# INDUCTION OF IMMUNOLOGICAL TOLERANCE TO KIDNEY ALLOGRAFTS FOLLOWING DONOR-SPECIFIC BLOOD TRANSFUSION : EXPERIMENTAL STUDIES IN THE RAT

by

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# ABBREVIATIONS

| | |
|---|---|
| ADCC | Antibody dependent cellular cytotoxicity |
| APC | Antigen presenting cell |
| ATXBM | Adult thymectomised lethally irradiated and bone marrow reconstituted |
| Con A | Concanavalin A |
| Cr | Chromium |
| CTL | Cytotoxic T lymphocyte |
| CyA | Cyclosporin A |
| DEPC | Diethyl pyrocarbonate |
| DSBT | Donor-specific blood transfusion |
| DSM | Donor-specific microchimerism |
| DTH | Delayed-type hypersensitivity |
| ECACC | European Collection of Animal Cell Cultures |
| FCS | Foetal calf serum |
| GIC('s) | Graft infiltrating cell(s) |
| HBSS | Hanks buffered salt solution |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| IFN-γ | Interferon-γ |
| Ig | Immunoglobulin |

| | |
|---|---|
| IL | Interleukin |
| LCA | Leukocyte common antigen |
| LNC('s) | Lymph node cell(s) |
| mAb | Monoclonal antibody |
| mH | Minor histocompatibility antigen |
| MHC | Major histocompatibility complex |
| MLR | Mixed lymphocyte reaction |
| MRC | Medical Research Council |
| MST | Mean survival time |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| RBC('s) | Red blood cell(s) |
| RT | Room temperature |
| RTE('s) | Recent thymic emigrant(s) |
| SD | Standard deviation |
| TCR | T cell receptor |
| Th | T helper lymphocyte |
| TNF-α | Tumour necrosis factor-α |

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The rat thymectomy operation has also been published subsequent to this work.

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# SUMMARY

Donor-specific blood transfusion (DSBT) prior to renal transplantation has been known to have a beneficial effect on graft survival for over twenty years. In experimental models the magnitude of this effect is markedly strain-dependent. Two principal MHC disparate rat strain combinations were used to examine possible mechanisms of DSBT induced tolerance of renal allografts in this study. The first of these was an established model where a single pretransplant blood transfusion produces tolerance (DA into PVG: fully MHC disparate). The second (R8 into $RT1^U$: class I-disparate) involved the development of a new model where DSBT alone was ineffective but the addition of a short course of cyclosporin A (15mg/kg/day for 7 days) at the time of the first of 4 weekly blood transfusions induced tolerance.

In the first model, DA into PVG, over 80% of animals showed long-term acceptance of a kidney allograft following a single 1ml DSBT 7 days prior to transplant. The tolerance was not tissue-specific and was independent of the thymus gland. Graft adaptation was seen in this model as passenger leukocyte depleted grafts survived for approximately 100 days in unmodified hosts (normal rejection time < 10 days). Cytokine levels present in rejecting, enhanced and syngeneic grafts were examined by semi-quantitative PCR. Levels of IFN-γ, IL-4 and IL-10 were broadly similar in rejecting and enhanced grafts but the peak in IL-2 message around 4 days following transplant in the rejecting grafts was abrogated in the enhanced grafts.

In the second model, R8 into $RT1^U$, tolerance was again achieved in >80% of animals and was independent of the thymus gland. No evidence was seen for graft adaptation. Graft acceptance following DSBT/cyclosporin A-pretreatment correlated with low levels of cytotoxic alloantibody while DSBT alone led to high alloantibody levels and graft rejection. Interestingly the production of intragraft Th2 cytokines correlated with graft rejection rather than tolerance. This observation may reflect the immunological role of alloantibody in causing graft damage in this experimental model whereas in other models alloantibody may tend to favour tolerance rather than rejection. This protocol for tolerance induction was also shown to be effective in a fully disparate strain combination (DA into Lewis) where it was found that increasing the number of transfusions improved graft survival.

Both of these models suggest that DSBT-pretreatments have a potential role in clinical practice especially when cyclosporin A is likely to be used as the principle immunosuppressant. DSBT is currently used in a small number of transplant centres but a greater understanding of the underlying mechanisms and development of a more effective protocol is required if its use is to become widespread.

# CHAPTER ONE

## Introduction

## 1.1 A brief history of transplantation

The term transplant was initially used by John Hunter in 1778 when he described his work involving ovarian and testicular grafts between unrelated animals (Morris, 1982). However, the first successful series of grafts between unrelated humans was not achieved until as recently as the 1960's.

The earliest recorded attempt at human organ transplantion was by a Russian surgeon, Voronoy, who in 1933 attempted unsuccessfully to treat, by renal transplantation, a patient suffering from renal failure secondary to mercury chloride poisoning. The transplanted kidney which was harvested six hours after the donors death, probably never functioned, and the unfortunate recipient died within 48 hours (Voronoy, 1936) .

In the 1950's a successful series of human renal transplants were carried out in Boston, USA (Murray *et al.*, 1955). These were between identical twins, thus avoiding the immunological problems that had caused earlier transplants to fail acutely (Hume *et al.,* 1955). This highly successful programme served notice of the potential of transplantation if the rejection response of the immune system could be suppressed. At this time there was no available medical treatment that could achieve this and transplantation was limited to this small group of patients with an identical twin willing to donate one of their kidneys.

Preliminary reports of a drug with potent immunosuppressive effects, 6-mercaptopurine (Schwartz, 1959), were quickly followed by the demonstration of prolonged survival of renal allografts in dogs receiving the drug (Calne, 1960). The

development of azathioprine, an equally effective but less toxic metabolite of 6-mercaptopurine (Calne *et al.,* 1962), allowed successful human transplants to be performed and by 1963 the first patients to survive for a year following a cadaveric renal transplant were being reported (Murray *et al.,* 1963). Corticosteroids were usually used together with azathioprine and the combination of these two agents was to become the standard therapy until the early 1980's.

Also around the early 1970's the somewhat surprising observation was made that transplant patients who had required blood transfusions, because of anaemia, prior to their renal transplant appeared to have a better outcome than those who had not (Opelz *et al.,* 1973). Other transplantation centres confirmed this finding and the practice of random blood transfusion of potential cadaveric renal transplant recipients became established, although the number and frequency of transfusions varied from centre to centre. This use of blood transfusion as a beneficial treatment has to a large extent disappeared from current clinical practice due to the risk of sensitising the potential recipient and because the beneficial effect has become obscured by the use of better immunosuppressive drug therapy, notably cyclosporin A (Lundgren *et al.,* 1986).

Widespread introduction of the immunosuppressive agent cyclosporin A into clinical practice began in the early 1980's. This compound is a metabolite derived from a soil dwelling fungus (*Tolypocladium inflatum Gams*) and is an extremely potent immunosuppressive agent (Borel *et al.,* 1976). It was first shown by Calne and coworkers (1978) to be an effective immunosuppressive drug in renal

transplantation and soon became used widely in this area. Employment of cyclosporin A, usually in combination with steroids and azathioprine, led to a one year graft survival figure, following cadaveric kidney transplantation, of between 80-85% (Green, 1988). Little improvement upon this figure has subsequently been achieved (UKTSSA, Bristol, 1994).

Over the last five years a number of novel immunosuppressive agents have been developed some of which, e.g. FK 506, are now being used in clinical practice. FK 506 appears to be especially useful in the field of liver transplantation (Murase *et al.,* 1990). Other agents currently showing promise in animal models and competing for a potential role in the management of future patients include leflunomide (Williams *et al.,* 1994), rapamycin (Chen *et al.,* 1994) and brequinar sodium (Shirwann *et al.,* 1994).

All the agents mentioned above have a major drawback as their basic action is to cause non-specific generalised depression of the immune system. This leads to a significant risk of morbidity and mortality from infection and, to a lesser extent, malignancy. In addition low grade rejection still is the main cause of graft failure in the long-term (Orosz *et al.,* 1997). In the case of renal allografts approximately 3-5% of grafts functioning at one year following transplant will fail in each of the following years (Calne, 1987).

For these reasons a large volume of research, in the field of organ transplantation, is currently being directed at methods of manipulating the host immune system in a more selective manner with the aim of achieving specific

immunological tolerance, thereby obviating the dangers inherent in blanket immunosuppression. This concept of prolonged graft function without long-term drug administration has been shown to be possible in a variety of animal models. Some of these animal models of tolerance and their possible application to the clinical situation are discussed later in this chapter.

From a clinical viewpoint the recent reports by Starzl and coworkers of survival of some longstanding human liver allografts, after discontinuing all immunosuppressive drug therapy, provides hope that immunological tolerance may be an achievable clinical goal (Ramos *et al.*, 1995).

## 1.2 Immunogenetics of transplantation

### 1.2.1 The major histocompatibility complex (MHC)

At the start of the twentieth century it was demonstrated that acceptance or rejection of transplanted tumour tissue is under genetic control (Little and Tyzzer, 1916). This idea was subsequently shown also to apply to normal tissues i.e. allografts (Medawar, 1944). The term histocompatibility loci was coined by Snell (1948) to describe the location of the genes governing transplant rejection with a major histocompatibility locus and other minor histocompatibility loci being identified.This division of loci was made on the speed of rejection of skin grafts;

with major histocompatibility locus genes mediating a faster rejection response than minor histocompatibility loci genes.

This major locus is now referred to as the major histocompatibility complex (MHC) and the others are collectively called minor histocompatibility antigens. The basic concept of a MHC has been identified in all vertebrate species studied although most work has been carried out on the human, mouse and to a lesser extent the rat. In the context of organ transplantation, the role of the MHC is essentially an unfortunate complication of the true biological function of a system that has evolved to allow the recognition of foreign antigens, e.g. microbial and viral proteins/peptides. The unrivaled degree of polymorphism exhibited by the MHC (Bodmer *et al.,* 1994) provides a survival advantage for the species by maximising the probability that peptides originating from a pathogen can be presented by the MHC to allow the immune system to mount an effective response (Hill *et al.,* 1991). Despite the complex nomenclature that has built up to describe the MHC of each species there is remarkable similarity in the basic structure and function of MHC between species. Figure 1.1 shows a diagrammatic representation of the MHC of mouse, rat and human.

The publication of high quality three-dimensional electron density maps (Bjorkman *et al.*, 1987a) and the ability to identify and sequence the amino acids present has allowed the structure of the MHC molecule to be elucidated. This in combination with our rapidly increasing knowledge of the immune system, at the molecular level, has allowed an understanding of how the MHC system functions.

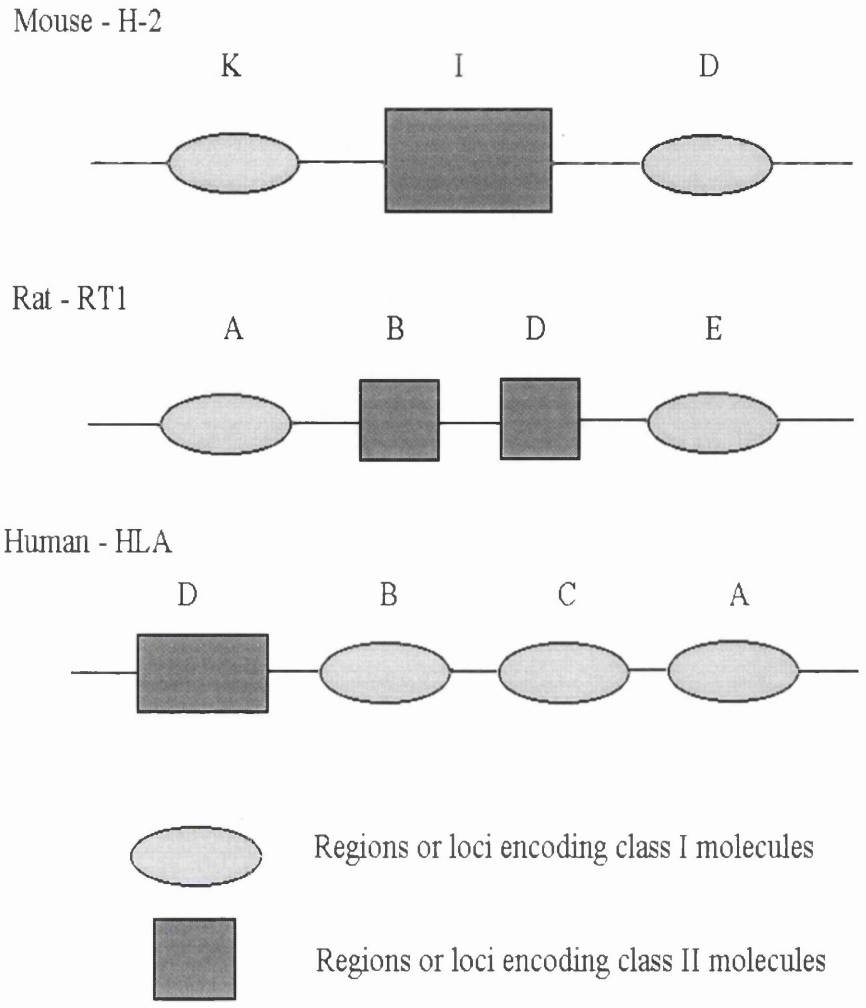



**Figure 1.1 A simplified linear map showing the organisation of the major histocompatibility complex (MHC)**

The remarkable similarity of the MHC in the mouse, rat and human genomes is shown in diagrammatic form above.

The class I MHC

Almost all nucleated cells in the body constitutively express polymorphic membrane glycoproteins known as class I MHC molecules on their surface although the level of expression can be up-regulated or down-regulated (Lew *et al.,* 1986). These molecules consist of two non-covalently linked chains, a polymorphic heavy chain with a molecular weight of approximately 45 kilodaltons (kd) and a non-variable lighter chain of 12 kd (β2 microglobulin).

The heavy chain is composed of three extracellular domains (α1, α2 and α3) linked by a transmembrane segment to an intracytoplasmic tail. The domains are arranged such that α1 and α2 form helical configurations on either side of a deep cleft, the floor of which is composed of 8 anti-parallel β pleated sheets. The cleft is occupied by a short peptide (most commonly a nonameric peptide) bound in an extended conformation (Madden *et al.,* 1991). Such peptides have been sequenced and appear to match known self-peptides derived from intracellular proteins (Jardetzky *et al.,* 1991). In the case of virally-infected cells, peptides derived from viral proteins have also been identified in the binding groove (Silver *et al.,* 1992). Most of the polymorphisms in the class I MHC heavy chain are clustered around the cleft and therefore influence the nature of the peptide that can bind there (Bjorkman *et al.,* 1987b). Peptides are provided via "the transporter associated with antigen processing" (TAP); a heterodimeric, MHC-encoded, ATP-dependent transporter molecule embedded in the endoplasmic reticulum membrane, with which newly

synthesised class I MHC heavy chain $\beta_2$-microglobulin complexes associate (Germain, 1995).

The class I MHC-peptide complex generally interacts with T lymphocytes only if they carry the CD8 costimulatory molecule (Monaco, 1992). This surface molecule is expressed on the T lymphocyte subpopulation responsible for cytotoxic killing of cells by way of direct contact and release of perforins and other toxins into the target cell (Berke, 1995).

Natural killer (NK) cells also exhibit specificity for MHC class I molecules in their interaction with target cells. These cells appear to be inhibited by the recognition of normal class I molecules and may play a role in regulating class I expression (Raulet, 1996). The role of these cells in relation to the rejection of organ allografts is poorly understood at the present time.

The class II MHC

In contrast to the widespread tissue distribution of class I MHC, class II molecules have a much more restricted distribution. Under normal circumstances these molecules are found predominantly on professional antigen presenting cells, notably B lymphocytes, macrophages, monocytes and dendritic cells. However, many other cell types can be induced to express class II MHC molecules given appropriate stimulation. Induction of class II molecules has been demonstrated in rejecting mouse skin grafts, on vascular endothelial cells and keratinocytes (De Waal *et al.,* 1983;

Dallman and Mason, 1983). Similarly donor tissue from directly vascularised rat heart and kidney allografts has been shown to express class II MHC molecules during rejection (Milton *et al.,* 1986).

The three-dimensional structure of the class II MHC molecule has been published and several such molecules sequenced (Brown *et al.,* 1993). They consist of two polypeptide chains of approximately 33 and 28 kd. Each chain is composed of an intracellular domain, a transmembrane domain and two extracellular domains. The extracellular domains, like the class I molecule, have a peptide binding groove, although, class II bound peptides are longer (~14 amino acids) and the peptide binding groove is open ended (Rudensky *et al.,* 1991). Class II MHC molecules present soluble exogenous foreign peptide antigens following internalisation and processing by the antigen presenting cell to the T lymphocytes. Only T lymphocytes with the CD4 accessory molecule recognise the class II MHC-peptide complex (Monaco, 1993).

### 1.2.2 Minor histocompatibility antigens (mH)

When Snell coined the term major histocompatibility locus he also recognised the existence of other genetic loci (encoded outwith the MHC) that appeared to cause a weaker immunological response; these he named minor histocompatibility loci. Later studies have shown that these minor histocompatibility antigens are themselves capable of provoking powerful immune responses

depending on the strain combination examined (Loveland and Simpson, 1986). In their study rapid rejection of skin grafts and cytotoxic T lymphocyte production was demonstrated in a mouse strain combination possessing identical MHC antigens. The minor histocompatibility antigens are widely scattered throughout the genome and are believed to be small endogenous peptides that occupy the antigen binding site of self-MHC molecules (Perreault *et al.,* 1990). The exact numbers of these antigens is unclear but is potentially very high with over forty mH incompatibilities found between a single strain combination of mice (Bailey, 1975). The best characterised of these antigens the mouse H-Y antigen is a male specific transplantation antigen which has recently been identified and studied in detail (Scott *et al.,* 1995).

### 1.2.3 High and low responder transplantation genes (Immune response genes)

Immune response genes were originally described prior to the identification of the MHC. The antibody response to a variety of synthetic polypeptides in the mouse was found to map to a region between H-2K and H-2D (McDevitt and Tyan, 1968) and the genes encoded in this area were termed immune response genes (McDevitt and Benaceraff, 1969). Subsequently these genes were shown to be a part of the MHC.

In the field of transplantation, Howard and Butcher (1981) proposed the concept of immune response genes, linked to the MHC, that control the strength of alloimmune responses as measured by skin grafting, alloantibody production and in vivo cytotoxic cell production. Using a series of congenic rats with recombinants in

their class I MHC (Butcher and Howard, 1977 and 1979) they later described the existence of a defect in antibody responses to transplantation antigens (Butcher *et al.*, 1982). On the basis of these immune responses they divided the recipient rats into "high" and "low" responder strain combinations. "High" responder genes led to high alloantibody levels and rapid graft rejection, while "low" responders did not reject their grafts and had undetectable alloantibody responses. Subsequently, using liver allografts, this division of immune responses was also seen to extend to a full MHC mismatch (Kamada *et al.*, 1988) with "low" responders enjoying complete tolerance induction following liver grafting. Indeed, these liver grafted animals would subsequently accept heart allografts long-term.

A possible mechanism for this subdivision was proposed by Stewart and coworkers (1985a, and b) when it was reported that five-fold higher cell numbers were required to restore rejection to irradiated heart transplant recipients if the recipient was of a "low" rather than a "high" responder strain. Further experiments with this model, using chimeric cells for reconstitution, showed that the antigen presenting cells of the "low" responder host were defective at presenting an isolated class I MHC disparity. In contrast, if a full MHC haplotype mismatch was present then vigorous rejection occurred and was thought to result from the ability of donor class II molecules to directly induce host helper T cells thereby avoiding this defective presentation. Since the main source of class II MHC molecules in organ allografts are passenger leukocytes, which migrate out of the graft following transplantation, the importance of this direct presentation presumably decreases with

time. This idea may explain why in "low" responder rodent models tolerance is often relatively easy to produce with short term courses of immunosuppressive therapies.

## 1.3 Mechanisms of allograft recognition and rejection

Classically the process of allograft recognition and rejection has been divided into three areas; an afferent phase where the graft releases foreign proteins into the host, a central phase where these proteins are processed and an efferent phase whereby the host immune cells primed by the foreign proteins attack their source.

As our knowledge of the complexity of the rejection process increases it has become obvious that this model is an oversimplification but it provides a useful framework for describing the stages of allograft rejection.

### 1.3.1 Afferent phase:-antigen release

For allograft rejection to occur the immune system of the recipient must recognise the presence of the graft. Early allograft experiments generally involved indirectly vascularised skin grafts and it was found that if the lymphatic drainage of these grafts was disrupted then rejection was dramatically delayed (Barker and Billingham, 1968). However, in directly vascularised organ grafts division of lymphatic drainage does not affect rejection (Pedersen and Morris, 1970). Splenectomy has been shown to prevent rejection of a renal allograft in a rat model (Fabre and Batchelor, 1975). These results led to the concept of graft antigens

causing activation of the hosts immune system in a central compartment rather than host immune system cells becoming activated within the graft. Following skin grafting this activation occurred in the regional lymph nodes while in directly vascularised grafts the spleen was also important in terms of initiating rejection.

The identification of the dendritic cell population and their powerful immunostimulatory properties supported the concept of the graft shedding antigens into the host (Steinman and Witmer, 1978). These bone marrow derived cells have a striking morphology and have been shown to migrate from allografts into the lymph nodes and spleen of the recipient animal (Larsen *et al.,* 1990). Elimination of these cells from the donor organ leads to a marked reduction of the rejection response in some animal models of allograft rejection. The most convincing demonstration of this effect involved retransplanting renal allografts, that have resided in a temporary host until these cells have left the graft (1 to 3 months), into a second unmodified syngeneic host. In some strain combinations such grafts show greatly prolonged survival but rejection was restored if donor-strain dendritic cells were administered at the same time as the retransplanted kidney (Lechler and Batchelor, 1982). Prolonged culture of pancreatic or thyroid tissues under conditions that are believed to destroy dendritic cells prior to transplantation also leads to prolonged survival of the grafted tissues (Lacey and Davie, 1984; Lafferty *et al.,* 1975). These dendritic cells are generally referred to as passenger leukocytes, a term coined much earlier to describe the effect that immunisation with donor splenocytes or lymph node cells had on transplanted tumour tissues (Snell, 1957).

Central sensitisation is not the only route for allograft recognition as elimination of passenger leukocytes does not always cause prolonged graft survival (Stuart *et al.,* 1971). Peripheral sensitisation is also reported to occur as the host lymphocytes pass through the transplanted tissue (Tilney and Gowans, 1971).

Lanzavecchia has recently reviewed much of the current knowledge of the various mechanisms of antigen uptake for presentation to the immune system (Lanzavecchia, 1996). In the case of dendritic cells macropinocytosis and cell surface receptor mediated mechanisms principally the mannose receptor (mannosylated or fucosylated antigens) and FcγRII (antigen-antibody complexes) are important although alternative mechanisms may also exist.

### 1.3.2 Central phase:-antigen presentation

Conventional immune responses to exogenous protein antigens require that the antigen is taken up by a professional antigen presenting cell (APC) and processed before being presented to the T cell receptor (TCR) as a peptide in association with class II MHC molecules. These APC's include dendritic cells, macrophages and B lymphocytes. In addition to presenting the processed antigen they also produce costimulatory signals that are required if the T cell is to produce its full range of functional and proliferative responses (Mueller *et al.,* 1990; Muller and Jenkins, 1995). One of the principle costimulators is the B7 molecule which interacts with the CD28 receptor leading to activation and increasing adhesion of the T cell (Harding *et al.,* 1992; Turcovskicorrales *et al.,* 1995). Several other T cell receptors including

CD27 and CD40 are also targets for costimulatory signals (Kobata *et al.*, 1994; Vanessen *et al.*, 1995). The particular APC involved may also affect the response seen, presumably as this will influence the costimulatory signals available. The role of secondary signals in the induction of T cells and graft rejection has recently been reviewed by Lafferty (1995).

Alloimmune responses are unique in that allogeneic class II MHC molecules are able to directly interact with and activate host T cells because they resemble self-MHC molecules carrying antigenic peptides in their binding grooves (Lechler *et al.*, 1990). This is referred to as the direct pathway of allorecognition. Dendritic cells express a very high density of class II molecules and are able to provide essential costimulatory signals. As such they are extremely potent at triggering immune responses and this is thought to be the major pathway involved in unmodified graft rejection (Braun *et al.*, 1993).

Other potential sources of allogeneic class II MHC molecules are vascular endothelial cells. Rodent vascular endothelium does not however readily express class II MHC. This has led to problems in comparing results from these models with the human situation where class II expression on vascular endothelium is much greater. This may also explain why rodent models permit long-term graft survival to develop with relative ease.

There is now evidence that allogeneic MHC molecules may be processed in a similar manner to conventional exogenous protein antigens if shed from the graft into the recipients bloodstream. In this case it is the recipient APC's that internalise donor

antigen and present it as peptides in the binding cleft of self class II MHC molecules. This process is generally assumed to be less efficient but may become increasingly important depending on the particular circumstances involved. This is called the indirect pathway of allorecognition. Strong evidence for its existence has been shown by experiments, in both murine and rat models, where host T cells primed to alloantigens subsequently respond to peptides corresponding to the donor MHC; i.e. the MHC molecule has undergone processing by host derived APC's (Benichou *et al.,* 1992; Watschinger *et al.,* 1994). A schematic model of allorecognition via the "direct" and "indirect" pathways is shown in Figure 1.2.

The final stage in allorecognition is when the specific MHC-peptide complex binds to the CD3/TCR complex in conjunction with the necessary accessory molecules. This leads to intracellular signalling and T cell activation. Much current research is being directed into this area. The first examples of the three-dimensional structure of TCR fragments have recently been published (Fields and Mariuzza, 1996). These confirm that the TCR is composed of $\alpha$ and $\beta$ (and also $\gamma$ and $\delta$) chains with variable and constant domains analogous to those of antibodies. Further the V domains include hypervariable regions of polypeptide which are probably equivalent to the complementarity determining regions (CDR's) of antibodies in that they mediate the peptide/MHC recognition. It is postulated that dimerisation of TCR's is required for activation (Kolanus *et al.,* 1993; Abastado *et al.,* 1995) and also that this leads to conformational changes prior to signal transduction (Karjalainen, 1994; Janeway, 1995).

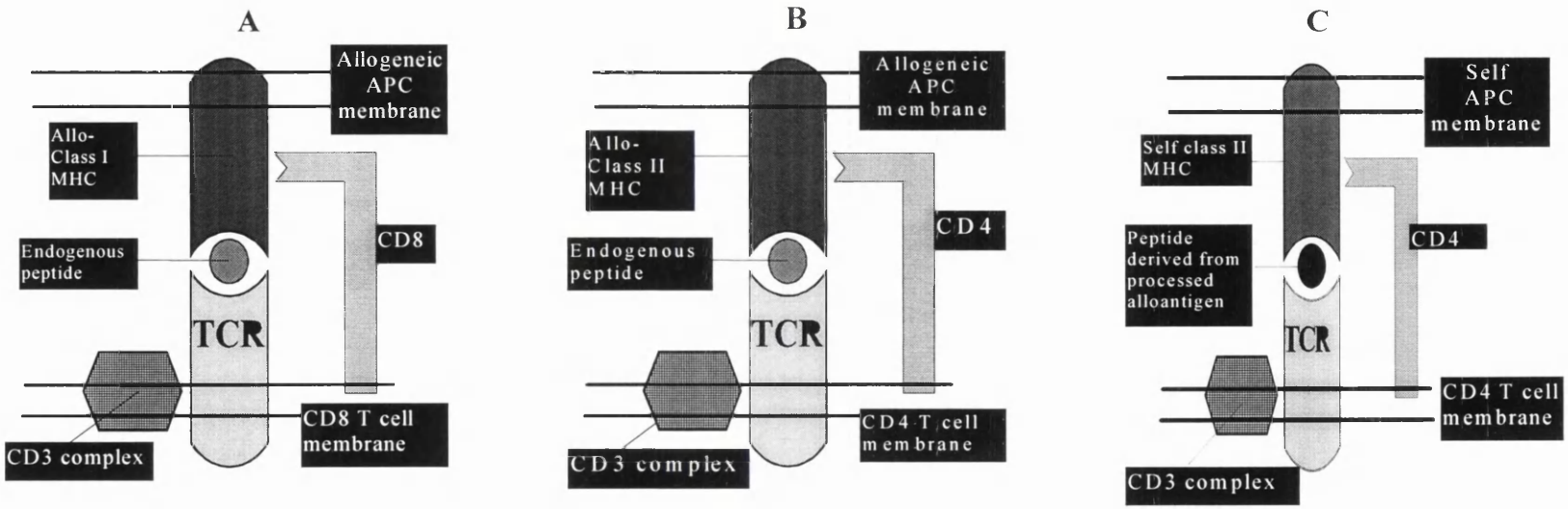



**Figure 1.2 Schematic model of allorecognition via "direct" (A and B) and "indirect" (C) pathways**

For direct allorecognition the CD8 and CD4 molecules on T cells bind to non-polymorphic regions of class I and class II MHC respectively on allogeneic APC's or target cells. The extent that the TCR recognises peptide in the binding groove of the allogeneic MHC and the extent that it is epitopes of the MHC molecule adjacent to the peptide binding groove that are recognised as foreign is unclear and evidence exists for both models of direct allorecognition (Lechler *et al.*, 1990). For indirect allorecognition $CD4^+$ T cells recognise allogeneic MHC that has been processed and presented as antigenic peptide in the peptide binding groove of class II MHC on self APC's.

### 1.3.3 Effector phase:-rejection mechanisms

The rejection of an allograft is characterised by a highly complex series of cellular and humoral interactions which combine to rapidly destroy an allograft in an unmodified host. There is now a consensus that the central and essential component of these responses is the T lymphocyte. The use of cloned cell lines and inbred animal models has allowed researchers to separate to some degree the different effector mechanisms which make up the immune response. The debate over the relative importance of delayed-type hypersensitivity reaction (DTH); cytotoxic T lymphocytes (CTL's); helper T lymphocytes ($T_H$); natural killer cells (NK) and alloantibody continues. The evidence for each of these potential mechanisms will now be discussed in turn.

Delayed-type hypersensitivity reaction

The historical similarities between the cells infiltrating rejecting skin grafts and those in DTH reactions led early workers to propose that DTH was responsible for allograft rejection (Brent *et al.*, 1958). The basic principle of the DTH reaction is that $T_H$ cells following activation produce lymphokines eg. IL-2 and IFN-$\gamma$ that stimulate the recruitment and activation of non-specific effector cells such as macrophages which in conjunction with the proinflammatory cytokine TNF-$\alpha$ cause localised tissue destruction. Local synthesis of these three cytokines has been shown by detection of their mRNA in rejecting allograft tissues (Dallman *et al.*, 1989).

It is difficult to show that an allograft has been destroyed by DTH since there is no in vitro assay for detection. Consequently, destruction by DTH is generally implied rather than directly proven. Much of the evidence from rodent models suggests that non-specific DTH mechanisms do not have a major role in acute allograft rejection, if the help from the $CD4^+$ T cell is excluded. DTH however may be more important in clinical organ graft rejection than in experimental models (Mason and Morris, 1986).

Cytotoxic T lymphocytes

The first direct evidence that CTL's may play a major role in acute allograft rejection came in 1960 when it was demonstrated that thoracic duct lymphocytes, harvested from dogs that had rejected an allograft, were able to specifically lyse donor type renal epithelial cells in vitro (Govaerts, 1960). Subsequent experiments have shown that mature CTL's cause cell killing by a direct action involving cell to cell contact without requirement for any lymphokines or help from macrophages or non-specific killer cells. The initial development of CTL's however does require help in the form of IL-2 from $T_H$ cells.

The precise nature of this killing was established using skin grafts from allophenic mice. Such mice were created by fusing embryos of two allogeneic strains at the blastomere stage of development. When allophenic skin was grafted to either of the parental strains, those hair follicles bearing MHC molecules of the foreign parent were destroyed without any non-specific damage of hair follicles syngeneic

with the recipient (Mintz and Silvers, 1970; Rosenberg *et al.,* 1987). The ability of cloned CTL cell lines to cause tissue necrosis when injected intradermally into mice only if they express the minor H antigen to which the cloned cells are reactive is clear evidence that CTL's can effect cell damage in vivo in such a precise manner (Tyler *et al.,* 1984).

By extracting graft infiltrating cells from rejecting and non-rejecting grafts (treated with cyclosporin A) it has been demonstrated that only the rejecting grafts contained specific CTL activity although similar levels of non-specific activity were present in both groups (Bradley *et al.,* 1985). This demonstration however, while being very suggestive of a role for the CTL, does not exclude other mechanisms and high levels of specific CTL activity can also be seen in some models where there is no apparent graft rejection (Armstrong *et al.,* 1987).

Early experiments involving adoptive transfer of T cells into rats rendered immunodeficient by sublethal total body irradiation showed that the restoration of rejection of skin grafts with only mH differences was delayed if the restoring inoculum was depleted of cytotoxic cell precursors (Mason *et al.,* 1984).

Other experiments using adoptive transfer of T lymphocytes into adult thymectomised, lethally irradiated and bone marrow reconstituted (ATXBM) mice and rats have shown that it is the $CD4^+$ and not the $CD8^+$ lymphocyte subpopulation which restores rejection (Loveland *et al.,* 1981; Dallman *et al.,* 1982). However $CD4^+$ reconstituted rats still had a dense infiltrate of $CD8^+$ T cells in rejecting grafts. Similar experiments in congenitally athymic rats restored with purified $CD4^+$ T cells

also found that $CD4^+$ cells alone were required to cause rejection (Bolton *et al.*, 1989). This gave rise to the suggestion that the $CD4^+$ $T_H$ cell is responsible for regulating graft rejection while the $CD8^+$ lymphocyte may simply be one potential effector mechanism. Experiments by Hall and coworkers (1985) lent further weight to this hypothesis. They showed that $CD4^+$ T cells alone could restore rejection of a cardiac allograft in acutely irradiated recipients. However transfer of both $CD4^+$ and $CD8^+$ cells together resulted in a faster rate of rejection.

Alloantibody

Antibody has long been accepted as the major cause of the severe endothelial damage that characterises the process of hyperacute rejection seen when a human kidney allograft is transplanted into a recipient with pre-existing cytotoxic alloantibodies (Kissmeyer-Nielsen, 1966). However the possible role of antibody in causing acute rejection in the non-sensitised recipient is less clearly defined. As recently as 1990 Colvin stated in a review of the literature that there was no obvious role for antibodies in primary graft rejection. Other workers however have reported a close correlation, in renal allograft recipients, between circulating anti-HLA-class I antibody and acute rejection episodes (Halloran *et al.*, 1992).

Experimental models in the past may have underestimated the potential of antibody as a cause of graft damage, since many rodents have a relative deficiency in the ability to fix complement (French, 1972). Also in some rodent models, administration of immune serum leads to a dramatic prolongation of graft survival, a

phenomenon known as passive enhancement (Fabre and Morris, 1974). These findings taken with the marked cellular infiltrate seen on histological examination of rejecting grafts and the ease with which such cells can be studied in vitro may have contributed to the potential role of alloantibody in graft rejection to be neglected.

Recent experiments have clearly demonstrated that, in a high responder class I-disparate rat strain combination, acute rejection is associated with the production of high levels of anti-class I antibodies (Gracie *et al.,* 1990a; Morton *et al.,* 1993). Furthermore in these experiments passive transfer of immune serum triggered acute allograft rejection in both congenitally athymic and anti-CD4 treated recipients. Rejection was dependent on the presence of $CD4^+$ lymphocytes to provide help for the production of anti-class I cytotoxic alloantibody.

Alloantibody could in principle lead to graft destruction either by complement fixation or by antibody dependent cellular cytotoxicity (ADCC). In the latter the specificity of the effector response is provided by antibody but the actual damage is mediated by killer cells that are otherwise non-specific. The role of ADCC in graft rejection is somewhat unclear since ADCC activity following transplantation has been observed both in stable and rejecting grafts (Gailiunes *et al.,* 1978).

### 1.3.4 The central role of the $CD4^+$ T lymphocyte

It is now clear that a variety of different effector mechanisms are responsible for graft rejection and that the relative importance of these may vary depending on factors such as the nature of the MHC disparity, the type of graft and the state of

sensitisation of the recipient (Swain and Cambier, 1996). The $CD4^+$ T lymphocyte plays a central role in orchestrating the various effector mechanisms. It does so by releasing a range of cytokines which provide help for generation of specific $CD8^+$ T cells, maturation of B cells (Steele *et al.,* 1996) and a variety of non-specific effector mechanisms. Because the $CD4^+$ T cell plays such a crucial role in the graft rejection response it has become an obvious target for monoclonal antibody therapy in an attempt to modify graft rejection (Shizuru *et al.,* 1990; Wood *et al.,* 1991).

## 1.4 Clinical experience of blood transfusion prior to transplantation

### 1.4.1 Random transfusion (unrelated)

The use of donor-specific transfusions 7 or more days prior to transplant in the clinical setting is clearly not possible, with the exception of living related kidney donors, due to the nature of organ donation and the limits on organ preservation. When it was reported in 1973, contrary to expectations, that cadaveric renal transplant recipients who had received "random" blood transfusions, on the basis of clinical need (usually anaemia), appeared to have fewer rejection episodes and longer graft survival, then interest in blood transfusion in the clinical setting became intense (Opelz *et al.,* 1973). The initial report was rapidly confirmed by other groups and the practice of deliberate preoperative transfusion became established for patients on renal transplant waiting lists. However the timing and number of transfusions

required for maximum benefit was not clear. In a large study (> 1300 cadaveric renal transplants) the number of grafts functioning at one year rose from 42% in recipients who had never been transfused to 71% in those who had received over 20 units of blood (Opelz and Terasaki, 1978). At the other extreme a single transfusion prior to transplant was reported to raise 1 year graft survival rates from 26% to 70% (Persijn *et al.,* 1984). Most transplant groups adopted transfusion protocols between these extremes with between two and five transfusions being most commonly employed. It was suggested that maximum benefit from blood transfusion was seen in those patients transplanted within three months of the transfusions (Hourmant *et al.*, 1979; Werner-Favre *et al.,* 1979).

This increased benefit from blood transfusion in terms of overall graft survival had however to be balanced against the dangers of sensitising the potential recipient against HLA antigens expressed by a potential future kidney allograft. Following the introduction of cyclosporin A as the main immunosuppressive agent and improved results from HLA matching (especially for HLA-DR antigens) the benefits of random transfusion in renal transplantation declined, as did its routine usage (Ting, 1988). Reports from the cyclosporin A era suggest that random transfusion prior to cadaveric renal transplantation has no measurable beneficial effect (Lundgren *et al.,* 1986) and may indeed decrease one year graft survival (Ahmed and Terasaki, 1992). The clinical need for blood transfusions has also declined in patients on long-term haemodialysis as the anaemia seen in these patients can be treated with recombinant erythropoetin. It is worth pointing out that the

kidney is currently the only organ where detailed HLA typing is practical and routinely carried out. In the case of other organs, limited supply, shorter preservation times and unstable potential recipients combine to make HLA matching impractical and blood transfusion may have some remaining role here.

Donor-specific transfusion is clinically possible in two settings; living related donors and cadaveric donor regimes where the transfusion is given at the time of the transplant or up to 24 hours before. Both regimes are being used clinically in a small number of centres and are discussed below.

### 1.4.2 Living related donor-specific blood transfusion

By preconditioning with three donor-specific blood transfusions prior to transplant, Salvatierra and colleagues (1980) were able to improve the outcome in HLA one- haplotype mismatches to that seen in HLA identical matches: around 90% one year graft survival. This transfusion regime however led to the exclusion of approximately one third of potential live transplants due to sensitisation and cytotoxic alloantibody formation. Interestingly, if the sensitised patients were given further blood transfusions, only a third appeared to show a further increase in cytotoxic alloantibody levels and a further third showed a marked decrease in alloantibody levels (Opelz *et al.,* 1981a). The latter group had a favourable graft outcome following transplantation and it has been suggested that this is due to

specific immunological unresponsiveness induced by the blood transfusions (Opelz *et al.*, 1981b).

### 1.4.3 Perioperative donor-specific blood transfusion

It is clear from animal models and also some clinical reports that blood transfusion has the potential to improve graft outcome in many cases. However the dangers inherent in random transfusion, as practised in the 1970's, from sensitisation and viral infections such as HIV, hepatitis and cytomegalovirus has made it unacceptable in current clinical practice. Recent reports have shown that in animal models donor-specific blood transfusion given in conjunction with immunosuppression either at the time of the transplant (with FK506: Fabrega *et al.*, 1993) or 24 hours before the transplant (with cyclosporin A: Brunson *et al.*, 1991) may have beneficial effects while avoiding sensitisation. Sensitisation is much less likely due to the close timing of the transfusion and the transplant. Furthermore as the blood transfusion is donor-specific there is not an increased risk of viral infection. A clinical trial of preoperative DSBT plus cyclosporin A reported a reduction in both severe rejection episodes (defined as requiring OKT3 treatment) and in donor-specific reactivity at six months post transplant measured by mixed lymphocyte reaction against stored donor spleen cells (Alexander *et al.*, 1992). This practice is still fairly new and as such is not widely used but is one reason for continuing

research into donor antigen as a means of inducing long-term donor-specific tolerance.

Perioperatively administered DSBT has also recently been shown to produce tolerance in a rat heart model if the immunoglobulin CTLA4-Ig is given 48 hours post transplant (Lin *et al.,* 1994). CTLA4-Ig is an antibody that appears to block activation of lymphocytes and is discussed later (Section 1.6.1).

## 1.5 Experimental models of tolerance induction using donor-specific blood transfusion pretreatment protocols

### 1.5.1 Tolerance

Clinical organ transplantation between genetically disparate individuals currently necessitates non-specific suppression of the recipient's immune system for as long as the graft is present. Even with the best current immunosuppresive therapy, graft rejection remains the major cause of graft loss and drug related morbidity and mortality cause considerable problems for the graft recipient. It would be far superior if a strategy could be developed which allowed an individuals immune system to accept an allograft as though it was "self" tissue and permit a normal immune response to potential pathogens without any requirement for continuing drug therapy. Such a state constitutes transplant tolerance in its truest sense.

Such tolerance was first seen in 1953 when it was shown that the delivery of foreign antigens into the developing immune system of neonatal mice, via the placental circulation, induced specific tolerance in the adult mouse. Skin allografts survived in tolerant animals only if related genetically to the cells in the neonatal transfusion

(Billingham *et al.*, 1953). Attempts to produce a similar state of transplant tolerance in adult animals, with a mature immune system, have also met with a considerable degree of success although this success has not yet been translated into clinical practice.

The main problem, as will be seen later, is that the species and genetic strain as well as the experimental protocol is extremely critical and no therapy has yet proved successful in an outbred population.

### 1.5.2 Donor-specific blood transfusion

Over 25 years ago it was shown that, in some rat models, tolerance to a vascularised allograft followed if the animal was transfused with donor-strain blood prior to transplant (Marquet *et al.*, 1971; Fabre and Morris 1972). This was known as donor-specific blood transfusion (DSBT) and was made possible by the development of inbred strains of rat with defined histocompatibility antigens. The ability of DSBT to prolong graft survival is however highly dependent on the rat strain combination and the type of graft, as well as the nature, dose and timing of the blood transfusion. In some rat strain combinations a single 1ml blood transfusion given 7 days prior to transplant prolongs kidney allograft survival indefinitely while in other strains little or no beneficial effect is seen (Stewart *et al.*, 1985a). Interestingly renal allografts appear to survive longer after donor blood transfusion than heart allografts, while skin allograft survival is not altered to any significant degree (Morris, 1980).

Many groups of workers have investigated which cellular components in the donor blood transfusion are required to produce transplant tolerance (see Table 1.1) and it is now apparent that rather than a specific cell type it is the donor major histocompatibility (MHC) antigens that are critical if tolerance is to be achieved. The demonstrations that tolerance can also be produced using cells from the spleen (Seki *et al.*, 1989) or the liver (Foster *et al.*, 1989) as a source of donor antigen and the elegant studies by Madsen (1988) using syngeneic fibroblasts transfected with donor class I and class II MHC alloantigens to produce prolongation of allograft survival reinforce this idea. Although intact donor cells are not required, the form the donor antigen is presented in is important; water soluble class I MHC does not readily produce tolerance while the same MHC molecules do when presented as insoluble protein micelles (Foster *et al.*, 1989).

DSBT can be viewed as a form of alloantigen pretreatment that stimulates the recipients immune system but renders it specifically unresponsive to the subsequent allograft. It is not a form of immunosuppression as DSBT-treated animals have a more rapid invasion of their grafts by leukocytes and a rapid induction of both class I and class II MHC relative to control animals (Armstrong *et al.*, 1987). Indeed when these graft infiltrating cells from transfused recipients are tested in vitro for specific cytotoxicity it appears to be equal to or greater than that shown by cells extracted from rejecting grafts in non-transfused recipients (Dalman *et al.*, 1987; Armstrong *et al.*, 1987).

Despite the accelerated cellular immune response to the graft following antigen pretreatment the grafts often enjoy prolonged function implying the existence

**Table 1.1**

**Ability of cellular components of allogeneic blood to enhance survival of organ allografts in the rat**

| Cell type | MHC expression | | Enhancement | |
|---|---|---|---|---|
| | Class I | Class II | Yes | No |
| B lymphocytes | + | + | [1-5] | - |
| T lymphocytes | + | - | [4,5] | [1,2,3] |
| $CD4^+$ T cells | + | - | [4,5] | - |
| $CD8^+$ T cells | + | - | - | [4,5] |
| Erythrocytes | + | - | [3,6,7] | [1,2] |
| Platelets | + | - | [8,9] | |

Renal allograft models: References [ 3,4,6,7,8,9 ]
Cardiac allograft models: References [ 1,2,5 ]

References:


1 Lauchart *et al.*, 1980
2 Nagata *et al.*, 1984
3 El-Malik *et al.*, 1984
4 Cranston *et al.*, 1987
5 Oluwole *et al.*, 1989
6 Majoor *et al.*, 1981
7 Wood *et al.*, 1985
8 Batchelor *et al.*, 1977
9 Hibberd and Scott, 1983

of a host regulatory mechanism(s) that can override the rejection mechanism(s) and allow the grafts to survive without any further treatment.

### 1.5.3 Immunosuppressive therapy combined with donor-specific blood transfusion

Evidence that a short course of cyclosporin A (7 days 15mg/kg) at the start of a series of weekly blood transfusions abrogated alloantibody production suggested possible advantages in this combination of therapies (Jones *et al.,* 1988; Cunningham *et al.,* 1988). These studies did not involve organ grafts but several other experimental models have been described using a variety of immunosuppressive strategies used in conjunction with DSBT including, cyclosporin A (Brunson *et al.,* 1991), FK506 (Fabrega *et al.,* 1991), anti-CD4 monoclonal antibody (Pearson *et al.,* 1992) and CTLA4-Ig (Lin *et al.,* 1993). The timing of the blood transfusion(s) and the immunosuppression relative to the transplant vary considerably but all these studies show that the combined strategy is more effective than either blood transfusion or immunosuppression used alone.

As newer, more potent immunosuppressive agents are introduced into clinical practice, the role of remote, random, or simple DSBT to enhance allograft survival will continue to diminish. However, as Fabrega and coworkers (1993) have pointed out these same agents may enhance the benefits of the administration of donor antigen in the form of DSBT's. The view of Starzl and coworkers (1993) that immunosuppressive drugs may lead to tolerance by permitting donor-specific

chimerism is a theoretical explanation for the synergistic use of immunosuppression and DSBT.

## 1.6 Potential mechanisms of unresponsiveness following donor-specific blood transfusion pretreatment

### 1.6.1 T cell anergy

Jenkins and Schwartz (1987) demonstrated that if T cells were cultured with modified syngeneic APC's (spleen cells that had been treated with a cross-linking chemical ECDI) the T cells were subsequently unable to proliferate in response to antigen presented by unmodified APC's. The fact that these T cells proliferated normally when presented with a different antigen on the surface of unmodified APC's showed that the cells were still potentially functional but they had become unresponsive to the original antigen. T cell anergy has been demonstrated in intact adult animals as well as in cloned cell lines (Dallman *et al.*, 1991). This study showed that graft infiltrating cells from alloantigen tolerised animals had an inability to produce IL-2 or proliferate in response to exogenous IL-2 in vitro. However administration of exogenous IL-2 to these animals was able to reverse this anergic state.

The mechanism by which the T cell enters the anergic or unreactive state is not yet fully understood. Two signals are required to fully activate a T cell. The first signal is engagement of the TCR with the peptide antigen plus host MHC molecules (or allogeneic MHC alone) while the second signal is the interaction of one or more

receptors on the T cell with appropriate ligands on the APC. Available evidence suggests that if the second signal is absent or insufficient then the signal arising from engagement of the TCR is a negative signal that effectively blocks further activation (Harding *et al.,* 1992).

One candidate for the second signal is the CD28 molecule on the cell surface of the T cell. This binds to the ligand B7.1/7.2 on the cell surface of APC's. Stimulation of CD28 by B7 when the T cell has been activated via its TCR leads to proliferation and lymphokine gene expression. There is also a further cell surface molecule CTLA-4 which is only seen following activation of the T cell via the TCR and this molecule also binds to B7.1/7.2 but with greater affinity than CD28. The physiological function of this molecule may be to prevent excessive immune responses to transient sources of stimulation (Schwartz, 1995). In terms of transplant rejection CTLA4-Ig is capable of blocking the interaction of CD28 with B7.1/7.2 due to its greater binding affinity and this has been shown to delay cardiac allograft rejection in the rat (Turka *et al.,* 1992), presumably through lack of costimulation leading to a relative deficiency of IL-2 (Linsley *et al.,* 1991).

### 1.6.2 Antibody

Antibody has long been suspected of having a role in the unresponsiveness seen following antigen pretreatment in some experimental models. An early finding was that administration of hyperimmune serum in many rodent models led, not to rapid graft destruction as expected, but instead to the development of specific

unresponsiveness towards the allografts. This phenomenon appeared most pronounced if the alloantiserum was primarily directed against donor class II MHC molecules (Fabre and Morris, 1974). The finding that tolerance following DSBT is associated with high levels of anti-donor class II antibodies (Marshall *et al.,* 1990) and the observation that serum from rat strains which spontaneously accept liver allografts (and will subsequently accept renal allografts of the same strain) contain anti-class II antibodies (Kamada *et al.,* 1986; 1988) is strong evidence that anti-class II antibodies may be tolerogenic. Such alloantibodies have the ability to block donor Fc receptors and this may interfere with the host's ability to mount a response against the graft or act by blocking direct allostimulation by sterically inhibiting antigen presentation (Marshall *et al.,* 1990).

Failure of the host immune system to switch anti-class I from IgM to IgG following transplant is reported to correlate closely with strain dependent variation in the efficacy of DSBT (Wasowska *et al.,* 1992). In this study rat renal allograft survival after DSBT was prolonged only if anti-class I IgG antibody levels remained low after transplantation. This finding fits well with reports that anti-class I antibody may have an important role in acute graft rejection (Oluwole *et al.,* 1989b; Gracie *et al.,* 1990).

Finally it is notable that the presence of anti-idiotypic antibodies following DSBT also correlates with tolerance induction (Ludwin *et al.,* 1986). Although the significance of this finding is not clear these immunoregulatory antibodies may, in principle, contribute to the enhancing effect of blood transfusion on allograft survival

either by directly blocking the TCR of antigen-reactive cells or by binding to the idiotype of alloreactive cells and facilitating their removal from the circulation by antigen-reactive cell opsonisation (ARCO) (Hutchinson, 1980; Bradley, 1991).

### 1.6.3 Clonal deletion

Clonal deletion of alloreactive T cells was an obvious early candidate mechanism to explain the induction of transplantation tolerance. This is undoubtedly the principle mechanism operating in the thymus to prevent the release into the periphery of large numbers of potentially auto-reactive T cells (Kappler *et al.*, 1987). Clonal deletion appears to be a major mechanism underlying the induction of neonatal tolerance (MacDonald *et al.*, 1988) but in several models where tolerance was previously believed to be due exclusively to clonal deletion it is now being suggested that clonal anergy may be partly responsible (Qin *et al.*, 1989).

Clonal deletion/anergy in the periphery may be reinforced if the thymus is incorporated into the treatment protocol. In a study by Sharabi and Sachs (1989) transplantation tolerance was produced by whole body irradiation and monoclonal antibody (Mab) treatment. The combined use of anti-CD4 and anti-CD8 Mabs caused effective depletion of peripheral T cells from the blood and spleen. Interestingly, thymic T cells were not depleted, even though they were coated with Mab. Further thymic irradiation increased the degree of tolerance suggesting that residual T cells were important. In a mouse skin graft model of tolerance induced by intravenous

alloantigen, surgical removal of the thymus 2-4 weeks prior to the experiment, was also shown to extend the period of tolerance produced (Kitagawa *et al.,* 1990).

The role of the thymus in overcoming peripheral tolerance, in addition to its role in deleting auto-reactive T cells, has led to extensive investigation of protocols involving direct injection of donor antigens into the thymus combined with systemic immunosuppression (reviewed by Posselt *et al.,* 1993). The concept behind these experiments is that if developing T cells in the thymus are exposed to graft antigens they may see these antigens as "self" when they meet them subsequently in the periphery. This model proposes that direct involvement of the thymus leads to clonal deletion and is claimed to be analogous to tolerance induction to self antigens (Markmann *et al.,* 1993).

### 1.6.4 Cytokines. The Th1 versus Th2 hypothesis

As discussed earlier the $CD4^+$ T cells can be divided into two functionally distinct sub-classes, Th1 and Th2 cells, based on the spectrum of cytokines they produce (Mosmann and Coffman, 1989). More recent studies have extended this concept and postulated that both $CD4^+$ and CD8 $T^+$ cells can be divided into type 1 and type 2 on this basis (Carter and Dutton, 1996).

Th1 and Th2 cells are mutually antagonistic i.e. IL-2 and IFN-γ promote Th1 and inhibit Th2 whereas IL-4 promotes Th2 and inhibits Th1. It has been postulated that the relative balance between Th1 and Th2 cells may be responsible for whether

an allograft is accepted or rejected. In this hypothesis Th1 cells are assumed to provide help for promoting graft rejection mechanisms while Th2 cells are believed to be involved in tolerance induction and maintenance (Lowry, 1993).

In so far as IL-2 is a Th1 cytokine and reduction in IL-2 levels either pharmacologically, (e.g. cyclosporin A) or by intervention through DSBT, leads to prolongation of graft survival, then depressed Th1 responses are associated with tolerance. The concept that raised Th2 cytokine levels are responsible for tolerance is more controversial. The persistent expression of IL-4 and IL-10 messenger RNA in the spleen and graft of mice (Takeuchi *et al.*, 1992) and rats (Papp *et al.*, 1992) bearing longstanding cardiac allografts has been taken as evidence that these cytokines are producing a tolerogenic effect within the microenvironment of the graft. It is important to note that cells other than T lymphocytes can also produce Th2 cytokines, e.g. eosinophils are able to produce large amounts of IL-4.

Preferential activation of Th2 cells in rats treated with rapamycin has been reported to explain the prolonged survival of cardiac allografts with a selective inhibition of cytotoxic antibody production and normal non-cytotoxic antibody production (Ferraresso *et al.*, 1994). Neonatal tolerance has also been recently reported to be associated with enhanced Th2 and diminished Th1 cytokine levels (Chen and Field, 1995).

It is important to point out that while cytokine release following T cell activation may be vital to the outcome of an allograft following transplantation this does not exclude other mechanisms of graft acceptance. Th1 but not Th2

lymphocytes have been reported to be rendered anergic following in vitro antigen-receptor cross-linking if costimulatory signals are absent (Williams *et al.,* 1990).

### 1.6.5 Suppressor cells including veto cells and infectious tolerance

Antigen pretreatment has been shown in a number of studies to lead to the development of a population of host cells with marked immunosuppressive properties. These cells appear able to prevent rejection of the graft. This is best demonstrated by the ability of T cells from tolerant animals to prevent allograft rejection when adoptively transferred to unmodified or "lightly irradiated" hosts. The first reports of such cells came from a mouse model involving lethal irradiation, syngeneic bone marrow rescue and a heart allograft (Jirsch *et al.,* 1974). Several groups have also reported the presence of such cells following donor antigen pretreatments (Dorsch and Roser, 1977; Bril *et al.,* 1985; Wood *et al., 1985*; Hall, 1985; Hutchinson, 1986). More recently, in a rat heart transplant model, peripheral tolerance seen following a short course of cyclosporin A has been claimed to be due to a transferable radiosensitive antigen-specific suppressor cell (Nisco *et al.,* 1995).

A veto cell, is a cell which, if recognised by an alloreactive cell, kills the cell recognising it. The concept of veto cells was first proposed as a mechanism for the suppression of murine auto-reactive cytotoxic T cell precursors, (Muraoka and Miller, 1980) and subsequently postulated to play a role in transplant tolerance (Fink *et al.,* 1988). Veto cells were later claimed to have a role in the prolongation of renal

allograft survival in a primate model where the recipient animals were pretreated with anti-thymocyte globulin followed by transfusion with donor bone marrow cells after transplantation (Thomas and Carver, 1991). In these animals in vivo anti-donor CTL activity was abolished although production of anti-donor alloantibody was merely delayed, suggesting that a split state of tolerance had been produced. The authors postulated that the donor bone marrow contained a population of cells that were effectively able to prevent (or veto) activation of the host CTL's. These donor cells may be able to interact with the host CTL's directly rather than by inducing the production of more traditional host derived suppressor cells (Martin and Miller, 1989).

The concept of infectious tolerance is an old idea and was first proposed by Medawar in the 1950's (Billingham *et al.,* 1953). They hypothesised that T cells, which are suppressed themselves, are able to confer their unresponsiveness to other naive immune cells. A recent report by Qin and coworkers (1993) showed that they were able to transfer tolerance by taking immune leukocytes from $CD2^+$ transgenic mice ($hCD2^+$/CBA), tolerant of mH antigens, and transferring them to a secondary host. This secondary host then became tolerant of the same mH antigens but only after they had been allowed to circulate in the second host for over 14 days. If they were eliminated before 14 days, in this case by administration of an anti-human CD2 Mab, then tolerance transfer was abrogated.

Suppressor cells and veto cells may exert their immunological effects via the cytokine network discussed in the previous section. Many of the classical studies of

these cells were carried out before the ability to measure and quantify cytokines became available.

## 1.7 Aims of this study

DSBT has been known to have beneficial effects on organ transplant survival in both clinical practice and in experimental models for over twenty years. However in current clinical practice blood transfusion is no longer used routinely, partly due to the significant numbers of patients who produce alloantibodies against future donor antigens rendering them unsuitable for transplantation (Burlingham *et al.,* 1988) and also partly due to the improved results seen with modern drug-induced immunosuppression. There are many drawbacks to current immunosuppressive methods in terms of the non-specific nature of their actions and toxicity. The major cause of graft loss remains rejection and cancer rates are very significantly raised in patients following organ transplantation. Both of these factors demonstrate the continuing need for a method of producing genuine tolerance to a graft rather than merely suppressing rejection.

The mechanisms that operate to produce both the beneficial and the detrimental effects of blood transfusion remain elusive reflecting the current uncertainty about the genetic and immunological mechanisms involved, although our understanding of these is rapidly increasing. The rat model allows the immune responses to defined histocompatibility differences to be examined. In addition, the

renal vessels in the rat are of a size that, with the aid of the dissecting microscope, renal transplantation can be carried out with a high degree of technical success. The work in this study has attempted to re-examine the "blood transfusion effect" and develop potential new strategies that could be used in current clinical transplantation. In addition this study has tried to use some of the recent developments in our ability to measure cytokine levels via the polymerase chain reaction to look at possible mechanisms behind the effect. Whilst care must be taken in extrapolating the findings in an animal model to the clinical situation any novel strategy for inducing tolerance will require such studies in the first instance before being applied to transplant recipients.

# CHAPTER TWO

# Materials and Methods

## 2.1 Animals

Inbred male rats were used throughout this work. DA, Lewis, and PVG rats were purchased as required from Harlan Olac Ltd. (Bicester, Oxon, U.K.) and used at 8 to 16 weeks of age (180 to 280g). PVG R8 and PVG $RT1^U$ rats were bred in the University of Glasgow animal facility from breeding pairs supplied by Harlan Olac. The MHC haplotype of the rat strains used is shown in Table 2.1.

Animals were housed in the animal facility of the University Department of Surgery, Western Infirmary, Glasgow. They were maintained under standard conditions with free access to fresh water and standard rat food.

## 2.2 Surgical procedures

### 2.2.1 Anaesthesia

All surgical procedures were performed under continuous inhalational anaesthesia using halothane and oxygen. Animals were not starved and no premedication was given prior to surgery.

Anaesthesia was induced by placing the animals in a perspex container filled with 4% halothane and oxygen. After shaving the operative site, anaesthesia was maintained by 2% halothane and oxygen delivered by an anaesthetic nozzle placed over the animals nose and mouth.

During transplant operations the recipient animal was placed on a heater board

**Table 2.1 MHC haplotype of rats used in this study**

| Rat Strain | RT 1 Region | | | |
|---|---|---|---|---|
| | A | B | D | C |
| DA ($RT1^a$) | a | a | a | a |
| LEWIS ($RT1^l$) | l | l | l | l |
| PVG ($RT1^c$) | c | c | c | c |
| PVG. R8 | a | u | u | u |
| PVG ($RT1^U$) | u | u | u | u |

(10 watt power) to maintain body temperature but this was switched off while the vascular anastomosises were fashioned to minimise potential warm ischaemic injury to the transplanted organ. Following completion of the operation the animals were placed in an incubator maintained at 37°C and observed until they were fully conscious. No post operative analgesia was required and the animals were able to return immediately to their preoperative diet.

### 2.2.2 Sutures

All sutures used during this work were purchased from Ethicon Ltd. (Edinburgh, U.K.). Types and sizes of sutures appear in the descriptions of the operations that they were used in.

### 2.2.3 Renal transplantation

All renal transplants were performed using the basic technique described by Fabre (1971) but with slight modification. The original bladder to bladder anastomosis was replaced by an end-to-end anastomosis of the donor and recipient ureters.

The donor rats abdomen was opened via a midline incision from xiphisternum to pubis and a self retaining retractor inserted. The intestines were wrapped loosely in a saline moistened swab and displaced to the right to expose the left kidney. If required the left lobe of the liver was also displaced from the operative field by a moist swab. The inferior vena cava was cleared of overlying fat by blunt dissection and the left

ureter divided transversely approximately 1cm from the renal pelvis . The renal artery and vein were then mobilised and side branches ligated with 8/0 silk and divided. Any remaining perinephric fat was divided to leave the kidney suspended on its vascular pedicle. A moist swab was used to cover the opened abdomen of the donor animal and attention turned to the recipient animal.

The abdomen of the recipient was opened through a midline incision and the intestines and liver displaced from around the left kidney. The left ureter was then divided just below the renal pelvis and the renal artery and vein prepared as described for the donor animal. In approximately 50% of the PVG animals used as graft recipients a double renal artery was encountered. In these cases the lower artery was ligated and the upper renal artery used for the transplant anastomosis. Fortunately double renal arteries were rarely encountered in donor animals. Non-traumatic vascular clips (Scovill-Lewis) were then placed separately across the renal artery and renal vein close to the aorta and vena cava respectively. The renal artery and renal vein were then divided close to the hilum and the recipients left kidney excised.

The swab covering the donor kidney was then removed and, after confirming that the kidney was well perfused and had not been damaged by the earlier mobilisation, 100 units of heparin were injected directly into the inferior vena cava via a 25 gauge needle which was left in situ. Two minutes were allowed for the heparin to fully circulate before non-traumatic vascular clips were applied to the renal artery close to the aorta and, after the kidney had blanched, to the renal vein close to the vena cava. The donor renal artery and vein were then divided next to the vascular clamps and the

excised kidney placed in ice cold saline. Gentle external pressure was applied to flush excess blood out of the kidney.

The donor kidney was placed in the fossa produced by the removal of the recipient animals left kidney. Vascular anastomosises were then fashioned between the donor and recipients renal arteries and veins using interrupted 10/0 Ethilon sutures. Normally 6 sutures were used for the artery and 12-14 sutures for the vein. During these anastomosises ice-cold saline was infused intermittently into the abdominal cavity. This helped to reduce warm ischaemic damage and aided separation of the renal vein walls thereby allowing accurate placement of vascular sutures. Small pieces of Surgicell (Johnson & Johnson Ltd., Maidenhead, U.K.) were placed around the vascular anastomosises and the vascular clips removed, first from the renal vein and then from the renal artery. During this procedure gentle pressure was applied to the anastamosises using cotton buds (Johnson & Johnson Ltd.) to assist haemostasis. In the vast majority of cases (> 95%), the transplanted kidney displayed rapid and complete reperfusion and urine was seen to flow from the cut ureter. The heater board was then switched back on. The respective ends of the donor and recipients ureters were trimmed to the appropriate lengths and joined end to end. Satisfactory ureteric anastomosis was usually achieved with 4 interrupted 10/0 Ethilon sutures.

The abdomen was irrigated with warm saline, the bowels replaced, and the abdomen then closed using two layers of continuous 3/0 chromic catgut. The warm ischaemic period was consistently between 20-25 minutes and the overall incidence of vascular anastomotic failure was <5%.

### 2.2.4 Contralateral nephrectomy

In survival experiments, unless otherwise stated, the native right kidney was removed on day 7 post transplant and survival of the animal after this time was dependent on adequate function of the transplanted kidney. Contralateral nephrectomy was normally carried out via a 2cm right subcostal incision. After delivering the kidney into the wound, the renal capsule was incised and reflected to expose the kidney extracapsularly. A clamp was then placed across the renal hilum, a single 2/0 silk ligature placed around the renal pedicle and the kidney excised. The nephrectomy wound was closed using two layers of continuous plain 3/0 catgut.

### 2.2.5 Cervical cardiac transplantation

Donor operation

The abdomen of the donor animal was opened and 100 units of heparin injected directly into the inferior vena cava. The heparin was allowed to circulate for one minute before the abdominal aorta and inferior vena cava were transected to exsanguinate the animal. The chest was then opened taking care to avoid damaging the heart. Cardiac arrest was achieved by placing crushed ice directly on the heart surface. The thymus was then excised to enable the great vessels to be visualised. 5/0 silk ligatures were placed around the left and right superior vena cavae as well as the inferior vena cava and the veins transected on the proximal side of the tie. The donor

aorta and pulmonary arteries were then transected immediately proximal to their first branches. Finally a 5/0 silk ligature was passed behind the heart and tied firmly to occlude any remaining vessels. All vessels entering or leaving the heart were thus ligated with the exception of the aorta and the pulmonary trunk. The heart was then excised from the chest and placed in a petri dish of ice cold saline.

Recipient operation

A 3cm longitudinal skin incision was made in the neck of the recipient animal to the right of the midline. The right external jugular vein was identified immediately before it passed underneath the clavicle and mobilised over approximately a 1cm length. Side branches were ligated with 8/0 silk and a non-traumatic vascular clamp applied immediately above the clavicle.The cranial end of the mobilised vein was then ligated with 5/0 silk and the vein divided just below the ligature. The sternomastoid and the strap muscles were retracted laterally and medially respectively and held with stay sutures to expose the right internal carotid artery. This was freed from its investing sheath and ligated with 5/0 silk immediately below its bifurcation. A non-traumatic vascular clamp was then placed across the artery low in the neck and the vessel divided obliquely (in order to increase the diameter for the anastomosis) immediately below the ligature.

The donor heart was placed in the neck such that the donor aorta and pulmonary artery were aligned with the recipient carotid artery and external jugular

vein respectively and the vessels anastomosed in that order using interrupted 10/0 Ethilon sutures. Surgicell was placed around the completed anastomosises, the vascular clamps removed and gentle pressure applied over the arterial anastomosis using cotton buds until any bleeding had stopped. The transplanted heart rapidly perfused and started to beat spontaneously, in sinus rhythm, after a brief initial period of fibrillation. The skin was closed over the heart using interrupted 3/0 plain catgut sutures.

Heart grafts were palpated daily and rejection taken as complete cessation of palpable impulses. Heart grafts which failed to beat during the first 24 hours after grafting (<5% of total grafts) were assumed to be technical failures and disregarded.

In later experiments a technical modification was made. Instead of the donor aorta being anastomosed directly to the recipients common carotid artery, the aorta was ligated just distal to its first branch and the smaller branch anastomosed to the recipient common carotid artery. Because the donor and recipient vessels were of similar size the vascular anastomosis was technically less demanding. This modification increased the operative survival rate but did not alter the fate of the grafts in any other way.

### 2.2.6 Skin grafting

The abdomen of the donor animal was shaved before the abdominal skin was excised in full thickness strips 1.5cm wide. All the subcutaneous fat was carefully

removed and the strips trimmed to produce 1.5cm x 1.5cm grafts which were placed into chilled saline before application.

The recipient animal was shaved between its shoulder blades and a full thickness disc of skin 1.5cm in diameter was excised leaving the underlying fascial layer intact. The donor skin was then sutured into the resulting defect using interrupted 4/0 catgut sutures. The skin graft site was protected with occlusive bandages for 7 days and thereafter inspected daily for sign of graft rejection. Rejection was defined as >80% destruction of the graft.

### 2.2.7 Thymectomy

A 3cm midline skin incision was made over the neck and upper part of the sternum. After identifying the sternomastoid muscles the manubrium was split in the midline. With the aid of an operating microscope, the pretracheal strap muscles were separated to reveal the upper aspect of the thymus gland. Exposure of the upper pole of the thymus was best achieved using an assistant to retract the overlying tissues. The upper pole was then mobilised and with steady retraction on the thymus gland combined with gentle separation of surrounding fat and blood vessels the entire gland was removed, usually intact. The manubrium was then reapproximated with a single 5/0 silk suture and the skin closed with continuous 3/0 catgut. An overturned 2ml syringe was placed over the animals nose and mouth to allow the application of positive pressure air to the animals lungs and ensure full inflation. Mortality as a consequence of this procedure was <5%.

## 2.3 Cells and tissues

### 2.3.1 Blood transfusion

Collection of large volumes of donor blood for transfusion was via direct cardiac puncture, of animals under terminal anaesthesia, with a 21 gauge needle on a 10ml heparinised syringe. The blood was then transferred to a heparinised vacutainer. Transfusion was performed as soon as possible after collection using a 23 gauge needle via the dorsal penile vein of the recipient.

### 2.3.2 Blood sampling

Samples of up to 1ml were obtained by transecting the tip of the animals tail which was then cauterised with potassium permanganate after sufficient blood had been collected. These samples were allowed to clot prior to separation of the serum by centrifugation for 5min at 2500rpm and storage at -20°C for future analysis.

### 2.3.3 Lymph node cells

Cervical and mesenteric lymph nodes were removed from individual rats and pooled prior to use. The nodes were forced through a fine stainless steel mesh using a syringe plunger into 10ml of washing medium*. These cells were centrifuged at

1300rpm for 5min and resuspended in 5ml of the same medium. This was carried out on two occasions to remove non-cellular contamination. The number of viable cells was counted using the trypan blue exclusion test; cells were resuspended in 1ml of phosphate buffered saline (PBS) at the desired concentration before being transfused into the recipient animal.

*Washing medium comprised Hanks buffered salt solution (HBSS) + 10mM Hepes + 2% heat inactivated foetal calf serum (FCS) (all from Life Technologies Ltd., Paisley, U.K).

### 2.3.4 Splenocytes

Spleens were placed in a petri dish with 10ml of washing medium (Section 2.3.3) and gently teased apart using sterile plastic forceps. The cell suspension thus produced was transferred to a plastic test tube and the debris allowed to settle out. The resulting cell suspension was spun at 1300rpm for 10min (room temperature). The pellet was gently resuspended and the erythrocytes lysed by the addition of 5ml of distilled water followed closely by 5ml of double strength saline. The cells were spun at 1300rpm and the pellet resuspended in washing medium on two further occasions to remove lysed cells and other debris. The viable cells were then resuspended in PBS, if they were to be returned to an animal, or in complete medium* if they were used for in vitro experiments.

*Complete medium comprised RPMI 1640 + 10mM Hepes + 10% foetal calf serum + 2mM glutamine + penicillin/streptomycin (all from Life Technologies Ltd.).

### 2.3.5 Concanavalin A stimulated lymphoblasts

Splenocytes from donor and third-party rat strains were stimulated with concanavalin A (Con A) to produce lymphoblasts for use as targets in chromium ($^{51}$ Cr) release assays.

Splenocytes at a concentration of $2.0x10^6$cells/ml in complete medium (Section 2.3.4) containing $2x10^{-5}$M 2-mercaptoethanol were incubated with 5mg/ml of Con A (Sigma-Aldrich Company Ltd., Poole, Dorset, U.K.) in 25ml tissue culture flasks (Nundon, Life Technologies Ltd.) for 48-72 hours at $37^0$C in a humidified 5% $CO_2$ incubator. Lymphoblasts were washed once and counted prior to use.

## 2.4 Monoclonal antibodies

MRC OX8 was used for the in vivo depletion of the $CD8^+$ subset of T lymphocytes. This is an IgG1 antibody specific for $CD8^+$ T cells and also the majority of natural killer cells (Gillman *et al.*, 1982; Dallman *et al.*, 1982; Cantrell *et al.*, 1982).

MRC OX8 was produced in mouse ascites, following intraperitoneal injection of appropriate myeloma hybridoma cells (ECACC, Porton Down, Salisbury, Wiltshire U.K.) into pristane (Sigma-Aldrich Co. Ltd.) primed (DBA/2 x BALB/c)F1 mice (Harlan Olac Ltd.). The immunoglobulin content of the resultant ascitic fluid was quantified using anti-mouse IgG1 immunodiffusion plates and known mouse Ig standards (Serotec Ltd., Oxford, U.K.). Antibody was diluted in PBS as required,

aliquoted and stored at -20$^0$C until used. It was administered to experimental animals by intraperitoneal injection immediately after thawing. Several mouse monoclonal antibodies (mAb) were used for in vitro experiments to label rat leukocytes as shown in Table 2.2.

## 2.5 Histology

### 2.5.1 Cryostat sections

Samples of freshly harvested renal allografts were embedded in OCT compound (Tissue-Tek, BDH Ltd., Poole, Dorset, U.K.), immediately snap frozen in liquid nitrogen and stored at -70$^0$C. Cryostat sections (5μm) were then cut at -20$^0$C onto gelatinised multispot slides (C.A. Hendley, Essex, U.K.).

### 2.5.2 Immunoperoxidase staining

Sections were fixed in acetone (BDH Ltd.) at room temperature for 10min, and washed in PBS. Excess moisture was wiped carefully from each slide and 50μl of primary antibody, appropriately diluted, was applied to each section. Slides were then incubated for 45min at room temperature in a humidified chamber before being washed three times in PBS. A second antibody was then applied. This consisted of peroxidase-

**Table 2.2 Monoclonal antibodies used for in vitro experiments**

| mAb | Specificity | Reference |
|---|---|---|
| **MRC OX1** | Leukocyte common antigen (LCA) | 1 |
| **ED1** | Most tissue macrophages, monocytes and dendritic cells | 2 |
| **R73** | Constant determinant of the rat TCR-α/β | 3 |
| **W3/25** | $CD4^+$ T lymphocytes and some macrophages | 4 |
| **MRC OX39** | IL-2 receptor α chain | 5 |
| **MRC OX21** | Human C3b inactivator | 6 |

All of the above mAb's were obtained from Serotec Ltd., Oxford, U.K.

References


1 Sunderland *et al.*, (1979).

2 Dijkstra *et al.*, (1985).

3 Hunig *et al.*, (1989).

4 Mason *et al.*, (1983).

5 Patterson *et al.*, (1987).

6 Hsiung *et al.*, (1982).

conjugated rabbit anti-mouse Ig (Dako Ltd., High Wycombe, Bucks, U.K.) with the addition of 10% normal rat serum to absorb any cross-reactivity. After a further 30min incubation at room temperature the slides were washed three times in PBS and the substrate, 0.6mg/ml 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Co. Ltd.) plus 0.01% hydrogen peroxide, applied for 5min. The sections were then lightly counterstained with Harris's haematoxylin, dehydrated using increasing alcohol concentrations and cleared in xylene before finally being mounted in DPX (BDH Ltd.) for microscopic examination.

### 2.5.3 Morphometric analysis of cellular infiltrate

The area of each immunoperoxidase labelled tissue section infiltrated by leukocytes of a particular phenotype was determined by morphometric analysis using a point counting technique (McWhinnie *et al.*, 1986). Sections were examined at a magnification of x400 in the presence of a microscopic eyepiece graticule bearing a squared grid with 745 intersections. For each of ten adjacent high power fields, the number of positively stained cells superimposed by an intersection was counted and the percentage area of each section occupied by cells of a particular phenotype was calculated as:

$$100 \times \frac{\text{number of positive cells under grid intersections}}{\text{total number of grid intersections}}$$

## 2.6 Functional assays

### 2.6.1 Complement dependent cytotoxicity assay

Serum samples were tested for the presence of cytotoxic alloantibody in a complement dependent cytotoxicity assay essentially as described previously (Winnearls *et al.*, 1979). Sera was heat inactivated at $56^0$C for 30min, centrifuged briefly to remove protein aggregates and then serially diluted in RPMI/5% FCS/10mM Hepes. 50µl of each dilution was then mixed with $5x10^4$ $^{51}$Cr-labelled Con A-transformed splenic blasts as targets (prepared from donor and third-party splenocytes as described previously in Section 2.3.5). Following incubation for 30min at room temperature, 100µl of freshly reconstituted guinea pig complement (Sera-Lab., Sussex, U.K.) diluted 1:5, in RPMI/5% FCS/10mM Hepes, was added and the cells incubated for a further 1h at room temperature. Finally 100µl of the supernatant was removed and counted in a gamma counter (LKB Wallac, Milton Keynes, U.K.). Target cells were also incubated with medium alone to determine spontaneous release and with 10% Triton X-100 (Sigma-Aldrich Co. Ltd.) to determine maximum release. A serum sample with known high cytotoxic alloantibody level was also tested in each assay as a positive control. The % specific $^{51}$Cr release was calculated by the formula:

$$\frac{\text{Experimental Release - Spontaneous Release}}{\text{Maximum Release - Spontaneous Release}} \times 100$$

Antibody titre was expressed as the lowest dilution that gave >20% specific killing.

### 2.6.2 The mixed lymphocyte reaction (MLR)

LNC's were used in allogeneic one-way mixed lymphocyte reactions (MLR's) to assess the degree of long-term donor-specific tolerance following DSBT (donor-specific blood transfusion) and transplantation. LNC's were used in preference to splenocytes since early experiments indicated the superior proliferative properties of these cells.

LNC's were prepared and resuspended at a final concentration of $8.0x10^6$cells/ml in complete medium (Section 2.3.4) containing $2x10^{-5}$M 2-mercaptoethanol and 5% normal rat serum. Irradiated (2000rads) donor-strain splenocytes ($8.0 \times 10^6$/ml in complete medium) were used as stimulator cells.

$4.0x10^5$ responder and stimulator cells were added to each well of a 96 well sterile round-bottomed microtitre plate (Nunclon, Life Technologies Ltd.), in triplicate, and incubated at $37^0$C in 5% $CO_2$ for 3, 4 or 5 days. Each well was pulsed with $^3$H thymidine (1μCi/well) for 16-18h prior to harvesting using a Dynatech Autowash Automatic Cell Harvester. Samples were then analysed in a liquid scintillation counter (LKB Wallac. Milton Keynes, U.K.).

## 2.7 Analysis of cytokine mRNA

### 2.7.1 Total RNA extraction

All tissues destined for RNA extraction were excised, weighed and immediately snap frozen in liquid nitrogen prior to storage at -70°C.

TRIzol (Life Technologies Ltd.) was employed for RNA extraction. Tissues were homogenised directly in TRIzol (1ml/100mg tissue) using a Polytron homogeniser. The homogenised samples were incubated (5min, room temperature) to permit complete dissociation of nucleoprotein complexes. Chloroform (0.2ml/1ml TRIzol) was then added and the samples vortexed prior to incubation (3min, room temperature). Samples were then centrifuged (12000*g*, 15min, 4°C) and the upper aqueous phase removed to a fresh tube.

RNA was precipitated from the aqueous phase by mixing with isopropanol (0.5ml/1ml TRIzol used for the initial homogenisation). Samples were incubated (10min, room temperature) and then centrifuged (12000*g*, 10min, 4°C) producing a gel-like pellet of RNA on the side and bottom of the tube. The RNA pellet was washed in 75% ethanol (1ml) and spun (7500*g*, 5min, 4°C). Having decanted the supernatant the pellet was air-dried. Solubilisation of the RNA was achieved by resuspending the pellet in DEPC-$H_2O$ and incubating (10min, 55-60°C).

The approximate quantity and purity of total RNA were determined by $OD_{260}$ and $OD_{260/280}$ readings.

### 2.7.2 First strand cDNA synthesis

First strand cDNA synthesis was performed using a Perkin Elmer DNA Thermal Cycler 480. 10μg of total RNA was primed with 1μg (2μl) of oligo dT (Life Technologies Ltd.) for 10min at 70°C. The mRNA was reverse transcribed in a 42μl reaction volume with 400u (2μl) of Superscript RNase H reverse transcriptase (Life Technologies Ltd.) for 1h at 37°C. The enzyme was then heat-inactivated by incubation for 5min at 90°C.

### 2.7.3 The polymerase chain reaction (PCR)

For PCR, 1μl of cDNA was amplified in a 25μl reaction volume containing 0.1μl Taq DNA polymerase, 0.4μl 10mM dNTP mix, 2.5μl 10x reaction buffer (10mM Tris-HCl pH 8.3, 1.5mM $MgCl_2$ , 50mM KCl)[all from Boehringer-Mannheim, Lewes, East Sussex, U.K.], 2.5μl each of sense and anti-sense primers (0.2μM final concentration [except β-actin, 0.1μM]), and DEPC-$H_2O$. A higher $MgCl_2$ concentration (2.5mM) was used when amplifying IL-4 message. Oligonucleotide sequences for sense and anti-sense primers are detailed in Table 2.3.

PCR was performed using a Perkin Elmer Gene Amp PCR System 9600. An initial cycle at 94°C for 5min was followed by multiple cycles of 94°C for 30s, 45-65°C for 30s and 72°C for 30s and concluded with a final cycle at 72°C for 5min. During the exponential phase of amplification PCR product was sampled at three - or

five - cycle intervals and analysed by electrophoresis on a 1.5% agarose gel and then stained with ethidium bromide. cDNA prepared from Con A-stimulated rat lymphoblasts served as positive control. Each PCR procedure also included negative controls in which cDNA and primer were omitted independently from the reaction mixture.

The identity of the PCR product was confirmed from the predicted size on gel electrophoresis and was further verified by Southern blot analysis using digoxygenin-ddUTP labeled (Boehringer-Mannheim) specific internal oligonucleotide probes selected from published gene sequences (Table 2.3).

The integrity of RNA preparation and reverse transcription was confirmed for each tissue by including a PCR for β-actin using three concentrations of cDNA (neat, 1/10 and 1/100) each amplified for 15, 20 and 25 cycles. Tissue cDNA's which did not generate the characteristic electrophoretic pattern for β-actin were prepared afresh prior to cytokine analysis. For all tissues each cytokine PCR was performed at least twice to confirm the electrophoretic pattern.

**Table 2.3 Oligonucleotide sequences for PCR primers**

| Cytokine or receptor | Oligonucleotide sequences-sense primer (s) anti-sense primer (a) and internal probe (p) | Product size (base pairs) | Annealing Temp. (°C) | Ref* |
|---|---|---|---|---|
| IL-2 | s 5' CATGTACAGCATGCAGCTCGCATCC 3' | 409 | 60 | (1) |
| IL-2 | a 5' CCACCACAGTTGCTGGCTCATCATC 3' | | | |
| IL-2 | p 5' TTACAGGTGCTCCTGAGAGG 3' | | | |
| IL-4 | s 5' TGATGGGTCTCAGCCCCCACCTTGC 3' | 377 | 60 | (2) |
| IL-4 | a 5' CTTTCAGTGTTGTGAGCGTGGACTC 3' | | | |
| IL-4 | p 5' GACTCCATGCACCGAGATGT 3' | | | |
| IL-10 | s 5' GTGAAGACTTTCTTTCAAA 3' | 372 | 45 | (3) |
| IL-10 | a 5' TGATGAAGATGTCAAACTC 3' | | | |
| IL-10 | p 5' CTGAGGCGCTGTCATCGATT 3' | | | |
| IFN-γ | s 5' ATGAGTGCTACACGCCGCGTCTTGG 3' | 405 | 60 | (4) |
| IFN-γ | a 5' GAGTTCATTGACAGCTTTGTGCTGG 3' | | | |
| IFN-γ | p 5' AGCATGGATGCTATGGAAGG 3' | | | |
| β-actin | s 5' ATGCCATCCTGCGTCTGGACCTGGC 3' | 607 | 60 | (5) |
| β-actin | a 5' AGCATTTGCGGTGCACGATGGAGGG 3' | | | |
| β-actin | p 5' AGCAAGAGAGGTATCCT 3' | | | |
| IL-2Rα | s 5' GTGGGGAGATAAGGTGGACGCAT 3' | 411 | 65 | (6) |
| IL-2Rα | a 5' GATCGAAAGGAGACAGGCACCC 3' | | | |
| IL-2Rα | p 5' TATCAGGTAGCAGTGGCCAG 3' | | | |
| IL-2Rβ | s 5' TACTGGTCCTCGGCTGCTTCTTTG 3' | 493 | 65 | (6) |
| IL-2Rβ | a 5' GTGAAAGGCAGCAGAGGTGGGA 3' | | | |
| IL-2Rβ | p 5'GACCATGCAGATGCTCCTGT 3' | | | |

* References


( 1 ) McKnight *et al.*, (1989).

( 2 ) McKnight *et al.*, (1991).

( 3 ) Feng *et al.*, (1993).

( 4 ) Dijkema *et al.*, (1985).

( 5 ) Nudel *et al.*, (1983).

( 6 ) Page and Dallman (1991).

# CHAPTER THREE

## Preliminary studies of donor-specific blood transfusion induced tolerance to renal allografts in a low responder rat strain combination

## 3.1 Introduction to the DA into PVG strain combination

The ability of donor-specific blood transfusion (DSBT), in some experimental transplant rodent models, to lead to prolonged allograft survival has been recognised since the early 1970's. This phenomenon is however markedly strain-dependent such that while no effect is seen in some strain combinations in others permanent graft acceptance follows (Fabre and Morris, 1972). In our early studies we sought to re-establish a previously published rat model (Fabre and Morris, 1972) where DSBT is effective in producing long-term graft survival. This model was then used in studies designed to characterise the nature of this effect.

The initial rat strain combination used in this work was DA (donor) into PVG (recipient). This combination has been widely used in the past, both in our own laboratory and elsewhere, as a model where a single DSBT administered 7 days prior to transplant produces prolonged allograft survival. Several groups have reported indefinite survival of renal allografts (Fabre and Morris, 1972; Quigley *et al.*, 1988) while for cardiac allografts survival is prolonged to ~20 days in most cases with a small number of grafts (20-30%) functioning for >100 days (Jenkins and Woodruff, 1971).

The phenotype and function of the cells infiltrating renal allografts, either enhanced following DSBT or undergoing unmodified rejection, in this strain combination is already well characterised; however, the exact mechanism of enhancement remains unclear and requires further study. What is most striking from earlier studies is the apparent similarities in the cells infiltrating rejecting and non-

rejecting grafts. Armstrong and coworkers (1987) showed that, in both cases, there was a heterogeneous mononuclear cell infiltrate that was composed of T cells, B cells, macrophages and natural killer cells. They also showed strong induction of both class I and class II MHC antigens in rejecting and non-rejecting grafts. The major difference found was the slightly accelerated time course of infiltration and MHC upregulation in the blood transfused animals (Milton *et al.,* 1986). Similarly when graft infiltrating cells (GIC's) were harvested from the transplanted organ in these animals and tested in vitro for both specific and non-specific cytotoxicity no differences were apparent. Wood and colleagues (1989) also found the cytotoxicity of GIC's to be similar in both groups when tested in vitro. Adoptive transfer of GIC's (harvested on day 5 post transplant) to fresh DSBT-treated animals (day 1 post transplant) indicated that GIC's from tolerant grafts were functionally incapable of causing rejection whilst those from rejecting grafts were able to cause rejection despite DSBT-pretreatment of the second animal. These results suggest that in vitro tests may not reflect the complex interactions that can occur in vivo. For this reason much of the current study has examined the nature of the tolerance following DSBT using in vivo methods where possible.

In preliminary experiments we first sought to develop the microsurgical skills required for the renal transplant operation and used a conventional immunosuppressant drug, cyclosporin A, to prevent rejection. Once a high (>90%) rate of survival and graft acceptance was achieved we then attempted to confirm that i.v. administration of donor blood, 7 days prior to transplant, also led to long-term

graft survival and that this was a donor-specific effect as reported previously. Cyclosporin A was chosen, for these initial studies, as it is widely used in clinical practice and can be given in a convenient manner as a once-daily oral dose.

## 3.2 Results

### 3.2.1 Establishing the renal transplant operation using cyclosporin A immunosuppression

In order to establish the renal transplant operation a group of ten PVG rats were transplanted with a DA kidney and treated with cyclosporin A at a dose of 10mg/kg/day for ten days. Dosing was by oral gavage and was started immediately on completion of the transplant.

Cyclosporin A-treated rats showed dramatically enhanced survival relative to control animals (Figure 3.1). When sacrificed at 100 days for histological examination of their renal allograft 9/10 animals remained in good health. The renal function of these rats (serum urea and creatinine) was also examined sequentially during this experiment. Most rats showed a small (<5%) and transient rise in urea and creatinine levels in the early days following the contralateral nephrectomy (Figures 3.2 & 3.3). Possible reasons for this brief rise include non-specific damage due to ischaemia at the time of transplantation, or transient self-limiting rejection, or even simply the effect of the animal becoming dependent on a single kidney. Renal

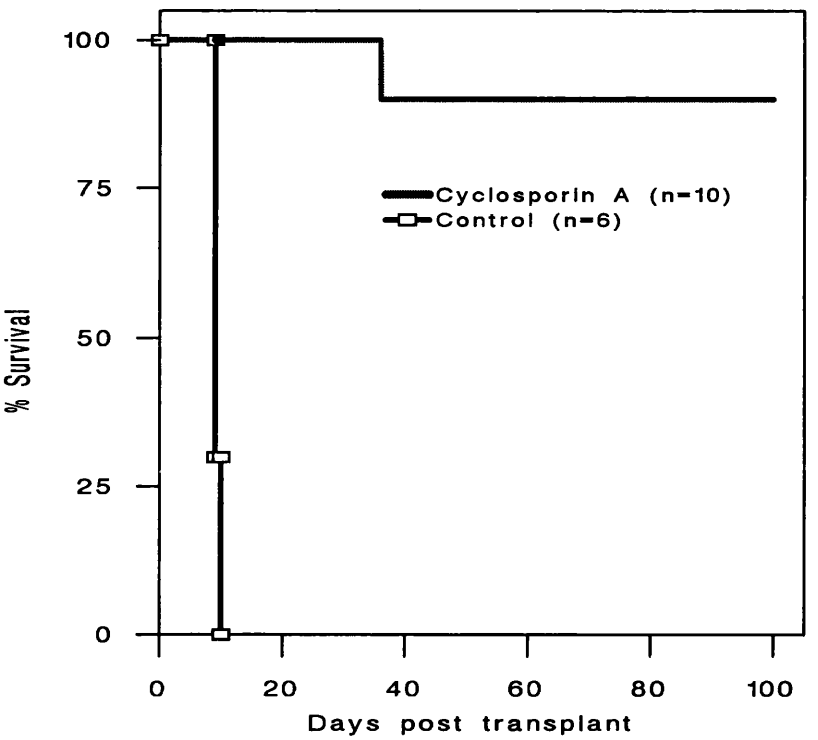



**Figure 3.1 Survival of PVG rats transplanted with DA kidneys and treated with cyclosporin A**

Cyclosporin A, dissolved in olive oil, was administered (10mg/kg/day) by oral gavage for ten days. The first dose was administered immediately on completion of the transplant. Control rats received olive oil only. Contralateral nephrectomy was carried out 7 days post transplant in all cases.

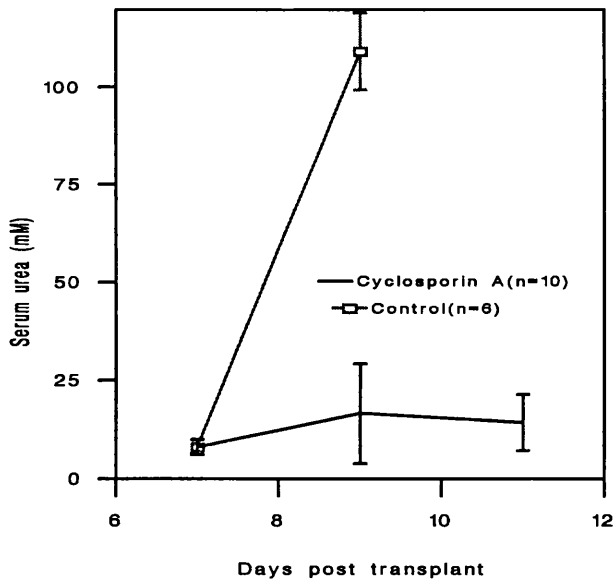



**Figure 3.2** **Serum urea levels of cyclosporin A-treated PVG rats transplanted with DA kidneys**

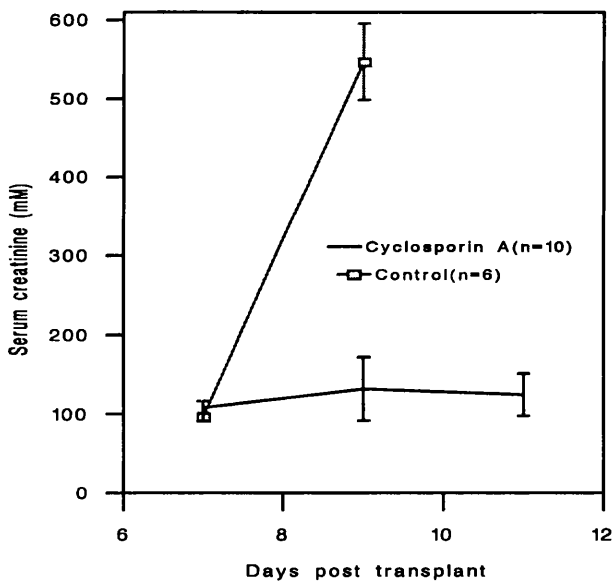



**Figure 3.3** **Serum creatinine levels of cyclosporin A-treated PVG rats transplanted with DA kidneys**

Cyclosporin A, dissolved in olive oil, was administered (10mg/kg/day) by oral gavage for ten days. The first dose was administered immediately on completion of the transplant. Control rats received olive oil only. Contralateral nephrectomy was carried out 7 days post transplant in all cases. Serum urea (Fig 3.2) and serum creatinine (Fig 3.3) were measured on days 7, 9 and 11 post transplant. All values are plotted as mean +/- SD.

function was then monitored until the end of the experiment (day 100). In 8/10 rats normal values were recorded at all points measured (serum urea <15mM: serum creatinine <120mM). One rat died of renal failure at day 36 and the other exhibited a small rise in urea (27mM) and creatinine (145mM) at day 100.

The prolonged survival seen after stopping treatment with cyclosporin A is interesting and has been documented previously in a variety of rat models (Morris, 1981; Nisco *et al.,* 1995). Histology of the surviving grafts at day 100 showed focal perivascular infiltration and cellular damage in keeping with low-grade rejection at a level that did not measurably alter renal function.

The prolonged survival achieved indicated that the renal transplant operation was sufficiently reproducible to allow investigation of any immunologically protective effect of DSBT.

### 3.2.2 Donor-specific blood transfusion and renal allograft rejection in the DA into PVG rat strain combination

Having demonstrated that the renal transplant operation was technically sound the next objective was to confirm that i.v. administration of 1ml of donor-strain whole blood 7 days prior to transplant led to long-term graft survival and that this effect was donor-specific.

Unmodified rejection, in this model, was shown to be rapid with no rats surviving longer than 10 days (Figure 3.1). In marked contrast DSBT-pretreated rats (1ml DSBT day -7 relative to transplant) showed prolonged survival with overall

long-term graft acceptance of almost 80% (Figure 3.4). This effect was clearly donor-specific as if a third-party blood transfusion (1ml Lewis whole blood day -7 relative to transplant) was given instead of donor-strain blood survival was identical to the unmodified rejection time. Graft rejection did not account for any death between days 50 and 100 post transplant. After 100 days a gradual loss of grafts was observed although survival times of >300 days were seen. Of these longstanding grafts around 30% were markedly hydronephrotic suggesting that some degree of ureteric obstruction or malfunction was present. The remainder were found consistently to be small in size with a white nodular appearance. These results would suggest that in DSBT-pretreated rats acute rejection has been replaced with a much more gradual process of chronic rejection.

Throughout the course of this work a small percentage of DSBT-pretreated animals died of histologically confirmed rejection at 12-14 days post transplant. For this reason the renal function of a number of allografts was followed closely in the first month following transplantation.

### 3.2.3 Early renal function following transplantation after DSBT

Survival alone is a rather crude method of assessing adequate renal function as any transient rejection episodes may not be noticed. The renal function of a group of 12 consecutively transplanted DSBT-pretreated rats was monitored by measuring the serum urea and creatinine over the first month post transplant. This was done to determine if all grafts underwent a degree of rejection that was spontaneously

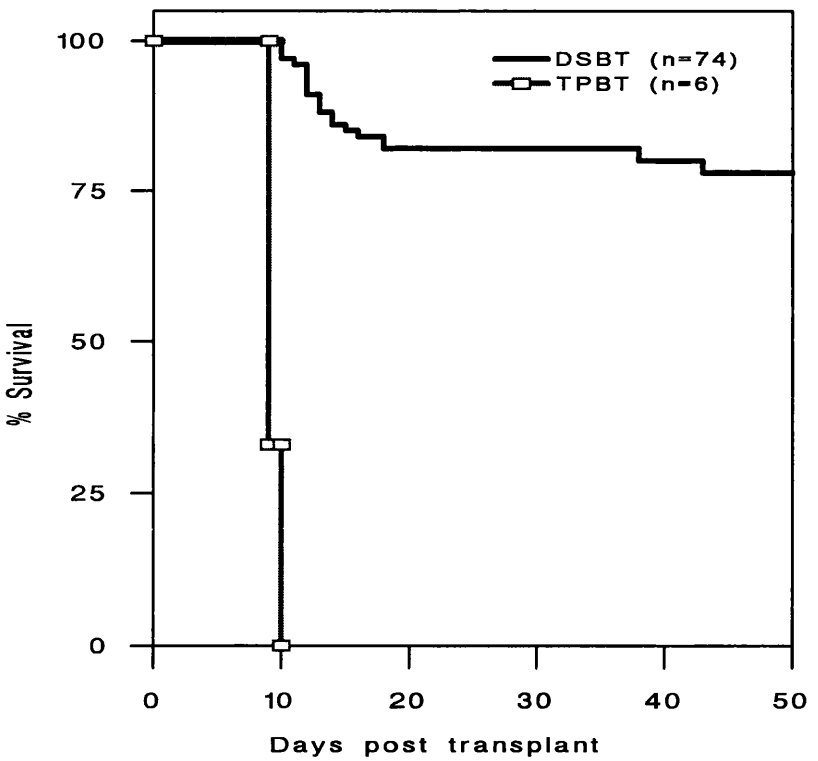



**Figure 3.4 Preoperative blood transfusion and survival of DA kidneys in PVG rats**

1ml transfusions of either donor-specific blood (DSBT) or third-party Lewis blood (TPBT) were administered 7 days prior to transplant and a contralateral nephrectomy performed 7 days post transplant.

resolved or if rejected grafts were responding in a different manner to the DSBT. Three patterns of renal function were seen. In half the rats (6/12) there was a limited rise in both urea and creatinine between days 12 and 18 post transplant to around double the expected levels. By the fourth week post transplant the renal function of these rats had returned to normal levels (serum urea <15mM; serum creatinine <120mM). In a small number of cases (2/12) the rise was much greater and one animal died of renal failure. The remaining rats (4/12) showed no rise in either urea or creatinine levels at any time measured post transplant.

### 3.2.4 The role of the thymus in DSBT-induced tolerance

Renal transplantation into thymectomised rats produced similar results to those obtained earlier in non-thymectomised rats. In the absence of DSBT, thymectomy did not appear to cause any reduction in the rate of rejection of renal allografts (Figure 3.5). This would indicate that the peripheral lymphocyte pool is sufficient to cause acute allograft rejection in these animals. Thymectomised DSBT-pretreated animal survival post transplant was also similar to that of animals with an intact thymus. The protective effect of DSBT does not appear to be diminished by prior thymectomy suggesting that this mechanism of tolerance production is peripheral in nature.

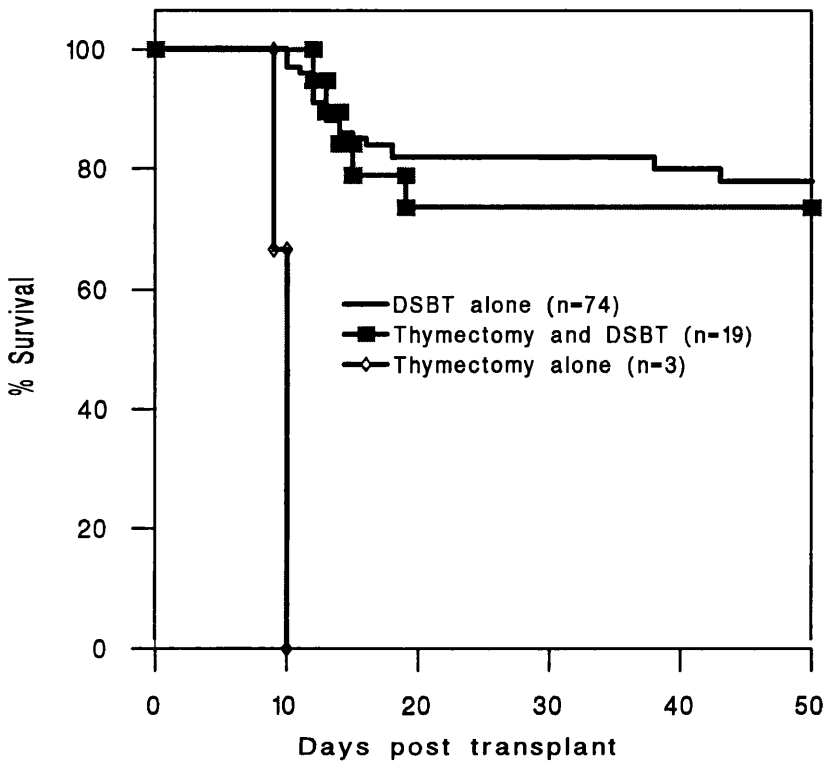



**Figure 3.5 Survival of adult thymectomised PVG rats transplanted with DA kidneys 7 days after donor-specific blood transfusion**

All PVG rats thymectomies were carried out at least 4 weeks prior to the start of the experiment. Donor-specific blood transfusions (DSBT) of 1ml were administered 7 days prior to transplant. Control thymectomised rats were transfused with 1ml of saline 7 days prior to transplant. Non-thymectomised DSBT-pretreated rats are also shown for reference. Contralateral nephrectomy was performed 7 days post transplant in all cases.

## 3.3 Discussion of the preliminary studies in the DSBT renal transplant model

Over the course of this work we were able to re-establish a rat renal transplant model and confirm that preoperative blood transfusion can lead to long-term graft acceptance in a donor-specific fashion.

The close monitoring of renal function in transfused animals following transplantation suggests that many grafts undergo an initial period of mild rejection around two weeks following transplant. This manifests itself as a transient rise in serum urea/creatinine. In general a prolonged period of stable renal function ensues however, in some cases, rejection is severe and the animal fails to survive. When tolerance induction was achieved by conventional immunosuppression such as cyclosporin A rather than DSBT smaller fluctuations in serum urea/creatinine were observed. This suggests that these rises were not due to the transplant operation but reflected some active immunological process. The period of transient rejection noted shortly after grafting is similar to the observation of Stuart and coworkers (1968) who found that up to 50% of rats apparently tolerant of a renal allograft, using a protocol involving alloantigen and/or alloantibody, exhibited an increase in urea that was resolved by the second week post transplant.

Such grafts have been shown previously (Armstrong *et al.*, 1987) to be heavily infiltrated by potentially damaging cells such as cytotoxic T cells and macrophages. This suggests that a balance exists between the rejection effector mechanisms of graft infiltrating cells and the tolerising effect of the DSBT.

It is possible that if two separate DSBT's had been used prior to transplant the number of tolerant animals obtained might have exceeded 80%. This was not tested during the current study, however another group (Bugeon *et al.,* 1993) routinely administer two blood transfusions prior to transplant in a cardiac allograft model of the same strain combination.

The central role of the thymus in establishing an animals T cell repertoire during the development of the immune system has been accepted now for many years. It is generally assumed however that any continuing influence of the thymus over the peripheral T cell pool is of little importance beyond the neonatal stage. Previously it was thought that a blood-thymus barrier existed thereby effectively preventing thymocytes from seeing antigens expressed in the periphery (Burnet, 1962); it was also presumed that mature T cells were excluded from the thymus (Gowans and Knight, 1964). A recent series of findings has suggested however that the adult rat thymus may not be as inactive as was first thought. Trafficking of thymocytes into cardiac allografts undergoing accelerated rejection and then back into the thymus has been described (Tanaka *et al.,* 1992) and activated, rather than resting, T cells have been shown to be capable of penetrating the thymus (Agus *et al.,* 1991). Injection of donor blood directly into the thymus one day prior to transplant has resulted reportedly in tolerance to a renal allograft (Perico *et al.,* 1992). Furthermore, the passive transfer of thymocytes from rats tolerised to vascularised organs into naive rats has been shown to prolong subsequent allograft survival in a strain-specific manner (Hendry *et al.,* 1979).

Thymectomy was carried out at least 4 weeks prior to either transfusion or transplantation. This time period was chosen to allow maturity of recent thymic emigrants (RTE's) as it has been suggested that these cells may be more easily tolerised (Miller and Morahan, 1992). Reports in the literature suggest that RTE's may account for ~20% of circulating T cells in LNC's of normal young adult rats (Hosseinzadeh and Goldschneider, 1993). Clearly this time delay is essential if the full effect of adult thymectomy is to be seen.

The efficacy of DSBT was identical whether or not the thymus was present with approximately 80% of thymectomised animals achieving long-term graft acceptance. Interestingly there was a group of animals that appeared to show reduced benefit from DSBT and rejected their graft around 12-14 days post transplant. Clearly this apparent failure of DSBT to achieve tolerance in these rats is not due to the emergence of a new population of cells from the thymus as has been reported in mice (Kitagawa *et al.*, 1990).

## 3.4 Summary

1 Unmodified rejection of DA renal allografts by PVG rats is rapid and survival is <10 days.

2 A 10 day course of cyclosporin A (10mg/kg/day) following transplantation produces long-term renal allograft survival.

3 A single 1ml DSBT given 7 days prior to transplant produces long-term allograft survival in 80 % of animals.

4 Transplant tolerance induced by pretreatment with blood transfusion is donor-specific.

5 A transient self-limiting rejection episode is frequently observed during induction of tolerance by DSBT in this rat strain combination.

6 The thymus gland does not play an essential role in tolerance induction by DSBT.

# CHAPTER FOUR

## Investigation into the nature of tolerance produced by DSBT in the DA into PVG rat renal allograft model

## 4.1 Introduction

It has been found that the immunogenicity of an allograft decreases with time following transplant. This may contribute to prolonged renal allograft survival in a DSBT rat model (Marquet *et al.,* 1985). In this study we have attempted to investigate the extent that the DSBT effect in the DA into PVG rat strain combination could be explained by such graft adaptation. We also tried to show that the immune system of the tolerant host was profoundly affected.

One possible mechanism of prolonged graft survival is that the graft itself undergoes a process of adaptation and modification following transplantation. This process effectively means that the host animal no longer recognises the graft as foreign tissue and the rejection response therefore declines as time passes despite the absence of any immunosuppression. One method of looking for graft adaptation is to retransplant the apparently tolerant graft into a fresh host without any form of immunomodulating therapy. This approach was first used by Batchelor and coworkers (1979) who were able to demonstrate prolonged survival of F1 (AS x AUG) kidneys retransplanted from tolerised primary (AS) hosts to untreated secondary (AS) hosts. They were subsequently able to restore rejection of these tolerised grafts if small numbers of donor-strain dendritic cells were administered concurrently with the retransplantation of the graft (Lechler and Batchelor, 1982). These findings have been corroborated by other workers (McKenzie *et al.,* 1984). It is now generally accepted that dendritic cells (passenger leukocytes), present within the allograft, form one of the main antigenic targets for rejection. An alternative test

for graft adaptation is to consider whether introducing fresh cells into a tolerised host can cause destruction of a stable graft. The disadvantage of this approach is that these cells may be modulated by the host immune system before they are able to exert an effect on the allograft.

The effect of DSBT on the immune system of the host rat can be investigated using in vitro methods such as the mixed lymphocyte reaction and also by a variety of in vivo experiments using long-term surviving allograft-bearing rats. Both approaches were used in this study. The mixed lymphocyte reaction (MLR), in which T cells are stimulated to proliferate in response to allogeneic cells, provides an in vitro model of cellular alloreactivity (Hayry and Defendi, 1970). Previous studies in the rat have shown that proliferation of lymphoid cells, in response to donor antigen, is decreased in rats pretreated with DSBT compared to lymphoid cells from naive rats (Quigley *et al.,* 1988). The reason for this effect is not entirely clear although there are probably both cellular and humoral factors involved.

## 4.2 Results: Graft related factors following transplantation

### 4.2.1 Passenger leukocyte depletion and graft survival

DA kidneys that had resided in a DSBT-pretreated PVG rat for at least 50 days (these grafts are sometimes referred to as being "parked" in their initial recipient) were retransplanted into a second PVG rat. The second host received no

treatment other than a contralateral nephrectomy 7 days post transplant. In all cases there was no evidence of acute rejection with grafts surviving at least 50 days in the second host. A severely attenuated rejection appeared to be occurring, in the longer timescale, with a mean survival time of 122 days (Table 4.1 and Figure 4.1). This late rejection of the grafts should also be considered in conjunction with the gradual loss in graft survival anticipated, with the course of time, had the grafts remained in their primary host. The fact that these grafts have now undergone a second transplant operation with a consequent further ischaemic period is also worthy of note.

### 4.2.2 The effect on graft function of transfusing sensitised lymphocytes into PVG rats bearing longstanding DA renal allografts following DSBT

Our earlier retransplantation experiments would suggest that the longstanding DA renal allograft in the PVG rat, following tolerance induction by DSBT, is a poor target for rejection due to the depletion of its passenger leukocytes. Consequently lymphocytes from PVG donors sensitised to DA antigens were transferred into PVG rats bearing DA renal allografts. Sensitised lymphocytes were produced by serial grafting of PVG rats with two DA skin grafts two weeks apart. Two weeks after the second skin graft lymph node cells (LNC's) were harvested from cervical and mesenteric lymph nodes of the PVG rat and counted for viability. These LNC's were transfused, in 1ml of PBS, into the PVG rat bearing the DA renal allograft via the dorsal penile vein. A total of $10^8$ unseparated viable LNC's were given to each

**Table 4.1 Survival of retransplanted kidneys DA into PVG**

| Residence in first host (days) | Survival of second host (days) | Total survival of graft (days) |
|---|---|---|
| 229 | 91 | 320 |
| 50 | 138 | 188 |
| 50 | 145 | 195 |
| 100 | >200 | >300 |
| 50 | 67 | 117 |
| 50 | 62 | 112 |
| 100 | 86 | 186 |
| 109 | >200 | >300 |
| 161 | 108 | 269 |

Mean survival time 122 days

Primary PVG recipients (n=9) of DA renal allografts were pretreated with a 1ml DSBT 7 days prior to transplant. Following survival of the grafted animals for at least 50 days post transplant the grafts were excised and retransplanted into second untreated PVG hosts. All host rats underwent contralateral nephrectomy 7 days post transplant.

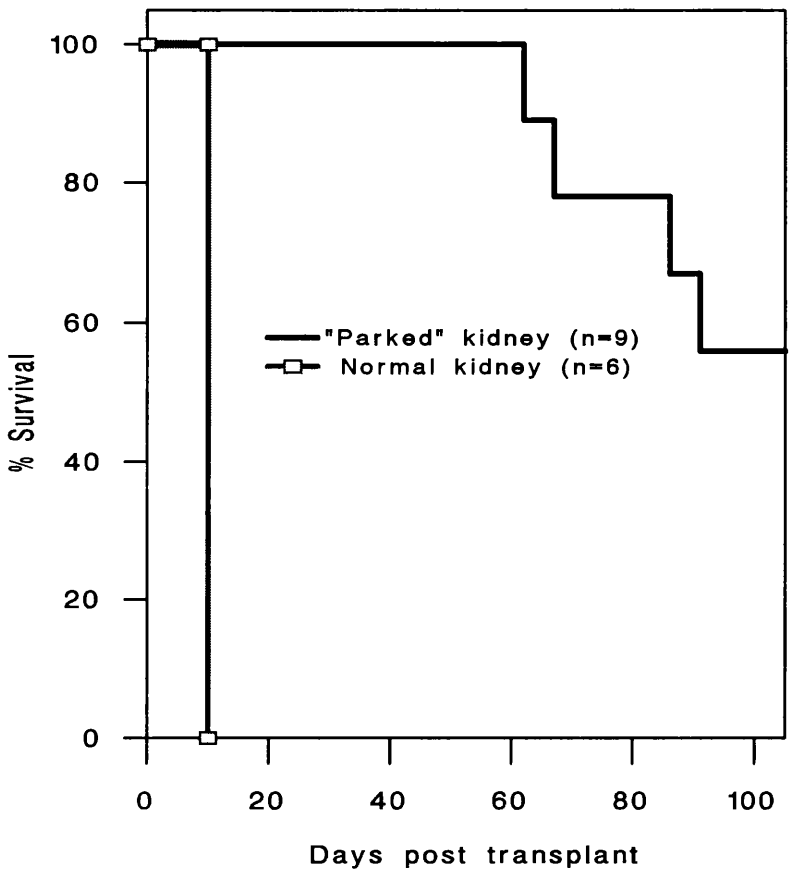



**Figure 4.1 Survival of naive PVG rats transplanted with either unmodified or previously "parked" DA kidneys**

A renal allograft modified by initial transplant into a PVG rat tolerised to DA antigens by a 1ml DSBT 7 days prior to transplant was retransplanted, after at least 50 days being "parked" in the first host, into a second PVG rat. The second recipient received no treatment either before or after receiving the retransplanted kidney. Contralateral nephrectomy was performed on all recipient rats 7 days post transplant.

of three PVG rats bearing a DA kidney grafted, following DSBT, >50 days earlier. All three rats survived for >100 days following this adoptive transfer of cells primed against their grafts. The serum urea and creatinine of the rats were measured at intervals following LNC transfer and failed to show any rise that would suggest graft damage (Figures 4.2 & 4.3). Grafts which are well established appear extremely resistant to damage by infusions of fresh lymphocytes even if the lymphocytes have been sensitised previously against the MHC antigens of the allograft. It is not clear however if host mechanisms are acting to disable these cells or if the graft is simply a poor target.

## 4.3 Results: Host related factors following transplantation

### 4.3.1 The mixed lymphocyte reaction in PVG rats bearing long-term DA renal allografts

Quigley and coworkers (1989a) have shown that at 4 days post DSBT, cells isolated from either thoracic duct lymph or the spleen of the transfused rat were capable of reducing proliferative responses in MLR reactions of naive cells in a strain-specific manner. They were also able to demonstrate that $CD4^+$ T cells were responsible for this effect. It was of interest therefore to see whether depression of the MLR response was sustained in the long-term by using LNC's from DSBT-pretreated PVG rats bearing DA renal allografts grafted at least 50 days earlier. Pooled cervical

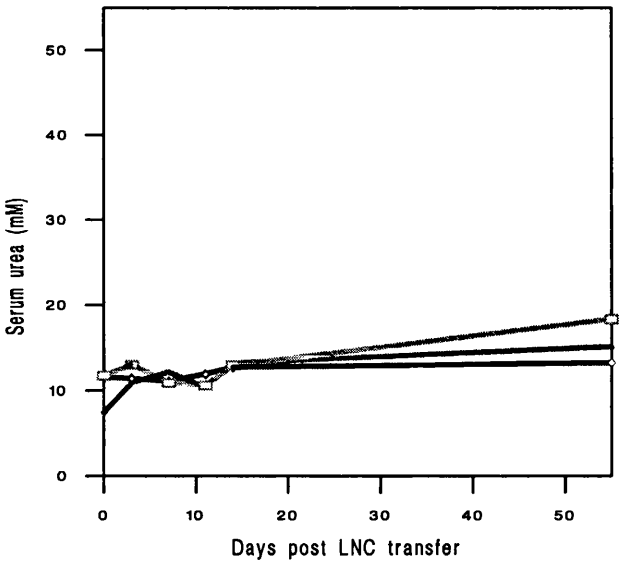



**Figure 4.2 The effect on renal function (urea) of transfusing sensitised LNC's into PVG rats bearing longstanding DA renal allografts**

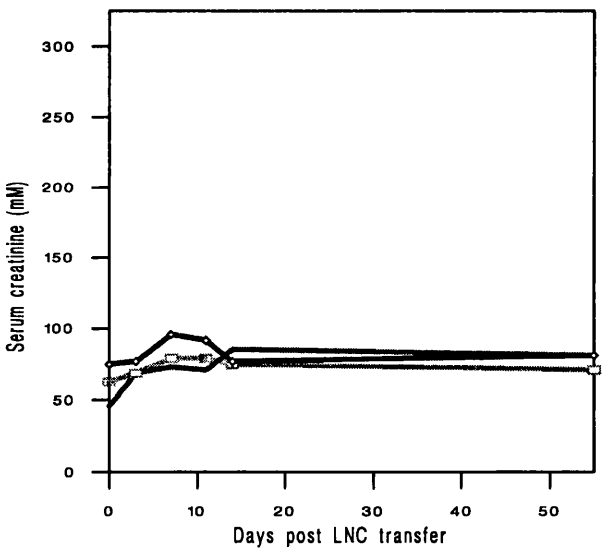



**Figure 4.3 The effect on renal function (creatinine) of transfusing sensitised LNC's into PVG rats bearing longstanding DA renal allografts**

DA kidneys were transplanted into PVG recipient rats (n=3) 7 days post DSBT (1ml). >50 days later sensitised LNC's ($10^8$) were transfused into the graft-bearing rats. Following LNC transfer, blood samples were taken at intervals from the recipient rats and analysed for serum urea (Fig. 4.2) and creatinine (Fig. 4.3).

and mesenteric LNC's ($2x10^5$) from either these "tolerised" rats or naive PVG rats were mixed with irradiated DA splenocytes ($2x10^5$) and proliferation measured by the incorporation of $^3H$ thymidine on days 3, 4 and 5 of the MLR (Figure 4.4). Similar results were obtained using LNC's from 3 other "tolerised" PVG rats.

Whilst there is still some proliferative response to donor-specific antigen, the proliferation of the "tolerised" LNC's was markedly reduced in comparison to that of naive LNC's. This finding is similar to the fall in the MLR response of LNC's taken from rats 7 days post DSBT treatment as reported previously (Quigley *et al.,* 1988). Our result would suggest that donor-specific response to antigen remains depressed in the long-term following transplantation.

### 4.3.2 Retransplantation of fresh renal allografts into tolerised rats

Before DSBT-pretreated PVG rats can be said to be tolerant of DA kidney antigens it must be shown that long-term survival is not simply due to the transplanted kidney having undergone a process of graft adaptation such that it no longer presents a target for the host's immune system: an effect which has already been shown to play a significant role in this strain combination (Section 4.2). On this occasion, the original DA renal allograft of a PVG rat "tolerised" by DSBT (1ml, day -7 relative to transplant) was excised 3-4 months after the original transplant and replaced immediately with a second DA kidney. No additional therapy was given to the PVG recipient after the original DSBT-pretreatment. On all four occasions in

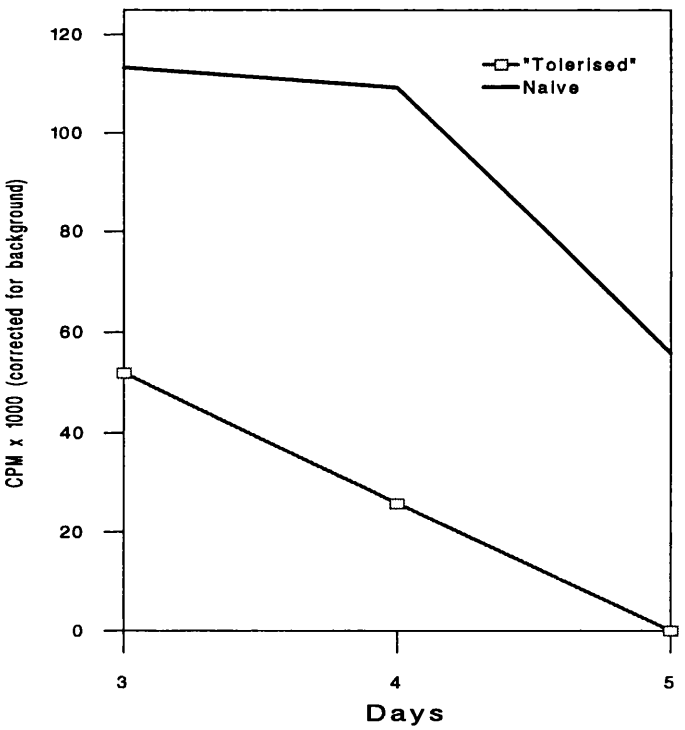



**Figure 4.4 MLR comparing the proliferation of LNC's from "tolerised" and naive PVG rats**

Unseparated LNC's ($2x10^5$) pooled from cervical and mesenteric nodes of either "tolerised" or naive rats were used as responder cells in the MLR. "Tolerised" PVG rats had been transplanted with a DA kidney 50 days earlier following DSBT-pretreatment; naive PVG rats had no previous exposure to DA antigens. Irradiated DA spleen cells ($2x10^5$) were used as stimulator cells. The proliferative response was assessed by measuring the incorporation of $^3H$ thymidine on days 3, 4 & 5 of the MLR.

which this procedure was carried out the new renal allograft also enjoyed long-term acceptance and in addition the renal function remained normal. There was no transient rise in serum urea (Figure 4.5) or serum creatinine (Figure 4.6) often seen following the original transplant (as described in Chapter 3; Section 3.2.3). This suggests that the long-term surviving PVG rats had achieved a state of tolerance towards DA strain kidneys. The second graft would have contained a normal complement of dendritic cells (passenger leukocytes) and would be expected to show the same transient rise in urea and creatinine as the first graft unless there had been some alteration of the host's immune system as a consequence of the first renal transplant.

It was also important to demonstrate that tolerance remained strain-specific and was not explained by some non-specific depression of the immune system such as exhaustion of the effector mechanisms of rejection caused by persistent antigen load. In order to achieve this, the original tolerant kidney was again excised after 3-4 months but this time it was replaced with a third-party kidney (Lewis strain). This procedure was again carried out on four occasions however, two very different outcomes were observed. As expected, two rats showed a period of normal renal function followed by a lethal rise in urea as the new graft was rejected (Figure 4.7). Interestingly the survival time of these rats was 17 and 18 days compared with a mean of 9 days (n=4 actual survival 9,9,9,9 days) for naive PVG rats transplanted with Lewis kidneys. This result suggests that some degree of protection also extends

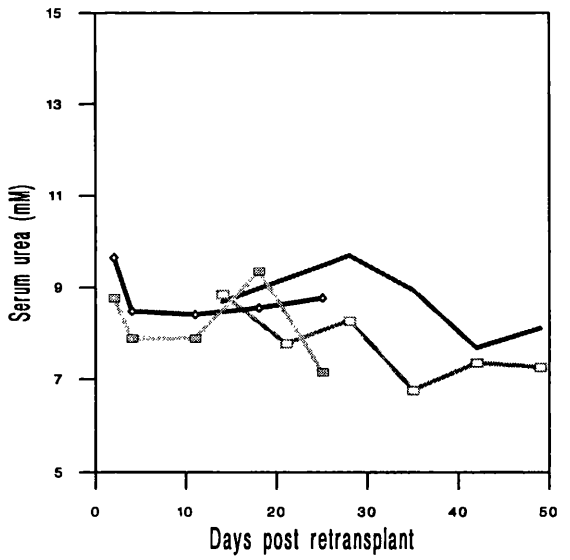



**Figure 4.5** **Renal function (urea) of a second DA kidney transplanted into a "tolerised" PVG rat**

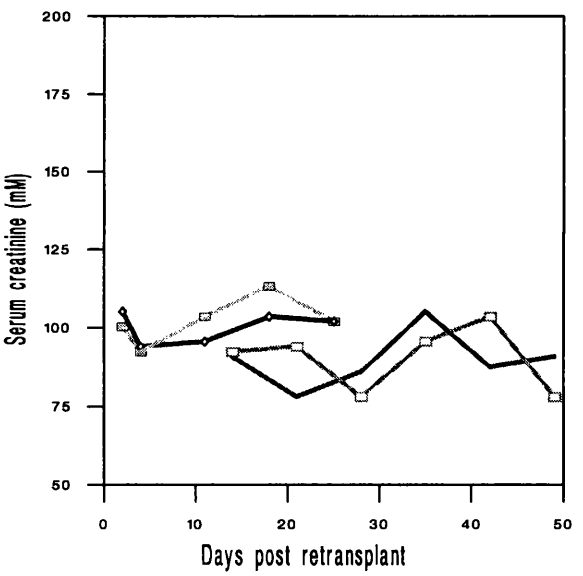



**Figure 4.6** **Renal function (creatinine) of a second DA kidney transplanted into a "tolerised" PVG rat**

"Tolerised" PVG rats were transplanted with a DA kidney following DSBT-pretreatment (1ml day -7 relative to transplant). >150 days later this graft was removed and replaced immediately with a second DA kidney (n=4). Regular blood samples were analysed for serum urea (Fig. 4.5) and creatinine (Fig. 4.6) post retransplant. Contralateral nephrectomy was performed 7 days after the first transplant and no further treatment was given other than retransplantation.

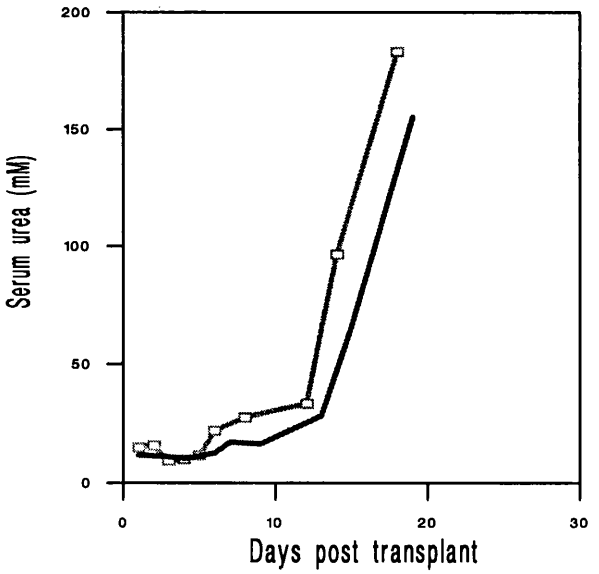



**Figure 4.7** **Urea levels after transplantation of Lewis kidneys into "tolerised" PVG rats: rejection**

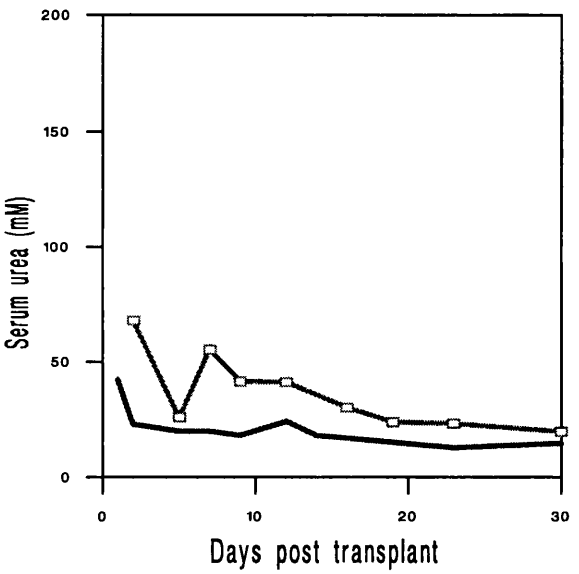



**Figure 4.8** **Urea levels after transplantation of Lewis kidneys into "tolerised" PVG rats: non-rejection**

"Tolerised" PVG rats were transplanted with a DA kidney following DSBT-pretreatment (1ml day -7 relative to transplant). >150 days later this graft was removed and replaced immediately with a Lewis kidney (n=4). Regular blood samples were analysed for serum urea post retransplant. Contralateral nephrectomy was performed 7 days after the first transplant. Two rats rejected their grafts (Fig. 4.7) whilst two rats survived for >50 days (Fig.4.8).

to the Lewis graft after tolerising the PVG rat to a DA kidney by DSBT pretreatment. What is not clear from this experiment however, is whether this reflects some cross-reactivity of the protective mechanisms to Lewis antigens or if there is a more generalised depression of the immune system in these long-term renal allografted rats. In contrast the remaining two rats in this experimental group failed to reject their Lewis allograft and both survived for >50 days. This suggests that these rats had developed a non-specific defect in their immune system. When the serum urea levels of these two rats were examined (Figure 4.8) a possible explanation became apparent. The first serum urea level measured post-operatively was markedly elevated. This implies that the renal function of the original allograft was declining and that the rats were markedly uremic at the time of the second transplant. The subsequent decrease in serum urea then witnessed lends support to the proposal of improved renal function upon transplant with a fresh kidney. The period of uremia post transplant may have played an immunosuppressive role and during this period the Lewis graft may have lost sufficient immunogenicity to allow long-term survival. This idea is analogous to the long-term graft acceptance seen following short term immunosuppression with cyclosporin A (Chapter 3, Section 3.2.1).

### 4.3.3 Organ specificity of DSBT-induced tolerance

Having demonstrated that DSBT-pretreated PVG rats were genuinely tolerant of DA renal allografts, the next objective was to determine whether tolerance was organ-specific or if it extended to other organs of the same donor MHC. This was investigated by grafting a heart onto the cervical vessels of PVG rats bearing longstanding DA kidneys by the method outlined previously (Chapter 2, Section 2.2.4).

Four DSBT-pretreated PVG rats bearing DA kidneys were grafted with DA hearts and all 4 animals accepted the new organ with no evidence of acute rejection of either the original kidney or the new heart (Table 4.2). Having transplanted the kidney (7 days after a 1ml DSBT) approximately 100 days prior to the heart graft, no further treatment (except a contralateral nephrectomy on day 7 post renal transplant) was given either before or after the cardiac allograft. All cardiac grafts pulsed strongly for >100 days post transplant, and were still beating immediately prior to the termination of the experiment when the animals developed renal failure.

Adult thymectomised PVG rats were shown previously to accept DA renal allografts after a 1ml DSBT (Chapter 3, Section 3.2.4). As an extension of this, the ability of such rats to accept a subsequent heart transplant was investigated. Four adult thymectomised PVG rats bearing DA renal allografts (transplanted >50 days earlier, following DSBT-pretreatment) were grafted with either a DA heart or a third-party Lewis heart. The results are shown in Figure 4.9. All the donor-specific DA

**Table 4.2 Outcome of DA cardiac grafts in PVG rats tolerant of DA kidneys**

| Original renal graft (days) | Renal plus cardiac graft (days) | Survival of animal (days) |
|---|---|---|
| 119 | 220 | 339 |
| 120 | 252 | 372 |
| 94 | 123 | 217 |
| 104 | 185 | 289 |

Tolerance to the original renal allografts (n=4) was induced by DSBT-pretreatment (1ml day -7 relative to transplant). No additional treatment (except for a contralateral nephrectomy day 7 post renal transplant) was given either before or after transplant of the the cardiac allograft ~100 days later. Cardiac graft survival was assessed by daily palpation and absence of palpable pulsation defined as rejection.

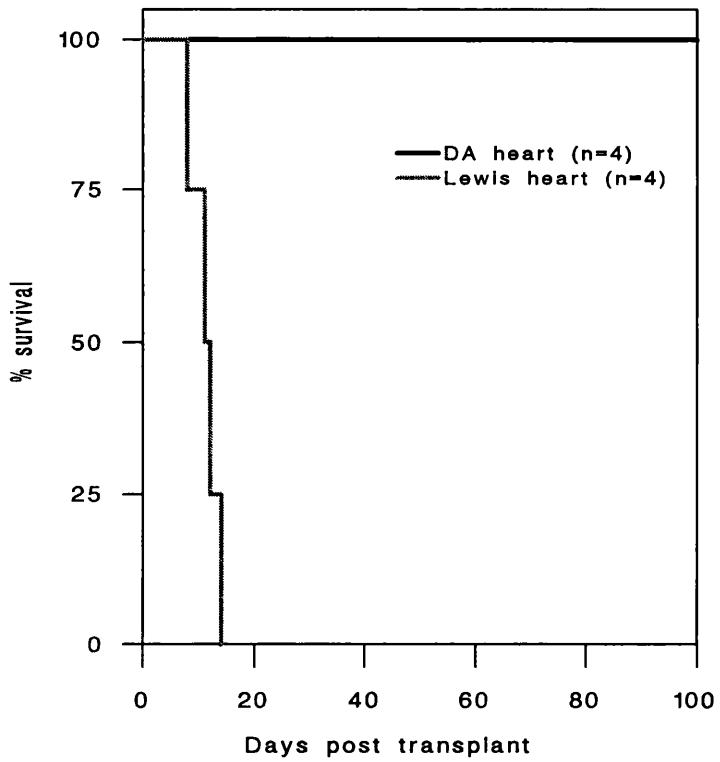



**Figure 4.9 Outcome of heterotopic cardiac allografts in "tolerised" thymectomised PVG rats**

Thymectomy was performed at least 6 weeks prior to the start of the experiment. "Tolerised" PVG rats were transplanted with a DA kidney following DSBT-pretreatment (1ml day -7 relative to transplant). Contralateral nephrectomy was performed 7 days later and >50 days after this a heterotopic cardiac transplant was performed with either a donor-strain (DA) or a third-party strain (Lewis) graft. All cardiac grafts were palpated daily and rejection defined as the absence of palpable pulsation.

strain cardiac grafts were accepted, beating strongly for >100 days, whilst third-party Lewis heart grafts were rejected in <20 days. This indicated that the extension of tolerance to a subsequent heart transplant does not require the presence of an intact thymus and again confirms the peripheral nature of tolerance in this model. The prompt rejection of third-party (Lewis) hearts excludes non-specific immunosuppression as an effector mechanism for tolerance production.

Having observed that the tolerant state clearly extended to another vascularised organ graft, several PVG rats (tolerised to a DA kidney by DSBT-pretreatment) were grafted with full thickness DA skin grafts as described previously (Chapter 2, Section 2.2.5). The skin grafts exhibited prolonged graft survival although the donor skin was eventually rejected. Skin graft rejection in these rats bearing renal allografts was manifested by a gradual loss of hair followed by slow de-epithelialisation and scabbing of the graft rather than the rapid inflammatory response seen with skin grafts in naive rats. The precise end-point of these grafts (>80% graft loss) was often difficult to define. One rat skin grafted 3 months following renal and cardiac allografts (performed sequentially at three monthly intervals) showed especially slow loss of its skin graft with donor epithelium still visible 100 days later.

It is worth noting that the survival of cardiac allografts(>100 days) in these DSBT-pretreated and renal transplanted rats is considerably greater than donor-specific blood transfusion alone can achieve in this strain combination. This suggests that the long-term presence of the renal allograft itself is also influencing the

outcome and correlates with the depressed alloreactivity of these rats suggested by the MLR data shown earlier (Figure 4.4).

### 4.3.4 Transfer of tolerance using splenocytes derived from long-term tolerant rats

If tolerance to an allograft is being maintained by an active mechanism then it should be possible in some instances to transfer tolerance by taking cells and/or serum from a tolerant rat and transferring these into a naive host which is then given an allograft with no further immunosuppression.

Consequently, unseparated splenocytes ($10^8$) were transferred from a PVG rat that had borne a DA renal allograft (>100 days) to a naive PVG rat which was then immediately grafted with a DA rat kidney in the usual manner. A contralateral nephrectomy was performed on the second recipient 7 days post transplant. Using this protocol no evidence was seen for the existence of a cellular protective factor since all rats rejected their kidneys in 9 days (n=6) as for unmodified rejection in this strain combination. In a single instance $3.3 \times 10^8$ splenocytes were transferred (i.e. all the viable splenocytes from one "tolerised" rat) and the recipient survived for 12 days. This increase in survival was minimal and the line of investigation was not pursued further.

## 4.4 Discussion of the DA into PVG DSBT renal transplantation model

This series of experiments demonstrated that a single DSBT of 1ml of fresh whole DA blood exerts a profound influence on the immune system of the PVG recipient. Given 7 days prior to transplant, donor-specific prolongation of survival is seen in all cases with around 80% of rats surviving for >100 days without any further treatment.

In many of these DSBT-pretreated rats close monitoring of their renal function post transplant indicated that there is often a period of transient rejection approximately two weeks after grafting. The spontaneous resolution of this rejection may be partially attributed to graft adaptation following transplantation. The extent to which graft adaptation tends to protect the grafted organ is witnessed by the continued function of the kidney when retransplanted into a fresh host. This presumably corresponds to the loss of donor dendritic cells since other possibilities such as the transfer of suppressor cells within the graft are, although theoretically feasible, highly unlikely. One interpretation of the prolonged survival of these retransplanted kidneys is that the direct route of antigen presentation by donor dendritic cells is the main route for sensitisation in the PVG host and that indirect presentation of other antigens contained within these grafts is considerably less important.

The survival of cyclosporin A-treated rats (Chapter 3, Section 3.2.1), long after any effect of the drug has presumably worn off, may also be explained by graft adaptation. These cyclosporin A-treated rats would be expected to reject a second DA

renal allograft (due to the dendritic cells within it) if graft adaptation is the sole reason for the prolonged survival of the original graft. It has also been proposed that dendritic cells themselves may influence the host immune system in a favourable manner, a concept known as donor-cell chimerism (Starzl *et al.,* 1993). This theory suggests that donor-dendritic cells are in fact vital for tolerance production and are not merely a stimulus for rejector mechanisms. If this is the case then it would be predicted that rats receiving a "parked graft" would reject a subsequent further graft carrying dendritic cells, even if given a DSBT prior to their first transplant. Such experiments were not attempted but would be of great interest in a further study.

The immune system of PVG rats transplanted with DA kidneys following DSBT-pretreatment does clearly undergo considerable modification. When such PVG rats bearing long-surviving DA renal allografts (> 50 days) were retransplanted with a fresh donor-strain kidney there was no evidence of rejection despite the fresh dendritic cells contained within this kidney. Partial protection also extends however to third-party grafts but the degree of protection is less than to the original donor antigens. i.e. the DSBT effect is still specific in the long-term but to a lesser degree than in the short-term. This protective effect has been shown to extend to a second directly-vascularised organ, the heart, and also to indirectly-vascularised skin grafts albeit to a lesser degree. Whatever the mechanism producing this prolongation of graft survival it is clearly not tissue-specific although it is MHC type-specific.

Despite our lack of knowledge of the underlying mechanism for graft prolongation some information has come to light. The MLR data obtained suggested

that some donor reactive cells were still present in the host LNC's, otherwise an abolition of proliferation would have been anticipated. This would indicate that clonal deletion is unlikely as an effector mechanism in producing prolongation of graft survival. In experiments using adult thymectomised animals it was shown that the absence of the thymus had no effect upon tolerance production by DSBT-pretreatment i.e. the effect is peripheral rather than central. In addition, splenocyte transfer studies provided no evidence to suggest the existence of a cellular transferable factor capable of prolonging graft survival. It should be noted however that this was a stringent test capable of detecting such factors only if they were exerting a powerful effect. Evidence for the existence of such cellular factors has been advanced by several experimental groups in a variety of models. Using splenocytes from mice rendered tolerant of cardiac allografts following combined anti-$CD4^+$ and anti-$CD8^+$ monoclonal antibody therapy, Chen and coworkers (1993) were able to transfer tolerance to a naive mouse in a donor-specific manner in the absence of any additional therapy. Other workers have only been able to demonstrate such tolerance transfer if the new recipient was first treated with a sub-therapeutic dose of radiation (Hutcheson, 1986). Evidence for a serum transferable factor has been proposed by Kamada and coworkers (1986) who have shown that serum from DA liver grafted PVG rats is capable of specifically preventing rejection of (DA x PVG) F1 heart grafts in naive PVG rats. In addition Chen and coworkers (1994) were able to show recovery of specific rejection following removal of a long-standing graft. This they attributed to the presence of the graft itself producing a state of

chronic desensitisation as a consequence of prolonged engagement of host TCR's by persistent alloantigens.

Graft adaptation corresponding to the loss of dendritic cells partly explains the prolonged survival of grafts in this model however, changes in the host's immune system caused by both the DSBT and the graft itself are also important. The nature of these changes remain elusive.

## 4.5 Summary

1 Depletion of passenger leukocytes in the transplanted kidney leads to prolonged survival and implies that indirect presentation is weak.

2 Longstanding transplanted kidneys are resistant to damage by infusions of sensitised lymphocytes implying that they are a poor target for rejector mechanisms.

3 The MLR of lymphocytes of animals bearing longstanding grafts is depressed implying that some unknown change in the host immune system is occurring.

4 Tolerised hosts will accept fresh kidneys in a strain-specific manner.

5 This tolerance is not organ-specific.

6 Thymectomy does not prevent DSBT induced tolerance to renal allografts subsequently extending to later heart allografts.

7 Tolerance was not transferable using splenocytes of animals bearing longstanding grafts when administered at the time of transplant.

# CHAPTER FIVE

## The role of cytokines in rejection and donor-specific blood transfusion induced-tolerance in a low responder strain combination

## 5.1 Introduction

Cytokines have a central role in the regulation of the immune response that occurs following organ transplantation. They are known to be involved in both the differentiation and proliferation of leukocytes (O'Garra, 1991a and b). In addition, it is now recognised that cytokines can cross-regulate each others production and function (Fiorentino *et al.,* 1989; Llorente *et al.,* 1989).

It has been very difficult to establish the precise function of individual cytokines in the immune response to an allograft due to the complexity of interactions taking place. In the past, bioassays have been employed to measure key cytokines such as interleukin-2 in both the blood stream and urine (Johnston *et al.,* 1990; Simpson *et al.,* 1989) of renal transplant recipients. However, such bioassay techniques suffer from poor specificity and sensitivity and it is unclear to what degree these measurements reflect what is occurring within the graft.

Alternative methods of investigating cytokine production have utilised cell culture systems, often involving cloned cell lines. These have given a great deal of information as to the functions particular cytokines may perform but the true physiological role of a cytokine may be quite different from that seen in such artificial systems. In alloimmune responses there are potentially multiple cell types releasing a complex mixture of cytokines and the final outcome may be very difficult to predict. Furthermore some cytokine mRNA's are reported to be highly unstable due to AU-rich sequences in their 3' untranslated regions which will cause the level

of transcripts to decay rapidly if the stimulus for their continued production is lost by removal from the local environment (Shaw and Kamen, 1986).

The polymerase chain reaction (PCR) offers a method ideally suited to detection of relatively low levels of mRNA. Tissue is snap-frozen as soon as it is excised from the animal thereby ensuring that the mRNA extracted accurately reflects what is occurring within the tissue. The semiquantitative nature of the PCR performed in this study does not allow precise quantification of the absolute cytokine level to be made. It does however allow comparison of the relative level of individual cytokines to be made between tissues; differences of around tenfold are detectable (Dallman and Porter, 1991).

An alternative method of attempting to investigate the role of an individual cytokine in DSBT-induced tolerance is to administer the cytokine exogenously at the time of transplant and monitor the effect on the anticipated outcome. Some investigators believe that a deficit of IL-2 and/or IFN-γ may be critical for tolerance induction by DSBT-pretreatment (Dallman *et al.,* 1991; Bugeon *et al.,* 1992). These workers have reported that both cytokines have the potential to restore rejection of allografts in models of DSBT-induced tolerance. Consequently we attempted to break DSBT-induced tolerance in the DA into PVG renal allograft model by intraperitoneal injections, of the recipient rat, with purified IL-2 and IFN-γ cytokines in the post transplant period.

## 5.2 Results

### 5.2.1 Cytokine mRNA levels in syngeneic, rejecting and enhanced renal grafts

Using the DA into PVG rat renal transplant model allogeneic grafts were examined at varying time points following transplantation in both naive (rejecting) and DSBT-pretreated (enhanced) recipients. In addition, normal non-transplanted kidneys and syngeneic grafts were also examined; the former to determine baseline cytokine mRNA levels and the latter to highlight any changes arising from the transplant operation itself. Syngeneic grafts were of PVG into PVG as the host immune system was then the same as in the allograft model.

Tables 5.1 to 5.4 show the individual results at each time point for the four cytokines tested. IL-2 and IFN-γ were chosen as representative of a Th1 response and IL-4 and IL-10 as representative of a Th2 response. Extra grafts were examined on day 4 as this time point appeared critical from the preliminary results.

In syngeneic PVG grafts low levels of IL-2 and IL-10 were detectable at all time points but no peak was noted. In contrast IL-4 and IFN-γ were not detected at any time point. In normal DA kidney very low levels of IL-2 and IL-4 were detected however neither IL-10 or IFN-γ were detectable.

When allografts were examined the following patterns emerged. IL-2 transcript levels rose to a peak on day 4 post transplant and this rise appeared to be

**Tables 5.1 and 5.2 Levels of IL-2 and IFN-γ (Th1 cytokines) mRNA detected by PCR in kidney tissue**

**Table 5.1 Interleukin-2**

| Time post transplant (days) | Syngeneic graft (PVG) | Rejecting allograft (DA) | Enhanced allograft (DA) |
|---|---|---|---|
| 2 | ND | (+) , - | + |
| 3 | + , (+) | + , ++ | +, + |
| 4 | ND | ++, +, ++, ++ ++ | (+), +, ++, + |
| 5 | + , (+) | +, +, ++ | +, +, + |
| 6 | ND | +, (+) | (+), - |
| 7 | + , (+) | +, (+) | (+),(+),(+) |
| 21 | ND | NA | +, +, + |
| 100 | (+) (+) | NA | (+), +, (+) |

**Table 5.2 Interferon-γ**

| Time post transplant (days) | Syngeneic graft (PVG) | Rejecting allograft (DA) | Enhanced allograft (DA) |
|---|---|---|---|
| 2 | ND | (+), (+) | + |
| 3 | -, - | +, ++ | +, ++ |
| 4 | ND | ++, ++, ++, ++, ++ | +, +, ++, ++ |
| 5 | -, - | ++, ++,++ | ++, ++, ++ |
| 6 | ND | +, + | +, + |
| 7 | -, - | (+), (+) | +, ++, ++ |
| 21 | ND | NA | +, ++, ++ |
| 100 | -, - | NA | -, (+), - |

Semiquantitative analysis of cytokine mRNA in fully disparate kidney allografts. DA grafts were excised on the days shown after transplant into naive or enhanced PVG rats. Enhanced allograft recipients received a 1ml DSBT 7 days prior to transplant. PCR for individual cytokine mRNA was carried out on neat cDNA and dilutions of 1/10 and 1/100, products were sampled at sequential 3-cycle intervals. Products were then gel electrophoresed, stained with ethidium bromide and viewed by UV transillumination. The level of mRNA present was scored as;

++ cytokine message detected at lowest cDNA concentration or cycle number
+ cytokine message detected at intermediate cDNA concentration or cycle number
(+) cytokine message seen only at highest cDNA concentration or cycle number
- cytokine message not detected at any cDNA concentration or cycle number
NA not applicable as rejected grafts are destroyed by day 10. ND not determined

**Tables 5.3 and 5.4 Levels of IL-4 and IL-10 (Th2 cytokines) mRNA detected by PCR in kidney tissue**

**Table 5.3 Interleukin-4**

| Time post transplant (days) | Syngeneic graft (PVG) | Rejecting allograft (DA) | Enhanced allograft (DA) |
|---|---|---|---|
| 2 | ND | ++, - | ++ |
| 3 | - , - | +, (+) | ++, - |
| 4 | ND | (+), -, +, -,- | -, -, -, - |
| 5 | - , - | (+), -, (+) | -, -, - |
| 6 | ND | ++, - | ++, - |
| 7 | - , - | ++, - | (+), -, - |
| 21 | ND | NA | -,-,- |
| 100 | - , - | NA | (+), (+) |

**Table 5.4 Interleukin-10**

| Time post transplant (days) | Syngeneic graft (PVG) | Rejecting allograft (DA) | Enhanced allograft (DA) |
|---|---|---|---|
| 2 | ND | +, (+) | + |
| 3 | +, ( + ) | +, + | +, + |
| 4 | ND | ++, +, (+), +, + | (+), (+), ++ |
| 5 | +, ( + ) | ++, ++, ++ | ++, +, (+) |
| 6 | ND | ++, ++ | +, (+) |
| 7 | +, - | + | ++, +, + |
| 21 | ND | NA | +,+, (+) |
| 100 | +, + | NA | -, -, - |

Semiquantitative analysis of cytokine mRNA in fully disparate kidney allografts. DA grafts were excised on the days shown after transplant into naive or enhanced PVG rats. Enhanced allograft recipients received a 1ml DSBT 7 days prior to transplant. PCR for individual cytokine mRNA was carried out on neat cDNA and dilutions of 1/10 and 1/100, and products were sampled at sequential 3-cycle intervals. Products were then gel electrophoresed, stained with ethidium bromide and viewed by UV transillumination. The level of mRNA present was scored as;

++ cytokine message detected at lowest cDNA concentration or cycle number
+ cytokine message detected at intermediate cDNA concentration or cycle number
(+) cytokine message seen only at highest cDNA concentration or cycle number
- cytokine message not detected at any cDNA concentration or cycle number
NA not applicable as rejected grafts are destroyed by day 10. ND not determined

reduced by DSBT-pretreatment (4/5 rejecting grafts exhibited high levels of transcript compared to 1/4 in the enhanced group). By day 6 however, even in the rejecting grafts these levels had fallen to that of syngeneic grafts.

These day 4 grafts were re-examined in a further experiment to try and confirm that this apparent difference was real and not an artefact produced by comparison of PCR reactions carried out on separate days. cDNA's prepared from day 4 rejecting and enhanced grafts were examined in one PCR reaction at neat concentration only but over an extended range of 24,27,30,33 and 36 cycles of amplification. All products were then examined on a single gel (Figure 5.1). Message for IL-2 was consistently detected at cycle 24 in rejecting tissue but not until cycle 27 in the enhanced tissue. This confirms that IL-2 transcript levels are higher in the rejecting tissues.

IFN-γ was seen at high levels in both allogeneic groups, rejecting and enhanced, increasing from day three onwards. Interestingly in the enhanced grafts high levels continued to be detected on day 21 while in the rejecting grafts there was a fall after day 5 similar to that noted for IL-2. IL-4 and IL-10 were detectable in both the enhanced and rejecting groups but no consistent pattern was seen and no difference was apparent between the two groups.

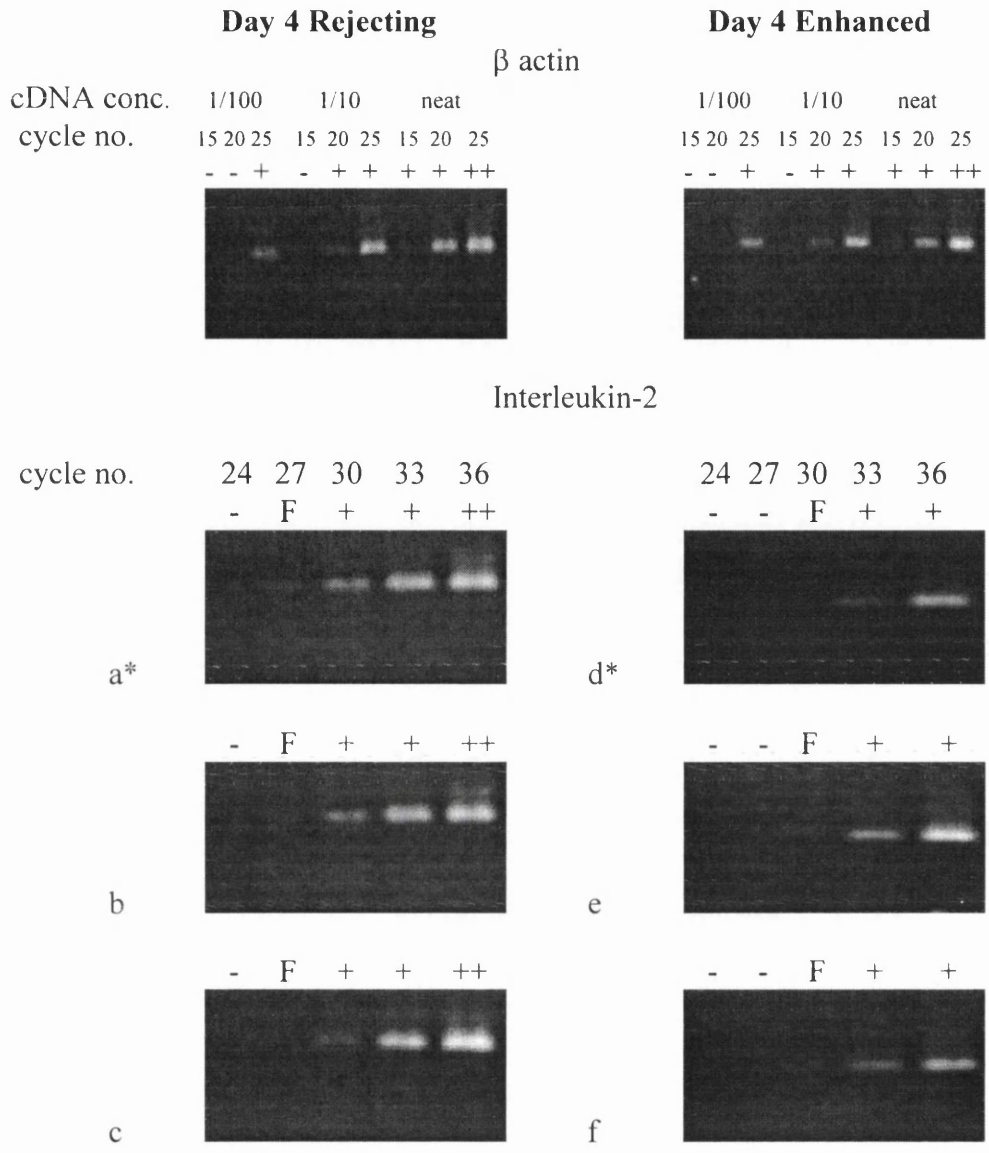



**Figure 5.1 Semiquantitative analysis of interleukin-2 mRNA in rejecting and enhanced kidney allografts 4 days post transplant**

DA grafts were excised 4 days after transplantation into naive or enhanced PVG rats. Enhanced allograft recipients received a 1ml DSBT 7 days prior to transplant. PCR for individual cytokine mRNA was carried out on neat cDNA prepared from individual allografts and products sampled at sequential 3-cycle intervals. Products were gel electrophoresed, stained with ethidium bromide and viewed by UV transillumination. Note that faintly visible bands on UV illumination (scored F) failed to appear on photographic reproduction. Gel electrophoresis of products from β-actin PCR (top) performed on 3 concentrations of cDNA show bands at 5-cycle intervals that are equivalent in tolerant and rejecting tissue (e.g., grafts a* and d*) demonstrating the integrity of RNA and cDNA preparation.

### 5.2.2 Attempts to abrogate DSBT-induced tolerance with exogenous IL-2 and IFN-γ

Exogenous IL-2

A total of 6 DSBT-pretreated rats were injected intraperitoneally with 3.6 x $10^5$ Cetus units of IL-2 on completion of the transplant operation and on days 1, 2, 3,4 & 5 post transplant. Contralateral nephrectomy was performed on day 7 post transplant. This dose of IL-2 was chosen as it had been found previously to completely abrogate the blood transfusion effect (Dallman *et al.*, 1991).

Survival of these animals was 7, 9, 19, 36, 45 & 150 days. As the animal that died on day 7 post transplant had not undergone contralateral nephrectomy this death was not a consequence of rejection. DSBT-pretreatment alone in this strain combination was shown previously to result in >80% survival to 100 days with control survival time being <10 days (Chapter 3 Figure 3.4). It is clear from our results that addition of exogenous IL-2 (3.6 x $10^5$ Cetus units) did not fully abrogate the effect of the earlier blood transfusion. Whilst there may be a reduction in the beneficial effect of the blood transfusion (only 1/6 survived to >100 days) it is difficult to draw any firm conclusion from the small number of rats involved. Unfortunately no further IL-2 was available to increase the number of rats treated or to try using higher dosages.

IFN-γ

Similar experiments were performed using a limited amount of recombinant rat IFN-γ. Rats were DSBT-pretreated 7 days prior to transplant and then injected on

the day of transplant and for 5 days afterwards with either $2x10^5$ international units (n=6) or $8x10^5$ international units (n=3) of IFN-γ.

This study was inconclusive. Three animals survived beyond the point where naive animals would have rejected their grafts (survival 18, 24 & 74 days). Of the other 6 animals whilst they died earlier (survival, 7, 9, 9, 9, 10 & 11 days) their grafts did not appear at post mortem to have undergone acute rejection and the suspicion was that these animals were succumbing to toxicity associated with IFN-γ treatment rather than graft rejection. No dose dependent difference in outcome was apparent although the numbers involved were small. As in the IL-2 study this line of investigation was curtailed due to a lack of IFN-γ.

## 5.3 Discussion of the role of cytokines in rejection and tolerance of allografts

A great deal of interest has been shown recently in determining the cytokines present within an allograft and postulating their role. This is partly a consequence of the division of T helper cells into subsets and the notion that one subset (Th1, including IL-2 and IFN-γ) may be responsible for rejection whilst another (Th2, including IL-4 and IL-10) may function in tolerance induction (Lowry, 1993).

Using PCR it was established that only low levels of cytokine message for IL-2 and IL-10 were detectable in syngeneic renal grafts. In addition IL-4 and IFN-γ were undetectable. Much higher levels of IL-2 and IFN-γ were found in rejecting allografts. These findings concur with the literature and in particular that documented

for IL-2 (Morgan *et al.*, 1993; Takeuchi *et al.*, 1992) and IFN-γ (Siegling *et al.*, 1994). Our results show that there is a peak in the level of IL-2 mRNA found in rejecting allografts at 4 days post transplant and these kinetics are in accordance to those published previously (Dallman *et al.*, 1991; Bugeon *et al.*, 1992). It has been shown in a murine cardiac allograft model that prior DSBT abrogates this peak (Takeuchi *et al.*, 1992) and reduced levels of IL-2, on day 4 post transplant, were noted in our study, supporting the proposal that peripheral tolerance induced by alloantigen results from an alteration in the IL-2 pathway. The relative failure to restore rejection with exogenous IL-2 was not pursued exhaustively in the current study. We were able to show some reduction in the beneficial effect of DSBT pretreatment. Further work might involve the use of higher doses of IL-2. Osmotic minipumps might also be implanted to ensure adequate blood levels of IL-2 thereby avoiding the problem of using intermittent injections as IL-2 has a short half life.

In the present study the apparent similarity in the IFN-γ transcript levels detected in both DSBT-enhanced and rejecting grafts is perhaps not surprising given that the main action of IFN-γ is to upregulate MHC expression. DSBT itself is known to lead to a marked increase in MHC expression (Armstrong *et al.*, 1987). Bugeon and coworkers (1993) have reported a reduction in IFN-γ in addition to that shown for IL-2 following DSBT-pretreatment and suggest that MHC expression has become uncoupled from IFN-γ in their model. In their study DSBT rats treated with exogenous IFN-γ in fact showed accelerated rejection compared to the control group

suggesting that IFN-γ was doing more than merely abrogating the blood transfusion effect.

The presence of significant amounts of IL-4 and IL-10 in rejecting as well as enhanced grafts implies that these cytokines are not responsible for the tolerant state seen following alloantigen pretreatment. Lowry (1993) has proposed that IL-4 and IL-10 (Th2 like cytokines) may mediate specific unresponsiveness and presented evidence of long intragraft persistence of these cytokines however our experiments clearly do not lend support to this theory.

One criticism of PCR analysis of homogenates of intact grafts is that the source of the cytokines being measured can only be inferred and need not originate from either CD4 or CD8 lymphocytes. IL-4 can be produced by cells of the basophil and mast cell lineages (Plaut *et al.*,1989) and IL-10 can be produced by non-lymphoid cells (Broski and Halloran,1994). It is possible therefore that these non-lymphoid cells are the source of the IL-4 and IL-10 message seen in rejecting allografts while in tolerised grafts the source is a tolerogenic lymphocyte. This would appear to be unlikely however given the similarity of the infiltrate seen when grafts from both groups were examined by immunohistochemistry. A definite answer to this question would require the in situ visualisation of the mRNA transcript concerned to permit the phenotype of the cytokine producing cell to be identified. This was attempted by in situ hybridisation using paraffin sections of formalin-fixed tissue but no successful specific labelling was achieved and this line of investigation was not pursued further.

## 5.4 Summary

1 mRNA for interleukins-2,4, 10 and interferon-γ were detectable using PCR technology in kidney tissues.

2 A rise in interleukin-2 transcript levels 4 days post transplant was seen in rejecting allografts.

3 DSBT-pretreatment reduces this rise suggesting an alteration in the interleukin-2 pathway is involved in tolerance induction by DSBT.

4 Interferon-γ mRNA levels were raised in both enhanced and rejecting grafts to a broadly similar degree.

5 Exogenous interleukin-2 and interferon -γ were both unable to fully break DSBT-induced tolerance.

6 No evidence was found to support the hypothesis that Th2 cytokines are tolerogenic as interleukin-4 and interleukin-10 were found at similar levels in rejecting and DSBT-enhanced grafts.

# CHAPTER SIX

## A model of tolerance involving DSBT combined with cyclosporin A-pretreatment in two high responder rat strain combinations

## 6.1 Introduction

One criticism of DSBT-induced tolerance is that it is restricted to specific strain combinations such as the low responder DA into PVG examined earlier. Other so called high responder strain combinations show little or no improvement in survival time when DSBT is used alone (Perloff and Barker, 1984; Robbins *et al.*, 1987). In these high responder strain combinations the use of a short course of immunosuppression in conjunction with DSBT-pretreatment has, in some cases, led to prolonged graft survival. Cyclosporin A has been used most commonly as a source of immunosuppression (Homan *et al.*, 1981; Yasumura and Kahan, 1984) and therefore was employed in this study. FK506 has also been reported to have a similar effect (Fabrega *et al.*, 1991). Cyclosporin A is widely used clinically and a combination of DSBT-pretreatment with cyclosporin A has been used in human cadaveric renal transplants. This appeared to cause a sustained reduction in the immunologic responsiveness to the donor antigens of the recipients (Alexander *et al.*, 1992).

Our initial studies employing combined immunosuppression and DSBT-pretreatment were carried out in the high responder R8 into $RT1^U$ strain combination. This combination was chosen as previous work in this laboratory had successfully used an anti-CD4 monoclonal antibody therapy to induce tolerance. We felt it was of interest to see if treatment involving donor antigen could also lead to tolerance induction and to compare this to the anti-CD4 model.

In the PVG-R8 into PVG-RT1$^{U}$ strain combination only the class I MHC is mismatched i.e. the class II MHC and the minor transplantation antigens are identical. Allograft rejection in this strain combination can occur in the complete absence of measurable $CD8^{+}$ cytotoxic T lymphocytes but the $CD4^{+}$ T cell is essential for rejection (Gracie *et al.,* 1990). It has also been demonstrated, by serum transfer experiments, that cytotoxic alloantibody alone can mediate graft destruction (Morton *et al.,* 1993). Together these findings suggest that a major mechanism of graft destruction in this strain combination is cytotoxic alloantibody and the role of the $CD4^{+}$ T cell is to provide help for the generation of this alloantibody.

The $CD4^{+}$ T cell can only recognise antigen when presented in conjunction with the class II MHC molecule. In this purely class I-disparate strain combination the class II MHC molecules in the donor organ are syngeneic with the recipient and therefore incapable of initiating a rejection response directly. It is thought that the antigenic class I MHC molecules from the graft are internally processed by recipient antigen presenting cells and a series of peptides produced. These peptides are then expressed on the cell surface of the recipients own APC's, in the peptide binding groove of host class II MHC molecules, where the $CD4^{+}$ T cell can now recognise the presence of foreign antigens and mount a response against them. This is referred to as indirect presentation and is often assumed to be less efficient than the more conventional direct presentation via donor antigen presenting cells. However as rejection in this strain combination can proceed rapidly even if direct presentation to

$CD8^+$ lymphocytes by class I MHC has been prevented by anti-CD8 monoclonal antibody therapy this indirect presentation is clearly not always weak or inefficient.

In subsequent experiments we employed the fully disparate DA into Lewis combination to ensure that the results obtained were not unique to the R8 into $RT1^U$ model. This fully disparate strain combination is also high responder. Donor-specific blood transfusion alone has been found to have no beneficial effect on renal allograft survival (Wasowska *et al.*, 1992) although an earlier report using affinity purified red blood cells did show some advantage (Wood and Morris, 1985).

## 6.2 Results: Development of a model of tolerance in a class I-disparate strain combination (R8 into $RT1^U$)

### 6.2.1 The effect of DSBT-pretreatment alone

DSBT-pretreatment alone was tested initially to see if any beneficial effect could be noted. A 1ml donor specific blood transfusion, when given 7 days prior to transplant, did not cause any increase in graft survival in this strain combination (Table 6.1). Increasing the number of transfusions to two or even four at weekly intervals was also ineffective at prolonging allograft survival with the mean survival time remaining ~9 days as for untreated controls.

**Table 6.1 Survival of $RT1^U$ rats transplanted with R8 kidneys following DSBT**

| DSBT (days) | Recipient survival (days) | Mean survival time (days) |
|---|---|---|
| None | 9,9,9,9,10 | 9 |
| -7 | 9,9,9,9,9 | 9 |
| -14,-7 | 9,9,9 | 9 |
| -28,-21,-14,-7 | 9,9,10,10 | 9.5 |

DSBT (1ml R8 whole blood) was administered via the dorsal penile vein of the recipient rat ($RT1^U$) on the days shown. All time points are relative to the day of transplant (R8 kidney day 0). Contralateral nephrectomy was performed on day 7 post transplant.

### 6.2.2 The effect of combined DSBT-pretreatment and perioperative anti-CD8 treatment

As blood transfusion alone did not promote graft survival in the R8 into $RT1^U$ strain combination we chose to add the monoclonal antibody OX8 to the treatment protocol. Although previous work in this strain combination has shown that the $CD8^+$ T lymphocyte is not essential for rejection it did not exclude these cells as potential effectors. In this study, by excluding any contribution from these cells, any beneficial effect of blood transfusion masked by these cells would be exposed.

In addition to receiving donor-specific blood (1ml days -14 , -7, relative to transplant) three rats were treated with OX8 (2mg intraperitoneal injection days -2, 0, +2, +4, +6). All rats were sacrificed on day 9 post transplant since they were showing severe signs of uremia. Immunohistology of the grafts was carried out to confirm the efficacy of the anti-CD8 treatment. Only a minimal infiltrate of $CD8^+$ T lymphocytes was seen in the grafted kidney of rats treated with OX8 despite widespread graft damage being present (Figure 6.1). The much higher $CD8^+$ T lymphocyte infiltrate in unmodified rejection of an R8 kidney in an $RT1^U$ host is shown for comparison.

These results confirm that the $CD8^+$ T lymphocyte is not essential for rejection in this strain combination and that a combination of anti-CD8 therapy and DSBT is ineffective at prolonging graft survival. This result concurs with the report that in H-2-recombinant MHC class I-disparate mice there are DSBT "resistant" $CD4^+$ T cells that are capable of causing graft rejection (Kitagawa *et al.*, 1991).

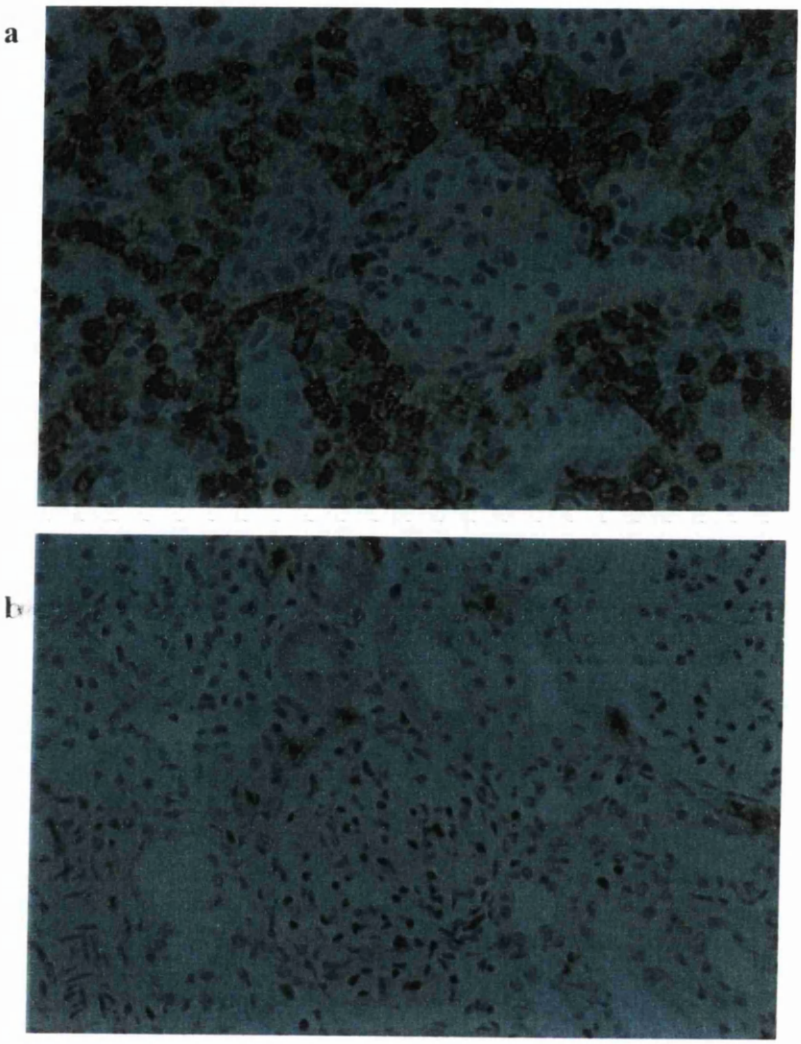



**Figure 6.1 Immunohistochemistry of R8 renal allografts in $RT1^U$ hosts**

R8 kidneys were transplanted into $RT1^U$ host rats either unmodified or pretreated with 2 DSBT's (1ml days -14 & -7; relative to transplant) and OX8 (2mg intraperitoneal injection days -2, 0, 2, 4 & 6). Grafts were removed for analysis 9 days post transplant. Kidney tissue was snap frozen in liquid nitrogen and cryostat sections (5μm) were cut at -20°C onto gelatinised slides. Monoclonal antibodies were used to label the slides employing an indirect immunoperoxidase technique. Dense staining for OX8 (CD8) is seen for the unmodified recipient (a) whilst minimal staining is evident for the pretreated recipient (b).

### 6.2.3 The effect of DSBT combined with short term cyclosporin A

Cunningham and coworkers (1988) have described a reduction in the complement-dependent cytotoxic antibody responses of F344 rats, following repeated weekly DA blood transfusions, if cyclosporin A is administered with the initial transfusion. Since antibody production has been shown to have a major role in causing acute allograft rejection in the R8 into $RT1^U$ strain combination (Morton *et al.*, 1993) we thought that this treatment strategy might be effective in producing tolerance. Consequently, a variety of treatment protocols were tested in this strain combination based upon single or multiple blood transfusions (1ml) with or without an accompanying 7 day course of cyclosporin A (15mg/kg/day) starting at the time of the first transfusion (Table 6.2).

In contrast to rats receiving DSBT-pretreatment alone rats treated with a combination of cyclosporin A and 4 DSBT's exhibited prolonged survival with 75% surviving >100 days post transplant. A single DSBT in association with cyclosporin A resulted in a moderate increase in graft survival (MST=50.5 days) however all grafts were rejected eventually. Cyclosporin A treatment alone also produced an increase in survival (MST=16.5 days) relative to control animals (MST=9 days). The tolerogenic effect of serial DSBT and cyclosporin A was found to be donor-specific since recipients receiving 4 third-party (PVG) blood transfusions and cyclosporin A all rapidly rejected R8 renal allografts (MST=10 days).

**Table 6.2 Survival of $RT1^U$ rats transplanted with R8 kidneys following blood transfusions with or without cyclosporin A**

| Pretreatment | | Survival | Mean survival time |
|---|---|---|---|
| Blood transfusion (days) | Cy A (days) | (days) | (days) |
| None | None | 9,9,9,9,10 | 9 |
| R8 -28,-21,-14,-7 | None | 9,9,10,10 | 9.5 |
| R8 -28,-21,-14,-7 | -28 to -21 | 18,31,>100 (x6) | >100 |
| R8 -7 | -7 to 0 | 10,48,53,75 | 50.5 |
| None | 0 to 7 | 9,15,18,22 | 16.5 |
| PVG -28,-21,-14,-7 | -28 to -21 | 9,10,10,10 | 10 |

Blood transfusions (1ml whole blood) were administered via the dorsal penile vein of the recipient rat ($RT1^U$) on the days indicated. All time points are relative to the day of transplant (R8 kidney day 0). Cyclosporin A (15mg/kg/day) was given by gavage once daily and contralateral nephrectomy was performed on day 7 post transplant.

## 6.3 Results: DSBT combined with cyclosporin A in a fully disparate high responder strain combination (DA into Lewis)

Initial experiments in this strain combination confirmed that DSBT alone had no positive effect on survival regardless if a single transfusion or multiple transfusions were administered prior to transplant (Table 6.3). If a 7 day course of cyclosporin A (15mg/kg/day) was incorporated at the time of the initial transfusion however, prolonged allograft survival resulted. This was shown to be the case for four transfusions in the first instance and subsequently the number of transfusions was reduced to two. Interestingly when two transfusions were employed the degree of increased survival was significantly reduced relative to that seen with four transfusions but remained well above the control group (Figure 6.2).

## 6.4 Discussion of the DSBT/cyclosporin A model of tolerance

A model has been developed in which cyclosporin A was used to influence the response to serial DSBT's such that a state of tolerance to a later allograft was produced in two high responder strain combinations. This tolerance is clearly donor-specific as when third party blood transfusions are given then rejection occurs at the same rate as in naive rats. Tolerance was not due to the non-specific immunosuppressive actions of cyclosporin A since the transplant was performed 21 days after cessation of treatment when any residual level of cyclosporin A would be extremely low. It was also shown in the R8 into $RT1^U$ combination that treatment

**Table 6.3 Survival of Lewis rats transplanted with DA kidneys following DSBT with or without cyclosporin A**

| Pretreatment DSBT (days) | Cy A (days) | Survival (days) | Mean survival time (days) |
|---|---|---|---|
| -7 | None | 9,9,9,9 | 9 |
| -28,-21,-14,-7 | None | 9,9,9,9 | 9 |
| -28,-21,-14,-7 | -28 to -21 | 31,43,50,62,>100(x6) | >100 |
| -14,-7 | -14 to -7 | 11,13,18,20,23, 25,31,48,100(x2) | 24 |

Donor-specific blood transfusions (1ml DA whole blood) were administered via the dorsal penile vein of the recipient rat (Lewis) on the days indicated. All time points are relative to the day of transplant (DA kidney day 0). Cyclosporin A (15mg/kg/day) was given by gavage once daily and contralateral nephrectomy was performed on day 7 post transplant.

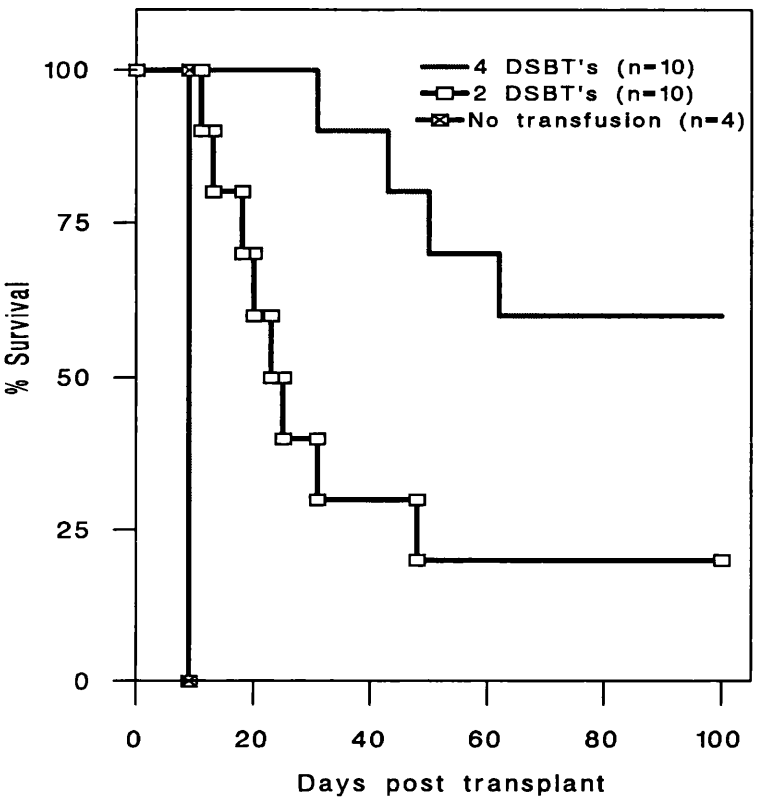



**Figure 6.2 Effect of decreasing the number of DSBT's used in conjunction with cyclosporin A on renal allograft survival (DA into Lewis)**

Lewis rats were transplanted with DA kidneys 7 days after pretreatment with donor-specific blood transfusions and cyclosporin A. DSBT's (1ml) were given at weekly intervals for either 4 or 2 weeks prior to transplant. Cyclosporin A (15mg/kg/day) was given by oral gavage once daily, after the initial blood transfusion only, for 7 days. Unmodified rejection time is shown for animals that did not receive any form of treatment. Contralateral nephrectomy was carried out on day 7 post transplant.

with cyclosporin A alone, for 7 days starting at the time of transplant, was insufficient to produce the prolongation in survival seen following combined DSBT/cyclosporin A therapy. Such animals did however show a modest survival advantage over the control groups.

It is of note that in the DA into Lewis strain combination four DSBT's appeared to confer increased benefit compared to two DSBT's. This was despite the greater time elapsed between administration of cyclosporin A and the transplant. There are two possible explanations. Repeated antigenic challenges in the form of DSBT's may serve to reinforce the developing tolerant state. Alternatively, the tolerant state may develop slowly and hence the increased time period between the cyclosporin A treatment and the transplant itself may be responsible for the improved outcome rather than the repeated antigenic challenges. This latter explanation is in keeping with the report that specific tolerance induced with donor antigens and anti-CD4 monoclonal antibody is seen at 28 days post transfusion (Pearson *et al.*, 1992).

The combination of repeated DSBT's and a short course of cyclosporin A, started at the time of the first transfusion, is capable of consistently producing long-term graft acceptance in strain combinations where neither treatment alone is effective.

## 6.5 Summary

1 DSBT-pretreatment alone does not increase allograft survival in either of the high responder strain combinations tested.

2 Anti-CD8 treatment in addition to DSBT does not improve allograft survival in the R8 into $RT1^U$ strain combination.

3 Serial DSBT's at weekly intervals with the addition of a 7 day course of cyclosporin A (15mg/kg/day) started with the first transfusion markedly prolonged survival in both high responder strain combinations.

4 Increasing the number of DSBT's from two to four increased the beneficial effect on graft survival in the DA into Lewis strain combination.

# CHAPTER SEVEN

## Investigation into the mechanism of DSBT/cyclosporin A-induced tolerance

## 7.1 Introduction

Having developed a model of DSBT/cyclosporin A-induced tolerance to renal allografts in two high responder rat strain combinations (Chapter 6) we investigated the nature of this phenomenon. It was of considerable interest to see how the results of these investigations compared to those obtained previously for the DA into PVG low responder strain combination (Chapter 4).

As this model had not been studied previously preliminary investigations involved examination of the cellular infiltrate in rejecting and non-rejecting grafts. Subsequently a series of retransplantation experiments were carried out to assess the degree of graft adaptation occurring in this model and also the extent of the tolerance achieved. Earlier experiments in the DA into PVG model showed clearly that the presence of the thymus was not required for tolerance induction following a single DSBT. Since DSBT alone does not lead to tolerance induction in either the DA into Lewis or the R8 into $RT1^U$ strain combinations it is possible that the mechanism involved may differ and might potentially involve the thymus. Cyclosporin A has been reported to lead to a dramatic increase in circulating recent thymic emigrants (Zadeh and Goldschneider, 1993), and it has also been suggested that these recent thymic emigrants may be more easily rendered tolerant than mature peripheral T cells. For these reasons it was considered worthwhile to question the role of the thymus in this model.

Cytotoxic alloantibody is believed to be a major cause of acute graft rejection in the R8 into $RT1^U$ strain combination (Morton *et al.*, 1993) and as such this

antibody titre should correlate closely with survival. Simple DSBT-induced enhancement of renal allograft survival (e.g. the DA into PVG model) has been shown to correlate with host MHC-linked inhibition of IgG anti-donor class I alloantibody (Wasowska *et al.,* 1993). In our model however, DSBT alone did not enhance graft survival unless cyclosporin A was also administered at the time of the first transfusion. The use of such combinations of DSBT and cyclosporin A has been shown to be effective in reducing cytotoxic alloantibody in strain combinations where DSBT alone is ineffective, although the study concerned did not attempt to establish any beneficial effect on vascularised organ grafts (Jones *et al.,* 1988). The level of cytotoxic alloantibodies was also measured in the DA into Lewis model. This was done to monitor the way in which antibody titres vary during tolerance induction in a strain combination where a role for cytotoxic alloantibody in acute rejection is less well established.

Antibody levels following blood transfusion alone were examined in adult thymectomised rats to determine if the presence of an intact thymus gland exerted any effect on antibody responses in these high responder strain combinations. In addition the IgM and IgG subclasses of anti-RT1A$^a$ antibody were studied in detail to assess whether any selective effect on individual alloantibody isotypes was identifiable.

Having carried out a detailed examination of cytokines detectable in grafts of DSBT-treated PVG rats transplanted with DA kidneys (Chapter 5), it was of great interest to see if the pattern obtained in these high responder rats was similar given

the greater role of antibody mediated graft destruction in this model. Several studies have reported important differences in cytokine expression between tolerant and rejecting organ allografts and this has given rise to the concept that transplant tolerance may, in part, be explained by polarisation of the alloreactive T helper response from a Th1 to a Th2 cytokine profile (Takeuchi *et al.*, 1992; Seigling *et al.*, 1993; Ferraresso *et al.*, 1994; Chen and Field, 1995). As before, semi-quantitative RT-PCR was employed to examine the presence of mRNA transcripts for Th1 and Th2 cytokines in rejecting and non-rejecting grafts. Due to the limited number of animals available (animals were bred in-house from a small number of breeding pairs) a single timepoint of day 5 post transplant was studied. Spleens were also examined as antibody production need not be an intragraft event. It remains possible that events in the spleen may have a bearing on the outcome in this model.

## 7.2 Results

### 7.2.1 Analysis of the cellular infiltrate in rejecting and non-rejecting R8 renal allografts in $RT1^U$ recipients

R8 kidneys were examined microscopically 5 days after transplant into either naive $RT1^U$ rats or those pretreated with DSBT/cyclosporin A. In the case of unmodified recipients there was a heavy interstitial cell infiltrate, widespread vascular damage, focal tubular necrosis and glomerular ischaemia. By day 7 this had progressed to extensive interstitial haemorrhage and often complete infarction. In

contrast, R8 grafts from $RT1^U$ recipients rendered tolerant by serial DSBT and cyclosporin A showed minimal evidence of renal parenchymal damage although there was still a dense mononuclear cell infiltrate.

Closer examination of the magnitude and phenotype of these cellular infiltrates was determined by morphometric analysis of immunoperoxidase labelled cryostat sections. The results are shown in Table 7.1. Both rejecting and non-rejecting grafts were infiltrated to a similar degree by a dense mononuclear cell infiltrate consisting mainly of macrophages distributed diffusely throughout the interstitium of the kidney. In both groups there were substantial numbers of $CD8^+$ cells although the distribution was different. In rejecting grafts the $CD8^+$ cells tended to be focally distributed perivascularly (and to a lesser extent periglomerularly) while the non-rejecting grafts contained a more diffuse $CD8^+$ infiltrate. Interestingly non-rejecting grafts contained approximately fourfold more $CD4^+$ cells, distributed diffusely throughout the kidney, than the rejecting grafts. $OX39^+$ cells were readily visible in rejecting grafts but were almost undetectable in non-rejecting grafts, in keeping with a reduced state of cellular activation. The mAb OX39 is directed against an epitope on the low affinity (p55α) chain of the IL-2 receptor, seen principally following cell activation (Paterson *et al.*, 1987).

**Table 7.1 Cellular infiltrate in class I $A^a$-disparate R8 kidney allografts in $RT1^U$ recipients**

Percentage area occupied by infiltrate

| | CD45 | CD8 | CD4 | αβTCR | MØ | IL-2R |
|---|---|---|---|---|---|---|
| Unmodified rejection | 21±9 (100%) | 7±4 (33%) | 2±2 (10%) | 6±3 (29%) | 13±3 (62%) | 3±3 (14%) |
| Tolerant (BT + CyA) | 25±9 (100%) | 10±6 (40%) | 9±3 (36%) | 9±4 (36%) | 20±9 (80%) | <1 (<5%) |

Tolerance to a R8 kidney was achieved by pretreating $RT1^U$ rats with four 1ml DSBT's at weekly intervals (days -28, -21, -14, -7 relative to transplant) together with cyclosporin A (15mg/kg/day) for 7 days after the first DSBT (days -28 to -21). Kidneys were excised from tolerised recipients and also from unmodified recipients 5 days post transplant and snap frozen. Cryostat sections from these kidneys were labelled using the immunoperoxidase technique. The percentage area infiltrate was determined by point counting with a microscope eyepiece graticule. Values shown are the mean ± SD of 4 allografts. Results in parentheses represent the phenotype as a percentage of the total area of cellular infiltrate ( i.e. percentage of CD45 infiltrate). αβTCR = T cell receptor, MØ=macrophage, IL-2R= interleukin-2 receptor.

### 7.2.2 Retransplantation experiments in the R8 into $RT1^U$ strain combination: second renal allografts of rats tolerant of an initial R8 kidney

Four $RT1^U$ rats bearing a longstanding R8 renal allograft (>100 days) were grafted with a fresh R8 kidney and at the same time the original graft was excised. These rats all survived for >100 days. This result showed that the long-term survival of the original graft was not simply due to graft adaptation and suggested that the host immune system had been tolerised towards R8 antigens. Unfortunately no third-party grafts were carried out to ensure that these animals were still capable of mounting an immune response against non-specific alloantigens hence caution is required in interpreting this result. It would appear however that DSBT combined with cyclosporin A does lead to tolerance of renal allografts in this model.

### 7.2.3 Retransplantation experiments in the R8 into $RT1^U$ strain combination: transplantation of grafts from tolerised rats into fresh naive hosts

As a test for graft adaptation R8 kidneys that had been resident in $RT1^U$ hosts for >100 days were retransplanted into naive $RT1^U$ rats. This was carried out in a group of four rats which underwent contralateral nephrectomy 7 days post transplant. Of this group, three rats died 9 days post transplant; inspection of the grafts at post mortem showed severe rejection. The remaining rat survived >100 days with normal renal function. It is unclear why this one animal should have a different outcome from the others. Rejection of "parked" grafts, at a similar rate to normal allografts would suggest that passenger leukocyte depletion, by "parking" in tolerised hosts,

does not usually lead to prolonged graft survival in the R8 into $RT1^U$ strain combination. This result differs from the DA into PVG study (Chapter 4, Section 4.2.1) where all "parked" grafts showed markedly prolonged survival (mean > 100 days).

### 7.2.4 The role of the thymus in DSBT/cyclosporin A-induced tolerance

Thymectomised rats were pretreated with four 1ml DSBT's (days -28,-21,-14, & -7 relative to transplant) and cyclosporin A (15mg/kg/day, days -28 to -21). All rats were thymectomised at least 4 weeks prior to the first transfusion. Both high responder strain combinations (R8 into $RT1^U$ and DA into Lewis) were examined. A total of four $RT1^U$ and five Lewis rats were treated in this experiment, survival times are shown in Table 7.2. Whilst it is possible that there was some reduction in the degree of prolongation of survival in the case of the thymectomised R8 into $RT1^U$ rats (survival of DSBT/cyclosporin A-pretreated non-thymectomised rats was shown in Chapter 6 to be 80% at 100 days) only small numbers were involved. It is clear however that in both strain combinations elimination of the thymus does not prevent DSBT/cyclosporin A-pretreatment prolonging allograft survival relative to that obtained in thymectomised but otherwise unmodified rats (survival <10 days in both strain combinations). This would suggest that a peripheral mechanism is responsible for the beneficial effect of DSBT/cyclosporin A-pretreatment in this model.

**Table 7.2 Survival following transplant of adult thymectomised rats pretreated with 4 DSBT's and cyclosporin A**

| Strain combination | Survival (days) |
|---|---|
| R8 / $RT1^U$ | 14*, 20, 42, >100 |
| DA / Lewis | 12, 39, 93, 103, >150 |

Thymectomy was performed at approximately 6 weeks of age and rats were not used for at least a further four weeks. All recipient rats received four 1ml DSBT's (days -28,-21,-14,-7, relative to transplant) and cyclosporin A (15mg/kg/day, days -28 to -21) by gavage. Contralateral nephrectomy was performed 7 days post transplant.
* This animal died due to an infrarenal abscess secondary to a ureteric leak and the graft appeared healthy at post mortem. Death was unlikely to be due to rejection.

### 7.2.5 The role of cytotoxic alloantibody in DSBT/cyclosporin A-induced tolerance

R8 into $RT1^U$

As can be seen in Figure 7.1 animals transfused in the absence of cyclosporin A show very high cytotoxic alloantibody titres which do not appear to decrease with repeated transfusion. When cyclosporin A was added to the protocol there was still a measurable response to the first transfusion but it was smaller and with repeated transfusions the titre fell almost to zero. Following grafting a very small rise in cytotoxic alloantibody was noted in some cases but this returned to zero. A late rise in antibody titre was exhibited by one rat that died 30 days post transplant. In general, low cytotoxic antibody levels are associated with prolonged graft survival as would be expected in a model where such an antibody is believed to cause acute graft damage.

The decline in cytotoxic alloantibody responses following tolerance induction by serial blood transfusion and cyclosporin A treatment was clearly demonstrated to be independent of the thymus gland (Figure 7.2). Rats having undergone thymectomy 30 days prior to transfusion and cyclosporin A treatment showed similar responses to rats having undergone only sham thymectomy.

The class and subclass of circulating anti-class I antibody in recipients of R8 renal allografts were determined by flow cytometric analysis (Figure 7.3). Whereas serum from unmodified recipients contained anti-class I antibodies of IgM and of IgG1, IgG2a and IgG2b isotypes, serum from tolerant animals contained minimal IgM alloantibody or IgG antibody of any subclass.

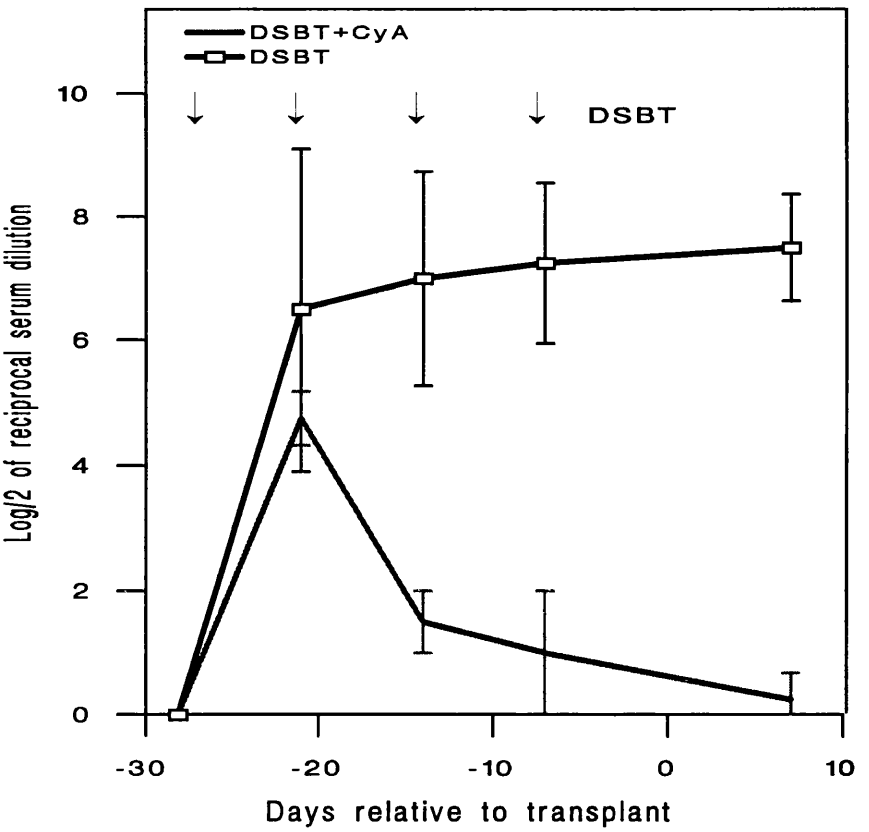



**Figure 7.1 Circulating cytotoxic RT1A$^a$ antibody responses in RT1$^U$ rats to serial R8 blood transfusions followed by a R8 renal allograft**

RT1$^U$ rats were given a series of four 1ml transfusions of R8 blood (DSBT) at 7 day intervals (arrows) followed by a R8 renal allograft on day 0. Recipients were given either DSBT alone or DSBT together with cyclosporin A (15mg/kg/day) for 7 days following the initial DSBT.Contralateral nephrectomy was carried out day 7 post transplant. Sera were assayed against $^{51}$Cr-labelled R8 Con A blasts in the presence of guinea pig complement. Values shown are the mean of 4 rats +/- SD.

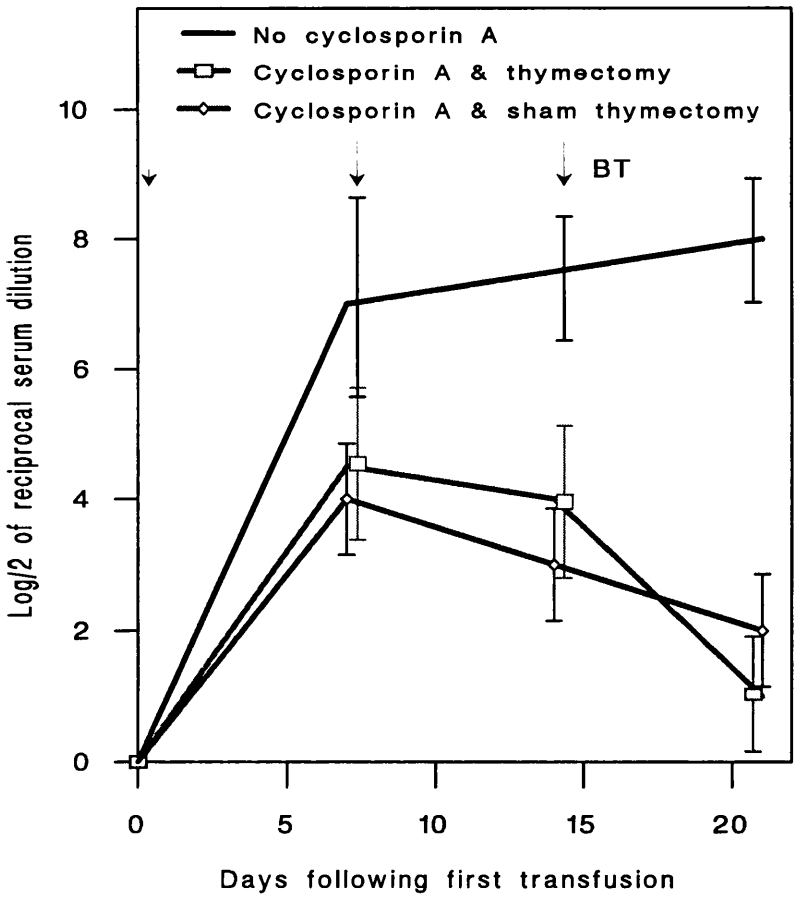



**Figure 7.2 Circulating cytotoxic RT1A$^a$ antibody responses in thymectomised and sham thymectomised RT1$^U$ rats to serial R8 blood transfusions**

RT1$^U$ rats both thymectomised and sham thymectomised were given a series of three 1ml transfusions of R8 blood (BT) at 7 day intervals (arrows). Recipients were given either R8 blood alone or together with cyclosporin A (15mg/kg/day) for 7 days following the initial transfusion. Sera were assayed against $^{51}$Cr-labelled R8 Con A blasts in the presence of guinea pig complement. Values shown are the mean of 4 rats +/- SD.

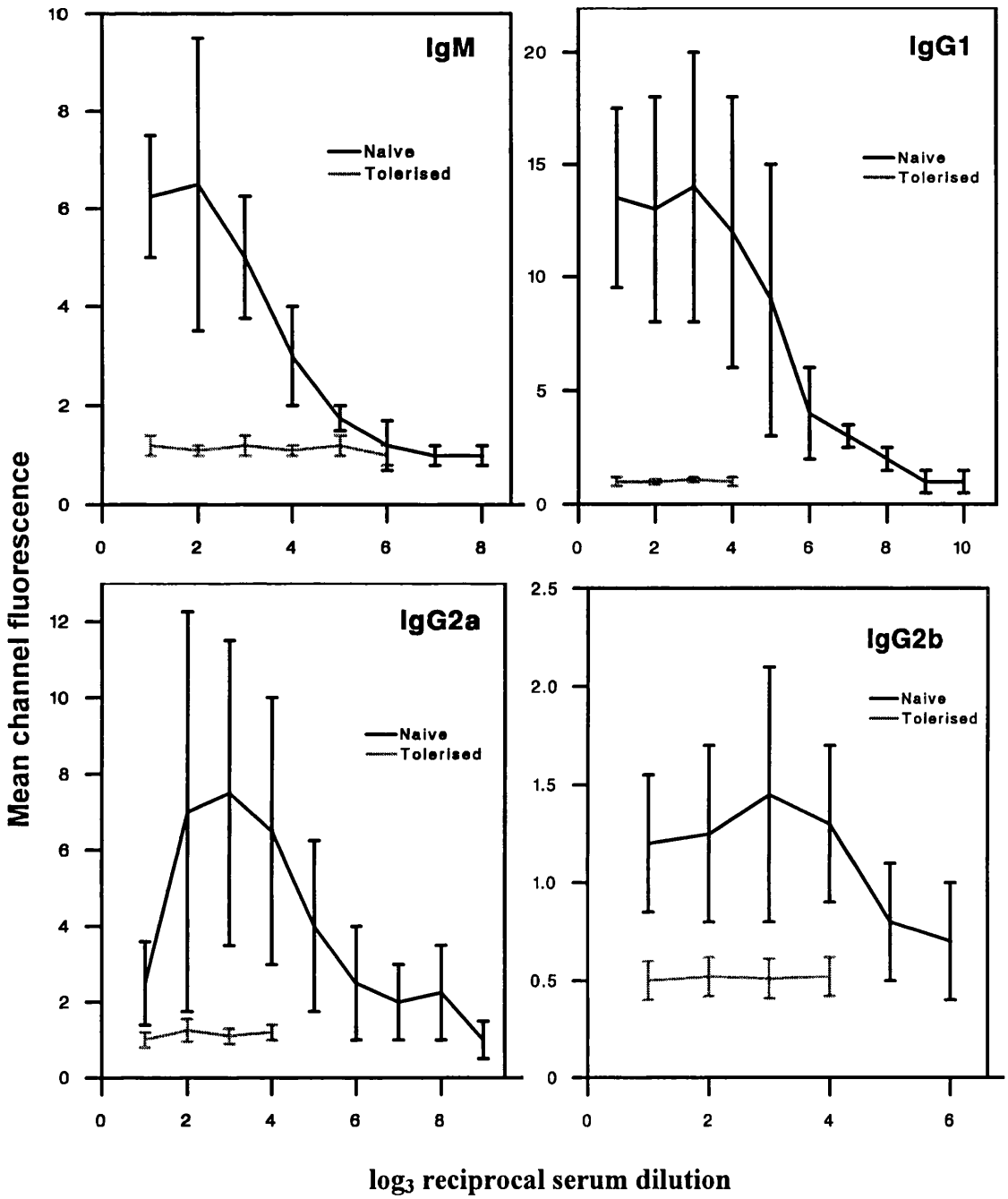



**Figure 7.3 Circulating anti-RT1A$^a$ antibody responses in RT1$^U$ rats 7 days after receiving a R8 renal allograft**

Graft recipients were either unmodified (naive) or had been rendered tolerant ("tolerised") by four 1ml DSBT's at 7 day intervals (days -28, -21, -14, -7 relative to transplant) together with cyclosporin A (15mg/kg/day) for 7 days after the first DSBT (days -28 to -21). The subclass and isotype of Ig were determined by flow cytometery using R8 lymph node cells as targets and mouse anti-rat Ig-specific monoclonal antibodies. Values shown are the mean of 3-4 rats +/- SD.

DA into Lewis

A role for cytotoxic alloantibody in acute rejection is not so clearly established in this strain combination. Cytotoxic alloantibody levels were shown to correlate however with the failure of a single DSBT to cause any positive effect in terms of graft survival. Figure 7.4 demonstrates that a single blood transfusion 7 days prior to transplant stimulates a strong response as measured by cytotoxic alloantibody titre. Grafts at this point are rapidly destroyed (Table 6.3) and the antibody titre remains high. While this does not prove that cytotoxic alloantibody is an important effector mechanism in this model (this would necessitate showing that serum transfer from rats bearing rejecting grafts could cause acute damage to stable grafts in a second tolerised rat) it does at least suggest that alloantibody may act as a marker of activation of the host immune system.

Repeated blood transfusions alone do not appear to lead to any significant decrease in antibody titre and grafts continue to be rapidly rejected (Figure 7.5). As in the previous R8 into $RT1^U$ experiments, the addition of a short course of cyclosporin A at the time of the initial transfusion resulted in a much smaller rise in antibody titre that declined dramatically despite further antigenic challenges in the form of either blood or a graft. These grafts went on to enjoy long-term survival.

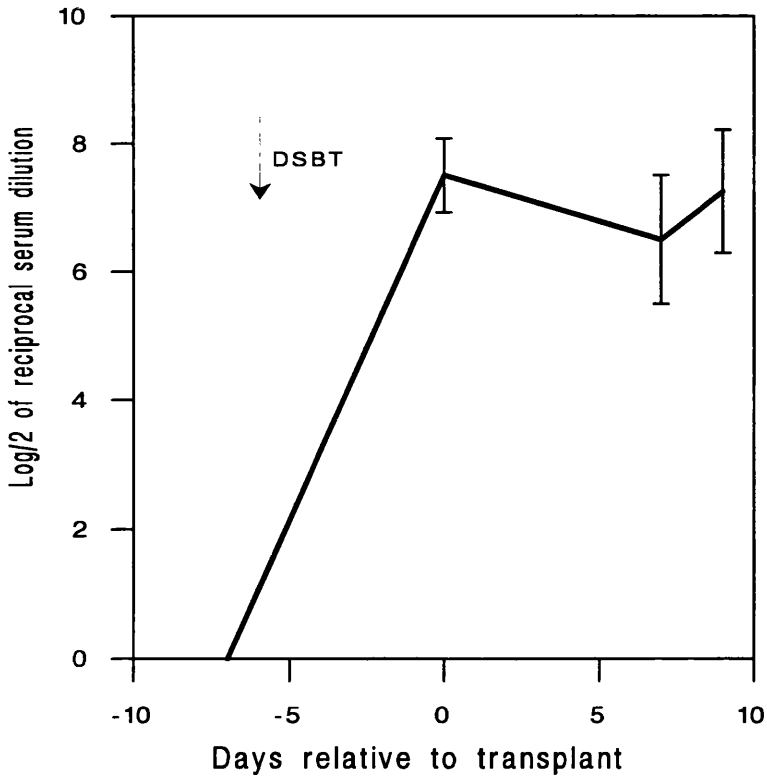



**Figure 7.4 Circulating cytotoxic alloantibody responses in Lewis rats to a DA blood transfusion followed by a DA renal allograft**

Lewis rats were given a 1ml transfusion of DA blood (DSBT arrow) followed 7 days later by a DA renal allograft. Contralateral nephrectomy was carried out day 7 post transplant. All rats were sacrificed on day 9 due to having developed severe uremia secondary to graft rejection. Sera were assayed against $^{51}$Cr-labelled DA Con A blasts in the presence of guinea pig complement. Values shown are the mean of 4 rats +/- SD. There was no lysis of third-party PVG target cells (data not shown).

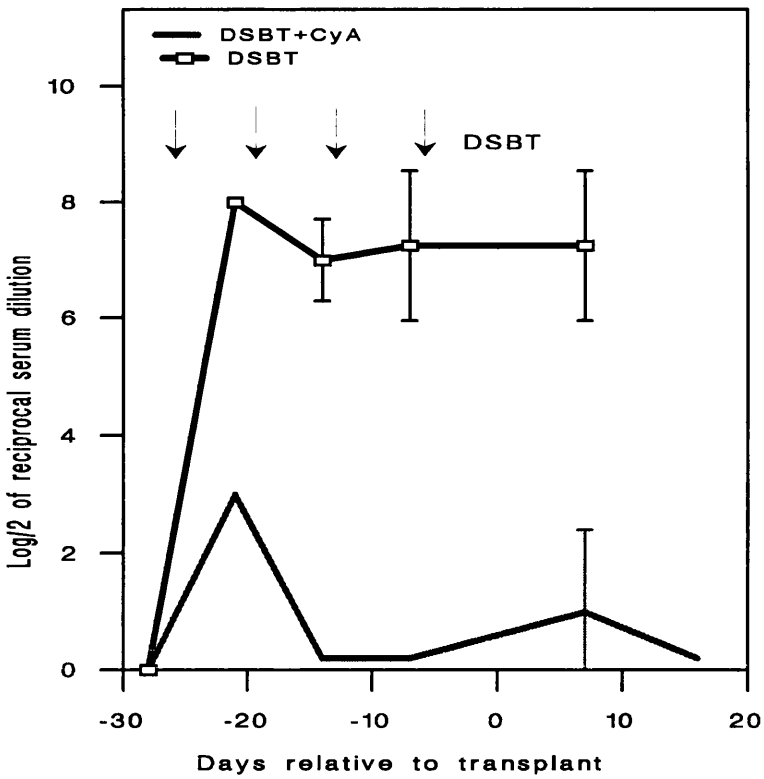



**Figure 7.5 Circulating cytotoxic alloantibody responses in Lewis rats to serial DA blood transfusions followed by a DA renal allograft**

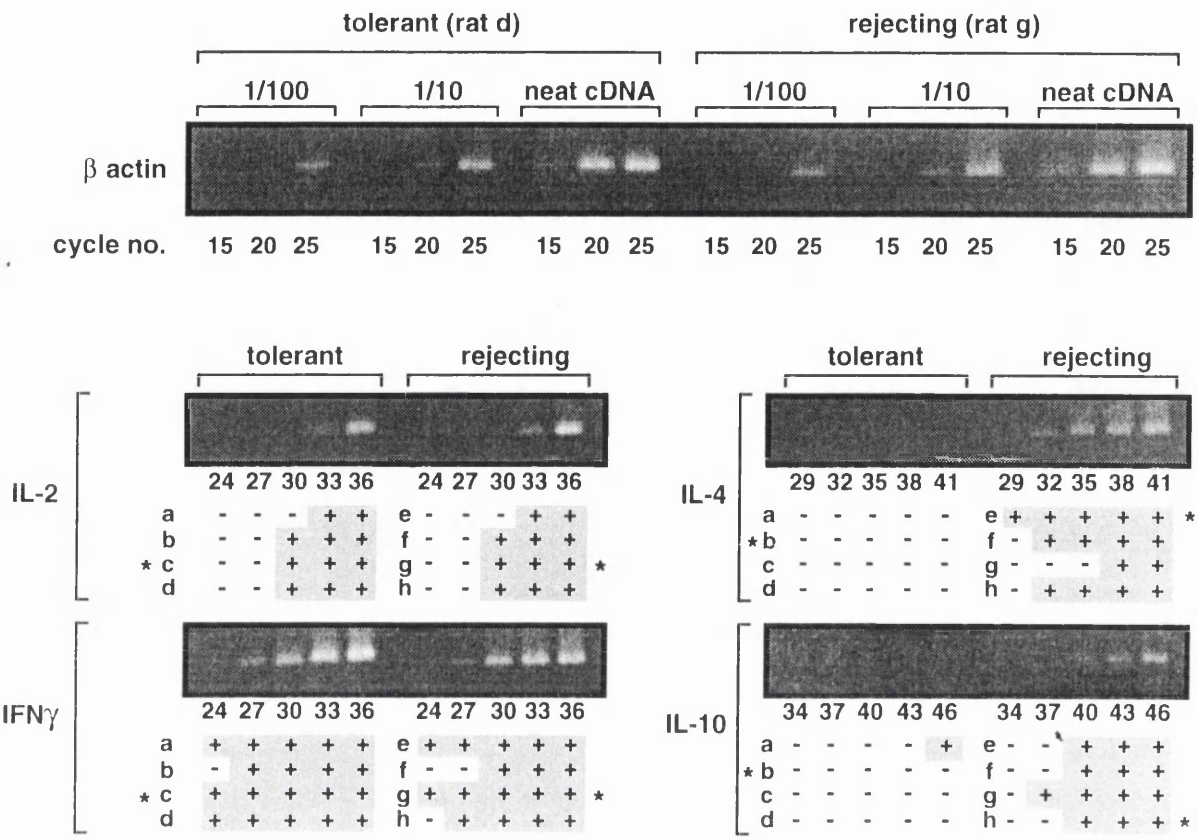
Lewis rats were given a series of four 1ml transfusions of DA blood (DSBT) at 7 day intervals (arrows) followed by a DA renal allograft on day 0. Recipients were given either DSBT alone or DSBT together with cyclosporin A (15mg/kg/day) for 7 days following the initial DSBT. Contralateral nephrectomy was carried out day 7 post transplant. All rats that received DSBT-pretreatment alone were sacrificed on day 9 having developed severe uremia secondary to graft rejection. Sera were assayed against $^{51}$Cr-labelled DA Con A blasts in the presence of guinea pig complement. Values shown are the mean of 4 rats +/- SD. There was no lysis of third-party PVG target cells (data not shown).

### 7.2.6 Cytokine mRNA levels in renal allografts and spleens of tolerised and naive rats 5 days post transplant. R8 into $RT1^U$

The presence of cytokine mRNA in class I-disparate kidney allografts 5 days post transplant was determined by semiquantitative RT-PCR (Table 7.3). Message for IL-2 and IFN-γ was detected at essentially similar levels in tolerant and rejecting allografts. Message for IL-4 and IL-10 was readily detected in the rejecting grafts but was barely present in the tolerant allografts of the DSBT/cyclosporin A-pretreated rats. The significance of this striking difference is discussed later.

The presence of cytokine mRNA in the spleens of those rats receiving class I-disparate kidney allografts was also determined by semiquantitative RT-PCR 5 days post transplant (Table 7.4). Message for IL-2 and IFN-γ was detected at broadly similar levels in the spleens of rats bearing either rejecting or tolerant grafts and similar levels were also detected in spleens removed from naive $RT1^U$ rats. IL-10 transcript was detected at low levels in the spleens of rats bearing rejecting kidneys but was found in none of the rats bearing tolerant kidneys. Where detectable, levels were substantially lower than those seen in the actual grafts themselves. Message for IL-4 was detected at varying levels in the spleens of most rats tested but there was no clear pattern and the significance of the IL-4 transcript in the spleen is unclear.

**Table 7.3 Semiquantitative analysis of cytokine mRNA in class I-disparate kidney allografts**




R8 grafts were excised 5 days post transplant into unmodified $RT1^U$ recipients (rejecting) and recipients pretreated with 4 R8 DSBT's together with cyclosporin A for 7 days following the first DSBT (tolerant). Results are shown for 4 tolerant (a-d) and 4 rejecting (e-h) kidney allografts.

PCR for individual cytokine mRNA was performed on neat cDNA prepared from each allograft (a-h) and products sampled at sequential 3 cycle intervals. Products were gel electrophoresed stained with ethidium bromide and viewed by UV transillumination. The presence (+) or absence (-) of a visible band was then recorded for each cycle number sampled. Note that at the lowest cycle numbers bands visible with UV and scored present (+) failed to appear on photographic reproduction. Asterisks identify individual tolerant and rejecting grafts for which gels of cytokine PCR reactions are shown. Gel electrophoresis of products from β-actin PCR (top) performed on 3 concentrations of cDNA show bands at 5-cycle intervals that are equivalent in tolerant and rejecting tissues (e.g., grafts d and g) demonstrating the integrity of RNA and cDNA preparation.

**Table 7.4 Semiquantitative analysis of cytokine mRNA in spleens of rats receiving class I-disparate kidney allografts**

IL-2

| | Tolerant | | | | | Rejecting | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| cycle no | 24 | 27 | 30 | 33 | 36 | 24 | 27 | 30 | 33 | 36 |
| a | - | - | - | - | - | e | - | - | - | + |
| b | - | - | - | - | + | f | - | - | - | + |
| c | - | - | - | - | + | g | - | - | - | + |
| d | - | - | - | - | + | h | - | - | - | + |

IL-4

| | Tolerant | | | | | Rejecting | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| cycle no | 29 | 32 | 35 | 38 | 41 | 29 | 32 | 35 | 38 | 41 |
| a | - | - | - | + | + | e | - | + | + | + | + |
| b | - | - | + | + | + | f | - | + | + | + | + |
| c | - | + | + | + | + | g | - | - | - | - | - |
| d | - | - | - | - | + | h | - | - | - | - | + |

IFN-γ

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| cycle no | 24 | 27 | 30 | 33 | 36 | 24 | 27 | 30 | 33 | 36 |
| a | - | - | - | + | + | e | - | - | + | + | + |
| b | - | - | + | + | + | f | - | - | + | + | + |
| c | - | - | + | + | + | g | - | - | - | + | + |
| d | - | - | - | + | + | h | - | - | - | + | + |

IL-10

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| cycle no | 34 | 37 | 40 | 43 | 46 | 34 | 37 | 40 | 43 | 46 |
| a | - | - | - | - | - | e | - | - | - | - | + |
| b | - | - | - | - | - | f | - | - | - | - | + |
| c | - | - | - | - | - | g | - | - | - | - | + |
| d | - | - | - | - | - | h | - | - | - | - | + |

Spleens were excised 5 days post transplant of R8 renal allografts into unmodified $RT1^U$ recipients (rejecting) and recipients pretreated with 4 R8 DSBT's together with cyclosporin A for 7 days following the first DSBT (tolerant). Results are shown for 4 rats bearing tolerant (a-d) and 4 rat bearing rejecting (e-h) allografts.

PCR for individual cytokine mRNA was performed on neat cDNA prepared from each spleen (a-h) and products sampled at sequential 3 cycle intervals. Products were gel electrophoresed stained with ethidium bromide and viewed by UV transillumination. The presence (+) or absence (-) of a visible band was then recorded for each cycle number sampled.

## 7.3 Discussion of the DSBT/cyclosporin A-induced model of tolerance in a class I-disparate rat renal allograft model

Using a pretransplant protocol comprised of 4 serial DSBT's combined with a 7 day course of cyclosporin A (given with the first DSBT) we were able to achieve long-term donor-specific renal allograft acceptance without the need for further treatment. The humoral and intragraft cellular events that accompany induction of tolerance by this protocol are of considerable interest since little is known in a model where alloantibody plays a key role in effecting acute rejection.

Microscopic analysis of the cells infiltrating these allografts 5 days post transplant showed that both rejecting and tolerant grafts were heavily infiltrated by mononuclear cells, including numerous $CD4^+$ and $CD8^+$ cells, although the distribution varied between the rejecting and tolerised grafts. In addition the virtual absence of $OX39^+$ cells (IL-2R) in tolerant grafts suggested a reduced state of cellular activation. No attempt was made in this study to determine the in vitro cytotoxic activity of these graft infiltrating cells as previous work had shown that the graft infiltrate from rejecting R8 grafts displays minimal cytotoxicity for R8 target cells (Gracie *et al.*, 1990).

The retransplantation of tolerised rats with fresh kidneys demonstrated that this protocol produced genuine tolerance as R8 kidneys showed long-term survival. The rapid rejection (3/4) seen when longstanding "parked" grafts were retransplanted into naive $RT1^U$ rats suggests that graft adaptation is minimal but prolonged survival is due to changes occurring within the host.

One interpretation of the rapid rejection of "parked" grafts is that the $RT1^U$ host's immune system is capable of processing the antigens remaining within the grafts and mounting an effective immune response without requiring professional antigen presenting cells (dendritic cells) contained in a fresh graft. This requires that the $RT1^U$ APC's are capable of presenting graft antigens (class I MHC derived as other transplant antigens are identical in this strain combination) to the effector cells involved in graft destruction. This in turn implies that there is an effective route of indirect presentation of graft antigens in this model. An alternative, but perhaps less likely, explanation might be that there are still donor-derived APC's present in the graft following its period of residence in the primary host. The marked prolongation of survival of "parked" grafts in the DA into PVG model studied earlier (Chapter 4, Section 4.2.1) suggests that such indirect presentation is of little consequence in that particular model. The idea that the relative contributions of the direct and indirect routes of antigen presentation to rejection, may be responsible for the strain-dependent efficacy of DSBT-pretreatment protocols has been proposed previously (Parker *et al.,* 1992). Further work using T cell clones has suggested that indirect presentation is favoured if the class II MHC of the donor and recipient are well matched (Chen *et al.,* 1990). Clearly the common class II antigens of the congeneic rats used in this model fit this hypothesis very well. Retransplantation experiments in the DA into Lewis strain combination were not examined although it would be of interest to see which pattern they would follow.

As in the earlier study of DSBT-induced tolerance in the DA into PVG model this DSBT/cyclosporin A-induced tolerance appears to be peripheral in nature, despite the fact that cyclosporin A is known to have a central effect on the thymus gland. This finding is potentially important since the human adult thymus is believed to be much less immunologically active than the rodent thymus. Consequently a mechanism of tolerance that required an active thymus would be less likely to be significant in a clinical environment. Both models of transplantation tolerance discussed in this work are independent of the thymus and thus may be suitable for the clinical setting.

Given the known ability of cytotoxic alloantibody to destroy $A^a$-disparate grafts in $RT1^U$ recipients, it was not surprising that tolerance induction by serial DSBT and cyclosporin A was associated with abrogation of circulating cytotoxic alloantibody. Exposure of the recipient's immune system to DSBT, initially in the presence of cyclosporin A, appears to lead to a reduced production of all subclasses of IgM and IgG in response to further challenge with repeated DSBT's and a renal allograft. The absence of circulating anti-class I alloantibody could, in theory, be due to either B cell tolerance or to impairment of $CD4^+$ T cell help. The principle site of action of cyclosporin A is the T helper cell where it appears to prevent clonal expansion of activated T cells (Granelli-Piperno, 1990). Exposure of the recipient's immune system to donor alloantigen in the absence of appropriate $CD4^+$ T cell help is likely to play a key role in tolerance induction in this model. Further studies might involve the transfer of $CD4^+$ T cells from tolerant animals into congenitally athymic

nude $RT1^u$ rats (which have an intact B cell pool) to see if they provide help for rejection of $A^a$-disparate allografts.

Previous studies involving DSBT's and cyclosporin A have raised the possibility of suppressor cells (Jones *et al.,* 1988) or the involvement of anti-idiotypic antibodies (Cunningham *et al.,* 1988). These proposals were not considered in the current study and would be of interest for future investigations using this model.

The limited cytokine analysis carried out in this model is interesting since the reductions found in the levels of IL-4 and IL-10 mRNA are consistent with the earlier finding that reduced circulating cytotoxic alloantibody is associated with prolonged graft survival. IL-4 provides help for antibody production by B cells (Paul and Ohara, 1987) and IL-10 is a costimulatory factor for B cell growth, differentiation and Ig secretion (Defrance *et al.,* 1992). Some caution is required in equating detection of IL-4 and IL-10 mRNA with the presence or absence of Th2 $CD4^+$ cells: non-lymphoid cells may under certain conditions produce IL-10 (Broski and Halloran, 1994) and IL-4 may be produced by mast cells and basophils (Plaut *et al.,* 1989).

The ability of DSBT, used in conjunction with transient immunosuppression, to produce long-term tolerance to a subsequent allograft has considerable implications for clinical transplantation. DSBT alone has been abandoned almost completely in clinical practice due to both the theoretical risk seen in animal studies and the real risk, confirmed clinically, of sensitising the host to donor antigen. Such sensitisation leads to accelerated or hyperacute rejection of an allograft as a result of the recipient developing anti-donor HLA class I cytotoxic antibody. The use of

cyclosporin A simultaneous with blood transfusion has been shown, by measurement of antibody level, to reduce the risk of allosensitisation in the rat (Cunningham *et al.*, 1988). In the present study, this combination was shown to confer long-term donor-specific protection to both a fully disparate graft and also to a pure class I MHC-disparate graft in which alloantibody is known to be essential for graft destruction.

The precise mechanism underlying this effect is not yet clear but would appear to be peripheral in nature. It is likely that an alteration in the ability of the $CD4^+$ T helper cell to effectively orchestrate the immune response to the graft is central to this effect. This study does not support the belief that transplant tolerance is associated with polarisation of the cytokine network in favour of a Th2 response. Rather it would appear that the cytokine profile in tolerant grafts is variable and may well depend on such factors as the type of graft, the MHC disparity and in particular the strategy used for tolerance induction.

In clinical transplantation indirect presentation is believed to assume greater importance after the early post transplant period where direct allorecognition, driven by donor dendritic cells, is believed to predominate. This may suggest that the late graft loss, seen in clinical practice, from chronic rejection is due to indirect presentation by self-restricted alloreactive T cells (Bradley, 1996).

In rodent transplant models allograft rejection tends to be almost exclusively attributed to cellular effector mechanisms, notably cytotoxic T cells and DTH (Hall, 1991). In this model the undoubted role of alloantibody mediated rejection gives it considerable clinical relevance given the relatively greater role of alloantibody in the

human compared to the rat immune system (Bradley *et al.*, 1992). Whether this protocol has any effect on presensitised recipients who already have high levels of circulating anti-class I antibody remains to be addressed and is clearly of interest in a clinical setting.

## 7.4 Summary

1 Tolerised grafts are heavily infiltrated with mononuclear cells but these lack IL-2R expression suggesting that they have been inactivated in some way.

2 Graft adaptation is minimal and this may reflect an increased role for indirect presentation in the high responder rat strain models.

3 This model of tolerance appears to be peripheral in nature.

4 Graft acceptance correlates with a reduction in all classes of cytotoxic alloantibody.

5 High levels of intragraft IL-4 and 10 seen in rejecting grafts are absent in tolerised grafts.

6 No evidence was found to support Th2 cells being tolerogenic.

# CHAPTER EIGHT

## Final Discussion

Less than forty years have elapsed since early reports of successful renal transplantation between unrelated donors and recipients (Murray *et al.,* 1963). Progress in transplantation has led to it becoming the treatment of choice for end-stage renal failure with few absolute contraindications. Most contraindications to transplant are relative and a consequence of a critical shortage of cadaveric organ donors. The number of renal transplants performed annually in the U.K. grew rapidly from 1974 (<450) to 1990 (>1800) but has since plateaued (Moore, 1997). Currently the U.K. transplant waiting list is rising by some 250 potential recipients a year as demand exceeds supply.

Clinical outcome following renal transplant appears initially to be highly satisfactory with 1-year patient and graft survival in excess of 95% and 90% respectively (UKTSSA, Bristol, 1994). By 5 years post transplant however, graft survival is in the region of only 50-60% and continues to fall thereafter (Belger *et al.*, 1997). Thus rejection remains the main cause of graft loss despite an increasingly sophisticated array of anti-rejection drugs. In addition to being unable to fully protect the allograft such drugs have very significant side effects. The long-term incidence of malignancies, primarily skin and lymphoid cancers, present the greatest concern (Penn, 1990; Barroso-Vicens *et al.*, 1997). The ability to withdraw immunosuppression after an initial induction period designed to facilitate tolerance, would diminish these side effects. Currently this is not possible due to the risk of rejection and graft loss. Research into potential strategies for inducing tolerance is

vital if we are to be able to maximise the benefit of limited organ availability while avoiding immunosuppressive dangers.

Tolerance without the need for immunosuppressive drugs has been achieved in many animal models. One of the earliest of these models was donor-specific blood transfusion (DSBT)-pretreatment prior to renal transplantation in the DA into PVG rat strain combination (Fabre and Morris, 1972). This model was used in the early part of the current study. Despite the prolonged period since this model was first described the mechanism of tolerance induction remains elusive and worthy of further study. One criticism of the model is the relative ease of tolerance induction as the PVG rat is a low responder to DA alloantigens (Butcher and Howard, 1982). Consequently we sought to develop a model of DSBT induced tolerance that was effective in high responder strain combinations and so compare the two models. This was successfully achieved by the addition of a short course of cyclosporin A to the first of a series of DSBT's.

The experiments reported herein, generated several interesting findings which contribute to an understanding of the blood transfusion effect. The powerful responses to alloantigens and hence the mechanisms of graft rejection are now accepted to revolve around the interaction of host T cells and the allo-MHC molecules contained within the graft (Murphy and Sayegh, 1996). Two methods for recognition of such foreign antigens exist and are described as "direct" and "indirect"(Lechler and Batchelor, 1982). "Direct" recognition involves the recipient T cell recognising intact allo-MHC on the surface of donor cells while the "indirect"

pathway allows for recipient T cells to recognise processed allo-MHC presented by recipient antigen presenting cells. The relative importance of each of these pathways is under considerable debate but the "indirect" pathway may well prove to be of particular importance in terms of the chronic graft loss, despite immunosuppression, that is a major problem in clinical transplantation (Liu *et al.*, 1993). In contrast the "direct" pathway may be responsible for early acute rejection (Braun *et al.*, 1993) since the graft initially contains a significant number of donor-derived passenger APC's (such as dendritic cells) that are capable of providing the necessary costimulatory signals for full T cell activation (Larsen *et al.*, 1992).

In our study the low responder PVG rat would appear to be poor at mounting responses via the "indirect" pathway and therefore the rapid rejection of DA kidneys by unmodified PVG recipients is primarily achieved via the "direct" pathway. The prolonged survival of "parked" DA grafts when retransplanted into a second unmodified PVG rat and the inability of sensitised lymph node cells to damage longstanding grafts both point to such a deficit in "indirect" presentation. This apparent loss of immunogenicity of the transplanted organ has been referred to as graft adaptation by other workers (Marquet *et al.*, 1985). The long-term survival of the kidney allograft seen with a short course of cyclosporin A may be explained if the cyclosporin A prevents early "direct" initiation of rejection while passenger cells leave the kidney. Subsequently it no longer presents a target for "direct" rejection and in the absence of an active "indirect" pathway long-term survival occurs. The transient rise we often noted in serum urea and creatinine in DSBT-pretreated rats

could suggest that protection by DSBT alone is relatively limited but is sufficient to allow long-term graft survival because the rejection mechanisms in this strain combination are only operative until the passenger leukocytes have left the graft.

Despite the results cited above graft adaptation is not the only reason for the prolonged survival seen in the DA into PVG renal allograft model. We have shown that second grafts (kidney and heart) are also accepted and that proliferation in the MLR is decreased. Thus DSBT does lead to tolerance and the mechanism behind this effect is peripheral in nature as thymectomy does not alter these findings. Several mechanisms have been proposed to explain the tolerant state induced by DSBT although no consensus has yet been reached as to their relative importance.

In 1978 Lafferty proposed the "two-signal hypothesis". This stated that if the T cell encounters antigen in the absence of a second costimulatory signal from the antigen presenting cell then the T cell becomes functionally paralysed or "anergic". The later descriptions of the roles of the CD28 and B7/BB1 molecules in supplying such signals (Schwartz, 1995) gave the two signal hypothesis considerable impetus. Exogenous IL-2 has been shown to "rescue" cells from such an anergic state (Heath *et al.,* 1992) and further to be able to abrogate DSBT-induced graft tolerance (Dallman *et al.*, 1991). If DSBT is serving to block such second signals then this is an attractive concept by which to explain DSBT-induced tolerance. One criticism of anergy as a major source of tolerance is that if the host has to subsequently initiate immune responses against separate pathogens in tissues then these potentially reactive cells may be rescued by signals or cytokines from "bystander" cells

(Bretscher, 1992). If as implied above, the PVG rat can only mount a response by "direct" presentation while passenger leukocytes are still present in the graft then this potential late loss of tolerance is of little importance in this model. Exogenously administered IL-2 has been reported to abrogate DSBT-induced tolerance when administered at the time of transplant (Dallman *et al.,* 1991). We would hypothesis that in this model delayed administration of IL-2 would fail to bring about rejection of stable grafts, an area for future investigation. The rejection of grafts in high responder strain combinations treated with either a short course of cyclosporin or with DSBT-pretreatment alone, would imply that "indirect" presentation is also important in such cases and that any tolerogenic mechanism would require a long-term mode of action.

Donor-specific microchimerism (DSM), whereby low levels of donor leukocytes may persist and multiply in the periphery of the recipient, has been suggested as a possible mechanism for induction and maintenance of tolerance (Murase *et al.,* 1996). DSBT and bone marrow transfusions prior to transplant have been demonstrated to lead to DSM (McDaniel *et al.,* 1994; Sakrada *et al.,* 1997). What is not clear is whether chimerism is an essential requirement for tolerance as Starzl has implied previously (Starzl *et al,* 1993) or is simply an epiphenomenon of successful organ transplantation (Schlitt, 1997). More recent studies have tended to support the concept that DSM is associated with but is not essential for tolerance (Sivasai *et al.,* 1997; Sakurada *et al.,* 1997). The close connection between tolerance induction and microchimerism has led to several attempts to augment leukocyte

numbers at the time of transplant in the hope of achieving a tolerant state (Barber *et al.*, 1991; Tsaroucha *et al.*, 1997). The model of tolerance in high responder rat strain combinations that we developed using repeated DSBT given with cyclosporin A could be explained by microchimerism induction although we did not look specifically for this in our studies. Microchimerism can be viewed merely as a form of antigen persistence as is the continuing presence of the graft itself. The idea that persistent antigenic stimulation may be tolerogenic has been proposed by some investigators (Hamano *et al.*, 1996). Chen and coworkers (1993) demonstrated that the tolerant state could be lost following graft removal. They proposed chronic desensitisation of the T cell receptor by persistent alloantigen as a possible mechanism of tolerance since they were unable to demonstrate either a suppressive serum factor or any regulatory cell production. Several other groups however have been able to adoptively transfer tolerance in different models (Hall *et al.*, 1990; Qin *et al.*, 1993; Scully *et al.*, 1994). In addition Field and coworkers (1997) describe "immunoregulatory" CD4 cells with the capacity to maintain tolerance potentially via a variety of mechanisms depending on the given situation being studied.

The role of cytokines in the pathophysiology of acute and chronic allograft rejection is now widely debated (reviewed by Nadeau *et al.*, 1996) and a considerable amount of our study involved the examination of cytokine profiles seen in rejecting and tolerised grafts in both low and high responder models of transplant tolerance. The Th1/Th2 paradigm, with the Th2 phenotype being associated with tolerance and a Th1 phenotype closely linked with rejection has been reported by several groups

(Lowry, 1993; Chen and Field, 1996). Our studies of the kinetics of IL-2 production in rejecting allografts and the abolition of the IL-2 peak on day 4 post transplant by DSBT pretreatment are comparable with other reports (Dallman *et al.,* 1991; Bugeon *et al.*, 1993) and agree that prevention of Th1 responses is critical for tolerance induction. In contrast our finding of high Th2 cytokine levels in rejecting R8 grafts and their abolition by DSBT/cyclosporin A-pretreatment clearly falls outwith the current Th1/Th2 paradigm. The abolition of the alloantibody responses of all IgG isotypes, demonstrated following DSBT/cyclosporin A-pretreatment, correlates with the cytokine mRNA findings and also with the previously published finding that cytotoxic alloantibody is a major cause of graft rejection in this strain combination (Morton *et al,* 1993). At least one other study of DSBT-pretreated recipients has also reported a decrease in both Th2 and Th1 cytokines (Josien *et al.*, 1995). In addition several studies have shown high IL-4 and IL-10 mRNA levels in rejecting grafts (Dallman *et al.,* 1991; Chan *et al.,* 1995). While there are several studies that support the Th1/Th2 paradigm (Siegling *et al.,* 1994; Hamano *et al.,* 1997) it would appear that the intragraft cytokine profile in tolerant grafts is variable and likely to depend on such factors as the type of graft, the MHC disparity and in particular the strategy used for tolerance production.

It is clear that tolerance production can be achieved in the human situation. There are now several reports of long-term allograft survival after discontinuation of all immunosuppressive therapies following successful liver transplantation and also similar isolated reports of renal allograft survival (Starzl *et al.,* 1993). A study in

rhesus monkeys of pretransplant blood transfusions and cyclosporin A has shown long-term survival of renal allografts following withdrawal of all immunosuppression but only if the transfusion was donor-specific (Leonard *et al.*, 1996). The problem with either reducing or withdrawing immunosuppression is that with our current level of understanding of tolerance we are unable to predict which patients this might be appropriate for and such attempts have a substantial risk of leading to graft loss. Currently patient noncompliance with immunosuppressive regimes has been reported to be one of the major causes of kidney graft failure (Garcia *et al.*, 1997).

The potential for "indirect" pathways of rejection to play a major role in chronic rejection has been noted by several investigators (Bradley *et al.*, 1992; Braun *et al.*, 1993). This form of graft rejection primarily affects the arteries in the graft leading to vascular occlusion and graft loss. In clinical terms it has been suggested that periodic assessment of the indirect allorecognition pathways in renal transplant recipients, with long-term well-functioning grafts, may allow closer monitoring of the level of immunosuppression required to optimise long-term graft function and reduction of such levels where appropriate (Iniotaki-Theodoraki *et al.*, 1997). More interestingly, in animal models DSBT has been shown to prevent the onset of chronic (vascular) rejection (Wood *et al.*, 1996). The use of multiple blood transfusions has also been shown to have a profound effect on indirect T cell allorecognition even without the use of concomitant immunosuppression (Sawyer and Fabre, 1997). Chronic rejection is now believed to be the cause of graft loss more often than acute rejection (Orosz *et al.*, 1997). If peri/pre-transplant blood transfusions can be shown

to reduce chronic rejection then their use may become more widespread once again. Currently only a small number of transplant centres worldwide still include donor-specific blood or bone marrow transfusion in their protocols for living related renal transplants (Okazaki *et al.*, 1997) or living related liver allografting (Ricordi *et al.*, 1997). Both of these groups claim enhanced graft survival with their protocols and in liver transplantation increasing the number of transfusions appears to increase the beneficial effect. Similarly a small number of groups have given donor-strain blood or bone marrow at the time of cadaveric allografting and long-term results from these groups may reveal potential advantages to this strategy (Alexander *et al.*, 1992).

Clearly we would hope that as our understanding of both the mechanisms for rejection and also for tolerance induction continue to grow many of the tantalising prospects for organ transplantation shown in animal models will facilitate their use in the field of clinical transplantation.

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