rapidly rejected their heart grafts in times comparable to those observed in unmodified recipients (MST 9 days in both groups).

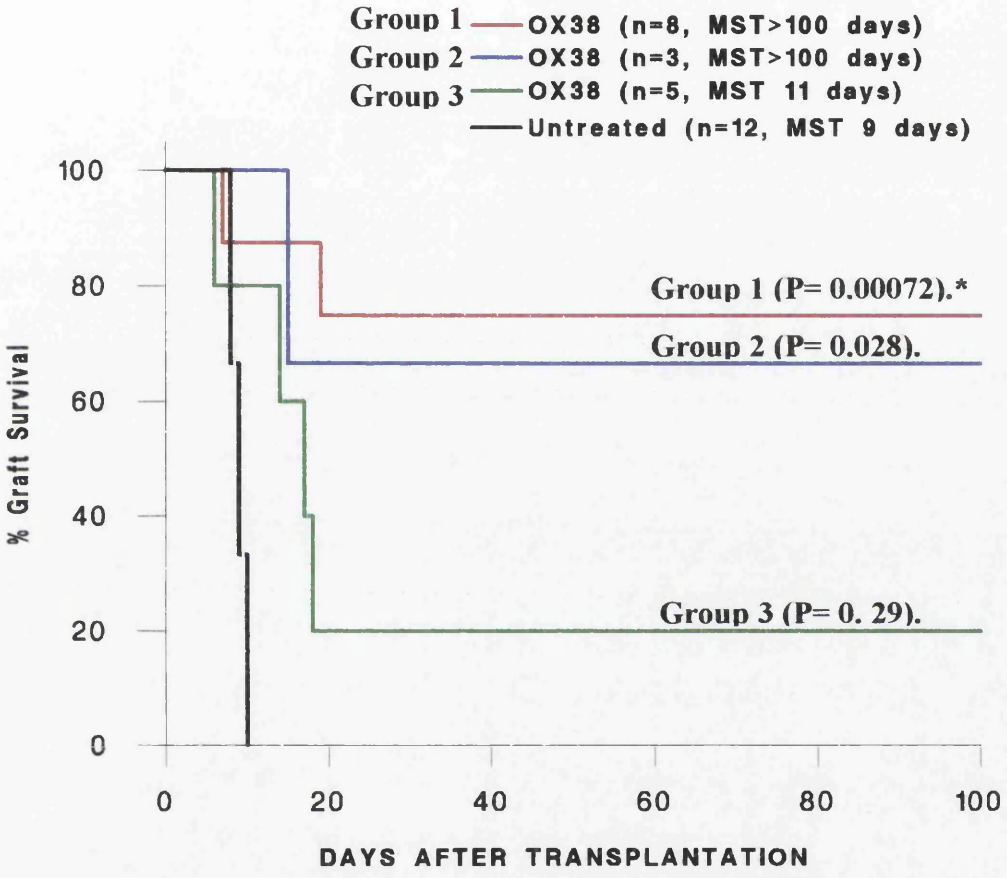
In further studies, a limited analysis in a small number of animals was performed to determine whether a reduced dose of OX38 mAb treatment was also able to prolong allograft survival. The results of this analysis are shown in figure 5:2. Treatment with 10 mg/kg OX38 on day -1 and 2 mg/kg on day 0 led to long-term graft survival in 2 of 3 treated recipients (P= 0.02857, Fisher's exact test in comparison with the untreated control group).

Administration of a single dose of OX38 mAb (10 mg/kg) on the day of transplantation led to long-term survival in only 1 of 5 treated recipients (MST 11 days, P= 0.29412) demonstrating that this treatment schedule is insufficient to induce transplant tolerance. On the basis of these results, therefore, the majority of further studies using OX38 were performed using the original regimen of anti-CD4 mAb treatment i.e. 10 mg/kg on day -3 and 2 mg/kg on days -2, -1 and 0 (the day of transplantation) which led to permanent allograft survival (MST> 100 days, P= 0.00072, by Fisher's exact test).

5:4 OX38 treated DA rats bearing long-standing (> 100 days) Lewis heart grafts display donor-specific immunological tolerance.

Following transplantation of a vascularised organ graft it is well known that the donor strain dendritic cell population within the graft is destroyed in situ and/or migrates to the recipients spleen (Larsen et al, 1990, Austyn and Larsen, 1990).

Figure 5:2 The ability of different doses of OX38 mAb to prolong survival of Lewis heart grafts in DA recipients.



DA rats were treated with anti-CD4 mAb (OX38) using different treatment schedules and then given a heterotopic Lewis heart allograft. This data was depicted in fig 5:1 and is shown here for further comparison.

Group 1 received (10 mg/kg on day -3 and 2 mg/kg on days -2, -1 & day 0).

Group 2 received (10 mg/kg on day -1 and 2 mg/kg on day 0)

Group 3 received (10 mg/kg on day 0 i.e. the day of transplantation).

P value refers to comparisons with untreated control group (Fisher's exact test).

*** A P value of less than 0.017 (0.05/3) was required for statistical significance (Bonferroni's correction for 3 simultaneous comparisons).**

Since dendritic cells are the most immunogeneic component of the graft, the prolonged survival of Lewis heart allografts in DA recipients following OX38 mAb treatment could, in principle, result from reduced immunogenecity of the Lewis heart with time and/or the development of specific immunological unresponsiveness in the recipient. To determine whether DA recipients with long-surviving Lewis heart allograft (i.e. >100 days) displayed donor-specific tolerance, they were transplanted with a second vascularised rat heart graft of either the original donor (i.e. Lewis- $RT1^l$), or third party (PVG-$RT1^c$) strain. The second heart graft was transplanted to the cervical site with end-to-end anastomosis of the donor common carotid artery and pulmonary vein to the recipient internal carotid artery and the external jugular vein respectively. Graft recipients received no additional anti-CD4 mAb immunosuppressive therapy at the time of retransplantation. As shown in table 5:1, all 8 DA recipients bearing a long-standing Lewis primary heart graft accepted a second Lewis heart allograft (MST> 100 days, P= 0.00004), whereas 9 comparable animals given a third party PVG heart graft all rejected their second heart graft rapidly (MST 8 days). This result demonstrates, therefore, that recipients of long-term heart grafts after OX38 treatment display evidence of donor-specific transplant tolerance (MST> 100 days, P= 0.00004, Fisher's exact test in comparison with recipients of third party heart graft).

Table 5:1 OX38 treated DA recipients with long-term Lewis heart allografts display donor-specific transplant tolerance.

Group	*Recipient*[a]	*Donor*[b]	n[c]	Graft survival (days)[d]	MST (days)
1[e]	DA	Lewis	8	all >100 days	>100
2	DA	PVG	9	7, 7, 7, 8, 8, 8, 8, 11, 12	8

(a) DA recipients had long-standing (> 100 days) heterotopic Lewis heart grafts (abdominal site) following perioperative treatment with OX38 mAb. No additional immunosuppression was given at the time of transplantation of the second heart allograft.

(b) Haplotype of second (cervical) heart graft.

(c) Number of recipients per group.

(d) Survival of second (cervical) heterotopic heart allograft.

(e) Group 1 versus group 2 (P= 0.00004, Fisher's exact test).

5:5 Ability of OX38 mAb to prolong allograft survival in additional rat strain combinations.

Although the Lewis to DA rat strain combination is fully allogeneic i.e. mismatched at major and minor histocompatibility loci, it is sometimes designated a relatively weak rat strain combination in the context of transplantation (McConnel and Hall, 1989; Ilano et al, 1989 & 1991). The ability of OX38 to prolong survival of heart allografts was also examined, therefore, in a number of other fully allogeneic rat strain combinations, namely: Lewis ($RT1^l$) to DA ($RT1^a$), DA ($RT1^a$) to PVG ($RT1^c$) (unpublished data Dr.BC.Jaques), DA to Lewis and BN [Brown Norway] ($RT1^n$) to Lewis, as shown in table 5:2. For these studies OX38 mAb was used at an identical dose to that used for the earlier studies in the Lewis to DA strain combination (i.e. 10 mg/Kg on day -3 and 2 mg/Kg on days -2, -1 & day 0). As shown in table 5:2, OX38 led to a significant prolongation of cardiac allograft survival in DA to PVG (MST=12, P= 0.0097 using Mann-Whitney test) and DA to Lewis (MST= 18, P= 0.0369) strain combinations studied but only one animal from this series of experiments (i.e. the DA to Lewis strain combination) accepted its heart allograft permanently. These studies demonstrate that the ability of OX38 mAb (when given as a brief preoperative course) is highly dependent on the rat strain combination used. OX38 mAb led only to a significant effective long-term cardiac allograft survival in the Lewis to DA rat strain combinations and not in any of the other three additional rat strain combinations tested (MST> 100 days, P= 0.0007 using Mann-whitney test and unmodified animals as a control group).

Table 5:2 Ability of OX38 mAb treatment to prolong cardiac allograft survival in different rat strain combinations.

Heart donor	Recipient	n	Graft Survival (days)	MST (days)	*P value	Treatment
DA	PVG	4	7, 7, 8, 9	8	-	Control (no treatment)
**DA	PVG	7	11, 11, 11, 12, 12, 17, 24	12	0.0097	OX38 (10 mg/kg on day -3 and 2 mg/kg on day -2,-1, 0)
Lewis	DA	12	8,8,9,9,9,9,10, 10,10,10	9	-	Control (no treatment)
Lewis	DA	8	11, 18, x6>100	100	0.0007	OX38, (10 mg/kg on day -3 and 2 mg/kg on day -2,-1, 0)
DA	Lewis	3	7, 8, 10	8	-	Control (OX21) (10 mg/Kg on day -3 and 2 mg/Kg on day -2,-1, 0)
DA	Lewis	5	13, 17, 18, 31 & 1>100 days	18	0.037	OX38 (10 mg/kg on day -3 and 2 mg/kg on day -2,-1, 0)
BN	Lewis	3	7, 8, 11	8	-	Control (no treatment)
BN	Lewis	4	15, 19, 21, 28	20	0.052	OX38 (10 mg/kg on day -3 and 2 mg/kg on day -2,-1, 0)

Recipients animals were treated with OX38 mAb as specified in the table. The survival of the heterotopic heart allografts in the OX38 treated recipients was compared with control animals which received either no mAb or control OX21 mAb which labels human C3 inhibitor.

*P-value refers to comparison with control animals in each group (Mann-whitney test). P- value of <0.05 was required for statistical significance

** Data kindly provided by Dr. BC.Jaques, Dept of Surgery, Western Infirmary, Glasgow (Unpublished data).

5:8 Discussion

The CD4 molecule is an attractive target for modulating the graft rejection response and the results in this chapter show that the mouse anti-rat CD4 mAb OX38 is able, when given as a brief perioperative treatment, to cause indefinite survival of Lewis heart allograft in DA recipients. From the different dose regimens used, only the four days treatment was significantly effective in inducing long-term graft survival. Moreover, such animals are rendered permanently and specifically tolerant to their grafts as shown by their ability to accept a second donor (but not third party) heart graft without further exogenous immunosuppression. These findings are consistent with those reported by other groups in the Lewis to ACI rat strain combination (Shizuru et al, 1990, Yin and Fathman, 1995). Interestingly, the efficacy of perioperative OX38 treatment to produce transplantation tolerance did not extend to any of the other fully allogeneic three rat strain combinations tested, although in all cases except BN to Lewis there was evidence of a significant increase in allograft survival. There is little previously published data on the efficacy of OX38 in other rat strain combination although Yin and Fathman also found this schedule of OX38 mAb treatment did not cause tolerance to cardiac allografts in the DA to Lewis rat strain combination (Yin and Fathman, 1995).

It is well known that there is very wide variability between different rodent strains combinations in their susceptibility to different immunosuppressive regimens (McConnel and Hall, 1989; Ilano et al, 1989 and 1991) and the Lewis to DA combinations is generally regarded as more susceptible to experimental

protocols designed to induce transplant tolerance. Nevertheless, unmodified DA rats reject Lewis heart allografts vigorously with complete destruction of the graft within 10 days and the ability of anti-CD4 mAbs to induce transplant tolerance in this strain combination may reflect a particular susceptibility to immunemodulation rather than a weak graft rejection response. It is worth emphasising that continued treatment with OX38 mAb was not used in any of the rat strain combination tested here. It may be that either a higher dose of OX38 or prolonged mAb therapy may have improved allograft survival further. However, the efficacy of long-term mAb use is likely to be limited by the fact that the xenogeneic mouse mAb provokes an anti-mouse Ig-response which likely neutralises mAb in the long-term. This problem could potentially be overcome by constructing chimeric mAb, using molecular biological techniques, in which the constant region of the Ig molecule has been substituted by rat amino acid residues.

5:9 Summary

5:1 Administration of a brief preoperative course of anti-CD4 mAb (MRC OX38) significantly prolonged survival of Lewis cardiac allografts in DA recipients (P= 0.00072) and in most animals promotes permanent graft survival (>100 days) .

5:2 The four days anti-CD4 treatment regimen (10 mg/kg on day -3 and 2 mg/kg on days -2, -1 & day 0) was the most effective for inducing tolerance.

5:3 Following anti-CD4 treatment, recipients of a long-standing cardiac allograft showed specific immunological tolerance when challenged with a second heart allograft.

5:4 The ability of OX38 mAb to induce long-term cardiac allograft survival in the rat was highly dependent on the rat strain combination used. Of the fully allogeneic strains tested, the Lewis to DA strain was the only one readily susceptible to tolerance induction.

CHAPTER SIX

The ability of different anti-CD4 monoclonal antibodies to prolong survival of rat cardiac allograft.

6:1 Introduction

In the preceding chapter, it was shown that the mouse anti-CD4 mAb OX38 is able to induce indefinite heart allograft survival in the Lewis to DA rat strain combination. The efficacy of a particular CD4 mAb to induce prolonged survival of an allograft is likely to be critically dependent on the isotype of the mAb in question and its precise antigen specificity for the CD4 molecule. The extracellular part of the CD4 molecule comprises 4 Ig-like domains, as already described in chapter two (figure 2:3C). The membrane distal or N terminus Ig-like domains (V1 and V2) of the CD4 molecule on the T cell bind to the β2 domain of MHC class II molecules on APC or target cells. The MHC contact sites of the CD4 molecule are in the CDR1-like and CDR3-like loops of the first domain (V1) and in the 'strand F-loop 6- strand-G' region of the second domains (V2). The contact point on class II MHC for the CD4 molecule is the loop three region of the β2 domain (Ryu et al, 1990; Parham, 1992; Fleury et al, 1991 and Halloran et al, 1990). As already noted, OX38 mAb is directed against an epitope on the distal 2 domains of the rat CD4 molecule and is of the IgG2a isotype.

There is little published information on the importance of epitope specificity of anti-CD4 mAb and its relationship to efficacy to prevent allograft rejection. In this chapter, studies were performed to assess the efficacy of anti-CD4 mAbs with different epitope specificity (and of different isotypes) for prolonging cardiac allograft survival in the rat. In addition to the IgG2a mAb OX38, four other mouse anti-rat CD4 mAbs were studied. One of these, the mAb W3/25,

also labels a determinant located on the distal two domains of rat CD4 and competes for binding with OX38. However, unlike OX38, it is of the IgG1 subclass. The other three anti-CD4 mAbs used in the following studies were raised and kindly provided by Dr Neil Barclay (Sir William Dunn School of Pathology, University of Oxford, Oxford). These mAbs (OX70, OX71 and OX73) were raised by immunising mice with a recombinant form of the soluble rat CD4 molecule and are all directed against epitopes located on the membrane proximal two domains (domains 3 and 4) of the rat CD4 molecule. The isotype and broad specificity of these anti-CD4 mAb is shown in table 6:1.

In additional studies in this chapter, the ability of anti-CD4 mAbs to induce indefinite allograft survival was examined in recipient rats which had been thymectomised (as adults) prior to heart transplantation. The latter studies were undertaken to determine whether the peripheral T cells pool could still be rendered unresponsive by anti-CD4 mAbs in the absence of new T cells emerging from the thymus. Finally, since the degree of CD4 T cells depletion following mAb treatment may make an important contribution to the efficacy of anti-CD4 mAbs, the ability of different CD4 mAbs to deplete T cells from the peripheral blood and lymph nodes of treated rats was examined.

6:2 Ability of different mouse anti-rat CD4 mAbs to prolong heart allograft survival.

Four additional anti-CD4 mAbs (W3/25, OX70, OX71 and OX73) were tested for their ability to prolong survival of Lewis ($RT1^l$) cardiac allografts in DA ($RT1^a$) recipient animals. As in the preceding chapter, donor heart grafts were

Table 6:1 Isotype and broad epitope specificity of mouse anti-rat CD4 mAbs.

Antibody[a,b]	Isotype	CD4 Domain	Membrane proximity
OX38	IgG2a	1 & 2	Membrane distal domains
W3/25	IgG1	1 & 2	Membrane distal domains
OX70	IgG2a	3 & 4	Membrane proximal domains
OX71	IgG1	3 & 4	Membrane proximal domains
OX73	IgG1	3 & 4	Membrane proximal domains

(a) The anti-CD4 mAbs OX70, OX71 and OX73 were a generous gift from Dr A.N Barclay (Sir William Dunn School of Pathology, University of Oxford, Oxford).

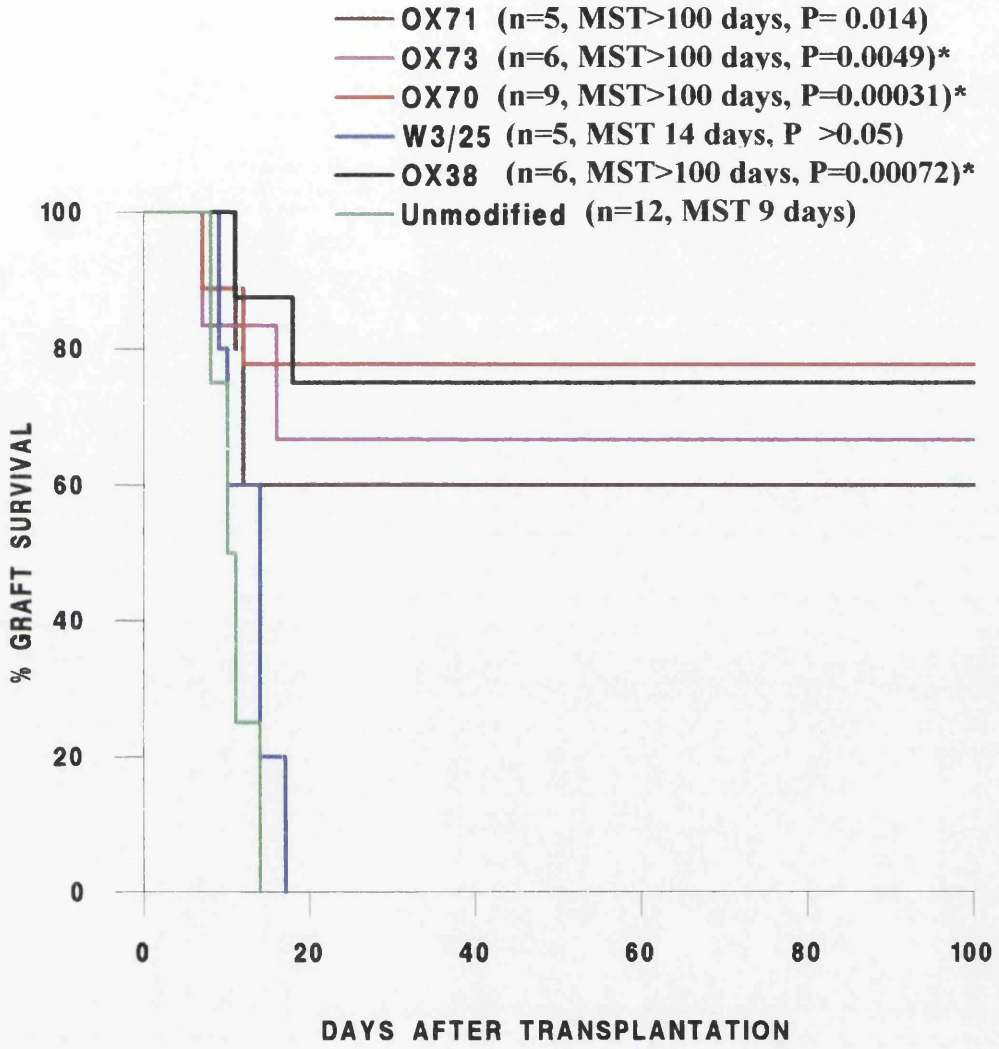
(b) Hybridoma cells secreting these monoclonal antibodies were grown as ascitis in BALB/C mice and the antibodies affinity purified prior to use.

transplanted heterotopically to the intrabdominal site and recipients were treated with anti-CD4 mAbs at a dose of 10 mg/Kg on day -3 and 2 mg/Kg on days -2, -1 and day 0 (i.e. the day of transplantation). All of the anti-CD4 mAbs were given by the intraperitoneally (I.P) route. Figure 6:1 summarises the results of the heart transplant studies using different mAbs to rat CD4. The survival data for OX38 has already been described and depicted in fig 5:1 and 5:2. It can be seen from figure 6:1 that all of the anti-CD4 mAbs directed against membrane proximal regions (domains 3 and 4) of the CD4 molecule (mAbs OX70 and OX73) significantly induced long-term heart allograft survival in the majority of treated recipients except OX71 mAb which was marginally significant (P= 0.014). Thus in DA recipients receiving the membrane proximal anti-CD4 mAb OX70, 7 of 9 treated animals retained their grafts indefinitely (MST> 100 days, P= 0.00031, using Fisher's exact test in comparison with the unmodified control group) whereas 3 of 5 recipients treated with OX71 (P= 0.0147) and 4 of 6 recipients treated with OX73 (P= 0.00049) retained their grafts indefinitely (MST > 100 days in both groups). Interestingly, treatment with the mAb W3/25 which is directed against the membrane distal domains of CD4 led to only a modest increase in heart allograft survival (MST 14 days, P>0.05) and none of the recipients treated with this particular retained their grafts for more than 17 days.

6:3 Role of the thymus gland in anti-CD4 induced prolongation of heart allograft survival.

Experiments were next performed to determine whether the presence of an intact thymus gland plays a critical role in prolongation of heart graft survival

Figure 6:1 The ability of different anti-CD4 mAbs to prolong survival of Lewis cardiac allografts in DA recipients.



DA recipients were treated with the different anti-CD4 mAbs at a dose of 10 mg/kg on day -3 and 2 mg/kg on day -2, -1 and day 0 (the day of transplantation).

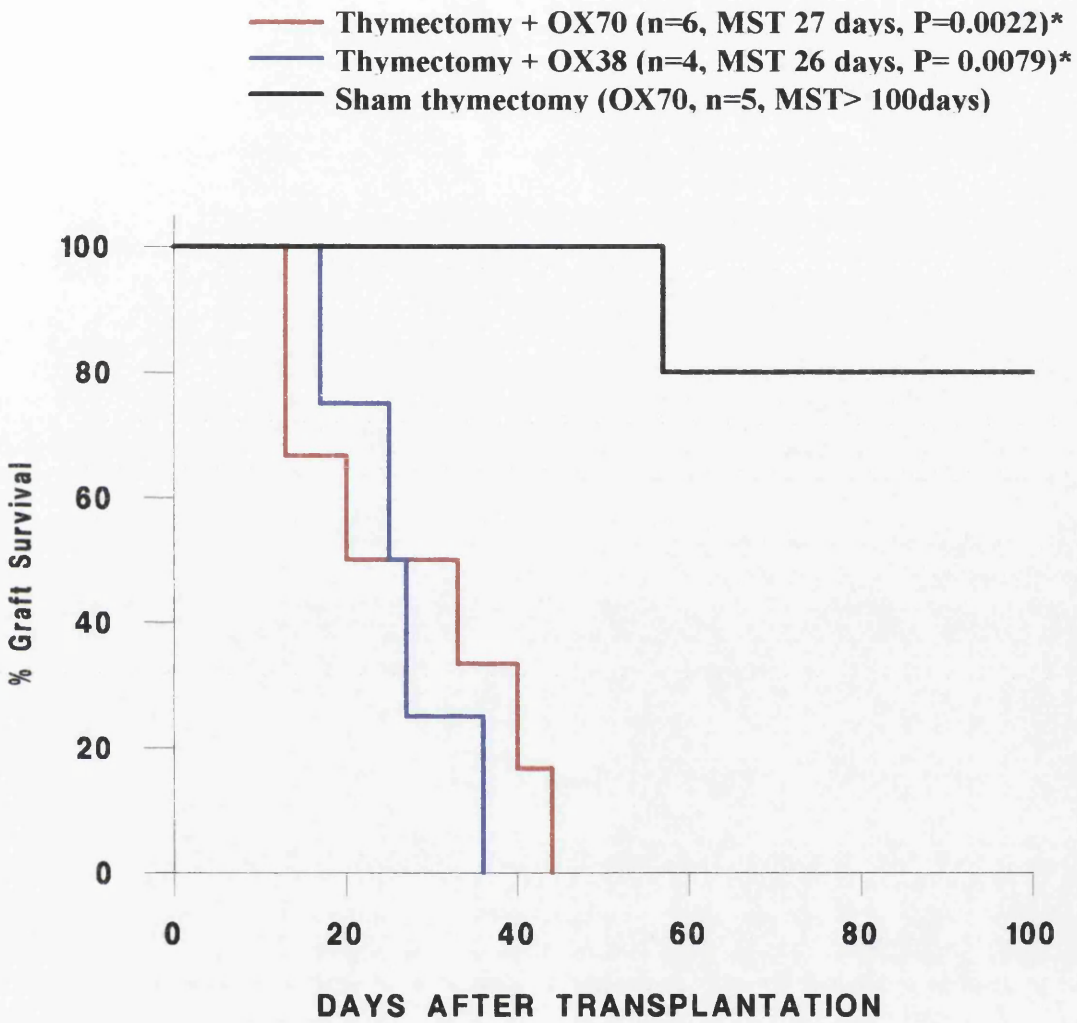
P value refer to comparisons with unmodified control animals (Fisher's exact test).

*** A P value of ≤ 0.01 (0.05/5) was required for statistical significance (Bonferroni's correction for 5 simultaneous comparisons).**

following anti-CD4 mAb treatment in the Lewis to DA rat strain combination. Adult thymectomy was performed 10 weeks prior to anti-CD4 mAb therapy and heart transplantation. Thymectomised recipients were treated with anti-CD4 mAb directed against either membrane distal (MRC OX38) or membrane proximal (MRC OX70) domains. MAbs were given at a dose of 10 mg/kg on day -3 and 2 mg/kg on days -2, -1 and day 0 (the day of transplantation). As noted earlier, this dose of anti-CD4 mAb (either OX38 or OX70) leads to indefinite allograft survival in the majority of euthymic treated DA recipients of Lewis heart allografts. The details of the surgical technique used for thymectomy are described in chapter 4 (material and methods). Fortuitously, recent thymic migrants in the rat express the cell-surface antigen Thy-1 for several days after emerging from the thymus and thereafter become Thy-1 negative (Mason and Williams, 1980; Campbell et al, 1980). Thy-1, which can be labelled using the MRC OX7, therefore provides a useful cell-surface marker with which to confirm that effective thymectomy has been accomplished. In the present experiments, flow cytometric analysis of PBLs from rats which had been thymectomised several weeks earlier confirmed in all animals tested the complete absence of Thy1 positive lymphocytes, therefore demonstrating the completeness of thymectomy (results not shown).

Interestingly, both OX38 and OX70 anti-CD4 mAbs led to a modest increase in cardiac allograft survival in thymectomised DA recipients when compared to unmodified graft recipients (fig 6:2) but none of the recipients became permanently tolerant to their allograft (median survival times of 26 to 27 days

Fig 6:2 Anti-CD4 mAbs (OX38 & OX70) treatment of pre-thymectomised DA recipients of Lewis heart grafts (at day 70).



DA recipients were treated with anti-CD4 mAbs at a dose of 10 mg/kg on day -3 and 2 mg/kg on days -2, -1 and day 0 (the day of transplantation). Recipients underwent thymectomy (or sham thymectomy) 10 weeks before transplantation.
P value refers to comparison with the sham thymectomised control group (Fisher's exact test).
*** A P value of ≤ 0.025 (0.05/2) was required for statistical significance (Bonferonni's correction for 2 simultaneous comparisons).**

respectively). The difference between the thymectomised/anti-CD4 treated and the sham thymectomysed group as a control was statistically significant (P= 0.00216 for OX70 treated group and P= 0.00794 for the OX38 treated group using Fisher's exact test). As noted earlier OX38 and OX70 mAbs both produce, when given to euthymic DA rats, indefinite survival of Lewis heart allografts. In keeping with these observations, a group of sham thymectomised DA recipients treated with OX70 mAb failed to reject Lewis heart allografts (MST>100 days). From these experiments, it can be concluded that the presence of an intact thymus gland is significantly important for the induction of tolerance by anti-CD4 mAbs (OX38 & OX70) in this particular rat transplant model.

6:4 Depletion of CD4 T cells by anti-CD4 mAbs

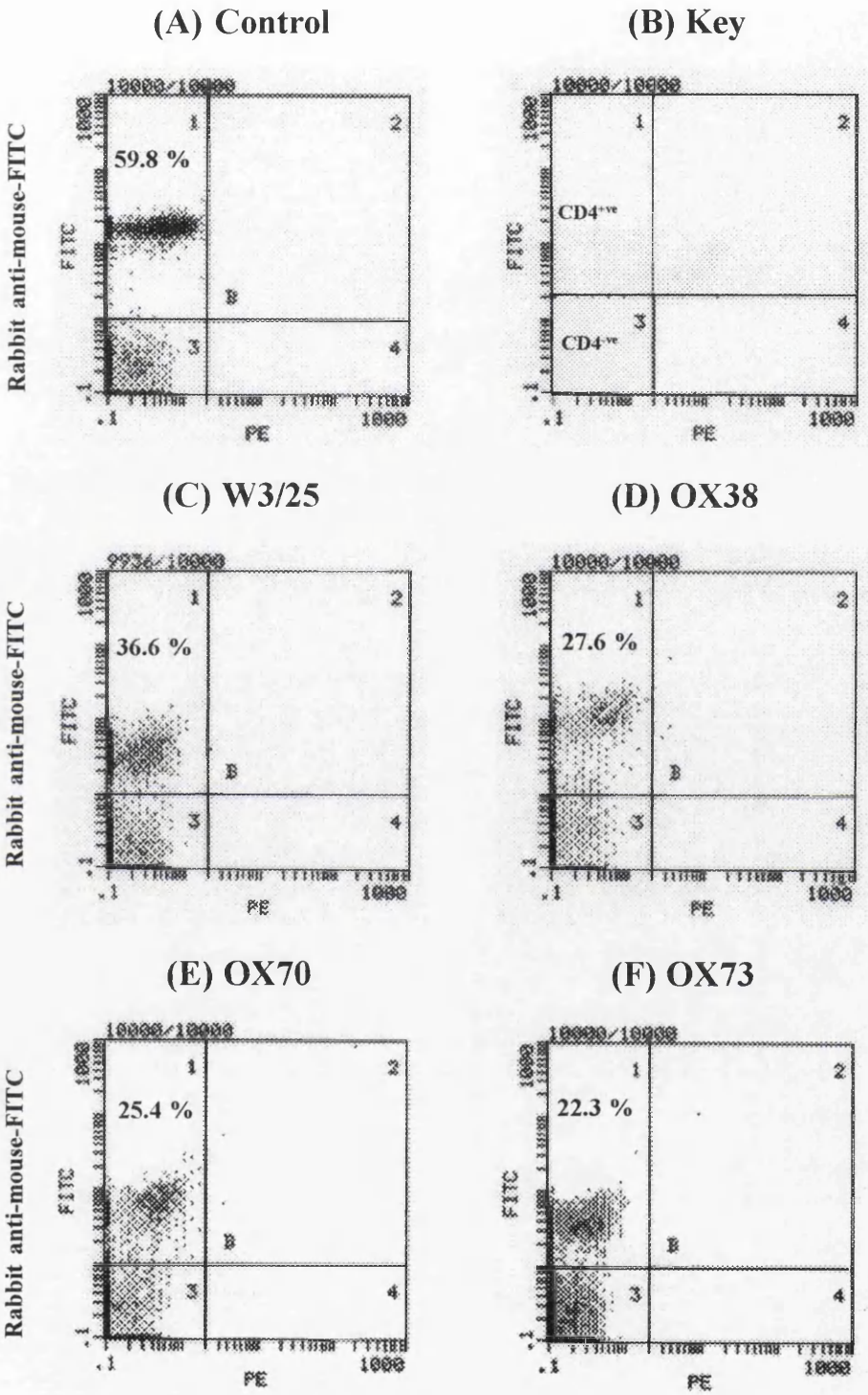
In the following studies, the ability of different anti-CD4 mAbs (i.e. W3/25, OX70 and OX73) to selectively deplete CD4 T cells from the peripheral blood (PBL) and lymph nodes (LNC) of DA rats was determined. Flow cytometric analysis was performed as described in details in chapter 4 (material and methods). The anti-CD4 mAbs were given at a dose of 10 mg/Kg on day -3 and 2 mg/Kg on days -2, -1 and 0 (the day of transplantation) and flow cytometric analysis was performed on day 0 (the day of administration of the last dose of mAb) and on day 4. The flow cytometric analysis performed on day 4 was carried out on animals given anti-CD4 mAb and a heart allograft on day 0 and also on animals given anti-CD4 mAb but not a heart allograft.

The following mouse anti-rat monoclonal antibodies were used to identify residual lymphocyte populations in treated animals: W3/25 (anti-CD4), OX8 (anti-CD8), R73 (αβ TCR) and OX22 (anti-CD45R). These mAbs were all FITC- or -PE conjugated. In addition, cells from anti-CD4 treated animals were labelled with rabbit anti-mouse-FITC directly so as to detect any residual anti-CD4 mAb on the lymphocyte cell surface or adding exogenous non-conjugated apprpopriate anti-CD4 mAb followed by rabbit anti-mouse-FITC to determine the total number of CD4 T cell population. In the rat, some activated monocytes/macrophages may also express the CD4 molecule (Brideau et al, 1980) and some natural killer (NK) cells may express the CD8 molecule (Gilman et al, 1982). Two colour fluorescence labelling was therefore carried using PE-labelled anti-CD4/anti-CD8 antibodies and FITC-labelled anti-αβ TCR (R73) so that the percentage of residual CD4 and CD8 T cells could be documented accurately. In the following figures, the FACS analysis data of individual experimental animals (of LNCs & PBLs) are depicted first followed by statistical analysis shown as bar chart on 4 animals in each anti-CD4 treated group. The percentage of CD4 and CD8 T cells in the LNC and PBL of anti-CD4 treated animals on day 0, i.e. after the completion of anti-CD4 treatment are shown in figures 6:3a, 6:3b and 6:4a, 6:4b. In normal unmodified DA rats, the percentage of CD4 T cells is 59.8% in LNC and 54.9% in PBL. There was an overall difference between the LNC groups and an overall difference between the PBL groups, as shown in figure 6:3c (P= 0.007, ANOVA) and, all the anti-CD4 treated groups (LNC & PBL) *except* the PBL/W3/25 treated animals were

significantly different from the control group ($P<0.05$, Dunnet's test). W3/25 mAb depleted CD4 T cells from 59.8 to 36.6 in LNC and from 54.9% to 41.7% in PBL as shown in figure 6:3a & 6:3b. The treatment with the anti-CD4 mAb W3/25 led to minimal CD4 T cell depletion which was only significant in the LNC treated group ($P<0.05$, Dunnett's test) as shown in figure 6:3c. In contrast, both OX70 and OX73 mAbs treatment led to partial (but statistically significant) depletion of CD4 T cells and depletion was most marked in the PBL ($P<0.05$, Student t test). OX70 and OX73 mAbs reduced CD4 T cells in the LNC from 59.8% to 25% and 22.3% respectively whereas in PBL CD4 reduced from 54.9% to 20.3% by OX70 and to 22.7% by OX73 as shown in figures 6:3a & 6:3b.

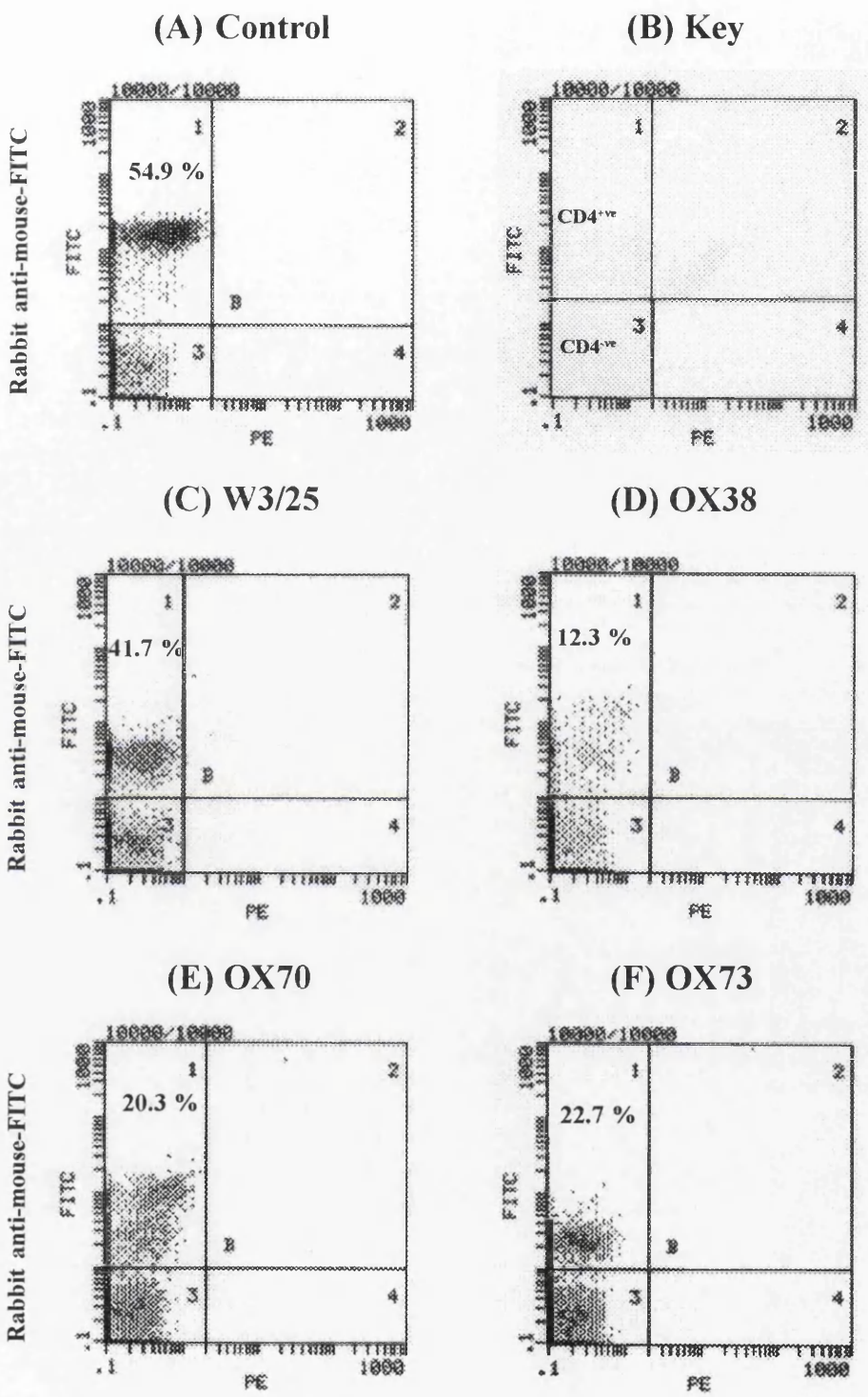
In normal untreated DA rats, the percentage of CD8 T cells is 13.7 in LNC and 14.1% in PBL as shown in figure 6:4a & 6:4b. As shown in fig 6:4c, there was a small increase in CD8 T cells after OX38, OX70 and OX73 mAb treatment. This increase was only significant after OX70 and OX38 mAbs in LNC group and significant only after OX38 treatment in PBL group ($P<0.05$, Dunnett's test). One exception was the lack of an increase in CD8 T cells in the PBL of OX73 treated animals. The effect of in vivo OX38 treatment was not included in this particular experiment, since many previous experiments in this laboratory (B Jaques) had already been performed. These showed that OX38 mAb, at the dose used here for anti-CD4 mAbs, depletes CD4 T cells to 27.6% in LNC and 12.3% in PBL, i.e. comparable to those reported here for OX70 and OX73. The unpublished data shown for OX38 mAb was kindly provided by Mr B.Jaques.

Figure 6:3a Flow cytometric analysis of LNCs showing the depletion of CD4 T cells on day 0 in animals treated by anti-CD4 mAbs.



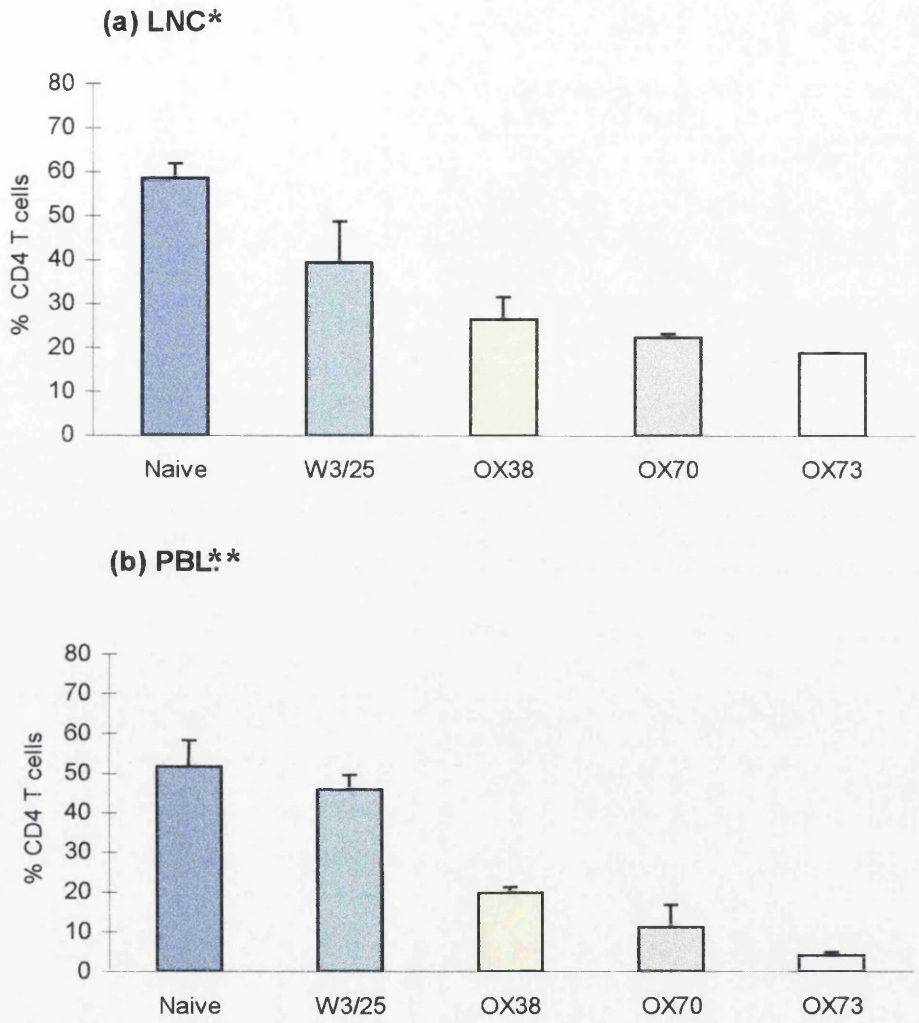
Single colour fluorescence analysis using FITC-conjugated rabbit anti-mouse Y-axis (log_{10} FITCsignal). LNCs obtained on day 0 following (C) W3/25, (D) OX38, (E) OX70, (F) OX73 mAb treatment. All LNCs were incubated with a saturating dose of appropriate anti-CD4 mAb [(A) Control cells had W3/25 added] followed by rabbit anti-mouse -FITC.

Figure 6:3b Flow cytometric analysis of PBLs showing the depletion of CD4 T cells on day 0 in animals treated by anti-CD4 mAbs.



Single colour fluorescence analysis using FITC-conjugated rabbit anti-mouse Y-axis (log_{10} FITCsignal). PBLs obtained on day 0 following (C) W3/25, (D) OX38, (E) OX70, (F) OX73 mAb treatment. All PBLs were incubated with a saturating dose of appropriate anti-CD4 mAb [(A) Control cells had W3/25 added] followed by rabbit anti-mouse -FITC.

Figure 6:3c Flow cytometric analysis of PBLs and LNCs showing the depletion of CD4 T cells on day 0 (i.e. day of last dose of anti-CD4) by different anti-CD4 mAbs.



Peripheral blood lymphocytes (PBLs) and lymph node cells (LNCs) were harvested from unmodified (naive) DA rats and from DA rats treated with different anti-CD4 mAbs given at a dose of 10 mg/kg on day -3 & 2 mg/kg on day -2, -1 and 0 (the day of analysis). Results shown are mean and standard deviation of 4 treated animals in each group. The mAbs below each column are those with which the recipients were treated in vivo.

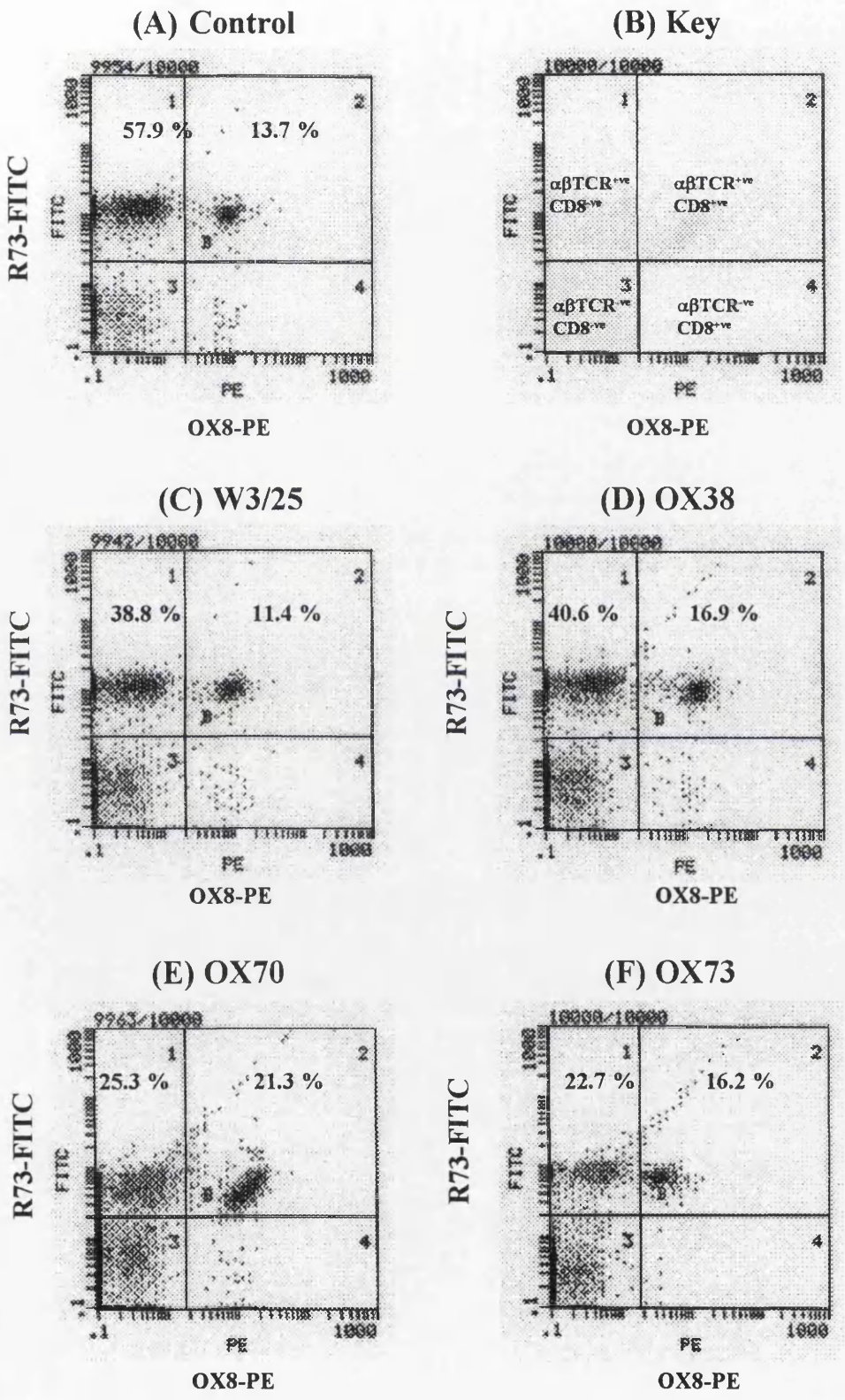
* There was significant overall difference between LNC groups (P= 0.007, ANOVA).

** There was also overall difference between PBL groups (P= 0.0007, ANOVA).

All the anti-CD4 treated groups (LNC & PBL) except the PBL/W3/25 treated animals were significantly different from the control group ($P<0.05$, Dunnett's test).

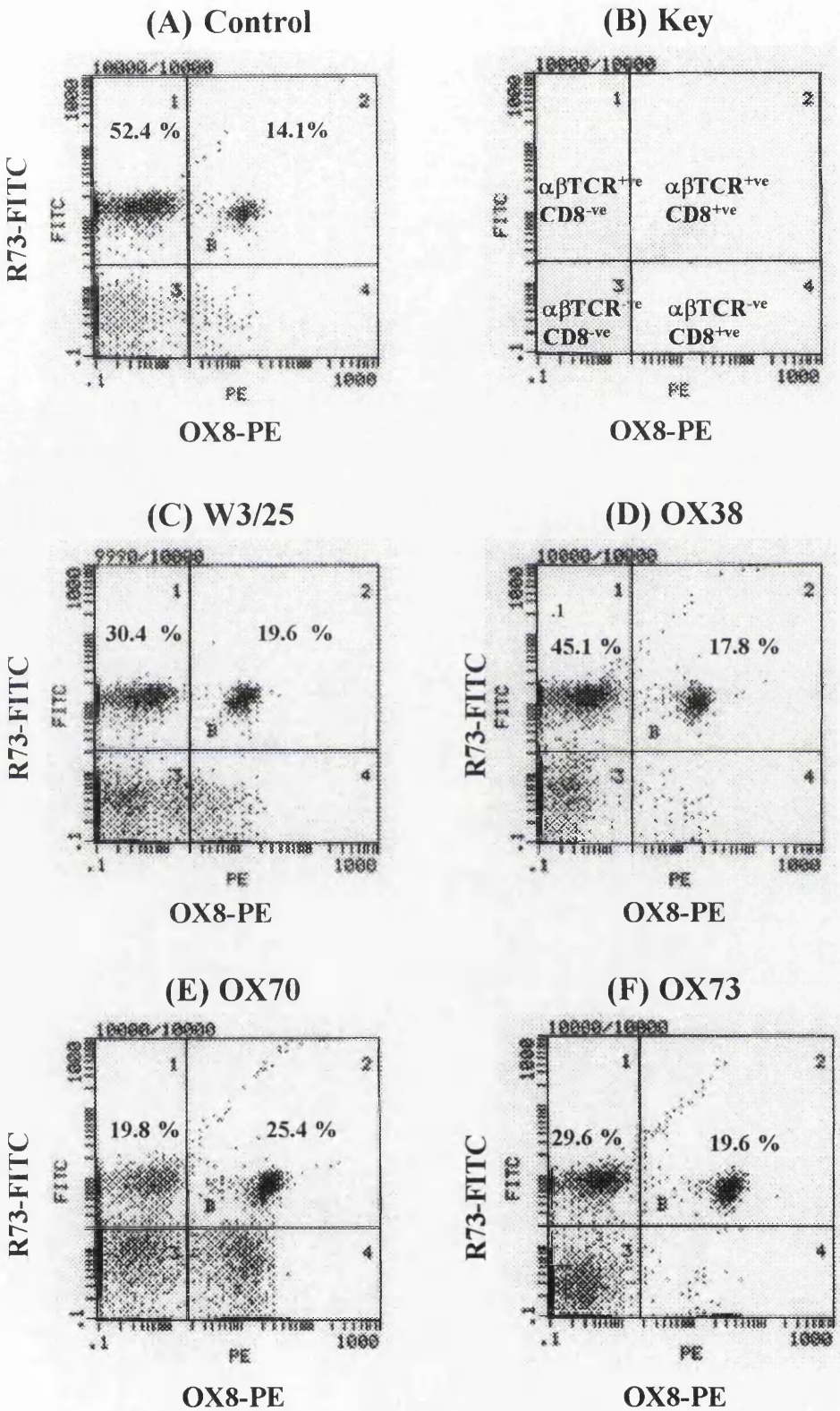
OX70 & OX73 treatment led to a greater depletion of CD4 T cells in PBL than LNCs ($P<0.05$, Student's t test).

Figure 6:4a Flow cytometric analysis of LNCs (labelling CD8 T cells) on day 0.



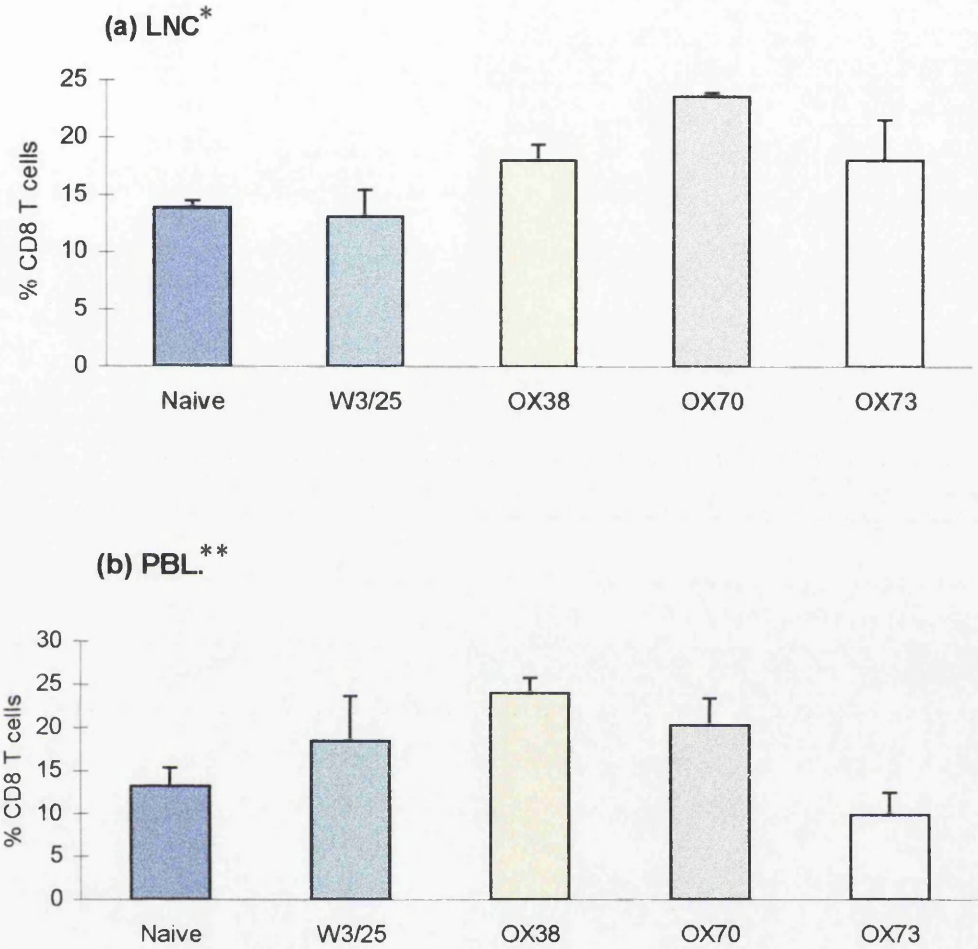
Two colour fluorescence analysis using FITC-conjugated anti-TCRαβ Y-axis (log_{10} FITCsignals) and PE-conjugated anti-CD8 X-axis (log_{10} PE signals). (A) Represents LNCs from a control naive untreated animal and (C), (D), (E) and (F) following treatment with different anti-CD4 mAbs.

Figure 6:4b Flow cytometric analysis of PBLs (labelling CD8 T cells) on day 0.



Two colour fluorescence analysis using FITC-conjugated anti-TCRαβ Y-axis (log_{10} FITC signals) and PE-conjugated anti-CD8 X-axis (log_{10} PE signals). (A) Represents PBLs from a control naive untreated animal and (C), (D), (E) and (F) following treatment with different anti-CD4 mAbs.

Figure 6:4c Flow cytometric analysis showing the percentage of CD8 T cells on day 0 (i.e. day of last dose of anti-CD4 mAb).



Peripheral blood lymphocytes (PBLs) and lymph node cells (LNCs) were harvested from unmodified (naive) DA rats and from DA rats treated with different anti-CD4 mAbs given at a dose of 10 mg/kg on day -3 & 2 mg/kg on day -2, -1 and 0 (the day of analysis).

* There was significant overall difference between LNC groups (P= 0.001, ANOVA).

* Only the OX38 and OX70 treated animals were significantly different from the control group (P< 0.05, Dunnett's test).

** There was significant overall difference between PBL groups (P= 0.007, ANOVA).

** OX38 treated animals were significantly different from the control group (P< 0.05, Dunnett's test).

Flow cytometric analysis of cells from animals 4 days after cessation of anti-CD4 mAb treatment showed that levels of CD4 T cell depletion were similar to those observed on day 0. As it can be seen from figure 6:5a & 6:5b, the data shown for day 4 is for W3/25 and OX70 mAbs only. There was significant difference between all the groups (LNCs & PBL) except the transplanted PBL/W3/25 treated animals in comparison with the control ($P<0.05$, Dunnett's test). In addition, there was no difference in the levels of CD4 T cell depletion between animals which had received a heart allograft and those which had received anti-CD4 mAb but had not been given a heart allograft except LNC/W3-25 treated animals ($P<0.05$, Tukey's test).

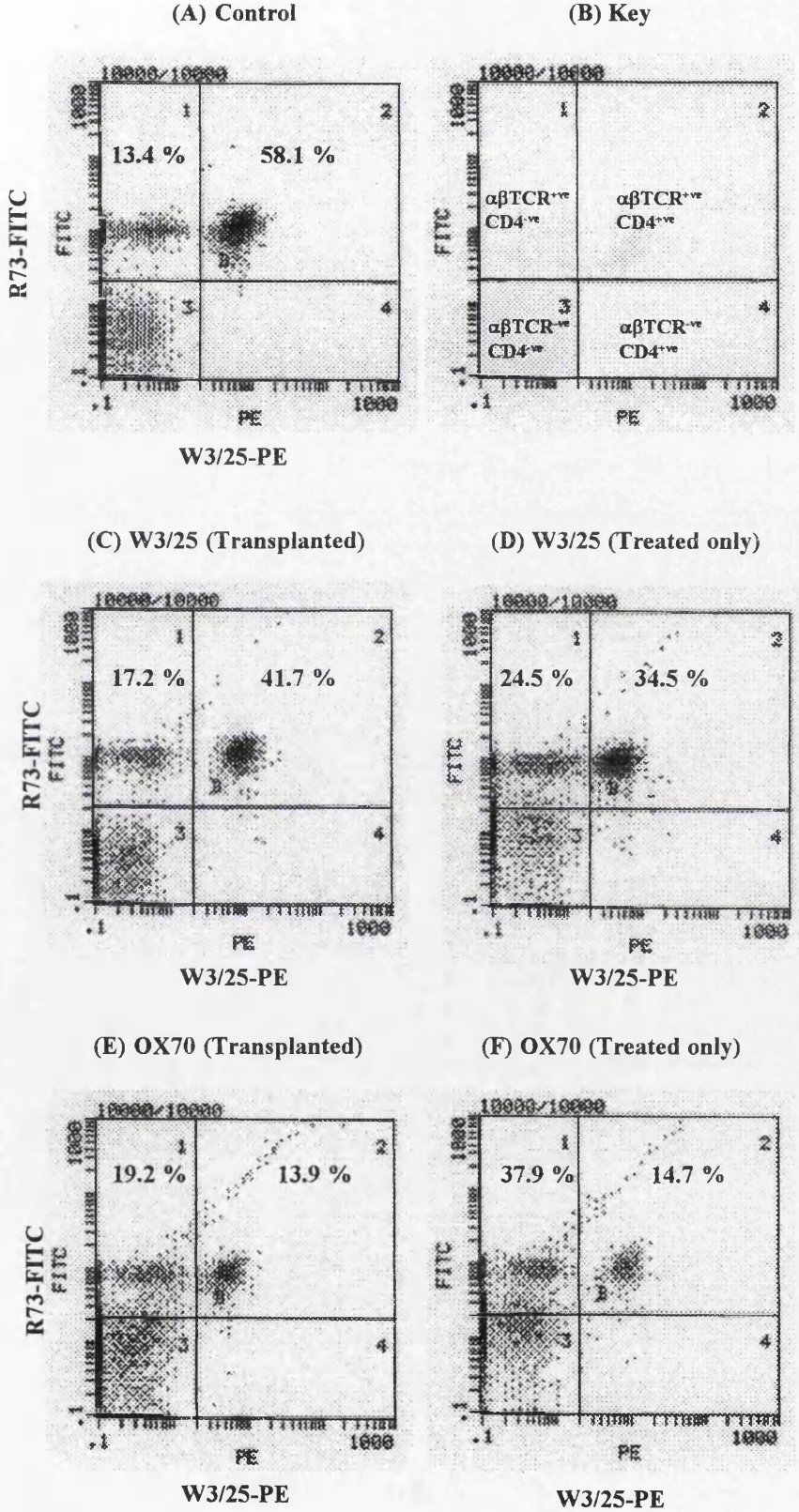
To determine the amount of bound anti-CD4 mAb, immunofluorescence analysis was performed on LNCs & PBLs obtained on day 0 (the day of last dose of anti-CD4 mAb treatment) from control and treated animals using FITC-conjugated rabbit anti-mouse polyclonal antibody as shown in figure 6:7a & 6:7b. The analysis showed that about 35% of PBL & LNC CD4 T cells are still coated by W3/25 mAb. In OX38 treated animals, only 8.6% of PBLs and 31% of LNCs CD4 T cells are still coated whereas in OX70 treated animals 18.2% and 20.2% of PBLs and LNCs respectively are still coated. In OX73 treated animals, 19.5% and 8.7% of LNC and PBL respectively are still coated too. By day 4, no residual anti-CD4 was detectable on the surface of either PBL or LNC, as judged by labelling of cells with rabbit anti-mouse-FITC antibody as shown in figure 6:8a & 6:8b. However, in vitro labelling of PBL and LNC from anti-CD4 treated animals, using the anti-CD4 mAb W3/25 followed by rabbit

anti-mouse FITC-labelled antibody, revealed that residual CD4 T cells showed comparable levels of fluorescence to cells obtained from normal animals i.e. there was no evidence of CD4 molecule modulation following prior in vivo treatment with anti-CD4 mAb.

In the rat CD4 T cells can be divided into two phenotypically distinct subsets according to their expression of the cell-surface molecule labelled by the mAb OX22. The OX22 mAb labels the high molecular weight isoform of the leukocyte-common antigen. Using this mAb lymphocytes can be divided into either $OX22^{low}$ ($CD45R^{low}$) or $OX22^{high}$ ($CD45R^{high}$) T cells (Spickett et al,1983; Sarwar et al, 1993). Recently, it has been reported that the cytokine production by $CD45R^{high}$ and $CD45R^{low}$ CD4 T cells appears to have characteristic cytokine patterns correlating to Th1 and Th2 cells respectively (Morrisely et al, 1993; Bushel and Wood, 1995). In normal untreated rats, the percentage of LNC $OX22^{high}$ and $OX22^{low}$ CD4 T cells from the total labelled lymphocyte are 30.7% and 21.1% respectively and in PBLs 36.2% are $OX22^{high}$ and 23% are $OX22^{low}$ CD4 T cells as shown in figures 6:6a & 6:6b. As shown in figure 6:6c, there was a significant overall difference between LNC $OX22^{high}$ groups ($P=0.007$, ANOVA) but no significant difference between LNC $OX22^{low}$ groups ($P=0.09$, ANOVA). A detailed analysis of OX22 high and OX22 low T cells in normal and anti-CD4 treated (W3/25 and OX70) animals revealed that both W3/25 & OX70 led to a significant preferential depletion of only $OX22^{high}$ cells from the LNC and only OX70 mAb depleted $OX22^{high}$ CD4 T cell in comparison with control cells ($P<0.05$, Dunnett's test). In addition, the

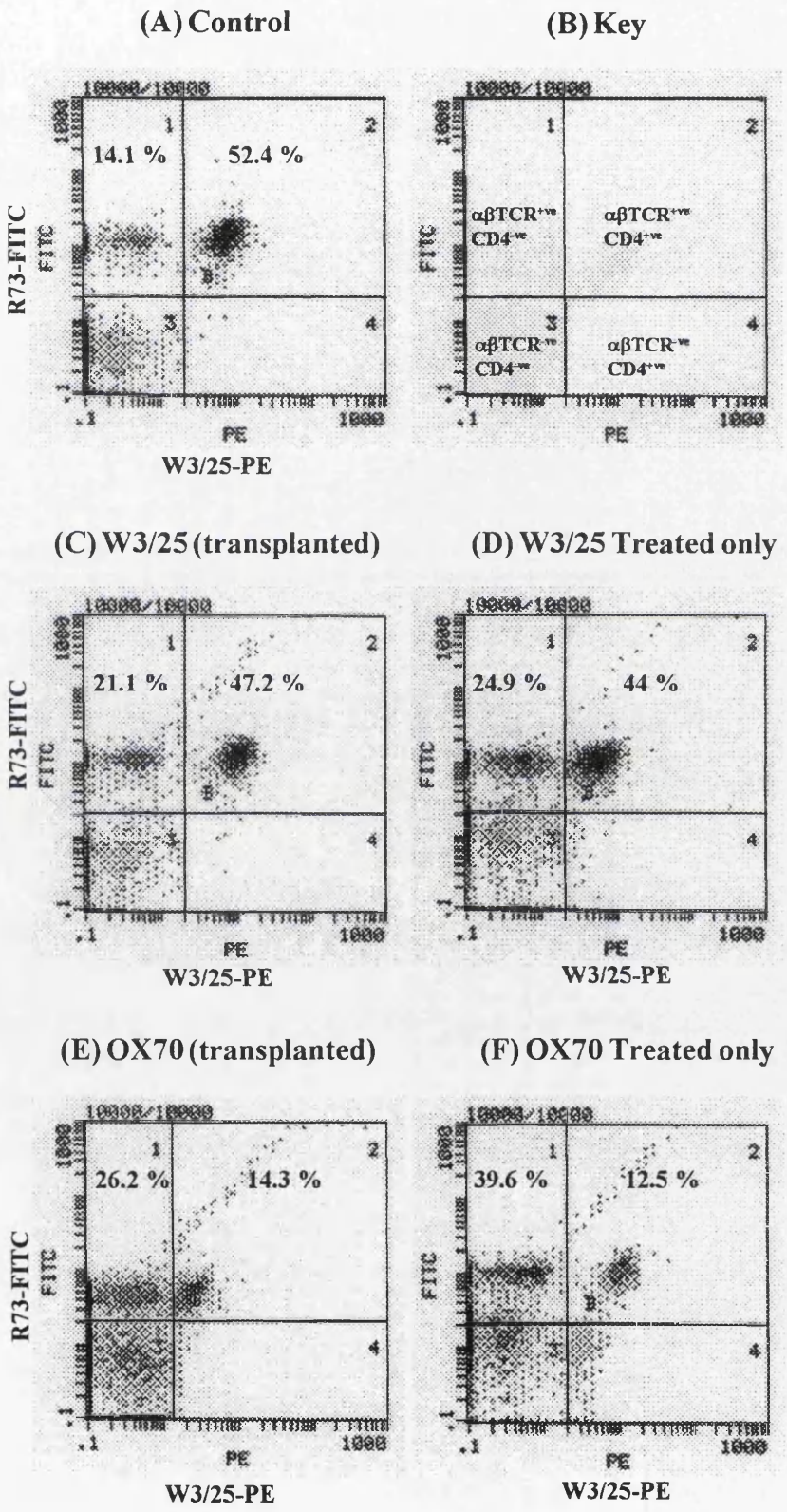
depletion of $OX22^{high}$ CD4 T cells was significantly higher by OX70 than by W3/25 mAb in either LNCs or PBLs ($P<0.05$, Tukey's test). As already noted, treatment with W3/25 had no significant effect on PBL CD4 T cells depletion on day 0.

Figure 6:5a Flowcytometric analysis showing the depletion of LNC CD4 T cells on day 4 in anti-CD4 treated and transplanted animals.



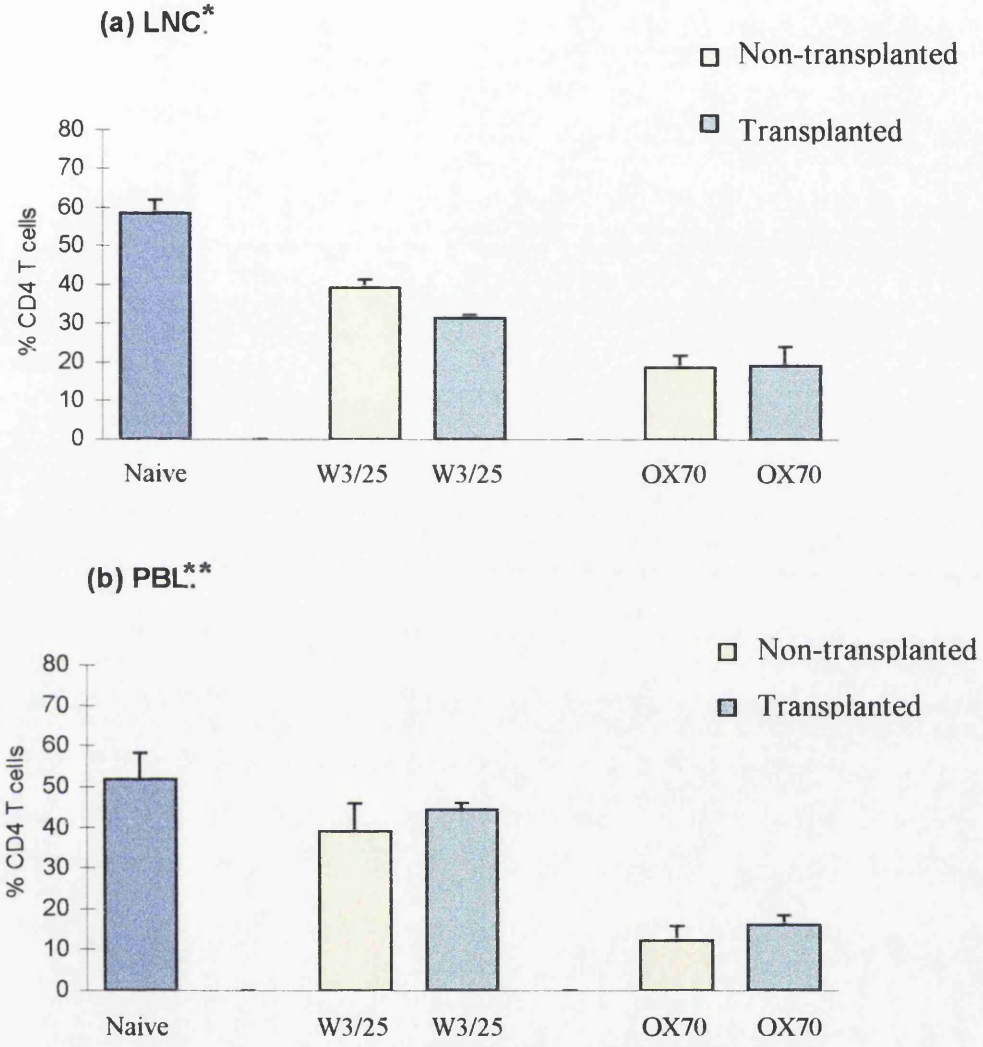
Two colour fluorescence analysis using FITC-conjugated anti-TCR αβ Y-axis (log_{10} FITCsignal) and PE-conjugated anti-CD4 X-axis (log_{10} PE signals). (A) Represents LNCs from naive control animals (C) Day 4 W3/25 treated and transplanted. (D) Day 4 W3/25 treated only (E) Day 4 OX70 treated and transplanted (F) Day 4 OX70 treated only.

Figure 6:5b Flowcytometric analysis showing the depletion of PBL CD4 T cells on day 4 in anti-CD4 treated and transplanted animals.



Two colour fluorescence analysis using FITC-conjugated anti-TCR αβ Y-axis (log_{10} FITCsignal) and PE-conjugated anti-CD4 X-axis (log_{10} PE signals). (A) Represents PBLs from naive control animals (C) Day 4 W3/25 treated and transplanted. (D) Day 4 W3/25 treated only (E) Day 4 OX70 treated and transplanted (F) Day 4 OX70 treated only.

Figure 6:5c Flow cytometric analysis showing the depletion of CD4 T cells on day 4 (i.e. the fourth day after the last dose of anti-CD4).



Peripheral blood lymphocytes (PBLs) and lymph node cells (LNCs) were harvested from DA rats 4 days after cessation of anti-CD4 mAb treatment. Animals were treated with anti-CD4 mAb alone or received a Lewis heart allograft on day 0 (last day of anti-CD4 mAb treatment). Naive animals were normal DA rats.

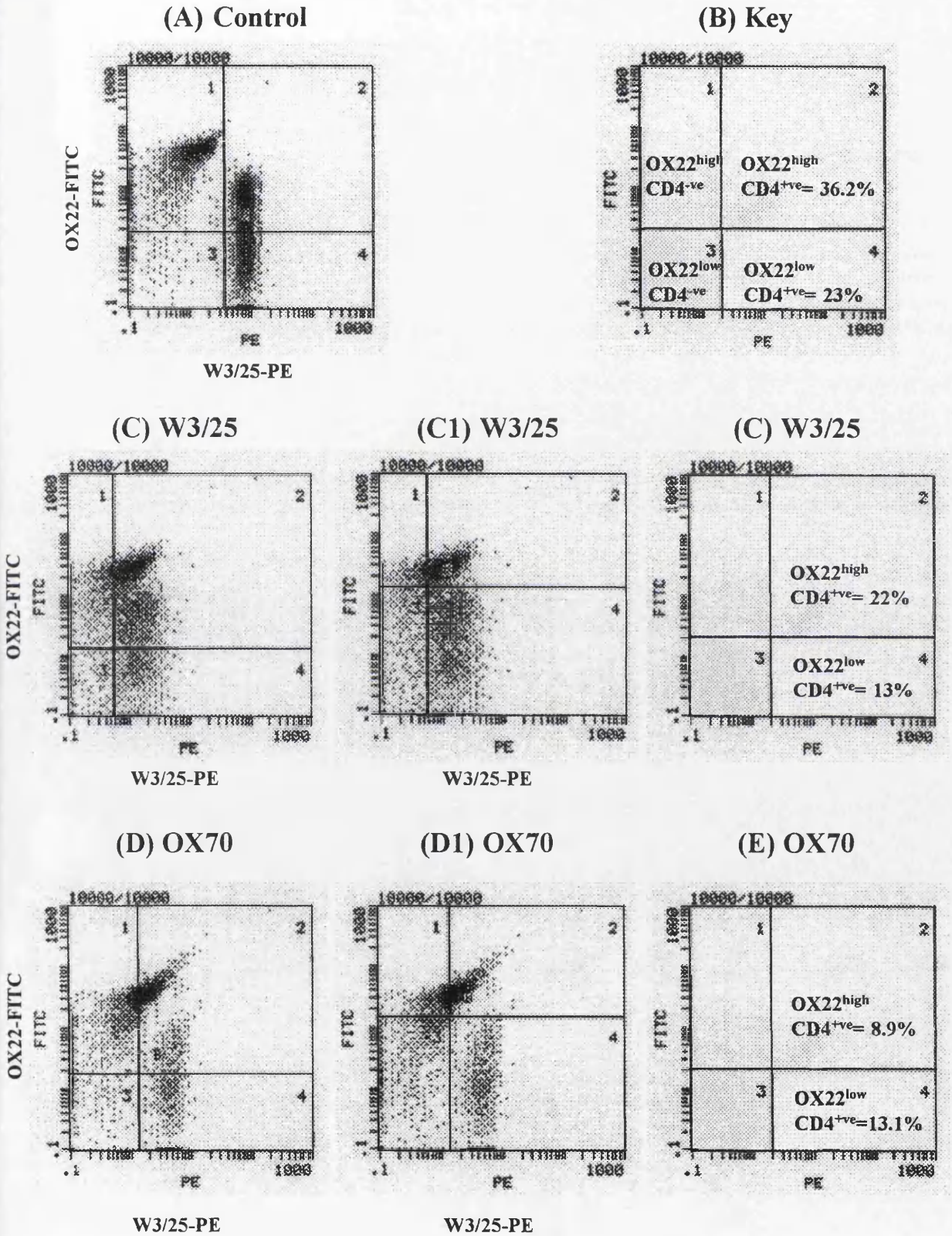
* There was significant overall difference between LNC groups (i.e. W3/25 & OX70 anti-CD4 treated) (P= 0.007, ANOVA).

** There was also overall difference between PBL groups except W3/25 treated group (P= 0.0007, ANOVA).

All the groups (LNCs & PBLs) except the transplanted PBL/W3/25 treated animals were significantly different from the control group (P<0.05, Dunnett's test).

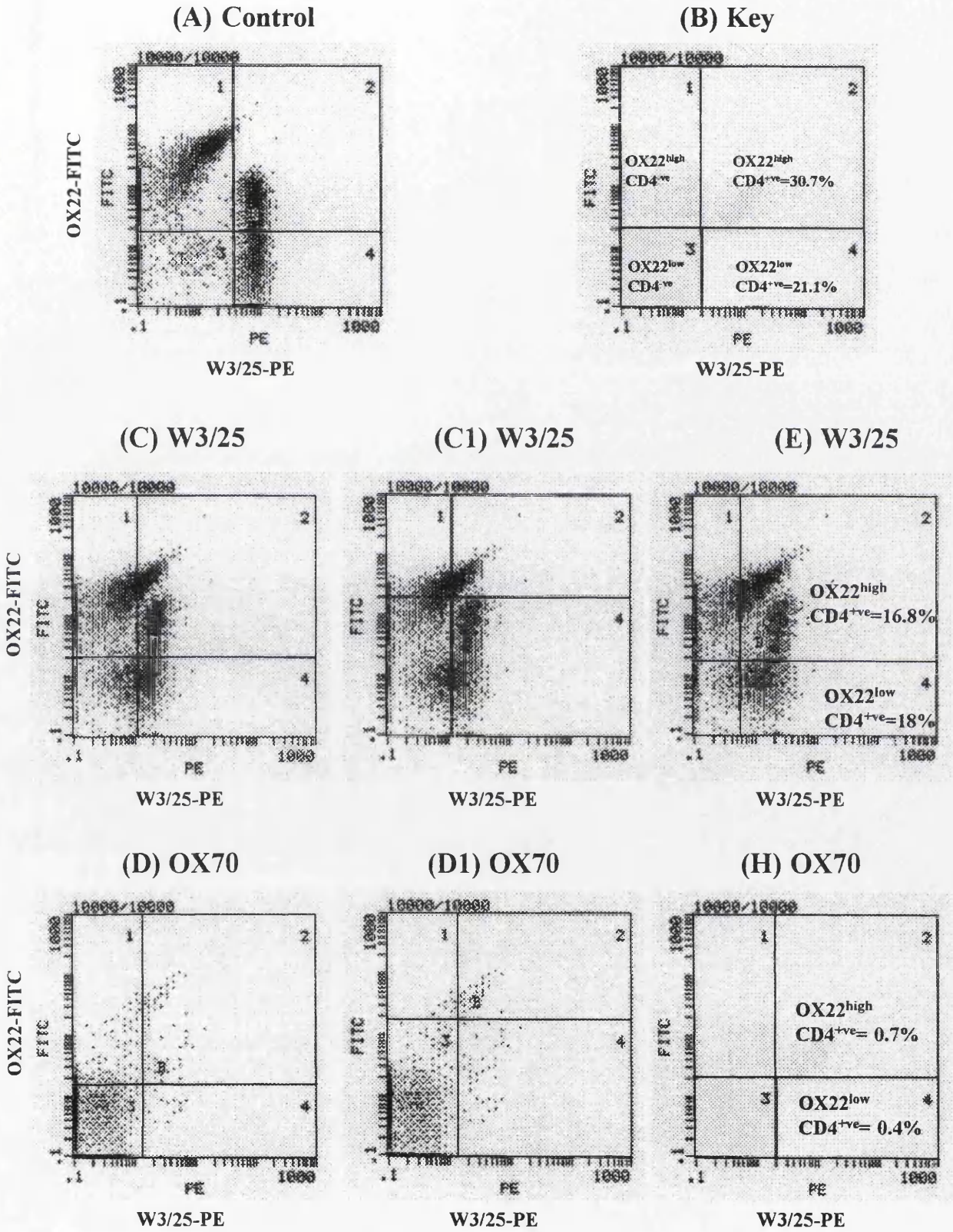
There was no significant difference between transplanted and non-transplanted except LNC/W3/25 treated animals (P< 0.05, Tukey's test).

6:6a Flowcytometric analysis of LNCs showing day 0 depletion of of OX22high and low CD4 T cells by anti-CD4 mAbs.



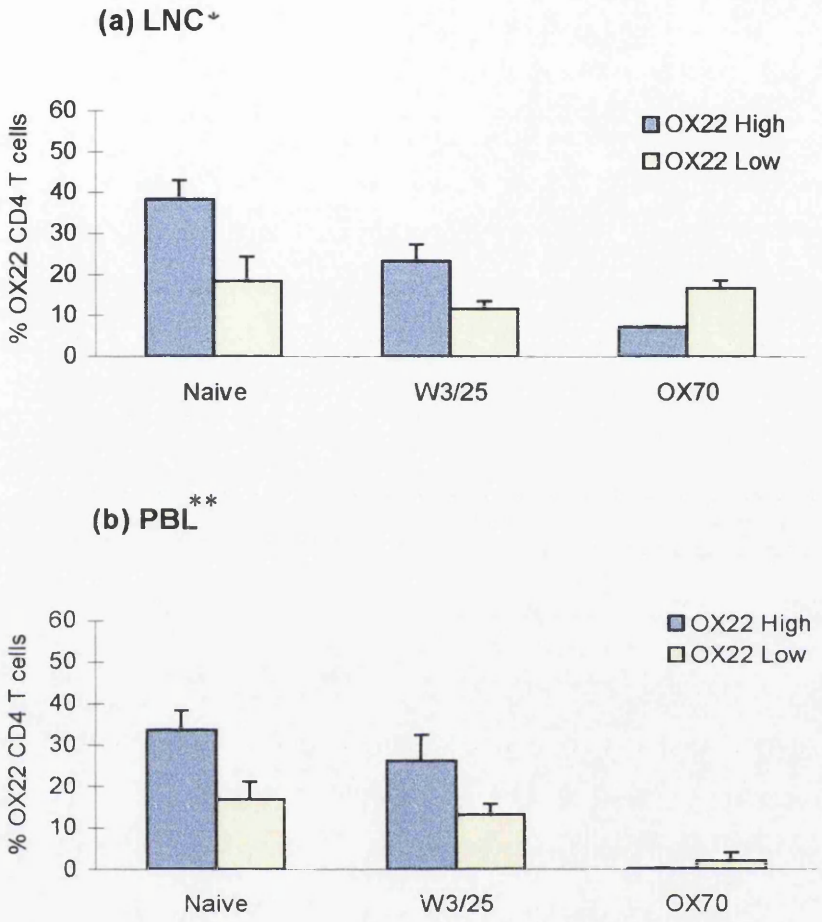
Two colour fluorescence analysis using FITC-conjugated anti-CD45R Y-axis (log_{10} FITC signal) and PE-conjugated anti-CD4 X-axis (log_{10} PE signals). Upper panel (A) represent LNCs from a control naive untreated animal. Middle panel (C) & (C1) Represent LNCs from a day 0 W3/25 treated animals. Lower panels (D) & (D1) represent LNCs from a day 0 OX70 treated animals.

6:6b Flowcytometric analysis of PBLs showing day 0 depletion of of OX22high and low CD4 T cells by anti-CD4 mAbs.



Two colour fluorescence analysis using FITC-conjugated anti-CD45R Y-axis (log_{10} FITC signal) and PE-conjugated anti-CD4 X-axis (log_{10} PE signals). Upper panel (A) represent LNCs from a control naive untreated animal. Middle panel (C) & (C1) represent LNCs from a day 0 W3/25 treated animals. Lower panels (D) & (D1) represent LNCs from a day 0 OX70 treated animals.

Figure 6:6c Flow cytometric analysis showing day 0 (i.e. day of last dose of anti-CD4) depletion of OX22 low and high CD4 T cells by the anti-CD4 mAbs.



Peripheral blood lymphocytes (PBLs) and lymph node cells (LNCs) were harvested from unmodified (naive) DA rats and from DA rats treated with different anti-CD4 mAbs given at a dose of 10 mg/kg on day -3 & 2 mg/kg on day -2, -1 and 0 (the day of analysis). Results shown are mean and standard deviation of 4 treated animals in each group.

* There was a significant overall difference between LNC $OX22^{high}$ groups (P= 0.007, ANOVA) but no significant difference between LNC $OX22^{low}$ groups (p= 0.09, ANOVA).

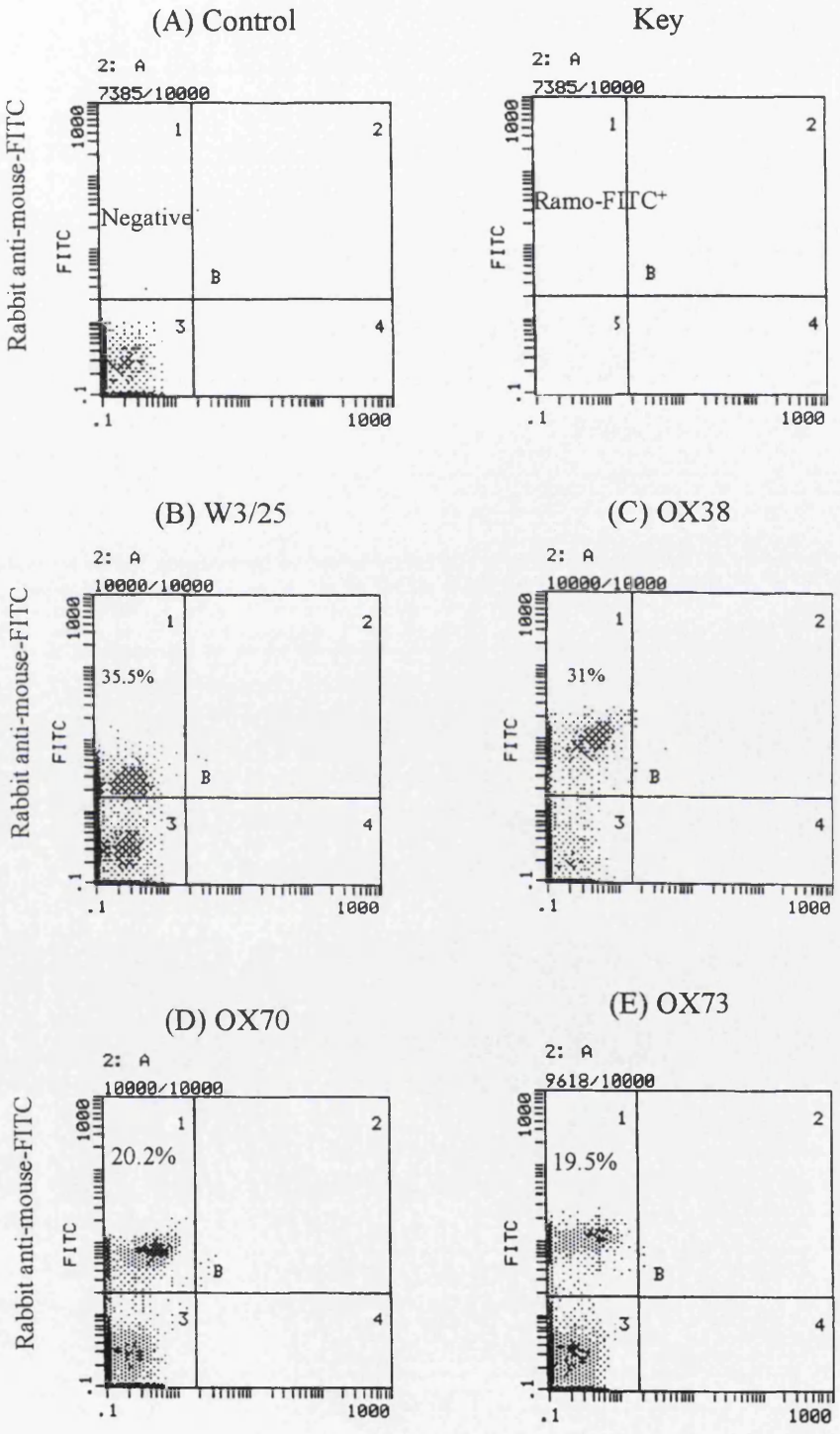
* Both W3/25 and OX70 LNC $OX22^{high}$ groups were significantly different from the control group (P< 0.05, Dunnett's test).

** There was a significant overall difference between both PBL $OX22^{high}$ & PBL $OX22^{low}$ groups (P<0.001 and P= 0.001 respectively, ANOVA).

** Only the OX70 treated animals were significantly different from control in both PBL $OX22^{high}$ & PBL $OX22^{low}$ CD4 T cells (P<0.05, Dunnett's).

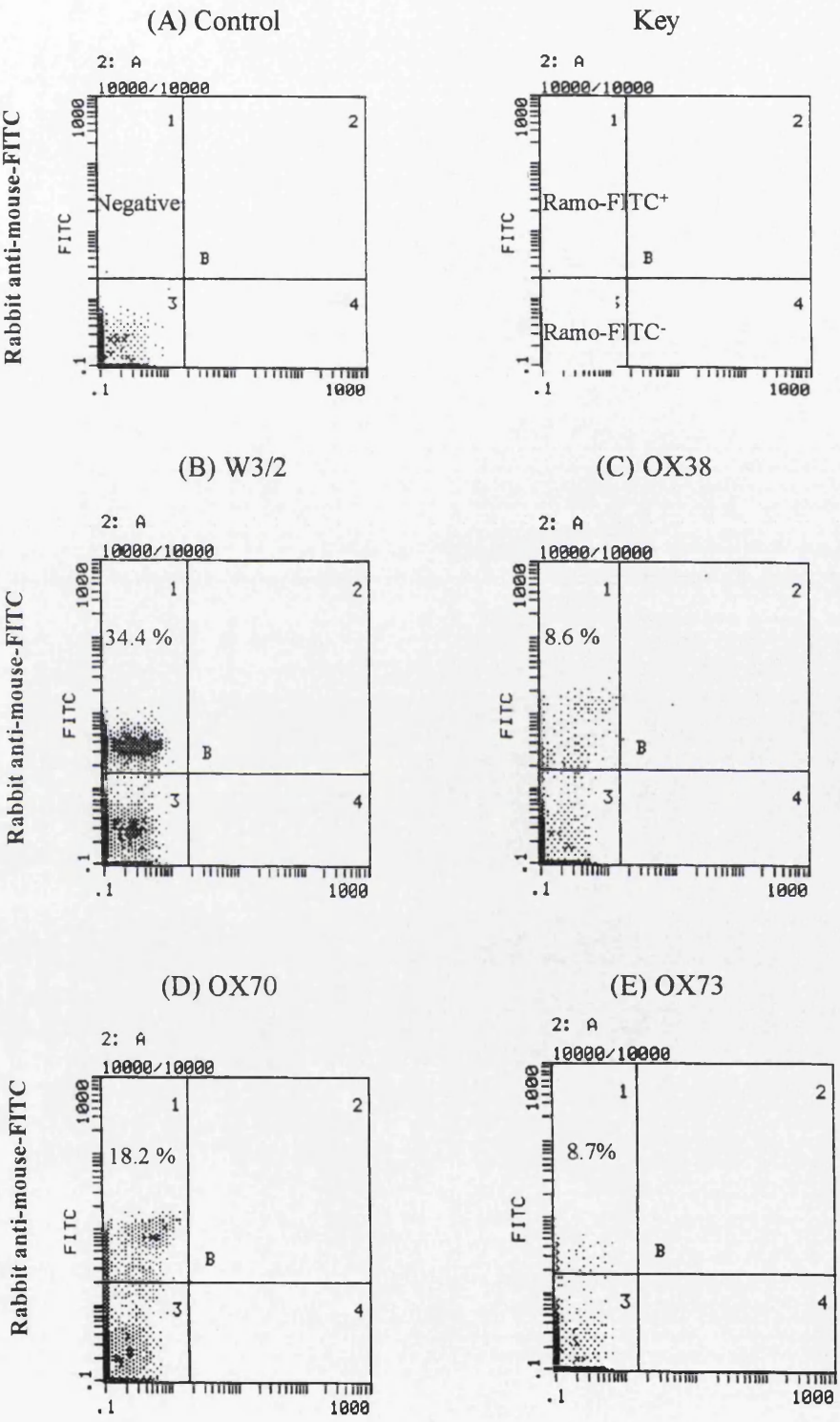
The depletion of $OX22^{high}$ CD4 T cells was significant for OX70 mAb than for W3/25 mAb treatment, in either LNC or PBL (P<0.05, Tukey's test).

Figure 6:7a Day 0 labelling of LNC CD4 molecules following treatment with different anti-CD4 mAbs.



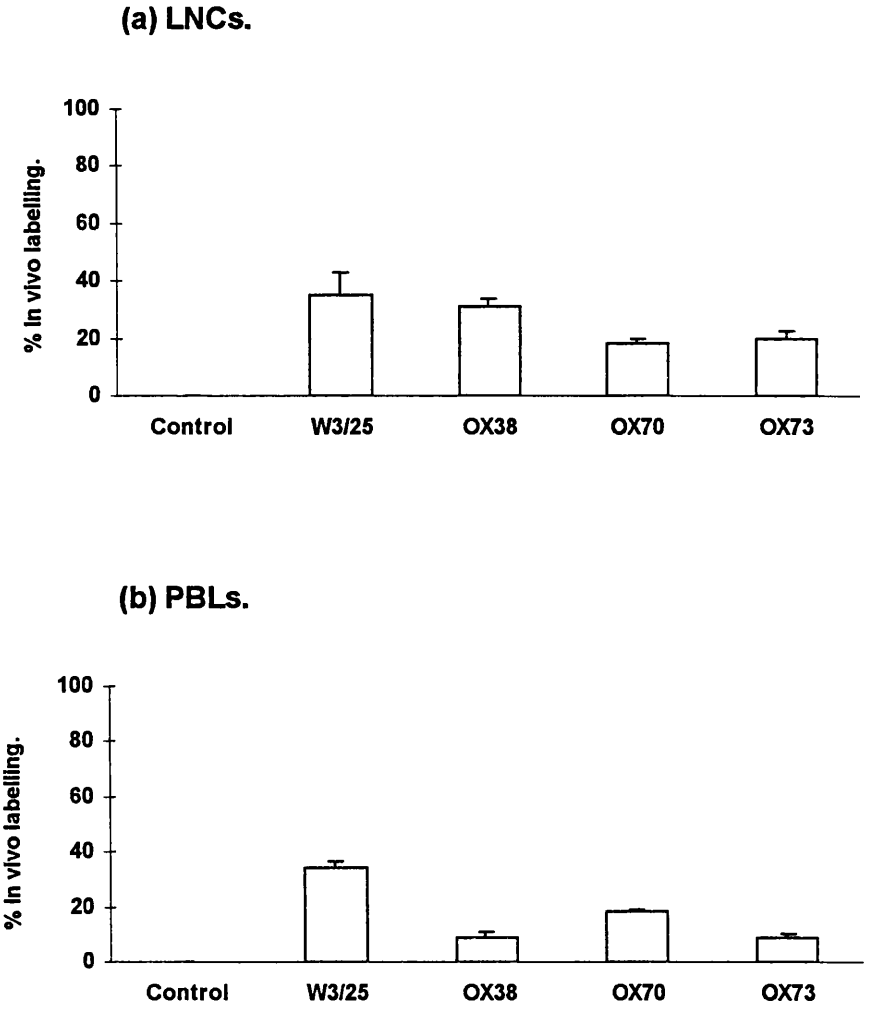
Single colour fluorescent analysis of LNCs using FITC conjugated rabbit anti-mouse (Y-axis). Rabbit anti-mouse-FITC was incubated with LNCs from (A) Control naive untreated animals (B) W3/25 (C) OX38 (D) OX70 and (E) OX73 treated animals.

Figure 6:7b Day 0 labelling of of PBL CD4 molecules following treatment with different anti-CD4 mAbs.



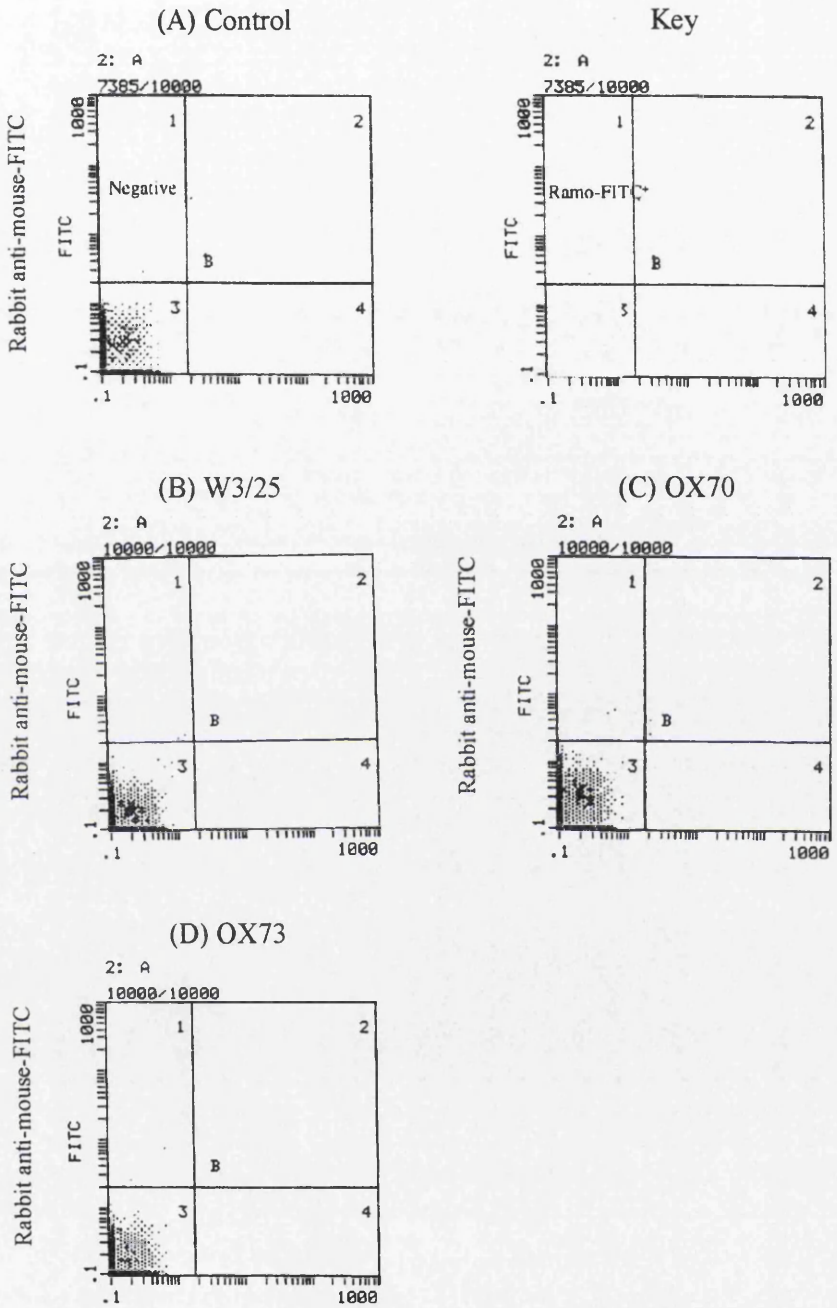
Single colour fluorescent analysis of PBL using FITC conjugated rabbit anti-mouse (Y-axis). Rabbit anti-mouse-FITC was incubated with PBLs from (A) Control naive untreated animals (B) W3/25 (C) OX38 (D) OX70 and (E) OX73 treated animals.

Figure 6:7c Day 0 *in-vivo* labelling of bound anti-CD4 mAbs (LNCs and PBLs obtained from treated animals after the last dose of anti-CD4 mAb)



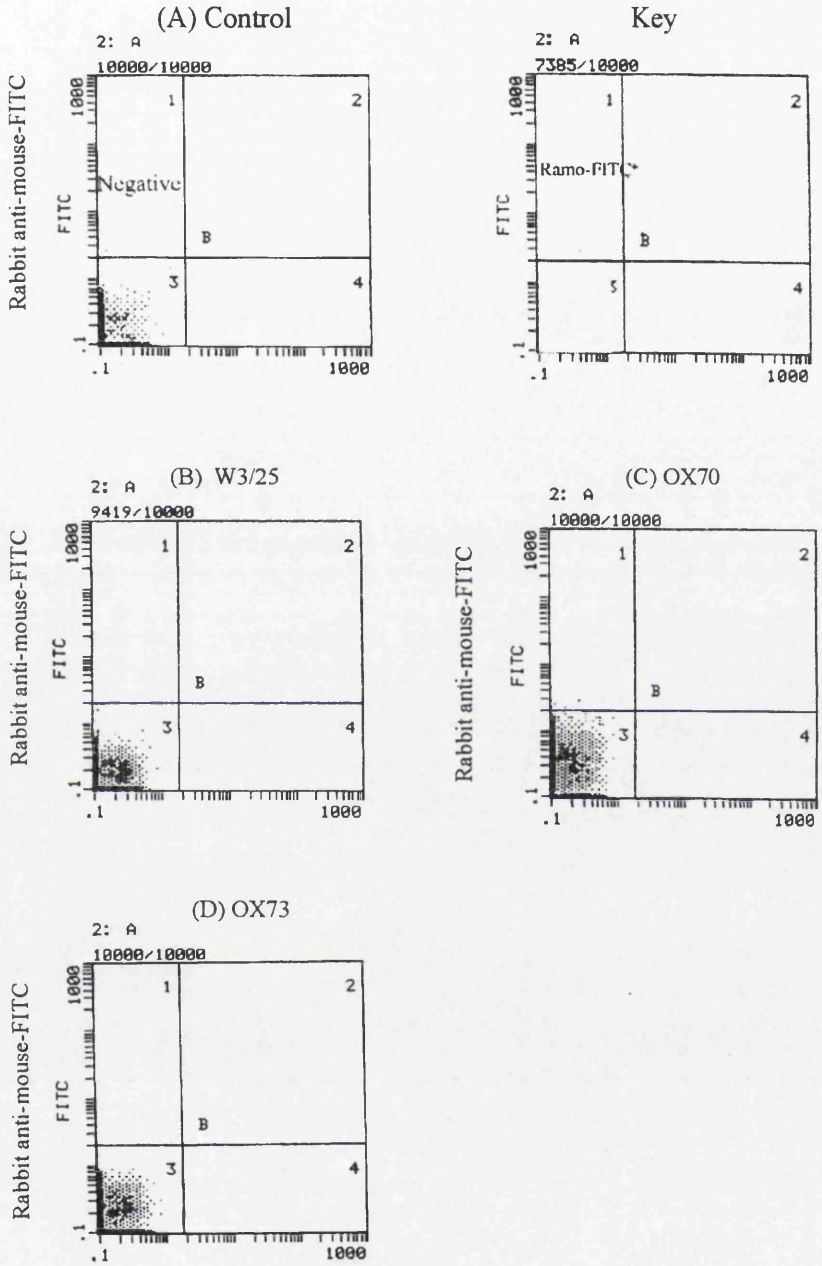
Results are expressed as the mean and standard deviation (from 3 treated animals in each group) of the fluorescence intensity of PBLs and LNCs following staining with rabbit anti-mouse-FITC of LNCs or PBLs from anti-CD4 treated animals (for residual in vivo anti-CD4 mAb labelling) or control which represent the untreated naive animals.

Figure 6:8a Day 4 labelling of LNC CD4 molecules following treatment with different anti-CD4 mabs.



Day 4 single colour fluorescent analysis of LNCs using FITC conjugated rabbit anti-mouse (Y-axis). Rabbit anti-mouse-FITC was incubated with LNCs from (A) Control naive untreated animals (B) W3/25 (C) OX70 and (D) OX73 treated and transplanted animals.

Figure 6:8b Day 4 labelling of PBL CD4 molecules following treatment with different anti-CD4 mabs.



Day 4 single colour fluorescent analysis of PBLs using FITC conjugated rabbit anti-mouse (Y-axis). Rabbit anti-mouse-FITC was incubated with PBLs from (A) Control naive untreated animals (B) W3/25 (C) OX70 and (D) OX73 treated and transplanted animals.

6:6 Discussion

The results from the experiments described in this chapter demonstrated that all of the anti-CD4 mAbs used had the ability to induce significant indefinite allograft survival (>100 days) in the fully allogeneic Lewis to DA strain combination with the notable exception of the anti-CD4 mAb W3/25. This is of interest since OX38 and W3/25 mAbs both label distal domains of the rat CD4 molecule and indeed they compete for the same epitope. However, whereas OX38 is a mouse IgG2a subclass antibody, W3/25 is a IgG1 subclass antibody. In vitro treatment with OX38 causes marked, but not permanent, CD4 T cell depletion (Jaques, unpublished data, Yin and Fathman et al, 1995) whereas in vivo treatment with W3/25 was found to produce very little T cell depletion in the present study, suggesting that CD4 T cells depletion may, at least in part, be an important factor in contributing to the efficacy of OX38 anti-CD4 mAb in inducing transplantation tolerance. There are no previous reports in the literature documenting the ability of mAb's known to be directed against membrane proximal domains of the rat CD4 to induce transplant tolerance. In the present study mAb's directed against the membrane proximal domains of CD4 (OX70, OX71 and OX73) all showed efficacy in promoting prolongation of cardiac allograft survival and this prolongation of graft survival was highly significant except OX71 mAb which its effect was just marginally significant. This is of interest since they label components of the CD4 molecule which do not interact directly with the class II MHC molecule. However, all three of these anti-CD4 mAbs also caused significant CD4 T cell depletion, supporting

the suggestion that, in this experimental model, depletion of CD4 T cells contributes to tolerance induction. However, it is important to note that because adult thymectomy prior to anti-CD4 treatment prevented effective induction of transplantation tolerance, CD4 T cell depletion can not be invoked the sole explanation to explain anti-CD4 induced tolerance. Cells newly emerging from the adult thymus gland appear to facilitate the emergence of the tolerant state. The reason for this phenomenon are not clear although it is reasonable to speculate that recently educated T cells may behave differently upon encountering graft alloantigen, perhaps, for example, showing preferential release of Th2 type cytokines (Onodera et al, 1997). If so, they may exert a regulatory effect on residual mature CD4 T cells which escape T cell depletion, and which in the absence of this regulatory effect are able to mediate allograft rejection. It was notable that anti-CD4 mAb therapy promoted preferential depletion $OX22^{high}$ rather than $OX22^{low}$ CD4 T cells. Again this might be broadly consistent with the idea that anti-CD4 mAb preferentially depletes Th1 CD4 T cells since $OX22^{high}$ T cells are reported to produce IL-2, IFNγ and TNFβ and $OX22^{low}$ T cells produce IL-4, IL-6 and IL-10. Recently reported in vitro studies by Mason's laboratory (Stumbles and Mason, 1995) have suggested that anti-CD4 mAb may preferentially promote Th2 T cells responses to alloantigens. Clearly, therefore, further examination of the role of Th1 and Th2 cytokine response is warranted.

6:6 Summary

6:1 Analysis of a panel of mouse mAbs (OX38, OX70 and OX73) directed against either membrane proximal or distal domains of the rat CD4 molecule revealed that all of these mAbs were able to induce significant prolongation of Lewis heart graft survival in DA rats.

6:2 In contrast, the anti-CD4 mAb W3/25 did not induce transplant tolerance even though it labels an epitope on the membrane distal domains overlapping with that labelled by OX38 mAb which, as shown in the preceding chapter, induced transplant tolerance.

6:2 The presence of thymus gland was essential for the induction of significant allograft survival by OX38 and OX70 mAbs in the Lewis to DA rat strain combination.

6:3 Flowcytometric analysis of anti-CD4 treated animals, showed that W3/25, in contrast to the other anti-CD4 mAbs studied, did not cause significant depletion of CD4 T cells from the peripheral blood of the treated animal.

6:4 On day 0 all of the used anti-CD4 mAbs are still bound to CD4 T cells but non was detectable on the surface of CD4 T cells by day 4.

6:4 More detailed flow cytometric analysis revealed that OX70 (but not W3/25) significantly depleted the $OX22^{high}$ subset of CD4 T cells in PBLs and LNCs. In addition, the depletion of $OX22^{high}$ CD4 T cells was significantly higher by OX70 mAb than by W3/25 mAb treatment in either LNCs or PBLs. This finding raises the possibility that anti-CD4 mAb may promote the dominance of CD4 T cells which preferentially produce Th2 cytokine patern.

CHAPTER SEVEN

Mechanisms underlying induction of transplant tolerance by anti-CD4.

7:1 Introduction

The immunological mechanisms responsible for inducing and maintaining transplant tolerance are undoubtedly very complex and are likely to depend on a variety of factors such as the strategy used for inducing tolerance, the type of tissue transplanted and the time following transplantation. In this chapter I chose to address further some of the immunological mechanisms which may operate to establish tolerance to rat cardiac allograft during the early or induction period after anti-CD4 mAb treatment. A number of studies have suggested that defective IL-2 production by T cells may be an important factor in some experimental models of transplant tolerance (Malkovsky et al, 1984; Nossal, 1989 and Dallman et al, 1991). Moreover, several authors have argued strongly that induction of transplant tolerance correlates with, and may be attributable to, a switch from a Th1 cytokine response (IL-2 and IFNγ) to a Th2 cytokine response (IL-4, 5 and 10) (Bugeon et al, 1992; Burdick and Clow, 1990; Papp et al, 1992 and Kupiec-Weglinski et al, 1993).

These issues were addressed here by determining first of all whether the administration of exogenous recombinant IL-2 to anti-CD4 treated rats bearing cardiac allografts was able to abrogate anti-CD4 induced tolerance during the early post-transplant period and, secondly, whether the administration of a neutralising mAb to rat IL-4 was able to prevent induction of transplant tolerance. Finally, in this chapter, I examined whether the presence of CD8 cells was important during the induction of transplantation tolerance by anti-CD4 mAb in the rat, since Fathman and colleagues recently suggested that CD8 T

cells may play a role in induction of anti-CD4 induced transplantation tolerance (Seydel et al, 1991; Alters et al, 1993 & Yin and Fathman, 1995).

7:2 IL-2 treatment of OX38 and OX73 treated DA recipient of Lewis heart graft.

In this experiment, naive DA ($RT1^a$) recipients were transplanted with Lewis heart grafts and animals were then treated with either OX38 or OX73 anti-CD4 mAbs. The dose of mAbs used was that which had been shown previously to induce transplantation tolerance in this strain combination i.e. 10 mg/Kg on day -3 and 2 mg/Kg on days -2, -1 and day 0 (day of transplantation). During the induction of tolerance, the recipient rats received either 20.000 Units or 400 Units of human rIL-2 daily by the I.P route for five consecutive days. The rIL-2 treatment was started on day 0 (the day of transplantation). Control animals received I.P injections of an equivalent volume of PBS instead of IL-2.

The results of this experiment are shown in table 7:1. It can be seen that administration of rIL-2, irrespective of the dose given, did not abrogate prolongation of cardiac allograft survival by anti-CD4 mAb, regardless of whether OX38 or OX73 anti-CD4 mAbs was used. An additional observation was that none of the rats treated with either dose of rIL-2 showed any signs of toxicity attributable to the cytokine administration.

In the animals receiving OX38 mAb and treated with 400 U/day of rIL-2, one of the five animals studied rejected its heart allograft 15 days after transplantation whereas the other four animals retained their heart grafts indefinitely (MST > 100 days, P= 1.00 using Fisher's exact test in comparison with PBS treated

Table 7:1 Effect of giving exogenous IL-2 on the ability of anti-CD4 mAb's (OX38 or OX73) to prolong cardiac allograft survival.

Group	Anti-CD4[a] mAb	rIL-2[b] (U/day)	Number of animals	Graft Survival (days)	MST (days)
1	OX38	$4x10^2$	5	15, > 100 days (x4)*	> 100 days
2	OX38	$2x10^4$	5	9, > 100 days (x4)*	> 100 days
3	OX38	None	8	11, 18, > 100 days (x6)	> 100 days
4	OX73	$2x10^4$	4	All > 100 days (x4)*	> 100 days

(a) DA rats were treated with anti-CD4 mAb (OX38 or OX73) prior to receiving a Lewis cardiac allografts.

(b) Animals were treated with rIL-2 (or PBS as control) daily for 5 days starting on the day of heart transplantation.

* P value was non-significant (P=1.00, using Fisher's exact test in comparison with PBS treated control group).

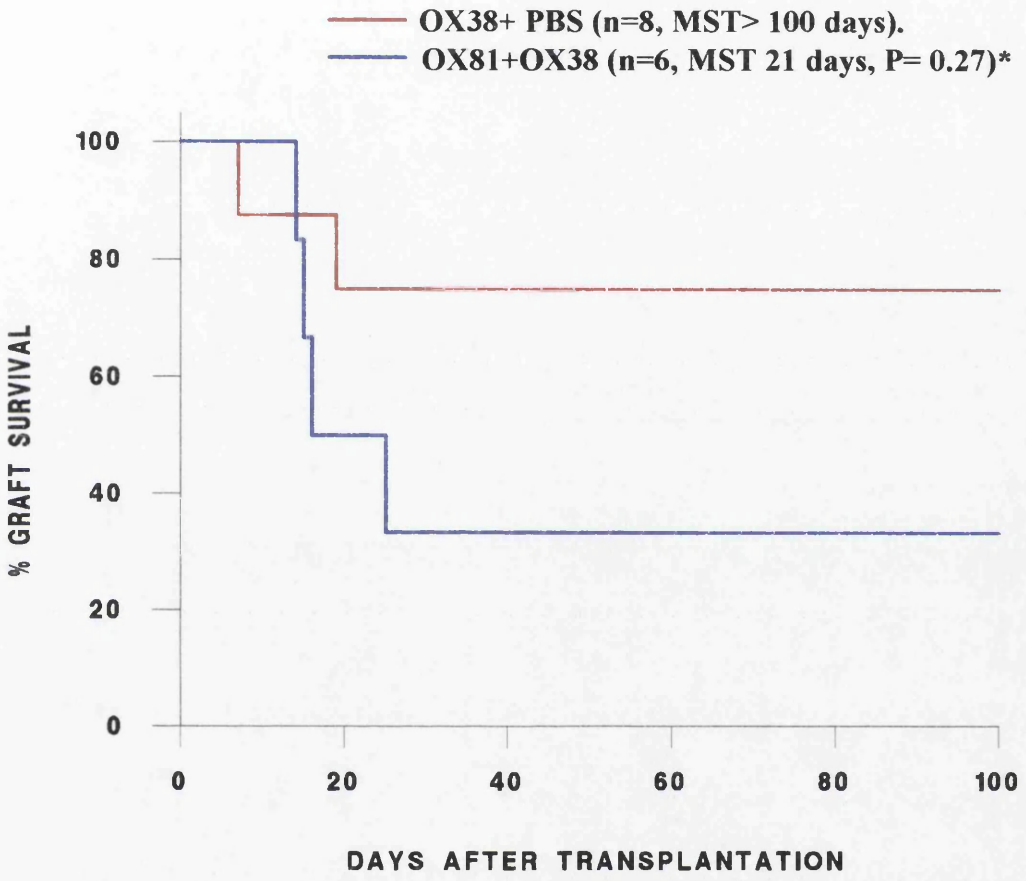
control group). The same result was obtained with the group of DA recipients given 20.000 U/day of rIL-2; one recipient rejected its heart graft on day 9 and 4 retained their heart for more than 100 days. Administration of 20.000 U/day of rIL-2 in OX73 treated DA recipients also failed to abrogate tolerance induction and all four experimental animals retained their heart grafts for more than 100 days (MST> 100 days, P=1.00, using Fisher's exact test). Therefore, the administration of rIL2 during the induction of tolerance by either OX38 or OX73 mAbs failed to induce rejection in this rat model.

7:3 Anti-IL-4 (OX81) mAb treatment of OX38 treated DA recipient of Lewis heart graft.

In this experiment, DA ($RT1^a$) rats were treated with anti-CD4 (OX38) mAb at a dose of 10 mg/Kg on day -3 and 2 mg/Kg on days -2, -1 and day 0 (the day of transplantation). In addition, however, they were also given an anti-IL-4 mAb (MRC OX81) to neutralise endogenous IL-4. OX81 is of the IgG1 isotype which has been shown in previous studies to neutralise the activity of rat IL-4 (Stumbles and Mason, 1995). It was given at a dose of 5 mg/Kg daily for four days (day 0, the day of transplantation and days 1, 3 and day 5 post transplantation).

The results of this study are shown in figure 7:1. It can be seen that four of the six DA recipients of Lewis heart grafts that were treated with OX81, during the induction of tolerance with OX38 mAb, rejected their heart graft (MST 21 days, P= 0.27 using Fisher's exact test in comparison with control group) and only 2 animals accepted their heart graft indefinitely. Control animals treated with OX38 received an equivalent volume of PBS instead of OX81 mAb and six of

Figure 7:1 The ability of anti-IL4 (MRC OX81)mAb to abrogate early induced tolerance by OX38 mAb in Lewis to DA strain combination.



DA recipients were treated with both OX81 (anti-IL4) mAb at a dose of 5 mg/kg on day 0, 1, 3 and 5 and OX38 mAb at a dose of 10 mg/kg on day -3 and 2 mg/kg on day -2, -1 and day 0 (the day of transplantation).

*** P = 0.27 (using Fisher's exact test in comparison with control group).**

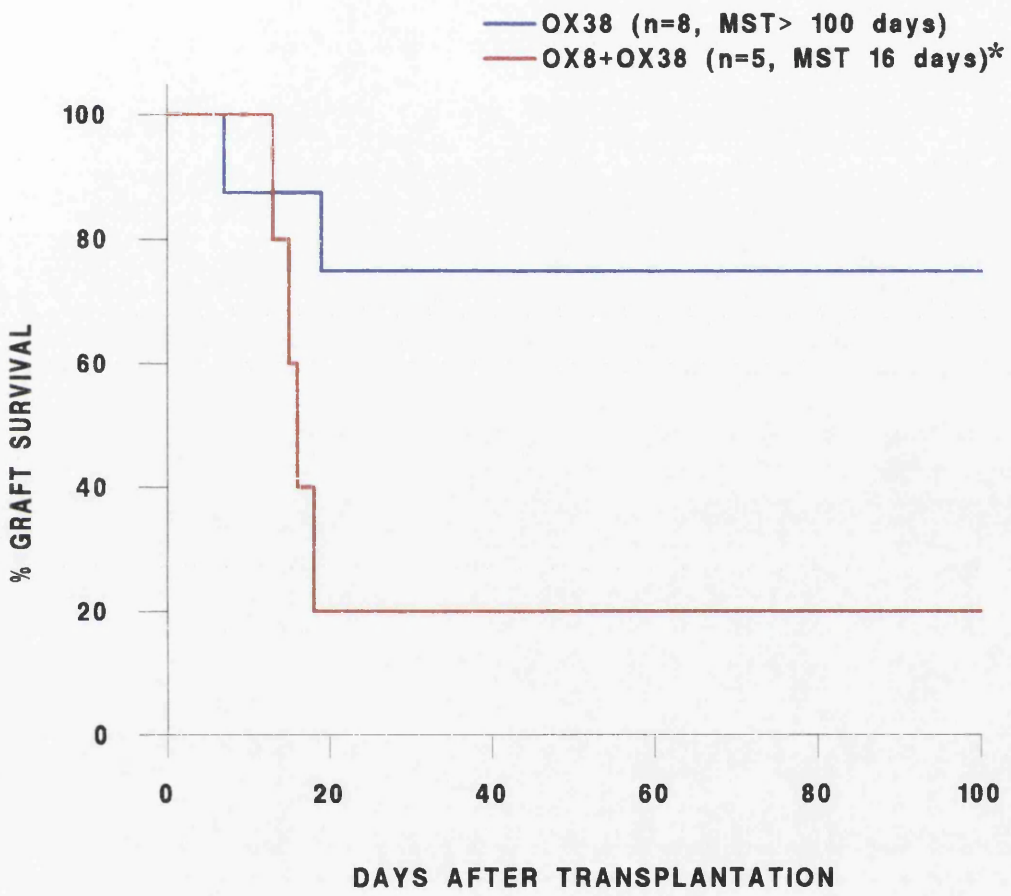
these animals retained their heart grafts indefinitely (MST> 100 days). This latter finding is in keeping with this thesis previous experiments, in demonstrating the efficacy of OX38 in inducing tolerance to Lewis heart graft in DA recipients.

7:4 Anti-CD8 mAb treatment of DA recipients of Lewis heart graft during the induction of tolerance by OX38 mAb.

To determine whether CD8 cells are critical during the induction of tolerance by anti-CD4 mAb in the rat, five DA ($RT1^a$) recipients were treated with a depletive regimen of MRC OX8 (anti-CD8) mAb at a dose of 5 mg/Kg on the day preceding transplantation (-1), the day of transplantation (0) and day 3 after transplantation. MRC OX8 has been shown in several studies, including studies from this laboratory, to be highly effective at depleting CD8 cells from blood and lymphoid organs of treated rats (Sedgwick et al, 1988; Gracie et al, 1990 and Bell et al, 1989). Anti-CD4 (MRC OX38) mAb treatment was given as before i.e. 10 mg/Kg on day -3 and 2 mg/Kg on days -2, -1 and day 0. Control animals received PBS during the induction of tolerance by OX38 mAb.

The results are shown in figure 7:2. Five of anti-CD4 treated DA ($RT1^a$) recipient rats, which were given anti-CD8 (OX8) mAb, four rejected their heart grafts and only one experimental animal retained its graft indefinitely (MST 16 days, P=0.102 using Fisher's exact test). Although the statistical analysis was not significant due to the small number of animals used in this experiment, the result still suggests that CD8 cells play a role in tolerance induction following OX38 mAb treatment.

Figure 7:2 The ability of Anti-CD8 (OX8) mAb to abrogate early induced tolerance by OX38 mAb in Lewis to DA strain combination



DA recipients were treated with both OX38 mAb at a dose of 10 mg/kg on day -3 and 2 mg/kg on day -2, -1 and day 0 (the day of transplantation) and OX8 mAb at a dose of 5 mg/kg on day -1, 0 (the day of transplantation) and day 3 after transplantation.

*** P = 0.102 using Fisher's exact test in comparison with control animals.**

7:5 Discussion

In several experimental models of transplantation tolerance induction, there is evidence of a defect in the IL-2 pathway (Dallman et al, 1991, Malkovsky et al, 1984; Nossal, 1989). The in vivo effect of exogenous rIL-2 treatment on tolerance induction in different mice and rat models of transplant tolerance described by other research groups is summarised in table 7:2. Administration of exogenous IL-2 (10^4 units/day for 4 days) has been shown to prevent prolongation of graft survival in blood transfused rats (Dallman et al, 1989) and of mice given anti-CD4 plus anti-CD8 mAbs and treated with rIL-2 (1600 Units/day for 6 days) (Chen et al, 1996).

It was therefore of particular interest to determine whether administration of exogenous IL-2 was able to prevent prolongation of heart allograft survival by OX38 mAb in the rat. Such studies are potentially problematical since the half-life of administered cytokine is usually very short. Nevertheless, the results obtained were unequivocal in that giving rIL-2 daily for 5 days after heart transplantation did not abrogate allograft survival. The amount of rIL-2 given was comparable to that used by other groups and probably close to the toxic dose in the rat. The findings with exogenous IL-2 suggest that deficiency in endogenous IL-2 is not the critical event in the induction of transplant tolerance following OX38 mAb treatment and in this respect, therefore, the model appears to differ from tolerance induced by donor-specific blood transfusion (Dallman et al, 1989).

Table 7:2 Reported effects of exogenous IL-2 on induction of transplant tolerance by different groups.

Immunosuppressive regimen	**Animal model**	**Dose of IL-2 U/day**	**Effect of IL-2**	**Reference**
1. Donor blood transfusion	Rat	10^4 (for 4 days)	Graft rejection	Dallman et al, 1989.
2. Adoptive transfer of splenocytes $5x10^7$ from tolerant mice	Mouse	1600 (for 6 days)	No effect on graft survival	Chen et al, 1996.
3. Anti-CD4 + anti-CD8 mAbs	Mouse	1600 (for 6 days)	Graft rejection	Chen et al, 1996.
4. Anti-CD4 mAb	Mouse	$10x10^3$ Units on the day of Txn & then $30x10^3$ U/day (for 5 days)	No effect on graft survival.	Pearson et al, 1993b.
5. Anti-CD28 (CTLA4Ig)	Rat	1000 (for 5 days).	Partial reversal.	Sayegh et al, 1995.

The studies performed in this chapter using the mAb OX81 to neutralise endogenous IL-4 in vivo are interesting but not conclusive. Overall, neutralising IL-4 by OX81 appeared to diminish the efficacy of tolerance induction by OX38 mAb but statistically was not significant. However, the OX81 hybridoma was found to be particularly poor at yielding significant amounts of immunoglobulin and this in turn limited the amount of material available for the study. Hence the amount of OX81 antibody which could be given per animal and the number of animals treated was suboptimal. In future studies it would be interesting to administer larger amounts of OX81 mAb, perhaps for a longer period to ensure complete blockade of IL-4. Another important point to consider in interpreting the present results is that IL-4 is also produced by other cell types in addition to Th2 T cells (Mosmann et al, 1986; Mosmann and Coffman, 1989). Eosinophiles and mast cells (Brown et al, 1988b; Rennick et al, 1987 and Zlotnik et al, 1987), for example may produce relatively large amounts of IL-4. It can not be assumed, therefore, that the experimental results obtained here necessarily suggest a role of Th2 T cells in transplant tolerance induction and further study of the this point is clearly worthwhile.

The observation that treatment with OX8 mAb abrogated tolerance induction after anti-CD4 mAb treatment is intriguing. This finding is consistent with that reported by other groups who carried out similar work in mouse (Seydel et al, 1991 and Alters et al, 1993) and rat models of transplantation tolerance (Yin and Fathman, 1995). Taken together, the results from these studies, indicate that the CD8 cell plays an important role in rendering or inducing potential

allograft specific unresponsiveness in DA recipients of Lewis heart graft at the early stages of tolerance induction by anti-CD4 mAb. It should be emphasised that in the rat the CD8 molecule is expressed not only on CD8 T cells but also by some NK cells. The effect of anti-CD8 mAb can not necessarily be attributed exclusively to CD8 T cells. Nevertheless, the above results raise the question as to how the presence of CD8 T cells promotes anti-CD4 mAb induced transplant tolerance. One possibility is that CD8 T cells may be acting directly as suppressor or regulatory T-cells during the early stages of the in vivo alloimmune responses. Second, the possibility arises that during the induction of tolerance with anti-CD4 mAb alloreactive CD8 cells may compete with the alloreactive CD4 T cells for the essential co-stimulatory molecules on allogeneic APC and this competition may lead to the failure of the second signal being delivered to residual CD4 T cells and therefore to the induction of specific CD4 T cell unresponsiveness. According to this hypothesis, on depleting the CD8 T cell population, competition for the co-stimulatory molecules on APC is reduced and this may, lead to the activation of residual CD4 T cells and hence rejection of the allograft. A third, possibility is that OX8 mAb may penetrate the blood-thymus barrier and eliminate double positive CD8CD4 T-cells which may if not eliminated, provide an immuno-regulatory signal for positive CD4 T cell subpopulation. Clearly, it will be important in future studies to address these possibilities.

7:6 Summary

7:1 Induction of tolerance to a Lewis heart allograft in DA rats following anti-CD4 mAb is not attributable to a lack of IL-2 alone, since provision of exogenous rIL-2 failed to prevent the development of tolerance.

7:2 Endogenous IL-4 may facilitate induction of tolerance by anti-CD4 mAb since administration of a neutralising antibody to IL-4 partially abrogated tolerance although the results were not conclusive.

7: Although OX8 mAb treatment appeared statistically not significant due to the small number of animals, CD8 cells might still play a critical role during the induction phase of anti-CD4 mAb induced tolerance.

CHAPTER EIGHT

Mechanisms underlying maintenance of transplant tolerance by anti-CD4 mAb.

8:1 Introduction

In this chapter some aspects of the immunological mechanisms responsible for maintaining transplant tolerance in anti-CD4 treated recipients with long-standing heart grafts were examined. As already discussed, the mechanisms which operate to maintain transplant tolerance are likely to be both complex and different from those responsible for initiation of the tolerant state. There is an increasing body of experimental evidence which suggests that regulatory or T suppressor cells may play a critical role in maintaining transplant tolerance (Matriano et al, 1982; Scully et al, 1994; Lombardi et al, 1994; Bushel et al, 1995, Chen et al, 1996, Onodera et al, 1996 & 1997). Since, in the preceding chapter, evidence was obtained suggesting that CD8 cells may be important for the **induction** of anti-CD4 tolerance to a heart allograft after anti-CD4, the question arose as to whether CD8 cells may also participate in the maintenance of the tolerant state. Several recent studies in mice made tolerant to allografts by a combination of anti-CD4 and anti-CD8 mAbs have demonstrated the presence of regulatory T lymphocytes which, on adoptive transfer to a second syngeneic recipient, are able to prevent allograft rejection (Chen et al, 1996; Chen and Waldmann, 1993). Such studies point to a role for a regulatory lymphocyte population in maintaining transplant tolerance. One of the aims of the work described in this chapter was, therefore, to determine whether evidence existed for such regulatory lymphocytes in anti-CD4 treated rats bearing a long-standing heart allograft.

Finally, in view of the large amount of recent interest in the concept that transplant tolerance may be associated with, and indeed partially attributable to, a deviation in the T helper cytokine repertoire from a Th1 to Th2 type cytokine profile (Mossman et al, 1989; Mottram et al, 1995 and Strom et al, 1996), the possibility that this phenomenon may offer an explanation for maintaining transplant tolerance after OX38 mAb therapy was examined. Analysis of the T cell response in long-term tolerant rats also enabled examination of the possibility that transplant tolerance in the present studies was attributable to clonal deletion of potentially alloreactive T lymphocytes. This last possibility was, however, not considered likely. Although clonal deletion is well known to be the principle mechanism governing the export of potentially autoreactive lymphocytes from the thymus gland, this mechanism is not generally thought to be instrumental in anti-CD4 induced transplant tolerance (Shizuru et al, 1987).

8:2 The role of CD8 cells in maintaining tolerance.

To determine whether CD8 cells were important in the maintenance of transplant tolerance induced by anti-CD4 (MRC OX38) mAb, four OX38 treated DA rats bearing long-term (MST>100 days) Lewis abdominal cardiac allografts were treated with a short course of the depleting anti-CD8 mAb (OX8) and then immediately engrafted with a second Lewis heart graft to the cervical site.

In this study, OX8 mAb was given at a dose of 5 mg/Kg for a total of three days (on the day preceding transplantation [day -1], the day of transplantation [day 0] and day 3 after transplantation). As mentioned earlier (chapter 7), this

regimen of OX8 mAb treatment has been shown previously to be highly effective at depleting rat CD8 cells in vivo. Retransplantation of tolerant animals with a second Lewis heart graft was performed in this experiment, so as to exclude the possible contribution of reduced immunogenicity of the original Lewis heart graft to the experimental findings.

The results of this study are shown in table 8:1. All four long-term tolerant recipients treated with OX8 mAb during the engraftment of a donor-specific cervical allograft retained their second heart grafts indefinitely (> 100 days). The original abdominal heart allograft was also noted to continue beating throughout this period of time. Five long-term tolerant control OX38 treated recipients receiving a donor specific Lewis heart graft to the cervical site were given PBS instead of OX8 mAb. These animals, as expected, retained their cervical heart graft indefinitely (MST> 100 days) and their original abdominal heart graft continued to beat for the duration of the study. The results of this experiment indicate that CD8 cells do not, therefore, appear to play an essential role in the maintenance of transplant tolerance following anti-CD4 mAb treatment in the rat as non of the grafted animals rejected its heart graft.

8:3 Ability of spleen cells from tolerant animals to mediate suppression.

The adoptive transfer technique is a potent method for analysing lymphocyte cell function in the field of experimental immunology and has been used with great effect in many studies to assess the ability of lymphocytes and lymphocyte subpopulations to effect or regulate allograft rejection. In the following

Table 8:1 CD8 cells are not essential for maintenance of transplant tolerance in anti-CD4 treated DA rats bearing a long-standing Lewis heart allograft.

Group	Recipient[a]	mAb[b]	Number of animals	Graft survival[c] (Days)	MST (Days)
1	DA	OX8	4	all> 100 days	>100[d]
2	DA	PBS	5	all >100 days	>100

(a) DA recipients had long-standing (> 100 days) heterotopic Lewis heart grafts (abdominal site) following perioperative treatment with anti-CD4 mAb (OX38). All recipients received a second Lewis heart allograft to the cervical site.

(b) OX8 mAb was given at a dose of 5 mg/kg on day -1, 0 (the day of cervical heart transplantation) and day 3 after the second heart transplantation. Control animals were treated with an equivalent volume of PBS.

(c) Survival of second (cervical) heart allograft. All of the primary abdominal heart allografts continued to beat for the period of the study.

(d) Group 1 versus group 2 (P = 1.0, using Fisher's exact test).

experiment, spleen cells harvested from OX38 treated DA rats bearing long-standing Lewis heart allografts were tested for their ability to suppress allograft rejection when adoptively transferred to a second naive syngeneic recipient which was then challenged with a Lewis heart allograft. The secondary recipients in this study received spleen cells from tolerant rats the day before challenge with a heart allograft and were not given any supplementary immunosuppression following transplantation. The results of this study are shown in table 8:2. It can be seen that adoptive transfer of $2x10^8$ spleen cells from tolerant rats led, in 2 of the 4 secondary recipients tested, to the permanent acceptance (>100 days) of a Lewis heart allograft although the overall increase in graft survival failed to reach statistical significance, most likely because of the small numbers in experimental groups. The suppression observed in this experimental system was donor (i.e. Lewis) specific since when $2x10^8$ spleen cells from tolerant rats were transferred into naive DA recipients which were then given a third party PVG ($RT1^c$) heart allograft the following day, no increase in allograft survival was seen. The number of adoptively transferred spleen cells necessary to suppress allograft rejection in this study was more than $1x10^8$, since the transfer of $1x10^8$ tolerant spleen cells failed to extend the survival of a donor-specific (Lewis) heart allograft in a secondary unmodified DA recipient (table 8:2). These experiments suggest that cells within the spleen of long-term recipients bearing a cardiac allograft may exert a suppressive or regulatory effect which may be important in maintaining the tolerant state within such animals although caution is required in the interpretation of these results

Table 8:2 Spleen cells from tolerant animals may suppress rejection of a donor strain but not third party heart allograft.

Number of spleen cells[a]	Recipient strain[b]	Strain of heart graft	Number of animals	Graft survival (days)	P[c] value	MST (days)
$1x10^8$	DA	Lewis	3	9, 9, 10	0.76	9
$2x10^8$	DA	Lewis	4	10, 15, >100 (x2)	0.11	58
$2x10^8$	DA	PVG	5	7, 8,11,11,12		11

(a) Spleen cells from DA animals bearing long-standing Lewis heart grafts were adoptively transferred into secondary unmodified DA animals. Spleen cells from tolerant animals were injected IV at the dose stated.

(b) Recipients animals were given either a DA (donor-specific) or PVG (third party) heart allograft the following day.

(c) P value using mann-whitney test in comparison with the group receiving a third party (PVG) heart graft.

because of the small number of experimental animals involved.

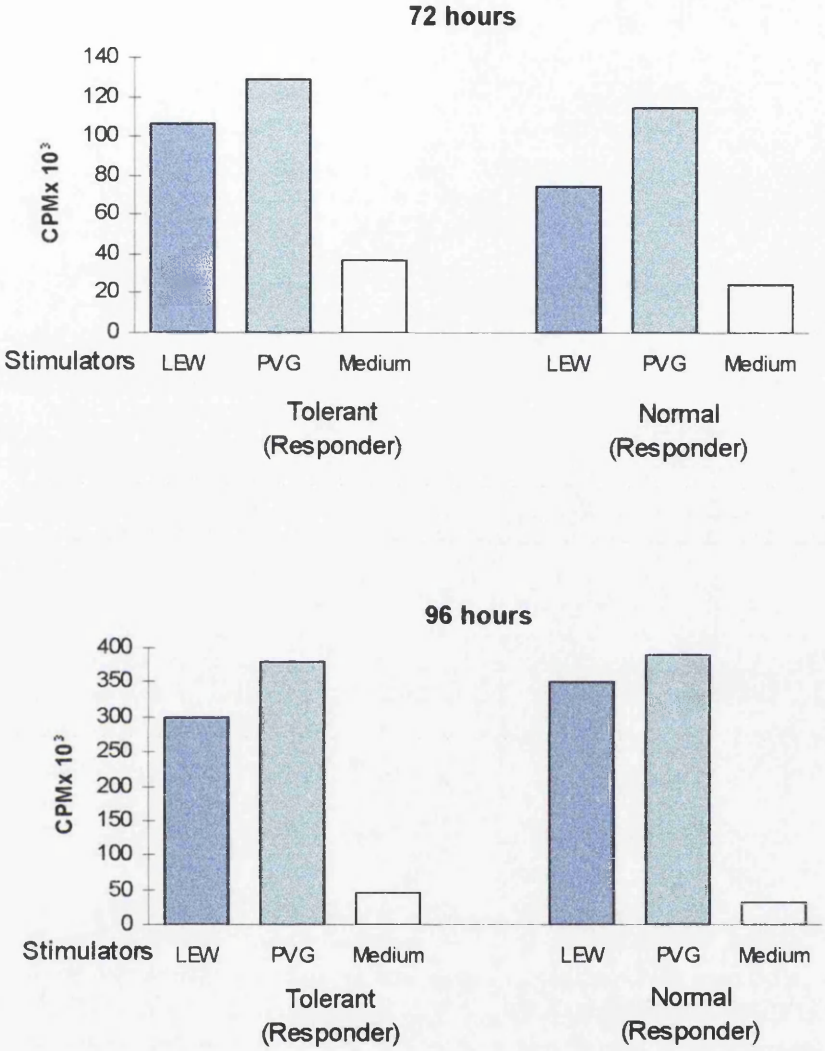
8:4 In vitro studies with purified CD4 T cells obtained from long-term tolerant OX38 and OX70 mAbs treated DA rats.

In the following studies, CD4 T cells obtained from anti-CD4 mAb treated DA bearing long-standing Lewis heart graft (> 100 days) were studied in vitro to determine their ability to proliferate and produce cytokines when re-stimulated in vitro with allogeneic APC. CD4 T cells were purified from the LNC of tolerant heart allograft recipients (and from normal control) animals by immunomagnetic beads (yielding a cell population > 95% of CD4 T cells) and stimulated in vitro with irradiated donor (Lewis) or third party (PVG) spleen cells. The following proliferation and cytokine data were not statistically analysed as the experiments were performed on one occasion.

8:4:1 Proliferative response of CD4 T cells from OX38 or OX70 treated tolerant DA rats.

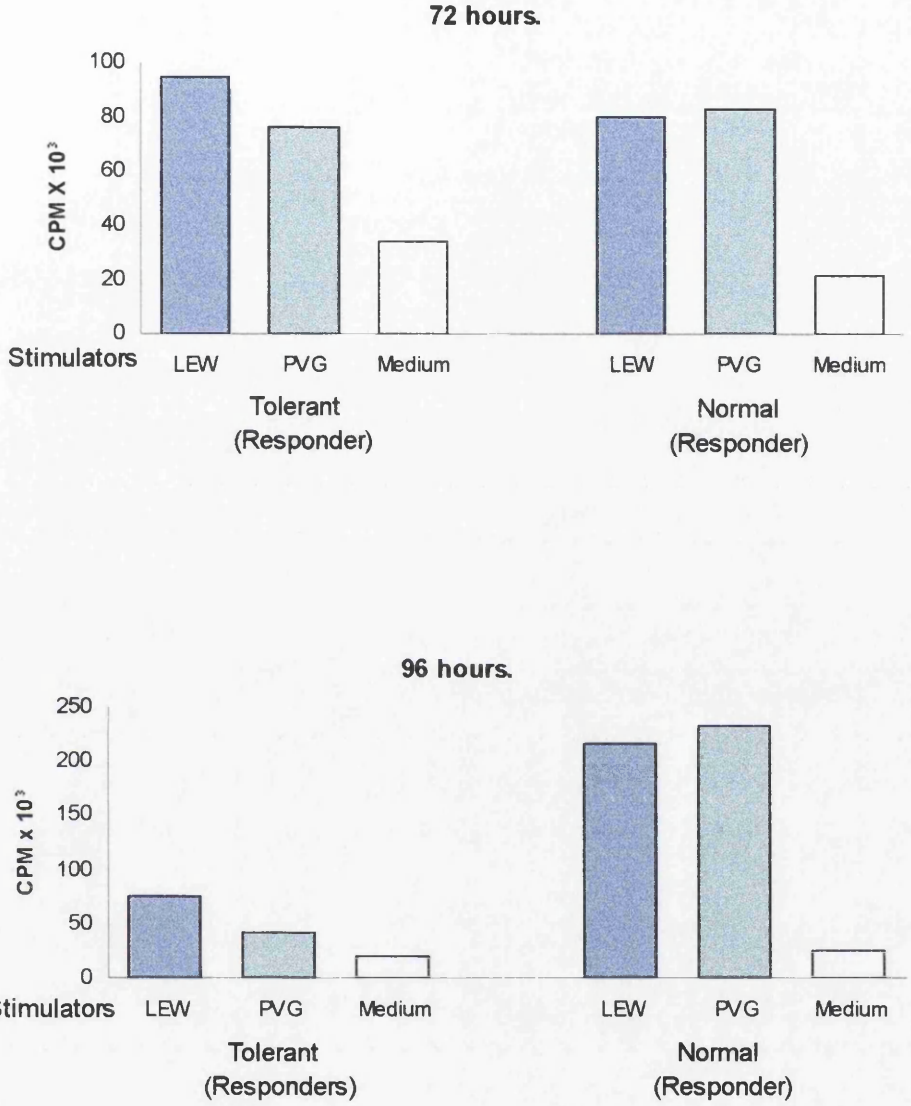
In this experiment, the proliferative response of purified CD4 T cells from both OX38 and OX70 treated long-term tolerant (>100 days) DA rats bearing Lewis heart grafts was determined. The results are shown in figure 8:1 and 8:2. It can be seen that purified CD4 T cells obtained from OX38 treated tolerant animals showed a good proliferative response (at both 72 and 96 hours of culture) to Lewis (heart donor strain) stimulator cells and the levels of proliferation observed against Lewis stimulators were only marginally lower than those seen in response to third party stimulator cells (fig 8:1). The ability of cells from tolerant animals to respond to donor specific stimulators indicates that major

Figure 8:1 Proliferative response of CD4 T cells from long-term tolerant (OX38) animals.



CD4 T cells were purified from the LNC of OX38 treated DA rats bearing long-standing (>100 day) Lewis heart allograft and used as responders in a MLR. Irradiated spleen cells from Lewis (donor-specific) or PVG (3rd party) rats were used as stimulators. Cultures were pulsed with [^{3}H]-thymidine 18 hours before harvesting. Cells were harvested after 72 hrs (top panel) and 96 hrs (bottom panel) of culture. Results are mean of triplicate determinations. LNC CD4 T cells were pooled from three OX38 treated tolerant rats.

Figure 8:2 Proliferative response of CD4 T cells from long-term tolerant (OX70) animals.



CD4 T cells were purified from the LNC of OX70 treated DA rats bearing long-standing (>100 day) Lewis heart allograft and used as responders in a MLR. Irradiated spleen cells from Lewis (donor-specific) or PVG (3rd party) rats were used as stimulators. Cultures were pulsed with [^{3}H]-thymidine 18 hours before harvesting. Cells were harvested after 72 hrs (top panel) and 96 hrs (bottom panel) of culture. Results are mean of triplicate determinations. LNC CD4 T cells were pooled from three OX70 treated tolerant rats.

deletion of donor alloreactive CD4 T cells has not occurred in long-term recipients after OX38 mAb treatment and that deletion of alloreactive lymphocytes is not, therefore, likely to be the explanation for transplant tolerance in this experimental model. Similarly, CD4 T cells from long-term tolerant recipients following OX70 mAb treatment (figure 8:2) proliferated as well to donor as to third party PVG stimulator cells and the proliferative response of CD4 T cells from tolerant animals was, at 72 hours, comparable to that shown by purified CD4 T cells from normal animals. After 96 hours of culture, however, the proliferative response of CD4 T cells from OX70 treated animals was less than that seen in cells obtained from normal animals, although the response to both donor and third party stimulators was diminished (i.e. not donor specific). The reduction in proliferation of CD4 T cells from tolerant animals at 96 hours reflects the fact that either the proliferation had already peaked at 72 hours or this generalised non-specific immunosuppression . Overall, therefore, there was no evidence for clonal deletion of donor-reactive CD4 T cells in long-term tolerant animals following OX38 or OX70 anti-CD4 mAb treatment.

8:4:2 Cytokine production by CD4 T cells from OX38 and OX70 treated tolerant rats.

To determine whether differential cytokine expression is likely to play a role in the maintenance of transplant tolerance induced by anti-CD4 mAbs, purified CD4 cells (from OX38 and OX70 treated tolerant and from naive control animals) were stimulated with irradiated donor (Lewis) or third party (PVG)

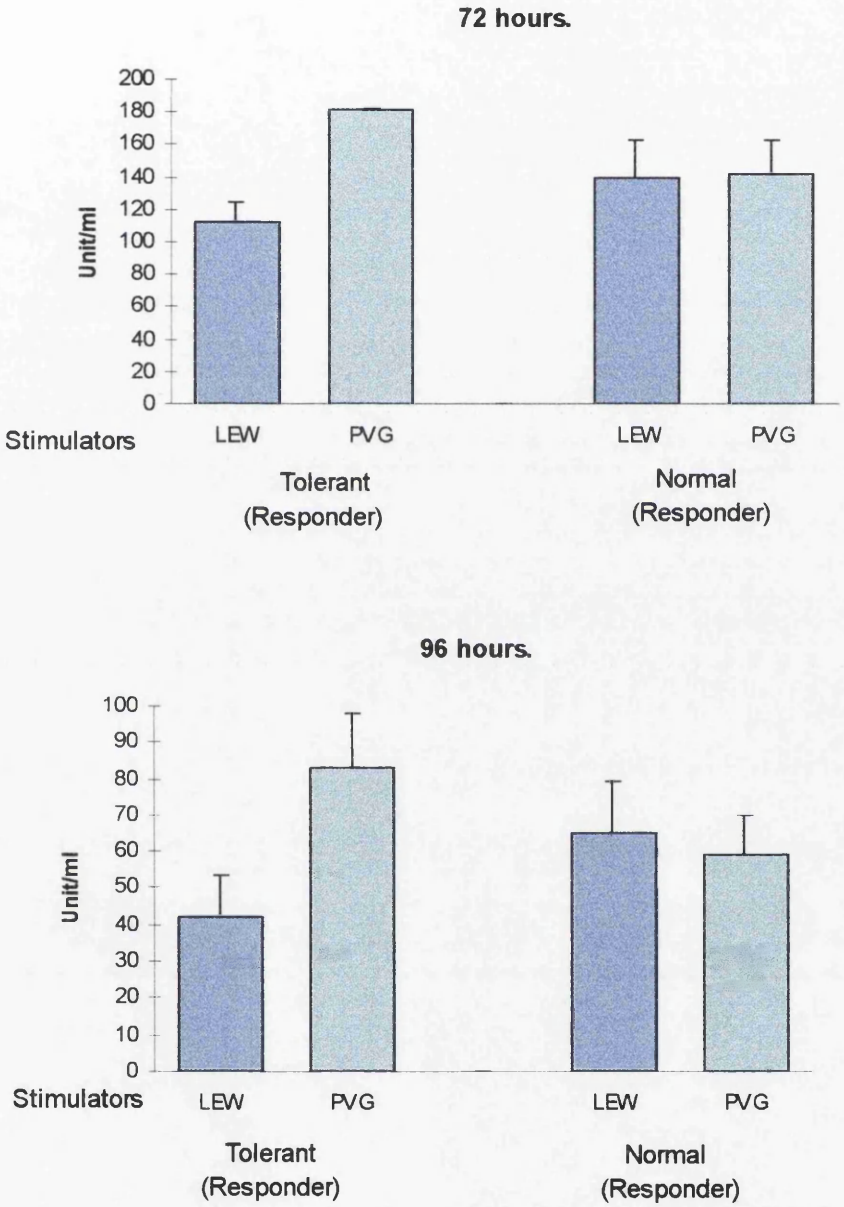
spleen cells, as already described. Culture supernatants (after 72 and 96 hours of culture) were examined for the presence of both Th1 (IL-2 and IFNγ) and Th2 (IL-4) cytokines.

The results obtained are shown in figures (8:3 to 8:7) and these will be described in further detail, considering, the individual cytokines separately for convenience.

Interleukin-2 (IL-2) production by tolerant CD4 T cells.

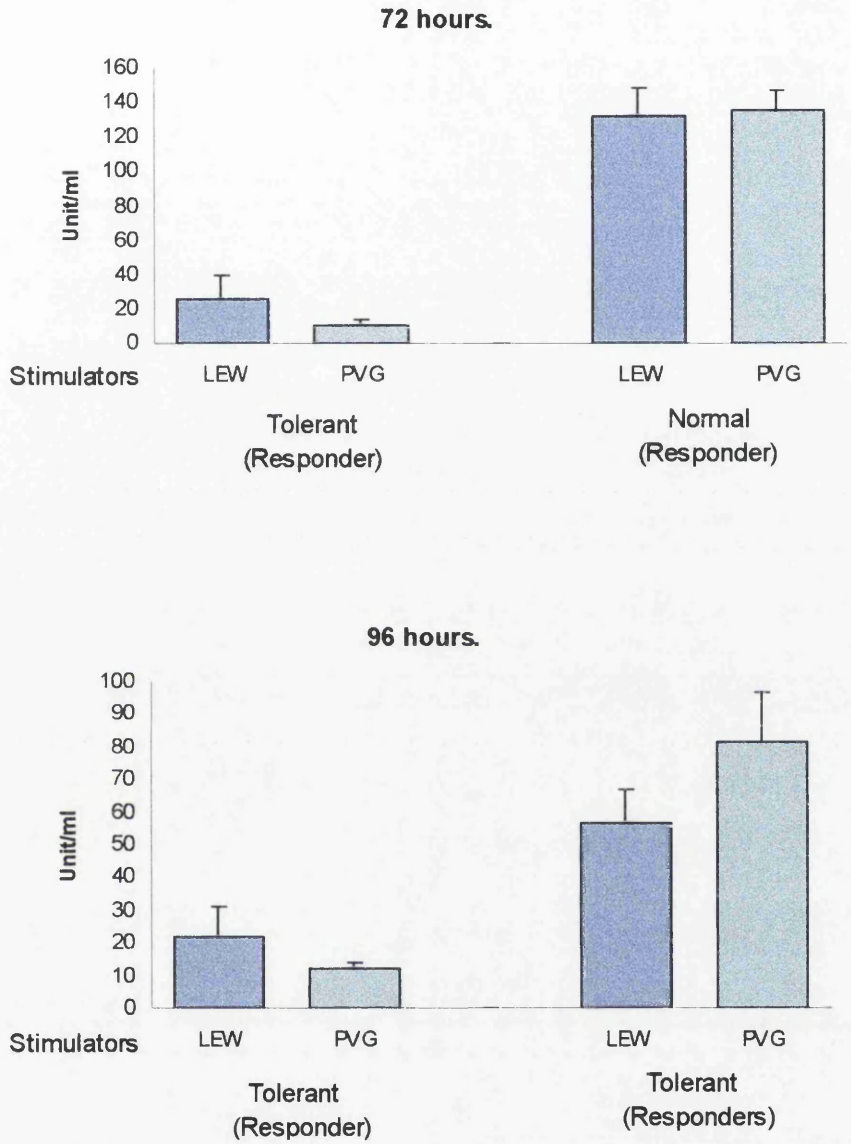
IL-2 production was determined by bioassay using the IL-2 sensitive CTL2 cell line (see material and methods). Purified CD4 T cells from OX38 treated animals released slightly less IL-2 in response to donor-specific (Lewis) than in response to third-party (PVG) stimulator cells at both 72 and 96 hours. However, although IL-2 production by tolerant CD4 cells was lower for Lewis than PVG stimulators, significant amounts of bioactive IL-2 were, however, detectable after both 72 and 96 hours in culture. Purified CD4 T cells from OX70 treated animals showed a marked reduction in IL-2 production at both 72 and 96 hours compared to normal CD4 T cells as shown in figure 8:4. However, there was no evidence for a donor specific defect since the low level of IL-2 produced by tolerant CD4 T cells were similar following both donor-specific (Lewis) and third party stimulation.

Figure 8:3 Production of IL-2 by CD4 T cells from long-term tolerant (OX38) animals.



CD4 T cells purified from the LNC of OX38 treated DA rats bearing long-standing (>100 days) Lewis heart grafts were stimulated with irradiated spleen stimulators and culture supernatants harvested at 72 and 96 hours to determine IL-2 content by bioassay using the IL-2 sensitive cell line CTLL2. Results are the mean and standard deviation of triplicate IL-2 determinations.

Figure 8:4 Production of IL-2 by CD4 T cells from long-term tolerant (OX70) animals.



CD4 T cells purified from the LNC of OX70 treated DA rats bearing long-standing (>100 days) Lewis heart grafts were stimulated with irradiated spleen stimulators and culture supernatants harvested at 72 and 96 hours to determine IL-2 content by bioassay using the IL-2 sensitive cell line CTLL2. Results are the mean and standard deviation of triplicate IL-2 determinations.

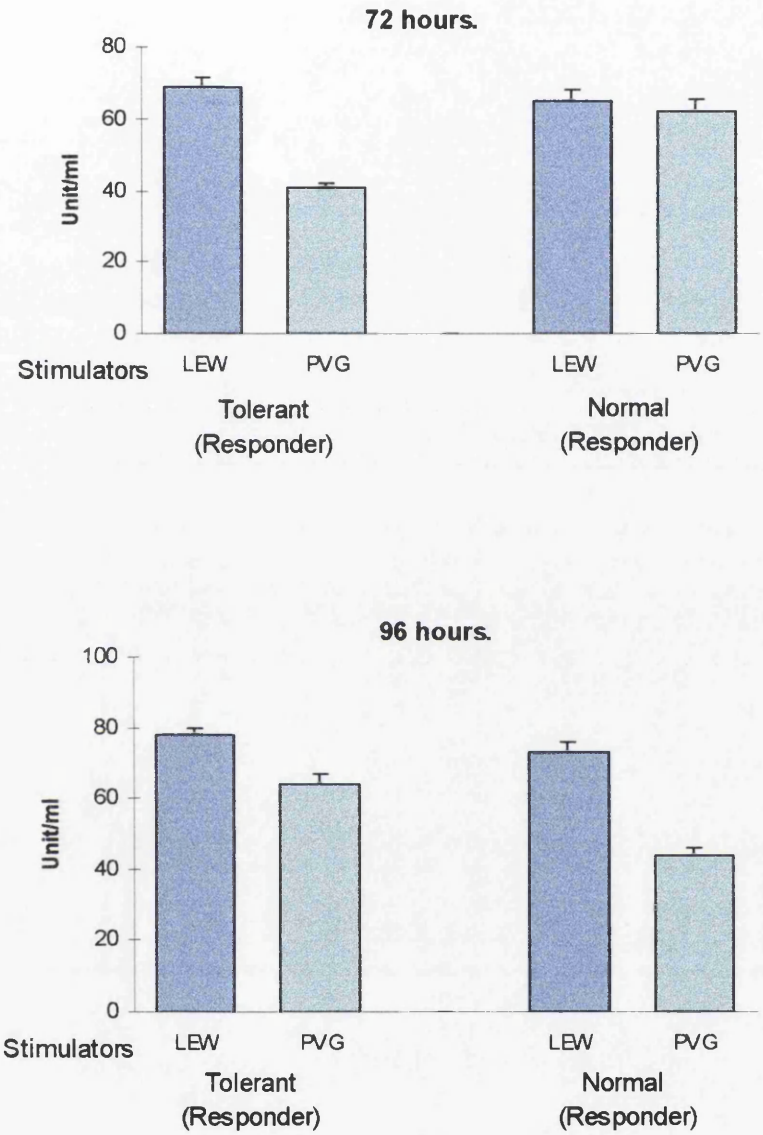
Interferon gamma (IFNγ) production by tolerant CD4 T cells.

The levels of IFNγ in MLR culture supernatants were quantified by ELISA. Purified CD4 T cells from OX38 treated animals produced similar amounts of IFNγ as normal CD4 T cells at both 72 and 96 hours of culture as shown in figure 8:5. After 96 hours of culture, IFNγ levels in culture supernatants of tolerant CD4 T cells were broadly similar after stimulation with donor-specific (Lewis) and third party (PVG) stimulation. In OX70 treated animals, levels of IFNγ were reduced in the supernatant from tolerant CD4 T cells at both 72 and 96 hours of culture as shown in figure 8:6. However, this reduction of IFNγ level was not donor-specific as tolerant cells produced a lower level on stimulation by third party (PVG) and donor specific (Lewis) stimulator spleen cells.

Interleukin-4 (IL-4) production by tolerant CD4 T cells.

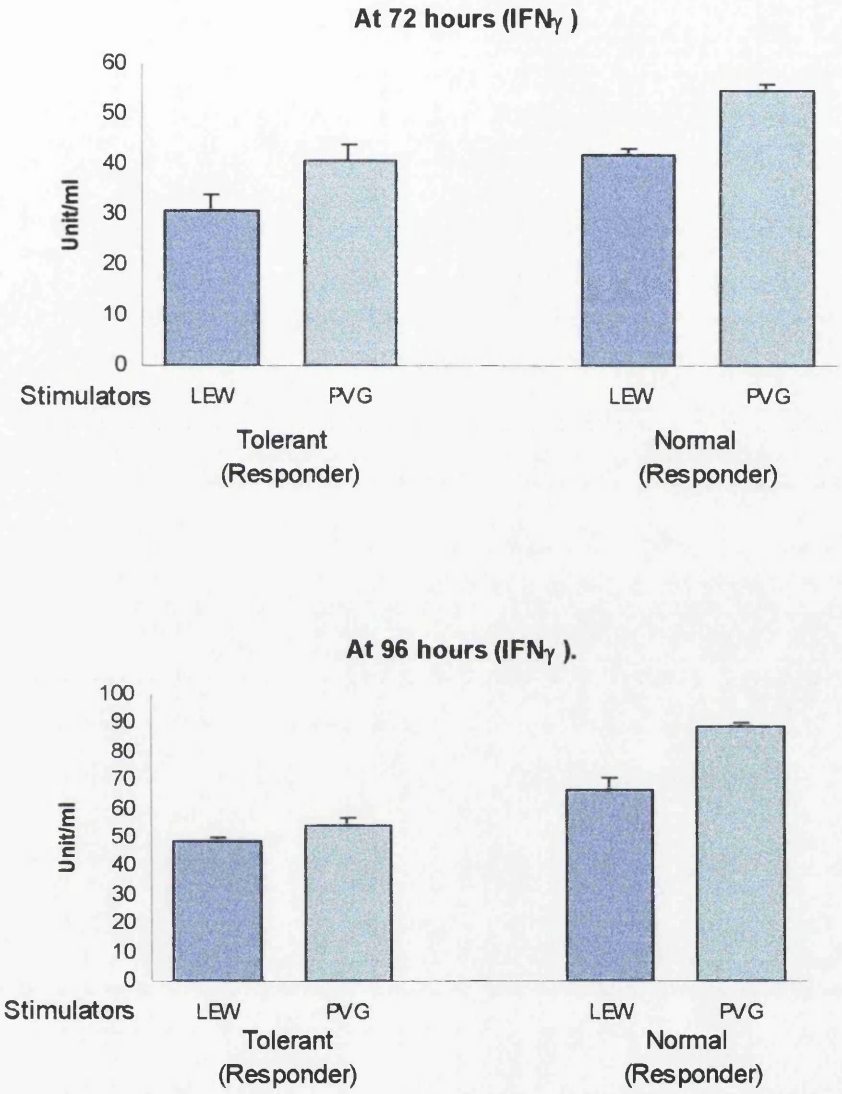
Attempts to establish an ELISA for rat IL-4 were not successful and, therefore, IL-4 in MLR culture supernatants was examined by a bioassay using the induction of class II MHC on B cells as an indictor of the presence of IL-4 (Stumbles and Mason, 1995). This assay is difficult and time consuming because of the need to prepare purified rat B cells. IL-4 levels were, therefore, only estimated for CD4 T cells obtained from OX38 mAb treated recipients. As shown in figure 8:7, the IL-4 levels were similar in CD4 T cells obtained from tolerant rats and CD4 T cells from normal animals. Levels of IL-4 production by tolerant cells stimulated by donor-specific (Lewis) and third party (PVG) stimulators were similar.

Figure 8:5 Production of IFNγ by CD4 T cells from long-term tolerant (OX38) animals.



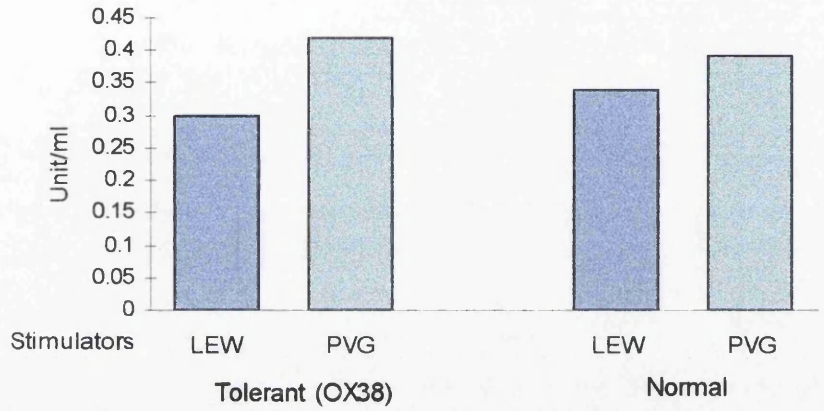
CD4 T cells purified from the LNC of OX38 treated DA rats bearing long-standing (>100 days) Lewis heart grafts were stimulated with irradiated spleen stimulators and culture supernatants harvested at 72 and 96 hours to determine IFNγ content by ELISA. Results are the mean and standard deviation of triplicate determinations.

Figure 8:6 Production of IFNγ by CD4 T cells from long-term tolerant (OX70) animals.



CD4 T cells purified from the LNC of OX70 treated DA rats bearing long-standing (>100 days) Lewis heart grafts were stimulated with irradiated spleen stimulators and culture supernatants harvested at 72 and 96 hours to determine IFNγ content by ELISA. Results are the mean and standard deviation of triplicate determinations.

Figure 8:7 Production of IL-4 by CD4 T cells from long-term tolerant (OX38) animals.



IL-4 was determined by bioassay to determine upregulation of expression of MHC class II molecules on B cells. Rat purified B cells were mixed with supernatants from CD4 T cells from long-term OX38 treated animals which had been cultured in vitro for 72 hours with irradiated Lewis stimulator cells. Values for IL-4 were derived from a standard curve constructed using serial dilutions of recombinant rat IL-4 obtained as tissue culture supernatant (10^4 U/ml) from an IL-4 transfected CHO cell line.

8:5 Discussion

The results from the studies described in this chapter provide further insight into the immunological mechanisms which contribute to maintenance of transplant tolerance after anti-CD4 mAb treatment. In marked contrast to the induction phase of anti-CD4 induced transplant tolerance (chapter 7), the experiments in which OX8 mAb treatment was used to deplete CD8 T cells from long-term tolerant recipients suggest that the CD8 T cell subpopulation does not play a critical role in the maintenance phase of tolerance. Interestingly, however, the adoptive transfer studies using splenocytes from long-term (MST>100 days) anti-CD4 treated animals suggested that a regulatory or suppressor T cells may be instrumental in maintaining transplant tolerance in this model. Although the number of animals studied was small, spleen cells from tolerant rats appeared capable of prolonging, and sometimes completely preventing, allograft rejection, after transfer into secondary naive allograft recipients. The regulatory effect of the transferred spleen cells in these studies was donor-specific which suggests it was attributable to T lymphocytes. As already emphasised, however, caution is required in the interpretation of the adoptive transfer results because of the small number of experimental animals involved. Since CD8 T cells are not critical it is most likely that the cell responsible is a CD4 T cell. It would be of interest, in future studies, to define more precisely the nature of the spleen cell subpopulation responsible for transferring suppression in this experimental model. This could be approached by purifying the T cell subsets prior to adoptive transfer.

The results of the adoptive transfer analysis obtained using cells from long-term tolerant rats are consistent with the studies of Waldman's group in mice rendered tolerant by anti-CD4 and anti-CD8 mAb (Cobbold et al, 1992, Qin et al, 1993; Chen et al, 1993). Waldman and colleagues proposed that tolerance is maintained by "infectiousness", and that a cohort of adoptively transferred tolerant T-cells are able to guide alloreactive naive T cells specific for the same antigen to become tolerant themselves (Cobbold et al, 1996). Waldmann has suggested that the prolonged blockade of important functional T cell surface molecules favours alloantigen presentation to T cells by non-professional APC and that this might be permissive for the emergence of regulatory T cells which, once established, become dominant and are able to induce tolerance in other T cells.

The demonstration that purified CD4 T cells from long-term tolerant rats after OX38 treatment are able to proliferate normally in the MLR argues strongly against a deletional mechanism for maintaining transplant tolerance in this model. Interestingly, cells from OX70 treated animals showed a non-specific reduction in proliferation after 96 hours in culture which may indicate that OX70 has a generalised immunosuppresive effect rather than a donor specific effect. This is an unexpected observation and one which is worthy of more detailed investigation in future experiments both to validate the observation and to explore further why OX70 should differ in this respect to OX38. Although cells from OX38 tolerant animals showed good proliferative response, such animals displayed donor specific tolerance by accepting second donor specific

(Lewis) heart graft and rejecting a third party cervical heart graft. Over the last five years or so, a number of authors have provided evidence that transplant tolerance correlates with a decrease in the Th1 cytokine response and, in some cases, an increase in the Th2 response. On the basis of these finding, some authors have suggested that a switch from a Th1 to Th2 cytokine pattern is instrumental in maintaining the tolerant state (Bugeon et al, 1992; Kupiec-Weglinski et al, 1993; Strom et al, 1996, Dallman, 1995 and Onodera et al, 1996 & 1997). However, the analysis of cytokine production by CD4 T cells obtained from tolerant anti-CD4 treated animals did not provide strong support for this idea. Overall, CD4 T cells from OX38 tolerant animals were able to produce relatively normal amounts of IL-2 and IFNγ after restimulation with donor stimulator cells in vitro. Moreover, CD4 T cells from OX38 treated animals produced levels of bioactive IL-4 which were comparable to those produced by normal CD4 T cells i.e. there was no evidence of over production of this Th2 cytokine.

As with the proliferative data, the results of the cytokine analysis from animals made tolerant by OX70 mAb were less straight forward. In contrast to OX38 treated recipients, animals made tolerant by OX70 mAb showed a rather different cytokine response. They produced less IL-2 and IFNγ proteins (i.e. Th1 suppression). Measurement of IL-2 protein production in vitro is complicated by the fact that the proliferating cells may consume the secreted product. However, the possibility that there is a genuine failure of CD4 T cells from OX70 treated animals to produce IL-2 protein and that there is Th1

suppression can not be excluded. This reduction of both IFNγ and IL2 proteins levels was non specific which may indicate that OX70 mAb has a generalised immunosuppressive effect rather than donor specifc tolerogenic effect. As already discussed, it would be interesting to examine, in vivo, whether, long-term OX70 treated animals can accept or reject third party skin or heart grafts. Unfortunately, there was insufficient supernatant from the OX70 CD4 T cell cultures to assess whether there is any increase of functional IL-4 protein. Similarly, no assay is yet available in the rat for measurement of IL-13 protein. Nevertheless, from the analysis of cytokine protein released from CD4 T cells in OX70 treated animals, immune deviation towards a Th2 response can not be excluded, and clearly further studies will be needed to clarify this. The differentiated cytokine response of CD4 T cells from OX38 and OX70 tolerant recipients raises the possibility that these two mAb's may achieve transplant tolerance, at least in part, through different pathways.

8:6 Summary

8:1 CD8 T cells did not play an essential role in the **maintenance** of transplant tolerance following anti-CD4 mAb treatment.

8:2 Spleen cells from long-term tolerant rats may be able, when adoptively transferred to naive animals receiving an allogeneic cardiac allograft, to induce transplant tolerance. This putative immunoregulatory effect was donor-specific and critically dependent on the total number of cells adoptively transferred.

8:3 CD4 T cells from OX38 treated tolerant recipients showed a good proliferative response on in vitro stimulation in the MLR. There was no evidence, therefore, of clonal deletion of alloreactive T cells. In contrast, CD4 T cells from OX70 treated recipients showed a generalised (i.e. not donor specific) reduction in proliferation.

8:4 There was a reduction in bioactive IL-2 in supernatants from both OX38 and OX70 treated tolerant CD4 T cells. Although marginally reduced in OX70 treated tolerant animals, IFNγ levels were reduced on stimulation with donor-specific and third party cells. CD4 T cells from normal and tolerant rats produced similar levels of IL-4.

8:5 OX38 mAb produces a donor specific tolerogenic effect whereas OX70 mAb appears at least on the basis of in vitro analysis to produce a non specific immunosuppresive effect. It must be emphasised, however, that further experiments are necessary to confirm these potential in vitro differences between CD4 T cells from OX38 and OX70 treated graft recipients.

CHAPTER NINE

FINAL DISCUSSION

Final Discussion

There is currently much interest, both experimental and clinical , in the use of anti-CD4 mAbs as a therapeutic tool for prolonging allograft survival and perhaps inducing transplantation tolerance. The experiments reported here were performed in the hope that they may provide further insight into the scope, limitations and possible mechanisms of anti-CD4 induced prolongation of allograft survival.

Several new finding emerged. In chapter 5, the anti-CD4 mAb OX38 was shown to be effective at inducing tolerance of Lewis heart grafts in fully allogeneic DA recipients. However, this tolerising effect of OX38 mAb was highly dependant on the rat strain combination used for transplantation. In none of the other rat strain combinations tested was OX38 found to extend heart graft survival permanently. This important influence of the rat strain combination studied on the efficacy of anti-CD4 mAb is consistent with that observed for other strategies for inducing transplant tolerance in the rat. For example, pre-transplant donor-specific blood transfusion (DST) promotes allograft survival in the Lewis to DA rat strain combinations, but it is completely ineffective in the DA to Lewis strain combination. The observation here that the effect of anti-CD4 is highly strain dependent does not necessarily diminish its biological importance, but it does indicate the need to understand the determinants which are responsible for such strain dependent effects. For example, one could speculate that DA recipients are more easily rendered

tolerant than Lewis recipients because the regulatory mechanisms responsible for tolerance are more easily activated in the DA rat strain.

The studies using anti-CD4 mAb against epitopes on proximal domains of the rat CD4 molecule were of particular interest, since there is little published information on whether such antibodies are effective in prolonging transplant survival. As was shown in chapter 6, such antibodies were as effective as OX38 at inducing tolerance. Interestingly, however, the W3/25 antibody which labels a distal CD4 determinant did not induce tolerance, even though it is cross reactive with OX38 mAb. W3/25 mAb was also notable by its failure, when given in vivo, to deplete CD4 T cells. All of the other anti-CD4 mAb used produced partial CD4 T cell depletion, suggesting that CD4 T cells depletion may be an important element of the ability of the anti-CD4 mAb tested to induce tolerance. It should be pointed out, however, that non-depleting anti-CD4 mAb's have been shown in some studies to be highly effective at promoting transplant tolerance (Darby et al, 1992 and 1994). Although CD4 T cells depletion may have helped to prevent graft rejection following OX38, OX70, OX71 and OX73 mAbs, the levels of depletion observed were not very profound and certainly not sufficient by themselves to ensure allograft survival. This was adequately demonstrated by the finding that removal of the thymus gland prior to transplantation or alternatively depletion of CD8 cells by anti-CD8 mAb at the time of transplantation were both able to abrogate tolerance induction in many of the treated animals. Clearly, therefore, sufficient alloreactive CD4 T cells remained to effect heart graft rejection in these situations.

The observation in chapter 6 that anti-CD4 mAb treatment preferentially depleted the $OX22^{high}$ subset of T cells was of interest since CD4 T cells of this phenotype have been shown to produce cytokines of the Th1 type (Powrie and Mason, 1990). Conversely, the $OX22^{low}$ subset, which were less effected by anti-CD4 mAb, have been shown to produce a Th2 cytokine pattern (Morrisey et al, 1993). This phenotypic difference in CD4 T cell subset depletion would be consistent with the view that anti-CD4 mAb promote a Th1 to Th2 shift in the cytokine response. However, there is another interpretation of the findings. CD4 T cells newly emerged from the thymus are $OX22^{low}$ and it may be that immature CD4 T cells are less susceptible to depletion by anti-CD4 mAb. In this respect, the observation that intact thymus gland is needed for induction of transplant tolerance by anti-CD4 mAb (chapter 7) was particularly intriguing. A possible role of the thymus in anti-CD4 induced transplant tolerance in the rat was also reported by Fathman's group (Yin and Fatman, 1995a+b)

These two explanations are not mutually exclusive, and it could be postulated that recent thymic emigrants may preferentially produce Th2 cytokines and thereby promote tolerance. Why CD8 cells should be helpful in the induction of tolerance by anti-CD4 mAb is less clear.

The experiments reported in chapter 7 and 8 were performed to address further the mechanisms contributing to the induction and maintenance phase of anti-CD4 transplant tolerance. In some models of tolerance induction, notably donor-specific blood transfusion in the rat, a defect in the IL-2 pathway has been demonstrated and this can be overcome by provision of exogenous IL-2.

This did not appear to be the case in anti-CD4 induced tolerance since administration of relatively large amounts of IL-2 failed to prevent induction of tolerance after anti-CD4 mAb treatment. Interestingly, however, the treatment with a neutralising IL-4 during induction of tolerance did, in some animals, abrogate tolerance induction. These experiments were problematical and further studies are needed before firm conclusions can be made. However, the experiment did suggest that endogenous IL-4 may facilitate anti-CD4 induced tolerance. It must be remembered that IL-4 may be produced by several cell types e.g. easinophiles and mast cells (Brown et al, 1988b; Rennick et al, 1987 and Zlotnik et al, 1987) in addition to Th2 T helper cells (Mosmann et al, 1986; Mosmann and Coffman, 1989). Perhaps the presence of IL-4 early during tolerance has a negative regulatory effect on Th1 T cells during a critical phase of the immune response to the allograft.

Investigation of the mechanisms responsible for maintenance of anti-CD4 tolerance (chapter 8) supported the view that a suppressor or regulatory T cells plays a critical role. Lymphocytes from long-term tolerant animals after OX38 mAb proliferated normally to donor alloantigen in MLR, excluding clonal deletion as a likely cause for transplant tolerance. There has been much recent interest in the idea that transplant tolerance may be attributable to a polarisation of the immune response from a potentially harmful Th1 response to a potentially less harmful or "neutral" Th2 response. However, the detailed analysis of the cytokine repertoire of CD4 T cells from tolerant animals did not provide strong support for this hypothesis.

In OX38 treated animals there was no evidence for a Th2 cytokine bias. Analysis of in vitro response of CD4 T cells from OX70 treated animals provided the unexpected finding that there was a generalised reduction in proliferation to both donor specific and third party alloantigen, suggesting that OX70 may have a different overall effect to OX38 mAb. CD4 T cells from OX70 treated rats also showed generalised impairment in IL-2 production in keeping with the proliferation data. Why the results after OX70 mAb should differ somewhat from those using OX38 mAb is not clear and it would be unwise on the basis of the limited amount of data to draw firm conclusions from this finding without further studies.

Clearly, the mechanisms underlying anti-CD4 Induced tolerance are highly complex and much further work is required to clarify them. The ability to transfer tolerance by spleen cells provides the most compelling evidence that a powerful immuno-regulatory lymphocyte is present in tolerant rats and this area is undoubtedly the most important area to pursue. Detailed analysis of the phenotype and function of the cells responsible for transferring tolerance is required.

Although there are still many unanswered questions, knowledge of the complex cellular interactions which underlie anti-CD4 induced tolerance will be of value in designing protocols for mAb treatment in clinical transplantation. If the immuno-regulatory mechanisms which anti-CD4 mAb induces can be better understood, perhaps these immunosuppressive pathways can be harnessed for clinical application.

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