CELLULAR AND HUMORAL EFFECTOR MECHANISMS OF ACUTE CARDIAC ALLOGRAFT REJECTION IN A RAT MODEL

by

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Abstract

This thesis examines the role played by $CD4^+$ and $CD8^+$ T cells in the rejection of a class I disparate cardiac allograft in a rat model (R8---$RT1^u$). The effector mechanisms involved in the rejection process are studied, and particular emphasis given to the possibility of specific alloantibody acting as an effector of acute allograft rejection.

Following the establishment of the rejection times of a class I disparate, heterotopic, intra-abdominal, cardiac allograft in unmodified animals, manipulation of T cell subsets in the recipient animals was achieved *in vivo* by the administration of mouse anti-rat monoclonal antibodies specific for either the CD4 or the CD8 molecule. Depletion of $CD8^+$ T cells had no effect on graft survival whereas $CD4^+$ T cell depletion resulted in prolonged allograft survival. In addition, the acceptance of the class I disparate graft following anti-CD4 monoclonal antibody treatment was associated with the abrogation of the specific alloantibody response against the donor tissue seen in unmodified rejection and in the anti-CD8 treated group.

Anti-class I alloantibody was shown to specifically lyse *in vitro* cultures of donor cardiac endothelial cells and passive transfer of immune serum *in vivo* results in the restoration of acute rejection in an animal depleted of $CD4^+$ T cells.

This calls into question the accepted belief that the $CD8^+$ T cell is of crucial importance in the acute rejection of a class I disparate allograft, by its role as a cytotoxic T cell. The $CD8^+$ T cell is <u>not</u> required for the rejection of such a graft in this model. On the contrary, the $CD4^+$ T cell was found to be essential to the rejection process and the findings of the alloantibody experiments suggest that the $CD4^+$ T cell is mediating acute rejection by providing T cell help for the production of specific, cytotoxic alloantibody.

The significant finding of the rejection of a class I disparate allograft in the absence of $CD8^+$ T cells provides further support for the indirect pathway of allorecognition.

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The design of the work presented in this thesis is that of the author and Mr. J.A. Bradley.

All of the cardiac transplants, and other animal experiments, were carried out by the author . The mouse anti-rat monoclonal antibodies were prepared by Dr. E.M. Bolton. Dr. H. Armstrong performed the *in vitro* assessment of specific alloantibody levels and its ability to lyse neonatal cardiac endothelial cells.

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The FACS analysis data refered to in 4:3.1 and illustrated in Figure 4:1 have been published by Gracie and co-workers (Gracie et al, 1990).

ABBREVIATIONS

ADCC	antibody dependent cellular cytotoxicity
Ag	antigen
APC	antigen presenting cell(s)
ATG	anti-thymocyte globulin
ATXBM	adult thymectomised, irradiated, bone marrow reconstituted
β2m	β-2 microglobulin
BSA	bovine serum albumin
CO_2	carbon dioxide
Con A	concanavalin A
CoVF	cobra venom factor
Cr	chromium
CTL	cytotoxic T lymphocyte
CyA	cyclosporin A
DAB	Dulbeccos A & B medium
DTH	delayed-type hypersensitivity
FACS	fluorescence-activated cell sorter
FCS	foetal calf serum
HLA	human leukocyte antigen
IFN-γ	interferon-γ
Ig	immunoglobulin
IL-2	interleukin-2
IVC	inferior vena cava
Kd	kilodalton
LNC	lymph node cells
mAb	monoclonal antibody(s)
MHC	major histocompatibility complex
mH	minor histocompatibility
MST	mean survival time
NK cell	natural killer cell

PBL	peripheral blood leukocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT	room temperature
TCR	αβ T cell receptor
T_H	T helper
TNF	tumour necrosis factor

CHAPTER ONE

INTRODUCTION

1:1 Historical aspects of transplantation.

Transplantation of viable tissue and organs from one person to another has been introduced and developed as a therapeutic tool during the Twentieth century.

The first human cadaveric renal transplant was carried out in 1933 by the Russian surgeon Voronoy. The recipient was in acute renal failure as a result of mercury poisoning. The donor had died following a severe head injury and there was a six hour warm ischaemic time before transplantation. Although the operation was a technical success, the kidney never functioned. did not reverse the patients condition and he died soon after the operation (Voronoy, 1936). In the early 1950's the first technically and therapeutically successful renal transplant was performed in Boston, U.S.A., between identical twins, thereby avoiding the insurmountable problem of graft rejection. Other renal transplants, performed in this centre, between donors and recipients who were not identical twins, all failed rapidly as a result of graft rejection by the recipient (Hume, Merril, Miller et al, 1955).

It was not until the early 1960's that it was discovered that treating transplant recipients with Azathioprine resulted in a highly significant reduction of rejection episodes (Calne, Alexander & Murray, 1962). This important breakthrough led to the increased use of renal transplantation as treatment for end-stage renal failure. The advances in treatment and control of graft rejection encouraged the development of techniques to transplant other organs and, in 1967, Barnard performed the first successful heart transplant in South Africa (Barnard, 1967). This success was repeated in centres in North America, particularly by

Shumway in Stanford who developed the technique used by Barnard, and the operation of cardiac transplantation has now become commonplace, throughout the world, in the treatment of various cardiac conditions such as ischaemic heart disease and cardiomyopathies (Yacoub et al, 1987).

Similarly, liver transplantation has been pioneered and developed in the latter part of this century, most notably by Professor Starzl in Pittsburgh U.S.A. (Starzl et al, 1963), and Professor Calne in Cambridge, U.K., and is now widely performed as treatment in cases of fulminant hepatic failure, of various aetiologies (Calne et al, 1981; Starzl et al, 1982; Starzl et al, 1985).

Other organs and tissues which are currently being transplanted, or are the subject of intensive research to assess their suitability for transplantation, include: Lung (double or single); Pancreas (whole organ or islets); Small bowel; Large bowel; Cornea and Bone (Green, 1988). In addition there is increasing interest in the possibility of using xenografts, in certain clinical applications, although the use of other animal species in transplantation into human recipients raises important ethical issues which will need to be addressed before such procedures gain widespread acceptance. Research into this area of transplantation is rapidly gaining momentum.

The primary problem which has had to be addressed to allow these advances in organ transplantation to be made is how to overcome, and control, the immune response with which the recipient of an allogeneic transplant attempts to destroy the graft, which is foreign to them.

Following on from the pioneering work of Calne in introducing azathioprine perhaps the greatest step forward in transplantation (other than the development of the operative techniques) was the introduction of Cyclosporin A (CyA) into clinical use in the late 1970's (Calne et al, 1978). This drug, which is a

metabolite derived from a fungus (Tolypocladium inflatum Gams), initially found in soil samples by the Department of Microbiology at Sandoz in Basle as an anti-fungal agent of limited activity (Dreyfuss, Harri, Hotmann et al, 1976), was subsequently shown to be an exquisitely potent immunosuppressive agent, both *in vitro* and *in vivo*, which blocked the activation of T cells at concentrations which were not toxic to other cell types (Borel, Feurer, Magnee et al, 1977). Its use, alone or in combination with more established drugs like Azathioprine and steroids, has allowed one year survival rates for renal transplants to climb as high as 85-90%. The main advantage of cyclosporin is that, unlike azathioprine, it is not an anti-metabolite but is specifically effective against T cells and does not prevent the normal proliferation of other cell types

Most recently, FK506, a macrolide antibiotic which differs considerably in structure to CyA, has been introduced and early work has suggested that it shows great promise in salvaging, and/or preventing rejection of liver grafts (Starzl et al, 1991). Both CyA and FK506 act largely by inhibiting production of the cytokine, interleukin-2 (IL-2), which is produced by helper T cells during the rejection response, and which recruits and activates cellular components of the rejection response. Both drugs act at a step distal to the cell membrane receptors but proximal to late signalling events such as the transcription activation of early genes. They bind endogenous intracellular receptors, the immunophilins, and the resulting complex targets the protein phosphatase, calcineurin, to exert the immunosuppressive effect by preventing the transcription of the cytokine genes (Shreiber & Crabtree, 1992).

The major drawback of these immunosuppressive modalities is that they are, to different extents, non-specific in their effects and result in significant side-effects related to their generalised immunosuppression. More specific types of

immunosuppressive therapy have, therefore, been sought, in the hope that they will allow adequate suppression of the immune response without compromising the patients defences against opportunistic infections.

Anti-Thymocyte Globulin (ATG) and OKT3 are polyclonal and monoclonal antibodies, respectively, which are currently being used clinically to target those T cells which are thought to play the most important role in graft rejection.

A different approach to immunosuppression was explored in the 1970s after it was noticed that pre-operative blood transfusions seemed to have a beneficial, immunosuppressive effect in renal transplantation (Opelz, Sengar, Mickey et al, 1973). This led to a policy of routine pre-operative transfusions which, initially, improved overall graft survival rates. However, the beneficial effect of blood transfusion in graft survival has become marginal in recent years, due possibly to impovement in immunosuppressive therapy. Additionally, because blood transfusion may result in sensitization, with high levels of panel reactive antibodies in the serum of potential graft recipients who are then more likely to reject an allograft, this form of "immunosuppression" has been largely abandoned. However, there remains much experimental interest in the unexplained phenomenon of "tolerance" produced in some animal models of rejection by administration of a donor-specific blood transfusion a week prior to transplantation.

It remains the case, however, that the exact cellular and molecular mechanisms involved in the rejection of transplanted tissues are not fully understood, and without such knowledge it will be difficult to develop more precise methods of immunosuppression.

Clinical observations of patient responses to grafts allowed a description of four different rejection tempos, each attributable to a different effector mechanism (Williams 1984) :

a) **Hyperacute rejection** occurs within 24 hours of transplantation of a graft bearing major histocompatibility antigens (MHC Ag) to which the recipient has already been exposed and is an antibody mediated response.

b) **Accelerated rejection** occurs within five days of transplantation and may be either cell or antibody mediated.

c) **Acute rejection** occurs within the first three months after transplantation and is probably due to a combination of cell and antibody mediated response. It is the clinical equivalent of a first set response in the animal model.

d) **Chronic rejection** occurs any time after the first three months as the result of prolonged exposure to the MHC antigens of the graft.

It is not possible to study the cellular and molecular mechanisms of rejection in the clinical setting as, by necessity, all transplant patients are maintained on immunosuppressive drugs and their immune responses are, therefore, compromised. That knowledge which we already have of the rejection process is based for the most part, on work done with animal models, in which it is possible to examine unmodified rejection of tissue transplanted between different strains of the same species (allografts) or between different species (xenografts).

In addition, these models allow us to manipulate the immune response in an attempt to clarify the mechanisms involved.

1:2 Initial Experimental Transplantation.

Current understanding of the immunological processes involved in the rejection of foreign tissue owes much to the pioneering work of the late Sir Peter Medawar. He had developed a keen interest in the treatment of severe burns, early in his career as a biologist and zoologist. This led him to spend a period of time working in the Burns Unit of Glasgow Royal Infirmary, collaborating with Mr. Tom Gibson, a local plastic surgeon.

At that time, it was know that treating severe burns by grafting skin from healthy donors would only work if the donor and the patient were identical twins. Grafts taken from unrelated donors were rapidly destroyed and would not "take".

Medawar and Gibson decided to treat a burns patient with skin taken from unaffected areas of the patients own body, and also with skin taken from her brother. In addition, she was given a further set of her brothers skin grafts, four days after the first.

It was noted that the first grafts from the brother were rapidly infiltrated with mononuclear cells and did not "take", while the second set of grafts were destroyed in an accelerated fashion, which has come to be known as "second set graft rejection". Medawar and Gibson suggested that this rejection was a specific response similar to that mounted by the body as a protection against infection. They observed that the rejection of the second set of grafts occurred in the absence of a cellular response and suggested that the "mechanism conforms to the general pattern of an antigen-antibody reaction" (Gibson & Medawar, 1943).

The patient and her brother were of the same blood group which led Medawar and Gibson to conclude that "blood compatibility is not sufficient, and is not known to be necessary, to ensure compatibility of the skin". It was apparent

that tissue incompatibility played an important role in determining the fate of a graft.

Back in England, Medawar continued his work in the field of transplantation with a series of experiments examining the rejection of skin grafts in rabbits (Medawar, 1944, 1945). This work demonstrated that the immune response to a graft was a systemic phenomenon; neither the site of origin, nor the site of transplantation of the graft had any influence on the rate of graft rejection. He also confirmed the results of his work on second set rejection with Gibson, and showed that this phenomenon was donor-specific, as it did not occur if the second graft was taken from a different donor from the first. Medawar went on to postulate the existence of tissue antigens which were capable of initiating a rejection response in rabbits, and suggested that graft survival time was dependent on the antigenic relationship between the donor and recipient.

This work laid the foundations of current transplantation immunology:

1) The graft rejection response is specific for the donor.

2) The response has memory and occurs in accelerated fashion when confronted with a second graft from the same donor (Second set rejection)

3) The first set response is primarily cell mediated.

Medawar was awarded the Nobel Prize for Medicine in 1960, in recognition of the importance of his early work in the field of transplantation.

Further fundamental contributions to the understanding of the processes involved in transplant rejection were to follow over the next twenty years.

Gowans and his co-workers demonstrated a role for recirculating lymphocytes in mediating both antibody responses to antigens, and cell-mediated responses to skin allografts (Gowans, McGregor, Cowen et al, 1962);

Snell proposed that the genetically determined histocompatibility antigens were responsible for the rejection of mismatched tissues (Snell, 1957);

The importance of thymically-processed T cells in a range of immunological responses was demonstrated by Miller (1962). These cells played a role in graft rejection, unlike the bursa-derived lymphocytes which had earlier been shown to be crucial in antibody responses, but not skin graft responses;

The clonal deletion theory of self tolerance was proposed and Billingham, Brent & Medawar (1956) who demonstrated the induction of neonatal tolerance.

1:3 Major Histocompatibility Antigens

It is now fully accepted that the fate of tissue grafted from one individual to another, is dependent upon the antigens expressed on the cell surface, which are encoded by that area of the genome known as the **Major Histocompatibility Complex** (MHC), as put forward by Gorer et al in 1948 (Gorer, Lymans & Snell, 1948). Incompatibility for MHC antigens between donor and recipient will lead to rapid rejection of transplanted tissue, whilst complete compatibility will afford a marked degree of protection to grafted tissue.

Although the MHC antigens are undoubtedly the most important determinants of graft survival or destruction, it is still possible for a graft to be rejected even when it is completely matched for MHC antigens. This results from incompatibilities of so-called **minor histocompatibility antigens** (mH) which are encoded outwith the MHC, but which have been shown to be capable of stimulating graft rejection in the presence of a full MHC match. This rejection is usually slower than that seen with an MHC mis-match but is no less destructive. The antigenic products of the mH loci are capable of activating either CTL or T_H cells. The mH antigens that stimulate CTL have been shown to be peptides derived from intracellular proteins (Wallny & Rammensee, 1990; Griem, Wallny, Falk et al, 1991). In a recent review, Roopenian suggests that mH genes that encode products which stimulate T_H cell reactivity will, typically, not in themselves constitute an effective transplantation barrier, possibly because the type or intensity of immunological activity induced is insufficient to lead to graft rejection even though the gene product is expressed by the allograft tissue. He argues that T_H cells and CTL commonly respond to antigens encoded by separate

mH genes and that effective immunity requires both types of antigen (Roopenian, 1992)

The first attempt to improve the results of transplantation by matching the donor and recipient tissues resulted in the identification of cell surface antigens on human leukocytes by Dausset in 1958 (Dausset, 1958). These Human Leukocyte Antigens (HLA) were first demonstrated on peripheral blood leukocytes and were found to be products of the MHC on chromosome six.

It was soon discovered that the antigens were not restricted to leukocytes, but were present on the surface on nearly all nucleated cells, and these are now known as class I MHC antigens.

Using serological techniques, clinical transplantation donors and recipients were matched for these antigens, with some improvement in results, but it was then found that cells which were apparently identical, as far as HLA was concerned, were capable of reacting with each other in mixed lymphocyte culture. This led to the discovery of a second group of surface antigens, also encoded by the MHC but with a much more restricted tissue distribution, which are now known as class II MHC antigens.

Our understanding of the roles played by these antigens in the rejection of transplanted tissue has been improved by the development and experimental use of inbred strains of laboratory animal, in particular the mouse and rat models. Much of the preliminary work was carried out using skin grafts in mice and, as the immunogenetics of the rat were unravelled, it became possible to perform vascularised organ grafts in the larger rodent. Further developments in microsurgical technique mean that it is now possible to perform whole organ vascular grafts between inbred strains of mouse (Corry, Winn & Russell, 1973).

It should always be borne in mind, however, that although these animal models have many similarities with respect to the MHC, there are also important differences between species and indeed between strains of the same species, and care should be taken in interpreting the relevance of experimental data to the clinical transplantation setting.

1:3.1 The Major Histocompatibility Complex

The MHC genes which encode for the HLA system in man are found on chromosome six. The mouse MHC is on chromosome 17, while the location of the rat MHC encoding the RT1 is on chromosome 20.

1:3.2 The class I MHC region

In man, class I molecules are encoded by the HLA-A, HLA-B and HLA-C locus genes. In the mouse, class I molecules are encoded by the H-2D, H-2K and H-2L regions whereas , in the rat it is the RT1A region which encodes for those class I molecules of importance in transplantation. RT1C and RT1E antigens appear to play a lesser role in transplantation as it proved difficult to generate *in vitro* cytotoxic T lymphocyte responses against RT1C differences (Stock & Gunther, 1982).

1:3.3 The class II MHC region

Human class II molecules are encoded by the DR, DQ and DP regions of the HLA system. Mouse class II molecules are encoded by H-2I region genes, including IE which is analogous to human DR, and IA which is analogous to DQ.

In addition, there is an IJ locus in the mouse which confers a suppressor function on T lymphocytes (Smith, Steinmetz & Hood, 1986)

The RT1B and RT1D regions encode for class II molecules in the rat, and are analogous to the IA and IE regions respectively (Blankenhorn, Cecka, Frelinger et al, 1980; Lobel & Cramer, 1981: Blankenhorn, Symington & Cramer, 1983).

1:3.4 The class III MHC region.

This region encodes for soluble proteins in all three species. These include complement components C2 and C4, and factor B, the serum-borne sex-linked protein in mice. Class III products play little part in initiating rejection of foreign tissue.

1:4 Structure of MHC antigens

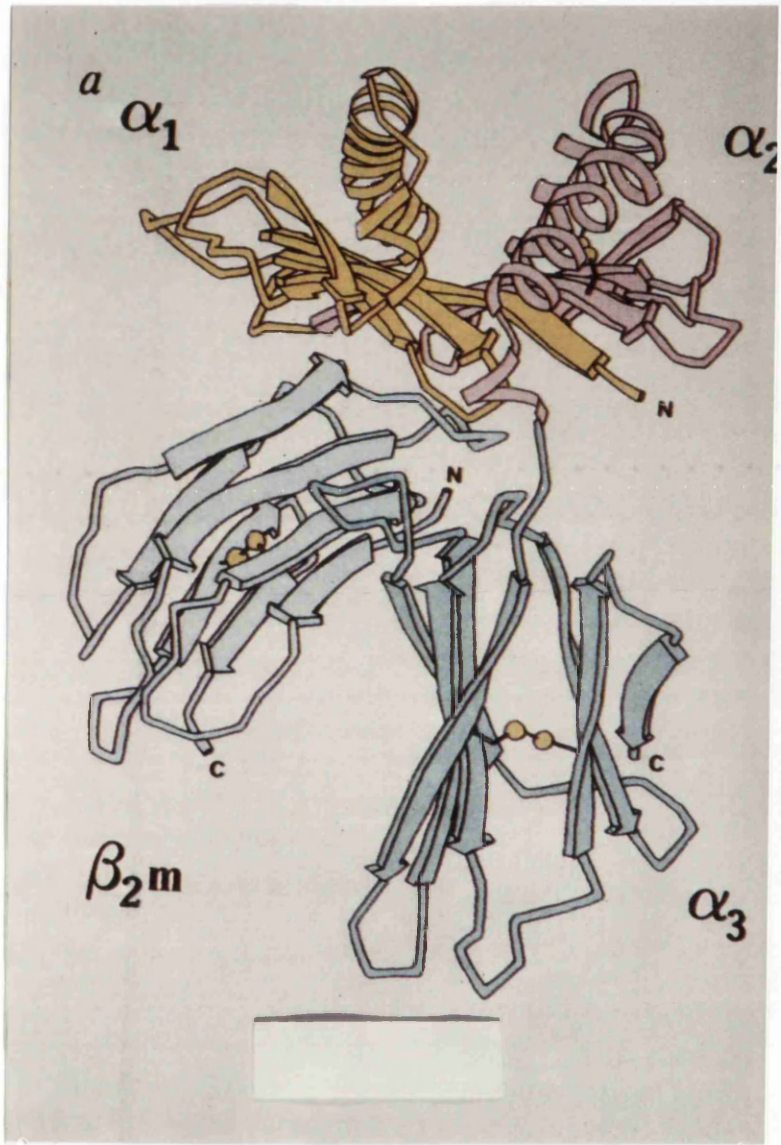
1:4.1 Class I antigens

The structure of class I antigens is similar in different species. They consist of two non-covalently linked glycoprotein chains with approximate molecular weights, in man, of 45 Kilodaltons (Kd) and 12 Kd.

The larger chain is a transmembrane protein with three extracellular domains, α1,α2 and α3, each approximately 90 amino acids long and encoded on separate exons. Proximally α3 joins the transmembrane section which links with an hydrophilic intracellular region. Distally, α3 associates with the smaller light chain, β2-microglobulin (β2m). The α3 domain and β2m are relatively conserved and show amino-acid homology to immunoglobulin constant domains. The α1 and α2 domains are polymorphic and show no significant sequence homology to immunoglobulin constant or variable domains. Bjorkman and co-workers studied the structure of crystals of purified HLA-A2 and found that the membrane-proximal α3 and β2m domains have tertiary structures resembling antibody domains, but are paired by a novel interaction not previously seen in immunoglobulin structures. The α1 and α2 domains are nearly identical to each other in structure, and are not similar to immunoglobulin variable or constant domains. The a1 and α2 domains form a platform composed of a single β-pleated sheet topped by α-helices with a long groove between the helices (Figure 1.1). X-Ray crystallography reveals electron density, which is not a part of the HLA molecule, in the groove between the helices (Figure 1.2) and this was presumed to represent an unknown bound antigen (Bjorkman, Saper, Samraoui et al 1987).

It has now become accepted that this configuration of the MHC molecule is accurate and that the groove formed by the α1 and α2 domains is the site at

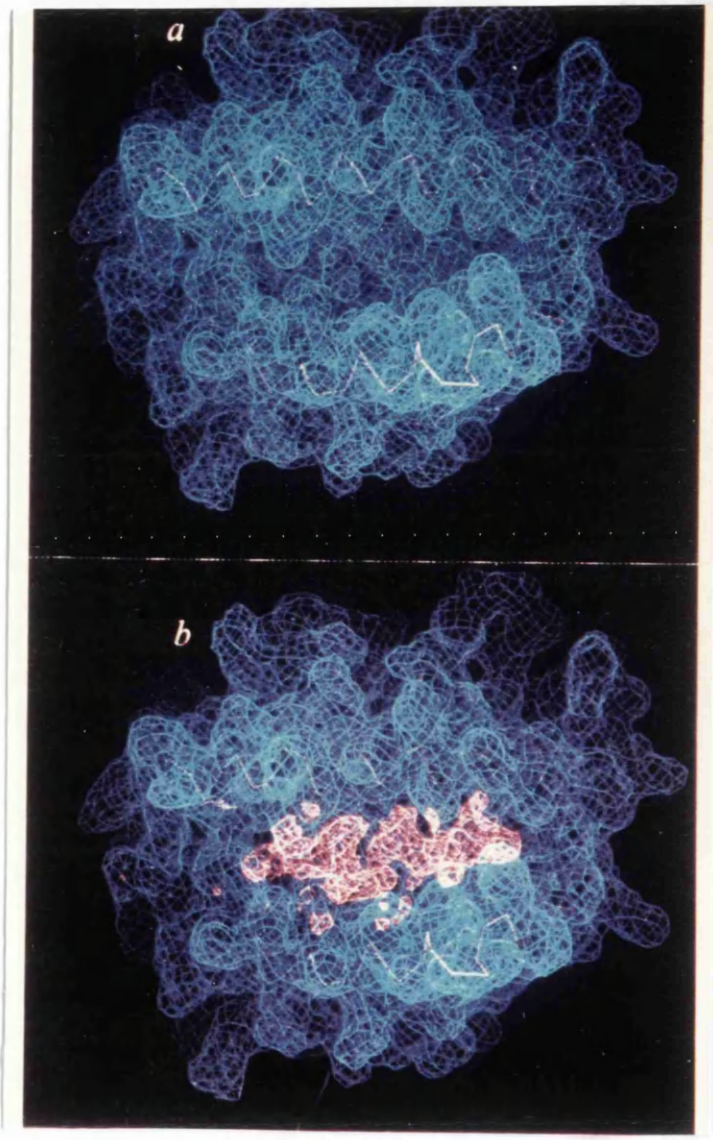
Figure 1.1 The polypeptide structure of the HLA-A2 class I molecule (Bjorkman et al, 1987)



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

* The antigen binding groove was thought to be between the two α-helices of the α1 & α2 domains.

Figure 1.2 The HLA-A2 molecule and antigen binding groove (Bjorkman et al, 1987).



* This Van der Waals surface representation of the top of the HLA-A2 molecule shows:

a) The deep groove of the antigen binding site.

b) electron density in the groove (red), thought to represent antigen peptide.

which the MHC molecule binds either endogenous (self), or exogenous (foreign) peptides. The complex of MHC antigen and peptide can then be recognised by a T cell receptor, in conjunction with a $CD8^+$ molecule.

1:4.2 Class II antigens

Class II antigens have a different, but in some respects similar, structure to class I antigens, in that they are composed of two polypeptide chains of approximately 33 and 28 Kd molecular weight.

Each chain has a short, hydrophilic, intracellular domain and a trans-membranous domain. In addition, each chain has two extracellular domains. Those domains next to the membrane are immunoglobulin-like with homology to class I $\alpha 3$ and $\beta 2m$. The N-terminal end of the class II chains are presumed to contain the binding site for antigenic peptides and one of the domains has weak sequence homology to class I $\alpha 1$ and $\alpha 2$ (Bjorkman et al, 1987). It is thought that, as in the class I molecule, the membrane-distal domains form the site for peptide binding and recognition by T lymphocytes, in this case of the $CD4^+$ phenotype. The alloantigenic specificity of the molecules is provided by the polymorphic nature of the membrane-distal domains.

1:5 Tissue distribution of MHC antigens

Class I antigens are expressed by nearly all nucleated cells, and in particular on interstitial dendritic cells and vascular endothelium, whereas Class II antigens have a much more restricted tissue distribution. They were originally thought to be restricted to B lymphocytes, macrophages, monocytes and dendritic cells.

It is now apparent, however, that the expression of both class I and class II antigens is not an all-or-none phenomenon, and either, or both, can be up-regulated (or down-regulated) under certain conditions. Lampert, Suitters & Chisolm (1981) demonstrated, in rats, the expression of class II antigen on epidermal cells, which do not normally express class II, during a graft-versus-host reaction, and this finding was confirmed by Mason et al (1981). Subsequent reports have shown the induction of class II expression on mouse and rat endothelial cells and keratinocytes in skin grafts undergoing rejection (de Waal, Bogman, Maass et al, 1983: Dallman & Mason, 1983). Induction of class I and class II antigens has also been described in vascularised heart and renal grafts in the rat model (Milton & Fabre, 1985; Milton, Spencer & Fabre 1986a). They demonstrated the specific induction of MHC antigens on donor tissue , and were able to show highly positive induction of class I and class II antigens on donor myocardial cells, which normally express very little class I, and no class II, antigen. In addition, they showed high levels of class II induction in both cardiac and renal vascular endothelium and it is well recognised that the endothelium of a vascularised graft is the initial interface between the recipient's immune response and the foreign tissue (Adams et al, 1989; Bishop et al, 1989).

The rejection of other whole organ grafts such as liver and pancreas is also associated with up-regulation of MHC products. In the rat, rejection of the pancreas is associated with induction of class II antigens on acinar cells, pancreatic duct epithelium and vascular endothelium, while the islets of Langerhans remain class II negative (Steiniger, Klempnauer & Wonigeit, 1985). Both human and rat liver allografts are rejected in unmodified hosts, with induction of class II MHC antigens on bile duct epithelial cells, whilst hepatocytes remain class II negative (Takacs, Szende, Rot et al, 1985).

1:6 The role of MHC antigens in transplantation

The MHC and its' antigenic products are the most important factor in determining whether grafted, foreign tissue is accepted by the recipient or is recognised as foreign and rejected by way of the immune response.

The biological function of both class I and class II MHC antigens is to present processed antigenic peptide fragments, of either endogenous "self" or exogenous "foreign" origin, to T lymphocytes, which recognise and respond to the complex of MHC molecule and presented peptide. In this process, cytotoxic T lymphocytes (CTL) of the $CD8^+$ phenotype recognise only class I MHC molecules, while T helper (T_H) lymphocytes of the $CD4^+$ phenotype interact only with class II molecules (Bach, & Sondel, 1976; Miller, Vadas, Whitelaw et al, 1976).

T cells also have the capacity to recognise and react to allogeneic MHC molecules on transplanted tissue and this results in a strong, and specific, immune response, leading to the destruction of the foreign tissue. Alloantigen will typically stimulate a response in approximately 10% of peripheral T cells as compared to less than 1% which are stimulated by exposure to nominal antigens. Complete MHC incompatibility leads to rapid graft rejection but it has also been demonstrated, using inbred animal strains, that isolated MHC disparities of either class I or class II can result in the rejection of the grafted tissue, albeit usually at a slower tempo than full MHC incompatible tissue (Katz, Liebert, Gill et al, 1983; Burdick & Clow, 1986).

Although the MHC antigens are of crucial importance in the initiation of the rejection process in transplantation, it is still possible for grafted tissue to be rejected in the presence of an identical MHC match, such as that between identical

twins (as discussed earlier). This rejection is as a result of incompatibilities of the minor histocompatibility (mH) antigens, which are encoded outwith the MHC.

An example of such a mH antigen is Epa-1 which is expressed only on epidermal cells of mice, rats and man (Steinmuller & Tyler, 1983). Incompatibility of this antigen leads to skin graft rejection and the generation of Epa-specific cytotoxic T lymphocytes. There are many other mH antigens whose ability to stimulate an immune response may be of clinical importance.

1:7 The role of cytokines in regulation of MHC expression

The alteration of cellular expression of MHC antigens is brought about, in transplanted tissue, by soluble mediators (cytokines) released by leukocytes infiltrating the graft. Foremost among these mediators is gamma-interferon (IFN-γ) which is produced by activated T lymphocytes and has been shown to induce the expression of both class I and class II antigens *in vitro* and *in vivo*. In addition, both alpha and beta interferon, which are produced by a wide variety of cells, are capable of inducing class I antigens *in vitro* (Fellous, Nir, Wallach et al, 1982; Wong, Clark-Lewis, Harris et al, 1984; Basham, Smith, Larnier et al, 1984; Halloran, Madrenas, 1990).

It is likely that the induction of class II antigens, or the up-regulation of their expression as a result of the influence of cytokine mediators, will facilitate the interaction of MHC molecules with $CD4^+$ T lymphocytes and thereby augment the production of an effective immune response against the foreign tissue.

This concept is strengthened by the fact that preventing the up-regulation of MHC antigens by, for example, Cyclosporin A (CyA) was associated with the acceptance of an allograft which would have been rejected by an unmodified

recipient (Milton, Spencer & Fabre, 1986b). Groenewegan and co-workers also demonstrated that class II expression was lost from kidney endothelial cells of dogs in which lymphokine production was inhibited by treatment with CyA (Groenewegan, Buurman & van der Linden, 1985). Steroids are also capable of preventing the induction of class II antigens by IFN-γ, as demonstrated by Leszczynski et al in 1986. They found that a single dose of IFN-γ markedly increased class II expression on rat renal endothelial cells, and that this effect was completely abolished by three daily doses of methylprednisolone (Leszczynski, Ferry Schellekens et al, 1986). It has also been shown that the induction of MHC antigens in rat renal allografts can be prevented by continuous local infusion of steroids (Ruers, Buurman, van Boxtel et al, 1987).

1:8 CD4 and CD8 cell surface molecules.

The expression of the CD4 or CD8 molecules on the surface of peripheral T cells is mutually exclusive. The presence of CD4 or CD8 molecules restrict the cells to recognition of antigens presented in the context of MHC class II and class I respectively. What is not fully understood is the exact role of these molecules in the recognition process and in the subsequent activation of the T cells. Biddison and co-workers first suggested that the CD4 molecule was separate from the TCR and had a binding site for a non-polymorphic epitope on class II MHC molecules (Biddison, Rao, Talle et al, 1982), and this was borne out by the finding that cells infected with a high expression SV40 vector containing a CD4 cDNA acquire the ability to specifically bind to cells that express various allelic forms of HLA class II molecules, and that this binding could be inhibited by anti-CD4 monoclonal antibodies (Doyle & Strominger, 1987). In this *in vitro* work, however, the CD4 expression was far in excess of physiological levels and while it demonstrates the

ability of CD4 to bind with class II MHC, it does not address the question of whether CD4 plays any further role in T cell activation. Subsequent work by Biddison and Shaw, looking at $CD4^+$ CTLs which were specific for class II MHC, showed that anti-CD4 mAb blocking could abrogate the action of these CTLs, further suggesting a role for CD4 in MHC recognition. In addition it was found that CD4 was of greater relative importance in facilitating interactions between TCR and MHC which were of "low-affinity". They concluded from this work, using human CTL clones, that the CD4 molecule had only a minor role in adhesion, per se, and that, as not all adhesions led to cell lysis, some further mechanism must be at play in the post-adhesional phase of cytotoxic T-cell recognition (Biddison & Shaw, 1989).

It is now apparent that the cytoplasmic domains of CD4 and CD8 have a physical association with a protein tyrosine kinase activity, known as p56lck, and as tyrosine phosphorylation is a major signal transduction system involved in the regulation of cell growth, it seems probable that it is acting as a signal for cell activation as a result of stimulation by CD4 or CD8 (Ruud, Trevillyan, Dasgupta et al, 1988; Veillette, Bookman, Horak et al, 1988). Other workers have demonstrated both stimulatory and inhibitory signals to T cells, mediated by the CD4 molecule, and independent of MHC recognition (Rosoff, Burakoff & Greenstein, 1987; Bank & Chess, 1985). These findings have led to the suggestion of the CD4 molecule being an integral part of the TCR (a co-receptor) for class II MHC, and not simply an accessory molecule which augments the interaction between the TCR and the MHC molecule (Janeway, Rojo, Saizawa et al, 1989).

The CD8 molecule acts in the similar fashion to CD4, but in association with its TCR it binds to a non-polymorphic region on class I MHC molecules.

1:9 Mechanisms of allograft rejection

The great increase in the number of clinical transplants, of various organs, now being performed world-wide is mirrored by the level of research into the exact mechanism, or mechanisms, by which the transplanted organs are being rejected by their recipients. It is recognised that the rejection process is extremely complex and multifactorial. In order to try to simplify matters it is convenient to consider the rejection of an allograft as consisting of three phases. These are:

1. The afferent phase
2. The central phase
3. The efferent , or effector, phase

1:9.1 The afferent phase of graft rejection

The afferent phase of allograft rejection refers to the process whereby the immune system of the recipient recognises the graft antigens as foreign, *i.e.* non-self, and becomes primed to initiate rejection. For this to occur, the alloantigens of the graft must be presented to the host lymphocytes. In the context of the normal physiology of the immune system, the T cell receptor recognises foreign antigens as peptide fragments in association with self MHC class I or II molecules. In allograft recognition, however, the recipient T cells recognise the whole foreign MHC molecule as being non-self, on the surface of allogeneic cells. In addition, it is now thought that these alloantigens can be processed by antigen presenting cells (APC), of donor or host origin, and presented by the APC to recipient sensitized T cells in a complex of MHC molecule and bound peptide. This complex is recognised by the T cell receptor (Shimonkevitz, Kappler, Marrack et al, 1983).

The most efficient APC belong to a group of bone-marrow derived, interstitial cells with a distinctive spicular morphology, known as dendritic cells. These cells are typically isolated from lymphoid tissues such as the spleen, are non-phagocytic and express the leukocyte common antigen (CD45). The lymphoid dendritic cells belong to a widely distributed and diverse lineage of dendritic leukocytes but the lymphoid subgroup appears to be a specialised immunostimulatory cell which, in addition to antigen presentation to sensitised T cells, has the ability to deliver an activation signal to resting T cells. The nature of this signal remains obscure.

Because of the immunostimulatory nature of the lymphoid dendritic cell and the fact that dendritic leukocytes are found in the parenchyme of all commonly transplanted tissues, and, indeed, are found in all tissues with the exception of the nervous system (Hart & Fabre, 1981; Daar, Fuggle, Fabre et al, 1984), it is now accepted that the non-lymphoid dendritic leukocytes may be important passenger leukocytes in transplantation. They express high levels of MHC class I and class II antigens on their surface and are potent stimulators of the *in vitro* mixed lymphocyte reaction (Steinman & Witmer, 1978; Mason, Pugh & Webb, 1981). They may present antigen to host circulating lymphocytes within the graft itself or following migration to the draining lymph nodes. It is also possible that the antigens, on donor APC, may be shed from the graft and interact with recipient APC and T cells within the draining lymph nodes. In a recent review of the migration patterns of dendritic leukocytes Austyn & Larsen point out that dendritic leukocytes **can** capture antigen in the periphery and transport it to draining lymph nodes, where they induce immune responses. in addition Austyn has shown, using double-labeling techniques with fluorochromes that, following intravenous administration, labelled dendritic cells migrate to the T

areas of the spleen but he was not able to find any migration of host dendritic cells into a vascularised allograft. This group has subsequently found that following a cardiac allograft donor-type dendritic cells were found in the recipient spleen, concomitant with a reduction in the number of dendritic leukocytes in the donor heart. Austyn is, therefore, of the opinion that sensitization to an allograft occurs centrally either via the lymphatics draining the graft or via the blood (Austyn & Larsen, 1990). Other cell types, such as endothelial cells, also have the capacity to act as antigen presenting cells, but this is usually less efficient than the "professional" APCs (Hirschberg et al, 1981).

Interestingly, it has been shown that, in the case of skin grafts, prolonged survival of an allograft can be achieved experimentally by preventing the restoration of lymphatic drainage (Barker & Billingham, 1968), whilst in primarily vascularised organ grafts lymphatic diversion had no effect on graft survival (Pederson & Morris, 1970). These results suggest that the lymphatic route is central in the sensitization against a skin graft, in which the vascular supply takes some time to mature, whereas it is of less importance in the rejection of a primarily vascularised organ graft.

The importance of the interstitial dendritic cell in the initiation of the rejection of an allograft was demonstrated by Lechler and Batchelor (1982). They transplanted a rat kidney into an intermediate host which was syngeneic with the eventual recipient, but which had been pre-treated with donor alloantibody to prevent rejection of the kidney (passive enhancement). The kidney was left in the intermediate host for several weeks to allow its bone-marrow derived dendritic cells to be replaced by those of recipient origin.

The kidney was then re-transplanted into the final host, which was syngeneic with the intermediate host, and it was found that the graft was not

rejected. Simultaneous injection of 10^4 donor strain dendritic cells was, however, sufficient to fully restore graft rejection. Although most of the re-transplanted hearts were rejected prior to reconstitution with donor-strain dendritic cells, some were not. The authors argued that this finding could be explained by the indirect pathway of antigen presentation, with the donor MHC being processed by the recipient strain dendritic cells in the intermediate host and presented by these recipient APCs to recipient T cells in the final graft recipient. A more detailed examination of the concept of indirect antigen recognition will be undertaken later in this thesis.

Prior to this work, blood-borne leukocytes of donor origin which had been trapped in the graft (passenger leukocytes) were suspected of being important in alloantigen presentation and sensitization against the graft, but it could not be demonstrated that elimination of these cells, or the use of radiation bone-marrow chimeric rat donors in which the passenger leukocytes were syngeneic with the recipients, could produce long-term graft survival, although some prolongation of survival was seen (Guttman, Lindquist & Ockner, 1969; Stuart, Bastein, Holter et al, 1971). More recently the possibility of targeting these passenger leukocytes with monoclonal antibodies prior to insertion of a vascularised graft, in an attempt to decrease the antigenicity of the graft, has become a reality, and is now undergoing assessment in clinical trials.

As the presentation of alloantigen is crucial in the development of the immune response to a graft, other cell lines have been examined to assess their ability to express class II MHC antigens. Pober et al showed in 1983, that human umbilical vein endothelial cells which had been cultured with IFN-γ developed an increased expression of Class II antigens which were able to interact with allospecific CTL (Pober, Collins, Gimbrone et al, 1983). It has also been

demonstrated that rat heart endothelial cells cultured *in vitro* with IFN-γ are induced to express increased class II, and these cells could sensitize an allogeneic host to rapidly reject an allograft (Ferry, Halttunen, Leszczynski et al, 1987).

Although it has become accepted that the vascular endothelium of an organ graft is the primary target for the effector arm of the rejection response, its role in the presentation of alloantigen, and thereby the *initiation* of the immune response, is much less clear and remains controversial. However, it is known that human umbilical vein endothelial cells can stimulate allogeneic lymphocytes in mixed cultures (Hirschberg et al, 1975), and can also present soluble protein antigens to stimulate purified T cells, in association with class II MHC antigen products on the endothelial membrane (Hirschberg, Scott, Thorsby, 1981). Hirschberg argues that antigen presentation may be the physiological role of MHC antigen on endothelial cells, and that allograft rejection by the destruction of a grafted organ's vasculature may be a by-product of it's role in antigen presentation. The potential role of vascular endothelium in antigen presentation remains the focus of much current research.

1:9.2 The central phase of graft rejection

The central phase of graft rejection is concerned with the initiation of effector mechanisms of rejection, as a result of interactions between APC and lymphocytes, and between lymphocyte subsets. This process takes place, in the main, in the draining lymph nodes and the recipient spleen. It has been shown that recirculating T lymphocytes play the central role in recognising antigen and stimulating the proliferation of B lymphocytes in lymph nodes and the production of allospecific lymphocytes, which then invade the grafted tissue (Gowans, McGregor, Cowen et al, 1962).

Bone-marrow derived cells with dendritic morphology and a high level of Class II MHC antigen expression have been demonstrated in the thymus dependent region of lymph nodes (Barclay, 1981). Recognition of the class II molecule by $CD4^+$ T lymphocytes results in the activation of these T_H cells, which play a central role in this phase of the rejection response (Mason & Morris, 1986). The exact mechanism of T cell activation is not yet fully understood, but will be discussed later in this thesis. Once activated, the T_H cell produces lymphokines, of which the best known and most studied is interleukin-2 (IL-2), which result in the proliferation and differentiation of various effector mechanisms involved in the immune response, such as the differentiation of cytotoxic T cells, the activation of macrophages and natural killer (NK) cells, and the clonal proliferation of allospecific B cells producing alloantibody which may lead to an antibody dependent cellular cytotoxicity reaction or complement mediated damage to the graft. In addition, as has been mentioned earlier, lymphokine production by T_H cells results in induction of Class I and class II MHC antigen expression on grafted tissue and increases their susceptibility to attack by effector limbs of the rejection process.

The nature, and relative importance, of the various effector mechanisms of transplant rejection will be discussed in detail in the remainder of this thesis.

1:9.3 The effector phase of graft rejection.

The rejection of an allograft by an unmodified recipient is a predictable and rapidly, destructive phenomenon. It does not occur, however, as the result of any one effector mechanism, but rather as a multifactorial, complex group of reactions, which can only be separated and studied in the experimental animal. In particular, the development of inbred animals has allowed us to greatly increase

our understanding of the individual effector limbs which make up the immune response to organ grafts.

There has been, and continues to be, much debate over the relative contributions of a delayed-type hypersensitivity reaction (DTH); specific cytotoxic T cells (CTL); helper T cells (T_H); antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent, alloantibody mediated, cell killing. Each of these will be considered in the following section.

1:9.3.1 Delayed type hypersensitivity (DTH).

When Brent, Brown and Medawar looked at the morphology of the cellular infiltrate in rejecting skin grafts in 1958, they observed that it was similar to the infiltrate seen in a classical delayed-type hypersensitivity reaction and they postulated that this was the likely mechanism involved in allograft rejection (Brent, Brown, Medawar, 1958).

In a DTH response, T_H cells which have been activated by recognition of donor class II MHC molecules, produce a number of lymphokines, notably IL-2, which have the effect of activating non-specific effector cells such as macrophages and NK cells. These cells will destroy foreign tissue but, in keeping with their lack of specificity, they will also cause some damage to surrounding "self" tissue.

It has been reported that both of these non-specific cells (macrophages and NK cells) are present in large numbers in rejecting grafts but, owing to technical difficulties in isolating them, it has proved difficult to closely examine their role in rejection, to the degree that has been possible with T cells. One striking piece of evidence which suggests that any role they play is entirely secondary, and dependent on the presence of T cell help, is the fact that congenitally athymic

animals, and ATXBM animals, are both unable to reject any kind of allograft, despite the presence of both NK cells and macrophages within the graft. These cells may play a role in rejection in conjunction with Th cells, or indeed antibody, as they both possess an Fc receptor and could be involved in ADCC, but they cannot reject an allograft on their own.

1:9.3.2 Cytotoxic T lymphocytes (CTL) and helper T lymphocytes (T_H).

There is now no doubting the central importance of the T cell in the regulation of rejection of an allograft. As has been mentioned previously, congenitally athymic rodents and ATXBM animals, which are devoid of T cells, are unable to reject an allograft although the grafts contain a heavy infiltrate of non-specific effector cells. Reconstitution of such animals with T cells restores their ability to reject the allograft. The difficulty arises in determining which type of T cell is more important and how do they interact with other cell lines to produce rejection of the graft. A great deal of time and effort has been put in to try to solve these problems in recent times, which have gradually increased our knowledge of the extreme complexity and adaptability of the immune system. The fact that much of this work has, by necessity, been carried out in laboratory animals must be borne in mind, as many of the results are species or strain-specific, and can be completely reversed by performing the same experiment in a different strain combination. In spite of these limitations, the rodent models have been invaluable as a tool to assist in the unravelling of the very complex cellular interactions which are taking place.

The main subdivision of T cells is into those cells which are able to directly lyse target cells, the $CD8^+$ CTL, and those which, by release of lymphokines, provide help for a variety of other cell types to differentiate and

become activated, the $CD4^+$ T_H cell. Both of these subsets express the T cell receptor (TCR) on their surface and through this they recognise MHC antigen presented to them on the surface of APC. It is known that the $CD4^+$ Th is restricted to class II MHC while $CD8^+$ CTL recognise only class I MHC. In the light of the knowledge that foreign MHC antigens present on an allograft can be processed by host APC and presented to the T cell as peptide in the groove of class II MHC, it seems possible that the $CD4^+$ T cell may be of greater importance in regulating the rejection of the antigen source.

In 1960, Govaerts demonstrated that thoracic duct lymphocytes taken from dogs which had rejected an allograft, had the ability to specifically lyse donor-type kidney epithelial cells *in vitro* (Govaerts, 1960). This was the first evidence that a CTL may be an effective mediator of graft rejection. It has subsequently been shown that such CTL cause cell killing by direct action, involving cell-to-cell contact, but without the involvement of lymphokines or of non-specific cells such as macrophages or NK cells. CTLs are of the $CD8^+$ phenotype and express the CD8 molecule on their cell membrane in close association with the T cell receptor (TCR). This complex of TCR and CD8 is restricted to recognition of class I MHC molecules. In the presence of a full MHC disparity, however, differentiation of CTL precursors is greatly enhanced by the action of $CD4^+$ Th cells which respond to the class II MHC molecules (Bach, Bach, Sondel, 1976).

Important demonstrations of the specificity of the graft rejection response came with the work of Mintz and Silvers (1970) and Rosenberg & Singer (1988). They developed allophenic mice by fusing blastomeres of two different H-2 strains and then grafted skin from an allophenic donor onto one of the parental strains. One of the parental strains used was white, while the other was black. They found that the hair follicles of the foreign parental strain were destroyed,

while those follicles that were syngeneic to the host, were undamaged, thus providing convincing evidence for a highly specific immune response which was assumed to be mediated by CTLs.

Following the work of Mintz and Silver, the concept of CTL rejection was a popular one. By examining the phenotype and functional capabilities of day 5 infiltrating cells from rat renal allografts, Bradley and co-workers demonstrated that in grafts undergoing unmodified rejection there was a high level of specific cytotoxic T cell activity, but this was lost in cyclosporin treated, and passively enhanced animals which did not reject their grafts (Bradley, Mason, Morris, 1985). In addition they found that the number of non-specific, cytotoxic cells was similar in rejecting and non-rejecting grafts. From this work they concluded that the effector mechanism in their model was the CTL, although it was pointed out that no other effector mechanisms had been evaluated.

Interestingly, Loveland & McKenzie (1981 & 1982) and Dallman, Mason & Webb (1982), found, in mice and rats respectively, that adoptive transfer of $CD4^+$, and not $CD8^+$, cells restored skin graft rejection in ATXBM animals. Dallman noted, however, the presence in the rejected graft of large numbers of $CD8^+$ cells and macrophages of host origin which gave rise to the theory that, in such animal models, the transfer of $CD4^+$ T cells may result in the maturation and activation of $CD8^+$ precursors of host origin, which may become the effector cells, acting as CTL. There is, however, increasing evidence that, although $CD8^+$ CTL may play a role as an effector cell, it is the $CD4^+$ Th cell which is central in regulating graft rejection. Adoptive transfer of $CD4^+$ T cells can restore the rejection of vascularised heart and kidney grafts in acutely irradiated rats across a full MHC disparity (Hall, de Saxe & Dorsch, 1983: Gurley, Lowry & Clarke Forbes, 1983) although, here again, there are $CD8^+$ cells of host origin found in

the rejecting graft which could be acting as effector cells, and the exact mechanism of Th effects remained unclear.

In keeping with accepted MHC restrictions, Lowry, Clarke Forbes, Blackburn et al (1985) showed, that the adoptive transfer of $CD8^+$ cells was required for the rejection of a class I disparate cardiac allograft. This evidence for a direct role for $CD8^+$ CTL was strengthened by the finding that the rate of rejection of an isolated class I disparate skin graft between Kbm and B6 mice, was dependent upon the number of Kbm-reactive, Lyt 2^+ (CD8) IL-2 secreting T cell precursors which could be generated in mixed Kbm x B6 lymphocyte cultures, and that the restoration of rejection of bm1 skin by a B6 nude mouse was dependent on the presence of Lyt 2^+ (CD8) cells alone (Rosenberg, Mizuochi & Singer, 1986). In contrast, however, using the congenitally athymic PVG nude rat, it has been shown that rejection can be restored rapidly by adoptive transfer of isolated $CD4^+$ T cells whereas it is significantly delayed by transfer of $CD8^+$ cells alone (Mason & Simmonds, 1988). Using a different model, namely an acutely irradiated DA rat recipient, Hall et al found that while rejection could be restored by $CD4^+$ cells alone, the effect of transfer of combined $CD4^+$ and $CD8^+$ T cells was an even more rapid rejection response (Hall, Gurley, Dorsch, 1985).

This finding adds weight to the argument of a central role for the $CD4^+$ T cell, with one of it's effects being activation of $CD8^+$ cells. Interestingly, in subsequent experiments, Hall and co-workers found that $CD4^+$ cells restored graft rejection in acutely irradiated rats which had additionally been treated with anti-CD8 monoclonal antibody (mAb) MRC Ox8 to deplete any residual $CD8^+$ cells, and they found that rejected grafts were completely free of $CD8^+$ cells but were infiltrated by $CD4^+$ cells and host macrophages. They also noted that the hosts did not produce alloantibody against the grafts (Hall, Pearce, Gurley et al, 1990). In

addition, they found that the acutely irradiated rats that were treated with MRC Ox8 but were not given any cells, were also able to reject their grafts and they argued that $CD4^+$ cells which remained following irradiation, or had regenerated, were alone sufficient to cause rejection, and that the $CD8^+$ cells, in this model, were acting as suppressor cells. An alternative explanation for this rejection has recently been put forward by Rosenberg et al (1991). They describe the possible existence of a tiny population of $CD8^+$ T cells which, instead of being depleted by anti-CD8 mAb therapy, are in fact activated by it and are, alone, capable of causing graft rejection even in the presence of massive, but they argue not complete, $CD8^+$ T cell depletion. The evidence for this is not convincing, as yet. It has also been demonstrated, in the rodent model, that $CD8^+$ cells can be activated independently of $CD4^+$ cells, and result in the rejection of a class I disparate graft (Rosenberg, Mizuochi, Sharrow et al, 1987). In direct contrast, however, Gracie et al found that kidney grafts bearing an isolated class I disparity, in a rat model, were rejected in the absence of $CD8^+$ cells or of specific CTLs. In addition, it was shown that these rats produced specific alloantibody against their graft, and transfer of rejecting serum could restore rejection to cyclosporin treated animals (Gracie, Bolton, Porteous et al, 1990). This result raises the possibility of an important role for alloantibody in the mediation of acute allograft rejection. This question will be discussed in greater detail later in this thesis.

In a recent review of the experimental evidence relating to allograft rejection, Hall concludes that the data from mAb depletion experiments suggest that $CD4^+$ cells mediate class II MHC-incompatible graft rejection, and $CD8^+$ cells mediate class I MHC-incompatible graft rejection, with $CD8^+$ cell activation being dependent upon $CD4^+$ cells to varying degrees (Hall, 1991).

1:9.3.3 The role of alloantibody in acute rejection.

It is widely accepted that the uncommon clinical phenomenon of hyperacute rejection (i.e. within 24 hours of transplantation) of an allograft is mediated by pre-formed, circulating host antibody against the grafted tissue MHC antigens (Kissmeyer-Nielsen, Olsen, Petersen et al, 1961; Patel & Terasaki, 1969; Williams, Hume, Hudson et al, 1968). The antibody binds to the vascular endothelium of the graft and sets off a sequence of events including complement fixation, platelet aggregation, fibrin deposition and, ultimately, destruction of the microvasculature and the graft itself (Busch, Reynolds, Galvanek et al, 1971). In a recent review of the place of alloantibodies in transplant rejection Baldwin argues that irreversible antibody-mediated rejection of a large multicellular organ is not due to the immune lysis of individual cells, but is as a result of the damaging effect of multiple potent inflammatory cells and factors that antibody and certain complement fragments are capable of attracting and activating to cause tissue damage (Baldwin, Pruitt, Sanfilippo, 1991). In the clinical setting it is pre-formed antibodies to class I MHC and to ABO blood group antigens which are particularly implicated in hyperacute rejection (Baldwin et al, 1991), but the clinical incidence of this problem has been greatly reduced by pre-operative testing of the recipients serum for the presence of alloantibodies against a panel of standardised antigens (Patel & Terasaki, 1969). Although IgM is the most efficient class of antibody at activating complement *in vitro*, it is IgG which is more harmful to the transplanted organ (Baldwin et al, 1991). This incongruous finding may be due to the fact that many of the positive crossmatches to IgM are the result of autoantibodies (Barger et al, 1989; Lobo, 1981), or immune complexes (Baldwin et al, 1986; Wang, Terashita, Terasaki, 1989), neither of which cause any harm to allografts, and an artificial impression is given as to the

importance of IgM as a result of the in vitro assays. It remains an undeniable fact, however, that alloantibody can, and does, cause hyperacute graft rejection.

A second potential mechanism by which alloantibody could mediate a rejection response is by "antibody dependent cellular cytotoxicity", or ADCC. This involves adherence of specific alloantibody to the MHC antigens of foreign cells, and the Fc portion of the antibody is then recognised by host macrophages and NK cells, both of which have Fc receptors. These effector cells then bring about the specific destruction of the allograft. It has been shown that, in the rat, IgG can both activate complement and mediate target cell cytotoxicity through activation of killer cells by the Fc receptors (Baldwin, Rhoton, Sanfilippo, 1991; Chassoux et al, 1988). As Fc receptors, of one or more types, are present on the surface of platelets, neutrophils, eosinophils, macrophages, NK cells and B lymphocytes, it can be easily understood that many acute and chronic inflammatory events may be mediated by the interaction of antibody with the broad array of Fc receptor-bearing cells (Petty, Francis, Anderson, 1989). It remains controversial, however, whether alloantibody plays a significant role in the acute rejection of an allograft (which is a far commoner clinical problem than hyperacute rejection), and this question must be addressed prior to any consideration of the mechanisms by which alloantibody might be effective.

As the concept of a cellular basis of acute rejection has been popular, so the experimental investigation of the role of alloantibody in this type of rejection response has been rather neglected. It has been known for some time that the adoptive transfer of immune serum can result in enhancement of renal allografts (French & Batchelor, 1969), and that this was easier to obtain in an F1 graft than a homozygous graft, in which the amount of antibody required to produce an effect was likely to tip the balance in favour of hyperacute rejection (McDowall,

Batchelor, French, 1973; Fabre & Morris, 1974). Fabre and Morris also found that, in certain strain combinations, passive transfer of alloantibody only resulted in hyperacute rejection and, consequently, they advised caution in the interpretation of previous experimental findings relating to alloantibody-mediated enhancement, and any potential clinical application of such findings.

In 1984, Lowry & Clarke Forbes reported that humoral antibody was not required for the acute rejection of a cardiac allograft in the rat (Lowry, Clarke Forbes, 1984). This statement was based on ultrastructural observations of rejecting, irradiated hearts in irradiated recipients, which had been reconstituted with unfractionated spleen cells syngeneic with the recipient, or with spleen cells which had been depleted of B cells. They found that rejection of the graft took place, in a similar ultrastructural fashion to unmodified controls, in those animals given B cell-depleted splenocytes, and in which there was no demonstrable alloantibody response. Earlier, it had been found, in a sheep model of renal allograft rejection, that graft rejection was associated with high levels of specific alloantibody production, and that the passive transfer of rejecting serum, given at the time of transplantation, resulted in a more rapid rejection response (Pedersen & Morris, 1974). Further evidence for a role in acute allograft rejection for alloantibody was presented by Gracie and co-workers (Gracie et al, 1990), who showed that immune serum from RT1u rats rejecting a class I disparate kidney (R8), was able to restore specific rejection of a class I disparate graft to an RT1u recipient which had been treated with cyclosporin to prevent rejection. They also demonstrated that the cyclosporin treatment had abrogated the recipients own alloantibody response to the renal allograft. These interesting results suggest that, in this model at least, alloantibody is capable of effecting acute allograft rejection.

1:9.3.4 Summary of effector mechanisms

Whilst it is true to say that our knowledge of cellular and humoral interactions in the process of allograft rejection has greatly increased, it is also the case that the complexity and variability of these events is becoming ever more apparent.

There is general agreement on the crucial, and central, role of the $CD4^+$ T cell in the regulation of rejection events by providing T cell help, in the form of cytokines, for the differentiation and activation of both $CD8^+$ cells and of antibody-producing B cells.

Experimental evidence has confirmed that both $CD4^+$ class II-restricted, and $CD8^+$ class I-restricted T cells are capable of mediating rejection in strictly controlled laboratory conditions. Equally, there is increasing interest in the possibility that alloantibody may be a more important effector arm than has been thought up until now.

These experimental advances in examining the individual components of the rejection response mounted against an allograft have already led to the introduction of more specific forms of immunosuppression into clinical transplantation practice. The prevention of rejection still remains the greatest challenge in the field of organ transplantation, however, and we are still a long way from fully understanding the factors which influence, and control, the interactions between the various arms of the effector phase of the rejection process.

1:10 Aims of this thesis

The primary aim of this thesis is to address the mechanism of rejection of an isolated, class I disparate, cardiac allograft in a rat model, with particular emphasis on the role of specific alloantibody produced against the graft by the recipient animal.

To study cellular events involved in the rejection of such a graft, T cell subsets were depleted, in vivo, using monoclonal antibodies, against $CD4^+$ and $CD8^+$ T cells, injected intraperitoneally at, and following, the time of transplantation.

Finally, this thesis considers the mechanisms by which alloantibody might effect tissue damage in this model of cardiac allograft rejection.

CHAPTER TWO

MATERIALS AND METHODS

2:1 Animals

Inbred male rats were used throughout this work. These were obtained from Harlan Olac Ltd (Bicester, Oxon, UK), or were bred in our own animal facility in The Western Infirmary, Glasgow. The following strains were used; PVG(RT1c), DA(RT1a), PVG(RT1u) & PVG R8 (Laboratory report, 1979; Butcher & Howard, 1979). The MHC haplotype of the rats used in the experiments is shown in Table 1.

Male rats (8-16 week old), weighing 250-300g, were used throughout.

All animals were kept in the animal facility of the University Department of Surgery, Western Infirmary, Glasgow. They were fed with standard rat diet and water.

Table 1. MHC haplotypes of PVG congenic and recombinant rat strains.

Rat Strain	RT1 Regions A	B	D	C
PVG RT1c	c	c	c	c
DA RT1a	a	a	a	a
PVG R8	a	u	u	u
PVG RT1u	u	u	u	u

* PVG R8 and PVG RT1u differ only at the classical class I MHC locus

They are otherwise genetically identical, including mH antigens.

** RT1c & RT1u have fully disparate MHC antigens but common mH antigens

*** DA RT1a & PVG RT1u have no common histocompatibility antigens

2:2 Surgical Procedures

2:2.1 Cardiac Transplantation

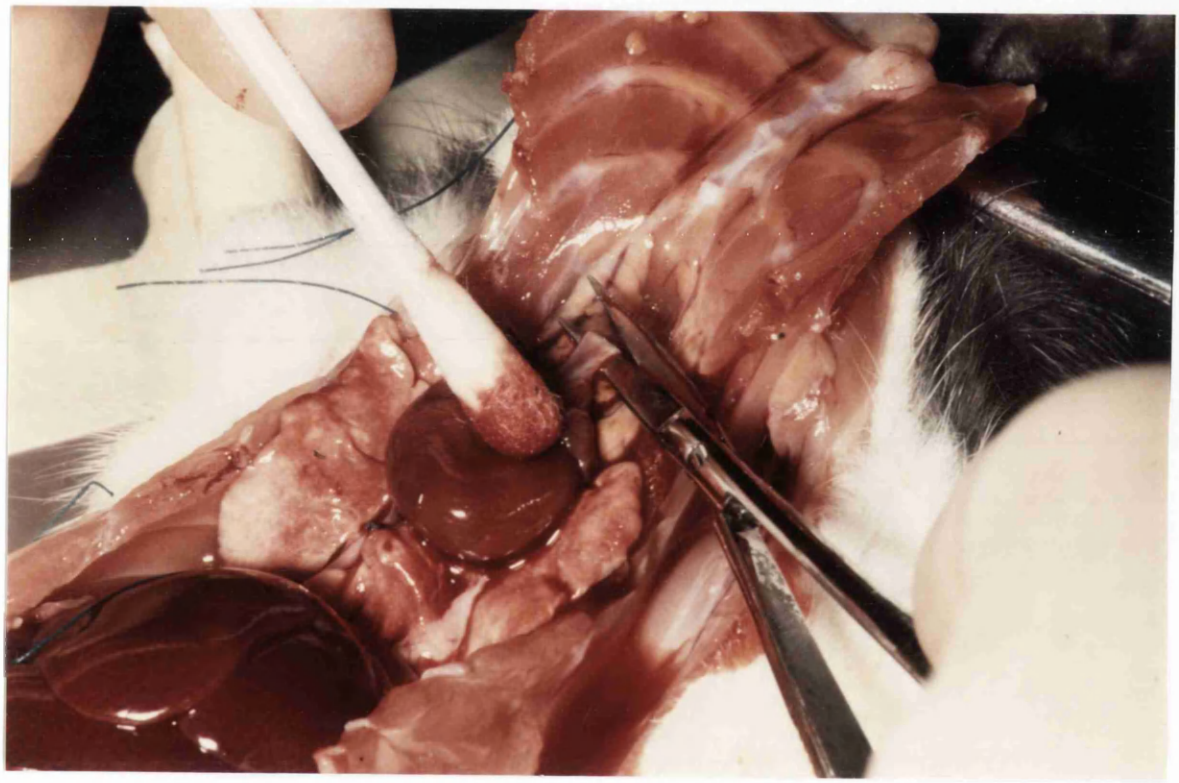
A heterotopic cardiac transplant to the intra-abdominal great vessels, as described by Ono & Lindsey (1969), was employed throughout the experiments described in this thesis.

Both donor and recipient rats were anaesthetised with 5% halothane and oxygen, and were then given an intra-peritoneal injection of 0.8-1.2 mls of 7.5% chloral hydrate in saline, dependent on their size. Towards the end of the experimental work presented in this thesis the anaesthetic technique was changed, on the advice of the supervising vet, to continuous halothane and oxygen delivered via a Fluovac system.

The rats were then shaved and the abdominal skin cleaned with chlorhexidine in alcohol.

The donor rat abdomen was then opened through a midline incision and the intestines swept cranially to expose the infra-renal inferior vena cava (IVC). 300 units of heparin were then injected into the IVC and, after two minutes, the infra-renal IVC and aorta were cut and the rat exsanguinated. The thorax was then opened with scissors and the diaphragm divided. The donor heart was stopped with a combination of topical ice and cold saline. The IVC was mobilised and tied with 4-0 silk, just as it entered the heart. The thymus was then excised, exposing the vessels above the heart. The right superior vena cava was tied with 4-0 silk as it entered the heart, and the left superior vena cava was then mobilised and tied close to the heart, and divided. Next, the pulmonary artery and aorta were freed and divided, (Figure 2.1.1) care being taken to leave them long enough to allow

Figure 2.1.1 Heterotopic cardiac transplantation in the rat.



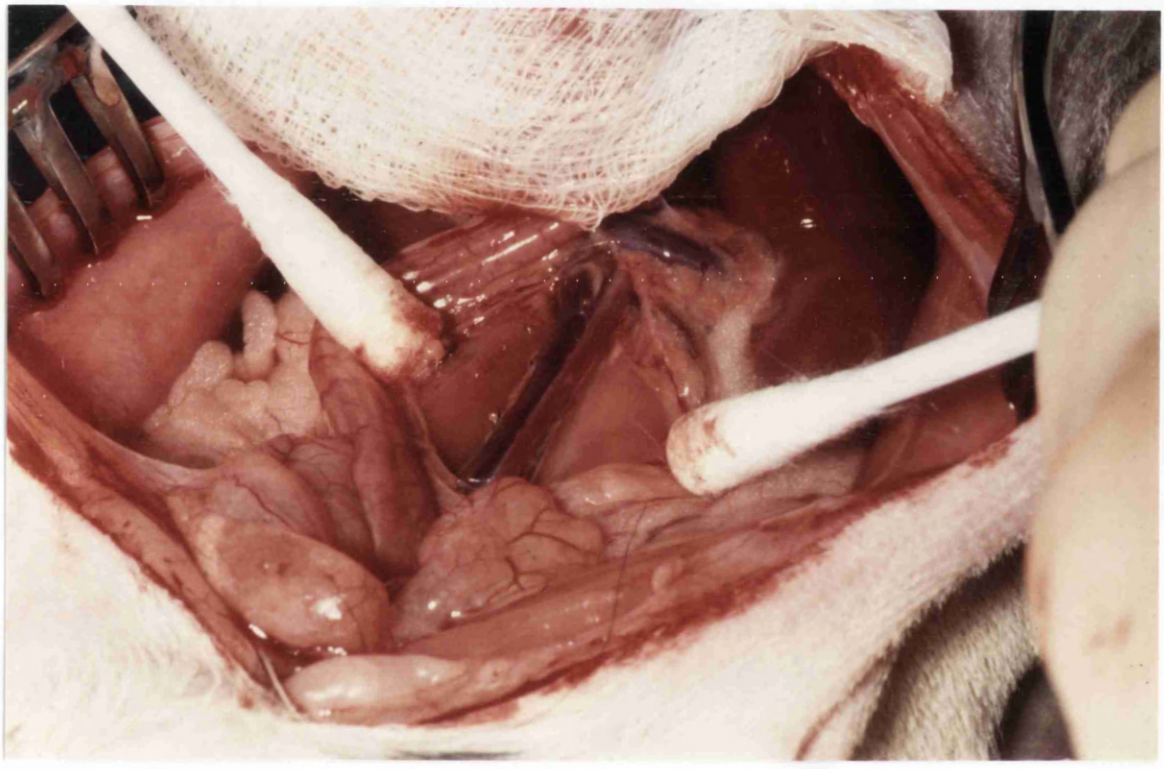
*** Donor aorta is being divided following ligation of the right and left superior vena cavae and inferior vena cava. Donor pulmonary artery is also divided and the remaining pulmonary vessels are tied. These attachments are then cut, as are the superior and inferior vena cavae, allowing the donor heart to be lifted free.

the later anastomosis. The right superior vena cava and the inferior vena cava were then cut, distal to their ties. The remaining pulmonary vessels were then caught by a 4-0 tie, placed around the heart, carefully avoiding the delicate atria. The donor heart was then removed, by cutting on the donor side of this final tie, and placed in a petri dish of cold saline, which was kept on crushed ice. The heart was gently squeezed to expel any remaining blood and returned to the ice.

The recipient rat was taped out on a heated plate and it's abdomen opened through a midline incision. The intestines were swept cranially and wrapped in a moist, saline swab. The infra-renal IVC and aorta were then dissected out and any venous or arterial branches were tied twice, with 8-0 silk, and divided. Once the major vessels were mobilised, great care was taken to identify and tie any lumbar vessels which entered behind the proposed site of the vascular anastomoses. The vessels were now ready to receive the graft (Figure 2.1.2). Occlusive vascular clamps were applied to the major vessels, immediately below the renal vessels and just above the bifurcation of the aorta, allowing as much vessel length as possible for the site of anastomosis. A puncture was made in both vessels with a fine needle and extended with scissors to form an arteriotomy and a venotomy of a size comparable to the donor aorta and pulmonary artery. The heated plate under the recipient was now switched off. The donor heart was removed from the cold saline and the aorta was anastomosed, end-to-side, to the recipient aorta, using a continuous 9-0 ethilon suture (Ethicon Ltd.) The donor pulmonary artery was then anastomosed, end-to-side, to the recipient inferior vena cava using the same suture (Figure 2.1.3).

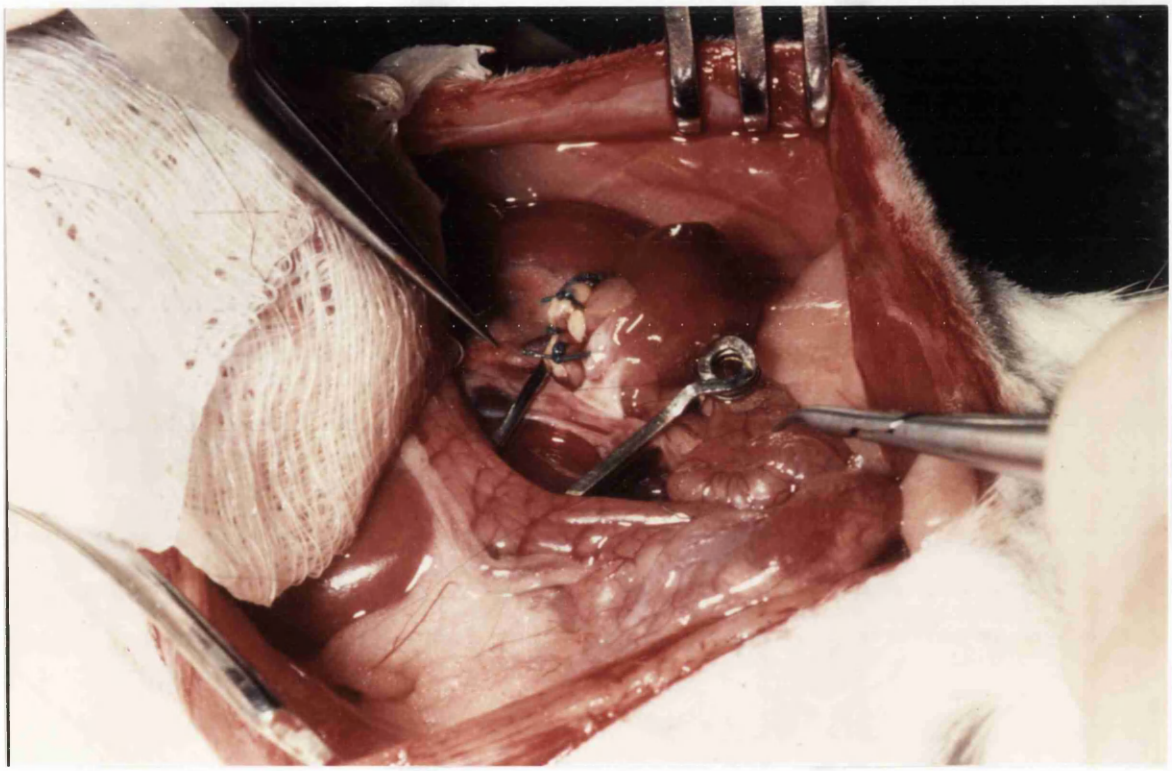
Following completion of the anastomoses, the distal, and then the proximal, vascular clamps were released, briefly, and re-applied. Any bleeding points were controlled with small pieces of Spongistan. The distal and proximal

Figure 2.1.2 Heterotopic cardiac transplantation in the rat.



*** Recipient IVC and abdominal aorta are visualised and prepared, below the renal vessels. Any branches are tied with 8-0 silk and divided. The abdominal contents are kept moist under a saline swab.

Figure 2.1.3 Heterotopic cardiac transplantation in the rat.



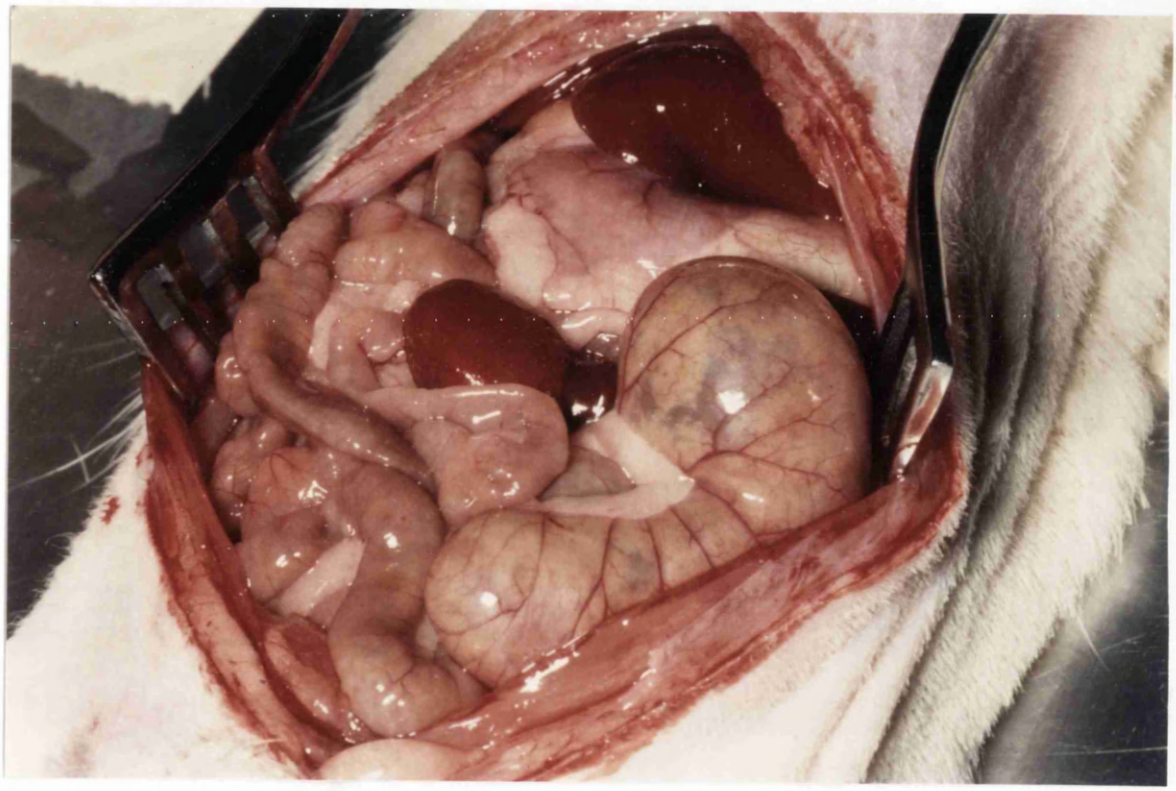
*** End-to-side anastomosis is performed between donor aorta and recipient aorta, using 9-0 nylon suture. The donor pulmonary artery is then anastomosed in a similar fashion to the recipient IVC.

vascular clamps were then removed and blood allowed to circulate through the graft.

The heart was observed until it had started beating normally and then the intestines were replaced, around the graft (Figure 2.1.4), and the abdomen closed in two layers, with continuous 3-0 cat gut suture (Ethicon Ltd.). The cold ischaemic time of the heart graft was approximately 20 minutes.

The viability of the heart grafts was assessed by daily palpation through the abdominal wall. Rejection was deemed to have occurred when the pulsation of the graft could no longer be felt by palpation of the abdominal wall. In doubtful cases the heart was examined by laparotomy and, if any pulsation was detected, the wound was closed and the rat woken up, to be re-assessed the following day.

Figure 2.1.4 Heterotopic cardiac transplantation in the rat.



*** The abdominal viscera are placed around the functioning heterotopic heart graft prior to closure of the abdominal incision. The pulsating heart can then be easily palpated through the abdominal wall.

2:3 Cells and tissues

All procedures with cells were performed in DAB containing either 2% foetal calf serum (DAB/FCS) or 0.2% bovine serum albumin (DAB/BSA).

2:3.1 Lymph node cells

Cervical and mesenteric lymph nodes were removed from rats, following cervical dislocation. The nodes were pooled, chopped with a scalpel and forced through a fine stainless steel mesh with a syringe plunger, in DAB/FCS or DAB/BSA. The cells were washed two or three times, in DAB/FCS, and dead cell aggregates removed with a pasteur pipette.

2:3.2 Spleen cells

Splenectomy was performed following cervical dislocation. The spleens were teased apart with sterile forceps in DAB/FCS or DAB/BSA. The cells were washed and dead cells removed. Erythrocytes were removed by hypotonic lysis using 5 mls distiled water followed by 5 mls double strength saline. Splenocytes were washed three more times before use.

2:3.3 Concanavalin A stimulated lymphoblasts

51 Chromium (^{51}Cr) labelled Concanavalin A (ConA)-stimulated lymphoblasts were used as target cells in lymphocyte mediated cytotoxicity assays. Lymphocytes were prepared from rat spleens, as described above, and adjusted to a concentration of 2-2.5 $x10^6$ cells/ml in RPMI containing 10% FCS, glutamine, penicillin, streptomycin and 2 x 10^{-5} M 2-mercapto-ethanol (BDH Ltd.). 10 mls cell suspensions were incubated in tissue culture flasks with 5 µg/ml Concanavalin A (Sigma Chemical Co.) for 72 hours at 37°C in 5% CO_2 in air. Lymphoblasts were washed once and counted before use.

2:4 Monoclonal antibodies

For the *in vivo* depletion of T cell subsets, the following monoclonal antibodies were used:

a) MRC OX8 - an IgG_1 antibody specific for $CD8^+$ cytotoxic/suppressor T cells and the majority of natural killer cells [Gillman, Rosenberg & Feldman (1982), Dallman et al (1982), Cantrell, Robins, Brooks et al (1982)].

b) MRC OX35 & MRC OX38 - both anti-$CD4^+$ T cell monoclonal antibodies of the IgG_{2a} subclass, which bind to different epitopes of the CD4 molecule, and do not crossblock each others binding (Jeffries, Green & Williams, 1985: Roser, 1989)

c) MRC OX21 - mouse mAb against human C3b inactivator, which was used as a negative control (Hsiung, Barclay, Brandon et al 1982).

The antibodies were produced in mouse ascites, following intra-peritoneal injection of appropriate myeloma hybridoma cells (European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wiltshire), into Pristane (Sigma Chemicals) primed (DBA/2 x BALB/c)F1 mice (Harlan Olac Ltd.). The immunoglobulin content of ascites was quantified using anti-mouse IgG_1 immunodiffusion plates and known mouse Ig standards (Serotec Ltd.). Antibodies were diluted in PBS, as required, and stored at -20°C.

They were administered to the experimental animals by intraperitoneal injection.

In addition, the following mouse mAbs were used to label rat leukocytes:

MRC OX1 (leukocyte common antigen [Sunderland, McMaster & williams, 1979]);

MRC OX18 and MRC OX6, which recognise polymorphic determinants of MHC class I and class II antigens, respectively (Fukomoto, McMaster & Williams, 1982);

ED1, which labels most tissue macrophages, monocytes and dendritic cells (Dijkstra, Dopp, Joling et al, 1985);

R73, which recognises a constant determinant of the rat TCR-α/β (Hunig, Wallny, Hartley et al, 1989);

W3/25, which labels $CD4^+$ T helper lymphocytes and some macrophages (Mason, Arthur, Dallman et al, 1983).

The above were obtained from Serotec Ltd., Oxford, U.K.

2:5 Histology

2:5.1 Cryostat sections

Cardiac cross-sections were embedded in OCT compound (Tissue-Tek, BDH Ltd.), snap frozen in liquid nitrogen and 5μ cryostat sections were cut at -20°C onto gelatinised multispot slides (C.A.Hendley, Essex).

2:5.2 Immunoperoxidase staining

Sections were fixed in acetone (BDH, Ltd.) at room temperature for 10 minutes, then washed in DAB. Excess moisture was dried from each section and 50μl of primary monoclonal antibody, appropriately diluted, were applied to each section. Slides were incubated for 45 minutes at RT in an humidified chamber, then washed three times in DAB. The secondary antibody was applied, consisting of peroxidase-conjugated rabbit anti-mouse Ig (Dako Ltd.) containing 10% normal rat serum to absorb out any cross-reacting activity.

After 30 minutes incubation at RT, the slides were washed three times and the substrate was added [0.6 mg/ml 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) plus 0.01% hydrogen peroxide, for 5 minutes]. The sections were then lightly stained in Harris's haematoxylin, were dehydrated and cleared in alcohols and xylene, and were mounted in DPX (BDH Ltd.).

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 the point counting technique (Aherne & Dunnill, 1982; McWhinnie, Thompson, Taylor et al, 1986). Sections were examined at a magnification of 400

in the presence of a microscope eyepiece graticule engraved with a squared grid bearing either 121 intersections (1mm apart) or 745 intersections (0.5mm apart). For each high powered field, the number of positively stained cells superimposed by an intersection was counted, and the area of the field occupied by cells of a particular phenotype was calculated as:

$$\% \text{ area of infiltrate} = \frac{\text{no. +ve grid intersections} \times 100}{\text{total no. grid intersections}}$$

Ten consecutive fields were counted for each section, so that for all sections the total number of points observed was well in excess of that required to maintain the accuracy of the point counting technique (Aherne & Dunnill, 1982)

2:6 Serum antibody titres

To determine allospecific serum antibody titres, the rats were bled from the tail on selected days following transplantation. The samples were allowed to clot, and were spun down. The serum was heat inactivated in a water bath at 56°C for 30 minutes and then centrifuged briefly to remove protein aggregates. It was then serially diluted in RPMI/5%FCS/20mM Hepes and mixed with ^{51}Cr-labelled splenocytes of both donor and third-party strain rats.

After incubating at RT for 30 minutes, freshly reconstituted guinea pig complement (Sera-Lab, Sussex), diluted 1 in 5, was added, and the cells were incubated for one hour at 37°C. The ^{51}Cr released into the supernatant was counted in a gamma counter and titration curves of % ^{51}Cr release against serum dilution were made for each serum sample.

2:7 Cell mediated cytotoxicity

2:7.1 Effector cells

Splenocytes, for use as effector cells in cell mediated cytotoxicity assays, were obtained as described in section 2.3.2, and were resuspended in RPMI/5%FCS/20mM Hepes.

2:7.2 ^{51}Chromium release assay

A standard ^{51}Cr-release assay based on the experiments of Brunner et al (1968) was used. Target cells were labelled by incubation with 5 MBq ^{51}Cr-sodium chromate for 90 minutes at 37^{o}C with frequent mixing. Cells were washed five times and then resuspended in medium.

Serial twofold dilutions of effector cells were made, to give effector:target cell ratios of 100:1, 50:1, 25:1, 12.5:1, 6:1 and 3:1.

Equal volumes (75µl) of effector and target cells were mixed in wells of 96 V-well microtitre plates (Sterilin, Teddington) so that each well contained $5x10^3$ or 10^4 target cells, and each cell combination was set up in triplicate or quadruplicate. Spontaneous release from targets was determined using medium instead of effector cells, and maximum release obtained by adding 10% Triton X (BDH) instead of effectors. The plates were centrifuged briefly then incubated for 6 hours at 37^{o}C in 5% CO_2 in air. From each well, 75µl supernatant was taken and the amount of released isotope was determined by a Compugamma counter (LKB, London). The amount of isotope released as a result of effector cell activity was calculated as follows:

% cytotoxicity= (experimental-spontaneous release)x 100

(maximum-spontaneous release)

2:8 Cell preparation and fluorescence analysis.

Single cell suspensions of lymph nodes and spleen were prepared as described previously (Bolton et al, 1989). PBL were separated by centrifugation of heparinised whole blood over 75% iso-osmolar Percoll (Sigma Ltd., Poole, UK). For single colour fluorescence analysis, cells were incubated with the appropriate mAb for 1 hr at 4°C, washed twice in DAB/BSA, and incubated for 1 hr at 4°C with FITC-conjugated $F(ab)_2$ rabbit anti-mouse Ig (Dako Ltd., High Wycombe,UK) containing 10% normal rat serum to prevent cross-reaction of the antibody with rat Ig.

For two-colour fluorescence analysis, the first stage was as described above. For the second stage, unbound sites of the second antibody were blocked with MRC OX21 (mouse anti-human C3b) and the cells were incubated for 30 min at 4°C with biotinylated W3/25 or biotinylated MRC OX8 (a gift from Dr. E. Bell, Department of Immunology, University of Manchester, UK) followed by a phycoerythrin-streptavidin complex (Becton Dickinson, Mountain View, CA). Cells were washed twice in DAB/BSA to remove excess antibodies. The labelled cells were analyzed on a FACScan flow cytometer (Becton Dickinson).

2:9 Statistical Analysis

All of the statistical analyses in this thesis were performed using the Mann-Whitney Test.

CHAPTER THREE.

Unmodified rejection of an MHC class I disparate graft

3:1 The Unmodified Rejection of an Isolated Class I Disparate Cardiac Allograft.

As has been mentioned before, there has been considerable variation in the reported literature regarding the cellular and humoral requirements for allograft rejection in animal models. Many of the apparently contradictory findings of different laboratories may be attributable to variation of species, or strain combinations used.

In addition, it has been speculated that the type of allograft itself may have an effect on the mechanism of its rejection. In particular, much of the early experimental work on allograft rejection was performed using skin grafts, and it is now recognised that the mechanism of rejection is different when the graft is a primarily vascularised organ graft.

Previous work in this laboratory, aimed at elucidating the cellular requirements for renal allograft rejection, produced some interesting data to suggest a possible role for alloantibody in the acute rejection of an isolated class I disparate renal allograft, which challenged the widely held belief that rejection of such a graft is mediated by $CD8^+$ CTL (Gracie et al, 1990).

We have established a model of acute rejection of an isolated class I cardiac allograft (PVG R8---PVG RT1u) in the rat, to allow examination of the hypothesis that cytotoxic alloantibody is an important effector mechanism in the acute rejection of such a graft.

Before considering the evidence in favour of alloantibody, the cellular and humoral events taking place during the unmodified rejection of a class I disparate graft will be examined.

3:2 Cellular infiltrate in unmodified rejection.

PVG R8 hearts were grafted heterotopically into PVG RT1u recipients, as described in Chapter 2. The condition of the grafts was assessed by daily palpation, through the abdominal wall, by the author. The hearts were deemed to have been rejected when there was no palpable pulsation, and it was noted during this work that there was a consistently rapid cessation of contraction of the graft, rather than a slow deterioration over several days.

In this strain combination, the mean survival time (MST) of the grafts was 6.5 days

To assess the cellular infiltrate of the rejecting grafts, recipient animals were sacrificed on days 1,3,5 & 7 post-transplant, and their grafts were snap frozen and sectioned to allow direct staining, and indirect immunoperoxidase staining, to be carried out, as described in Chapter 2.

In keeping with similar experiments performed on rat renal allografts in this laboratory, it was found that there was a progressive cellular infiltration of the graft, between day 3 and day 5, and after this there was such extensive damage to the graft that useful analysis was not possible.

Using a panel of mouse anti-rat mAbs, day 5 heart grafts from both syngeneic controls and unmodified, rejecting animals, were sectioned and stained by indirect immunoperoxidase techniques. The mAb used, and their targets are listed in Chapter 2 (2.4).

The effects of the transplant operation itself were examined by comparing syngeneic grafts (R8---R8) with naive R8 hearts. The syngeneic graft was found to be almost identical to the normal R8 heart in this experiment. In both there was a moderate expression of class II MHC, which did not alter as a result of transplantation. The expression of class I was less, in both groups but, once again, was unaffected by the operative procedure. The morphology of the cellular infiltrate of the hearts was comparable. Both showed very light staining with OX 1 indicating the presence of a few leukocytes, but had no significant staining for TCR or for either CD4 or CD8. A slightly positive staining for ED 1 showed that those leukocytes present, were macrophages. When the unmodified rejecting grafts (day 5) were compared with the syngeneic grafts it was found that there was a significant increase in the expression of class II MHC in the rejecting graft. There was also an increase in class I MHC expression in the rejecting graft but the expression of class I remained weaker than that of class II.

With respect to the cellular infiltrate, the R8 heart being rejected by the RT1u rat showed an increase in the number of leukocytes (OX 1) in the graft as compared to the syngeneic R8 graft. There was a slight increase in staining for TCR (R 73), with an associated increase in CD8 positivity, but CD4 remained negative. The most striking appearance in the rejecting graft was the increase in the number of macrophages infiltrating the graft.

3:3 Alloantibody production in unmodified rejection.

It has previously been shown that, in a model of renal transplantation in the rat, acute rejection of the graft is associated with the production of high levels of specific alloantibody (Gracie et al, 1990). This finding leads to the hypothesis that alloantibody may act as an effector mechanism of such an acute rejection response. This remains controversial, however, with Lowry & Clarke Forbes (1984) stating conclusively that alloantibody and B cells were not required for the rejection of a cardiac graft in a rat model. They found that an irradiated Lewis rat could reject a heart from an irradiated Wistar-Furth rat when the recipient was reconstituted with primed splenocytes, from which B cells had been removed and which lacked a demonstrable alloantibody response. They reported that, in the absence of antibody, the histological microvascular damage was the same as that seen in unmodified rejection.

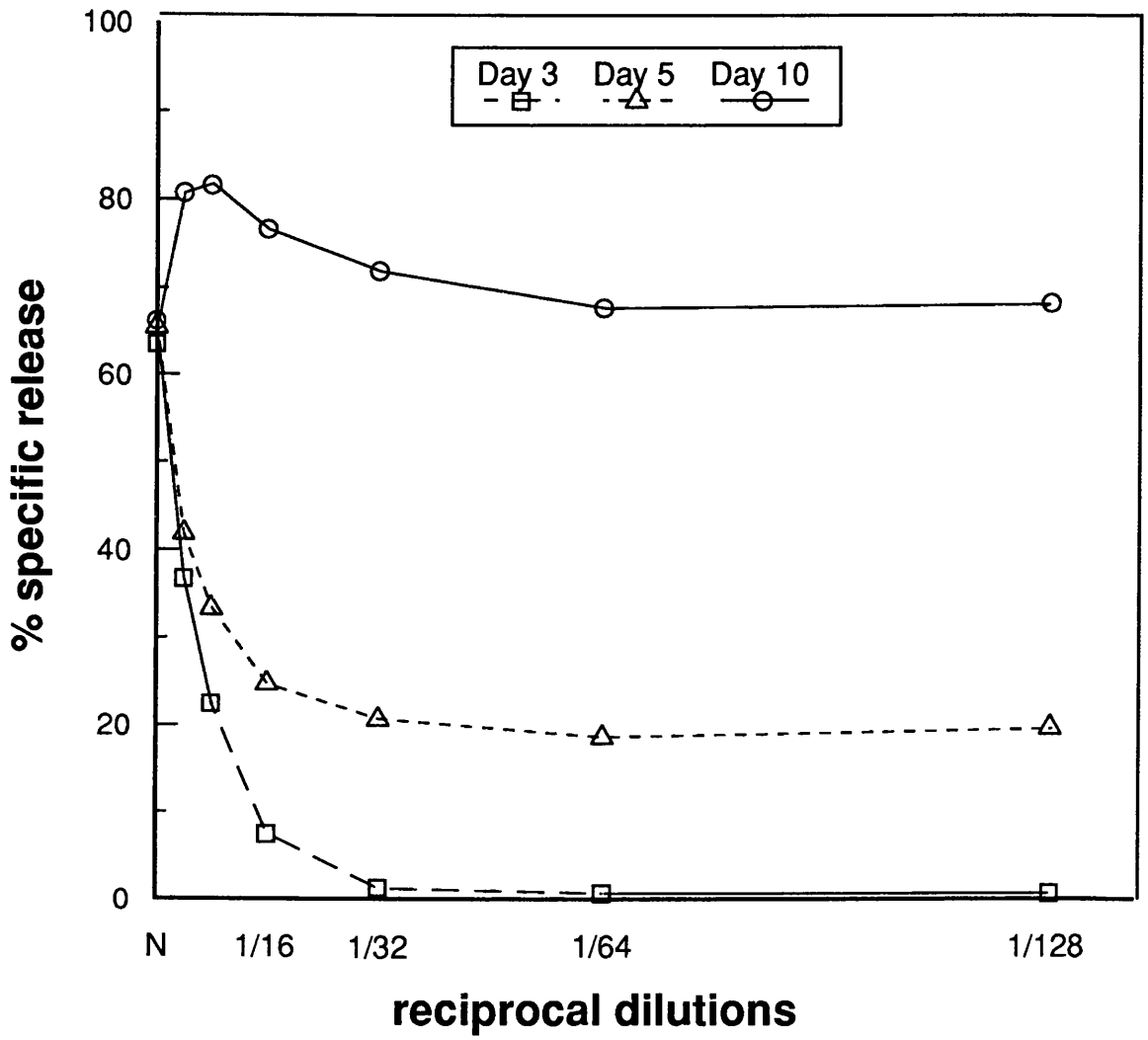
We determined the level of alloantibody produced by the RT1u rat strain, against an isolated class I disparate cardiac allograft of the R8 strain, by measuring the amount of ^{51}Cr release from ^{51}Cr labelled R8 blasts which had been combined with serum from RT1u rats which were rejecting R8 hearts. The results are shown in Figure 3.1.

It was found that there was a steady increase in alloantibody production from day 3 which was maximal on day 15, when the final test sera were taken.

The production of alloantibody is not surprising, in itself. Of greater interest, is whether this antibody is central to the acute rejection of the graft.

It is now generally accepted that the primary target of the rejection response against a vascularised allograft is the microvasculature of the graft, and

Figure 3.1 Alloantibody levels in unmodified rejection



Specific alloantibody produced against a class I disparate graft was determined by measuring the % release of 51Cr from labelled R8 lymphoblasts mixed with reciprocal dilutions of RT1u rejecting serum.

*** Significant levels of donor-specific alloantibody were detected from day 3 onwards.**

more specifically. the vascular endothelium (Forbes, Guttmann, Gomersall et al, 1983; Adams et al, 1989; Bishop et al, 1989).

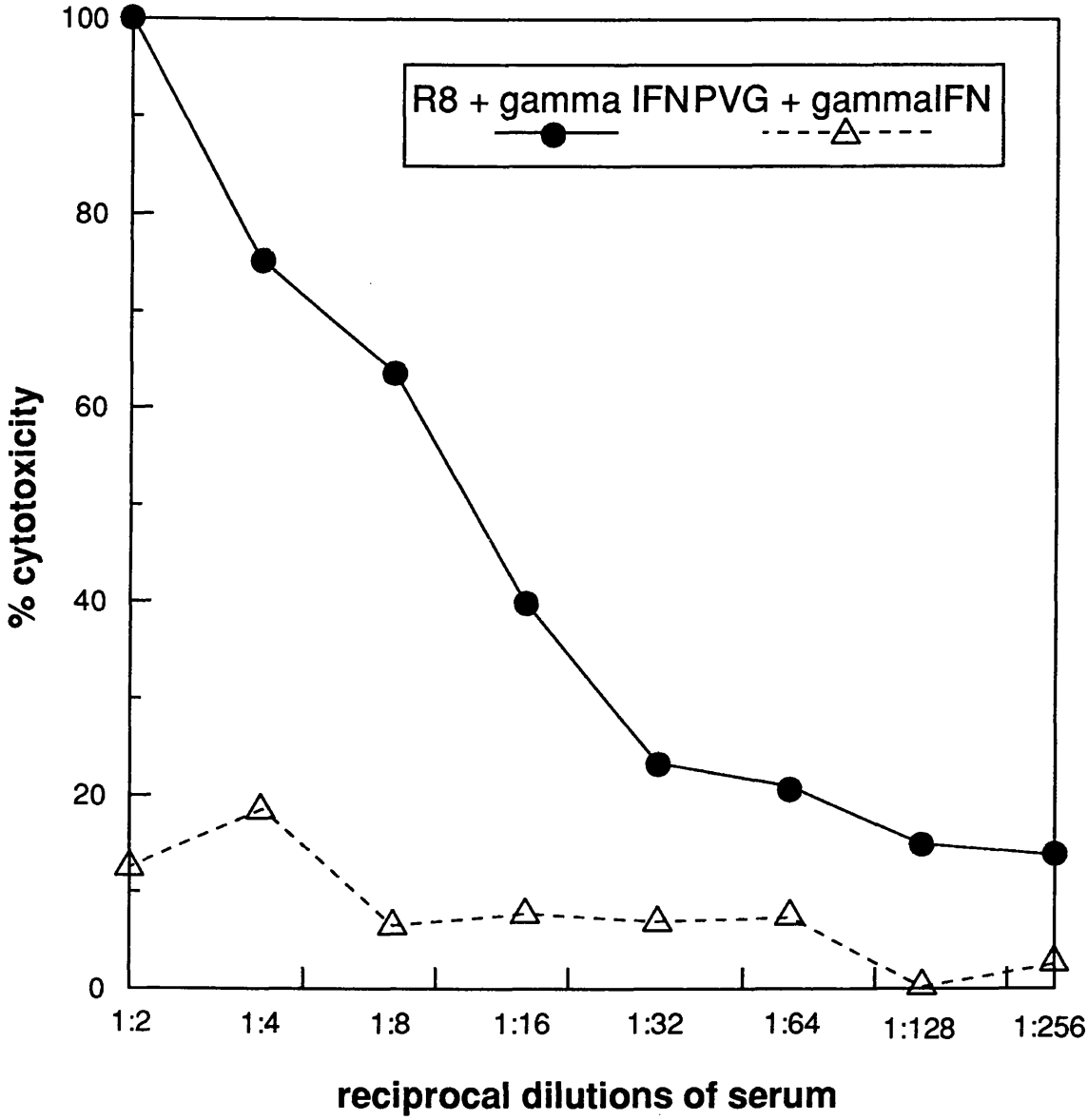
If it is being proposed that alloantibody may play a significant role as an effector mechanism in this response it is important to demonstrate the ability of alloantibody to cause endothelial cell damage.

To explore the cytotoxic properties of the alloantibody contained in rejecting serum, the ability of day 7 and day 10 rejecting serum to specifically lyse *in vitro* cultured monolayers of R8 neonatal heart endothelial cells, which had been prepared as described in Chapter 2., was examined. During these experiments, either guinea pig complement or RT1u rat serum alone was used as a source of complement. PVG RT1c endothelial cells were used as a third-party control.

It was found that when the R8 cells had been cultured in the presence of IFN-γ, the addition of day 7 or day 10 RT1u rejecting serum resulted in *in vitro* complement dependent cytotoxicity with endothelial cell lysis (Figure 3.2).

Contrary to previous reports of the deficiencies of the rat complement system in different rat strains (French, 1972; Oluwole, Tezuka, Wasfie et al, 1989), RT1u serum proved to be an adequate source of complement to allow cell lysis to occur. The specificity of the CDC reaction was shown by the fact that the RT1u rejecting serum, which was effective against R8 endothelial cells, was unable to lyse third-party endothelial cells cultured from neonatal PVG RT1c rats in the presence of IFN-γ. It was also found that if the R8 neonatal endothelial cells were cultured without IFN-γ, the addition of day 7 or day 10 rejecting serum, in the presence of either guinea pig complement or RT1u serum, did not result in a CDC response and there was no lysis of the endothelial cells.

Figure 3.2 CDC activity of rejecting serum vs R8 and PVG endothelial cells cultured in vitro with gamma IFN



* The only source of complement in this experiment was RT1u serum. Pooled sera from RT1u rats which had been transplanted with R8 hearts were tested for CDC activity against R8 and PVG neonatal cardiac endothelial cells, cultured in vitro with gamma IFN.

**** The rejecting serum was specifically cytotoxic to donor-type (R8) endothelial cells.**

*** When cultured without gamma IFN neither endothelial cell type was lysed by the rejecting serum.

The fact that co-culture with IFN-γ was required before the endothelial cells were susceptible to CDC with rejecting serum, supports the concept of the important role of cytokines in activating vascular endothelial cells and increasing their expression of class I and especially class II MHC antigens (Halloran & Madrenas, 1990). It is by recognition of, and binding to these surface antigens that alloantibody is able to initiate its cytotoxic effects. The absence of any non-specific effector cells from the transferred rejecting serum shows that the specific cytotoxicity in this *in vitro* experiment is as a result of CDC, and not an example of ADCC.

3:4 Discussion.

The acute rejection of a vascularised cardiac allograft is a predictable and consistent event, unless the recipient is immunosuppressed. The speed with which the heart is rejected is dependent upon the strain combination of the experimental animals used, and the MHC disparity between the donor and the recipient.

Complete disparity for both MHC and mH antigens results in rapid rejection of a rat heart graft in 6-8 days but, in certain strain combinations, an isolated RT1A subregion (class I) disparate graft survives indefinitely (Guttmann, Forbes, Fuks et al, 1985; Klempnauer, Steiniger, Wonigeit et al, 1985) whereas RT1B (class II) disparate grafts are rapidly rejected. However, use of the high responder PVG RT1u haplotype to PVG RT1a class I antigens has been shown to result in rapid rejection of a cardiac allograft (Lowry et al, 1985) and this has been confirmed by the results of the experiments described in this thesis, with unmodified RT1u recipients rejecting R8 hearts in 7 days.

The histological and immunohistological findings that are reported here are similar to those previously reported, using a similar model (Clarke Forbes et al, 1988), with a progressive infiltration of the rejecting graft by mononuclear cells, the majority of which are macrophages. Those few T cells which were found in the rejecting grafts were almost exclusively $CD8^+$, but this fact, in itself, is not significant evidence that the CTL is crucial to the rejection of this particular graft. It is , if anything, surprising, in the light of the accepted MHC restriction of $CD4^+$ and $CD8^+$ T cells, that a rejecting, class I disparate graft is not heavily infiltrated with $CD8^+$ cells. This finding is consistent with previous experimental results from this laboratory, when it was found that rejecting, class I disparate kidney grafts had a similar pattern of cellular infiltration as class I disparate grafts which were not being rejected because their recipients were "low responders". The

cellular infiltrate consisted predominantly of macrophages, and complete depletion of $CD8^+$ cells, by mAb OX 8, had no effect on the makeup of the infiltrate, nor on the survival time of the grafts which were all rejected by first-set rejection kinetics (Gracie et al, 1991). This work brings into question the widely accepted belief that the $CD8^+$ T cell is an absolute requirement for the rejection of an isolated class I disparate allograft (Lowry et al, 1985), and, furthermore, the associated finding of markedly elevated titres of specific alloantibody in the rejecting animals serum has stimulated closer examination of the role played by this antibody, and by the T cell subsets, in the rejection of a cardiac graft of the same MHC disparity, by our laboratory.

It was decided to approach the investigation of the contribution of each T cell subset in the acute rejection of a class I disparate cardiac allograft in the rat by selectively depleting them, individually, *in vivo*, using mouse anti-rat monoclonal antibodies, which were obtained from mouse ascites, as described in Chapter 2.

The results of these experiments form the next two chapters of this thesis.

CHAPTER FOUR

The role of $CD8^+$ T cells in the rejection of an MHC class I disparate graft

4:1 The $CD8^+$ T cell in the rejection of a class I disparate graft.

The fact that the $CD8^+$ T cell is restricted to recognition of, and interaction with, class I MHC and not class II, makes it seem a logical conclusion that it could be the predominant effector mechanism in the rejection of an allograft which differs from the recipient only at the classical class I MHC subregion. Indeed, there are many workers with an interest in this field who are convinced that this is the case. It is certainly true that specific CTLs have been shown to be present in both human and animal rejecting allografts (von Willebrand & Hayry, 1978; Hayry, von Willebrand & Soots, 1979), but although most reviewers propose that the CTL is the likeliest effector cell in allograft rejection, the crucial importance of cytokine help, provided by $CD4^+$ T helper cells, in the activation of these specific cytotoxic T cells is stressed (Bradley et al, 1985; Steinmuller, 1985; Mason & Morris, 1986; Hall, 1991).

It has also been shown, however, that unprimed Lyt-2^+ ($CD8^+$) cells can respond to class I antigen, *in vivo*, without help from $CD4^+$ T cells (Sprent, Schaefer, Lo et al, 1986) but this may be explained by the fact that in certain murine strains the $CD8^+$ T cells have the ability to produce their own Il-2, which is not the case in the rat model(Sprent, Schaefer, Lo et al 1986; Mizuochi, Ono, Malek et al, 1986).

Most recently, in an attempt to explain why mice which had been depleted of $CD8^+$ cells by mAb were still able to reject a class I disparate skin graft, Rosenberg and co-workers have proposed the existence of a unique subset of $CD8^+$ cells which are not only resistant to depletion by anti-CD8 mAb but are, in fact, activated by the monoclonal antibody (Rosenberg, Munitz, Maniero et al, 1991). This new concept will require further examination to determine its validity.

In the light of the surprising finding, in our laboratory, that complete depletion of $CD8^+$ cells had no protective effect on a class I disparate renal graft in a rat model, the experiment has been repeated, in the same high responder strain combination, using a heterotopic heart graft.

4:2 Depletion of $CD8^+$ cells by OX8 monoclonal antibody.

Recipient rats, of the RT1u strain, were treated with OX 8 mAb (2mg/ml), given by intraperitoneal injection in the following doses:

1.5ml on the day before transplant (D -1)

1.0ml on the day of transplant (D 0)

0.5ml on the third day post-transplant (D 3)

0.5ml on the sixth day post-transplant (D 6)

0.5ml on the ninth day post-transplant (D 9)

These doses have previously been shown to cause effective elimination of $CD8^+$ cells (Gracie et al, 1990). On Day 0 the RT1u rats were transplanted with an R8 heart. The heterotopic, intra-abdominal grafts were then palpated daily, in the standard fashion, to determine the time of rejection. As can be seen in Table 2 , there was no difference in the graft survival time between those recipients which had received the anti-CD8 mAb (mean 6.8 days) and the unmodified recipients which rejected their grafts in the expected manner (mean 6.7 days).

Table 2. Graft Survival Time (GST) in unmodified rejection of a class I disparate graft and following anti-CD8 mAb treatment.

Strain combination	mAb	GST (days)	Mean GST (days)
R8→RT1u	-----	6,6,6,6,7,7,7,8	6.8
R8→RT1u	OX8	6,6,6,7,7,7,7,7	6.8

☐**Treatment with anti-CD8 mAb OX8 had no effect on the rejection of this class I disparate heart graft.**

4:3 Efficiency of OX8 mAb.

With the findings of this experiment in mind, it is of obvious importance to determine whether OX8 is effective in its proposed role as a depleter of $CD8^+$ cells as, if this were not the case, it would not be possible to draw any valid conclusions from these data.

Three techniques were employed to investigate the extent of $CD8^+$ cell depletion, and these were:

a) Two-colour Fluorescence Activated Cell Sorter (FACS) scanning;

b) Indirect immunoperoxidase histology

c) Attempted *in vitro* generation of specific CTLs.

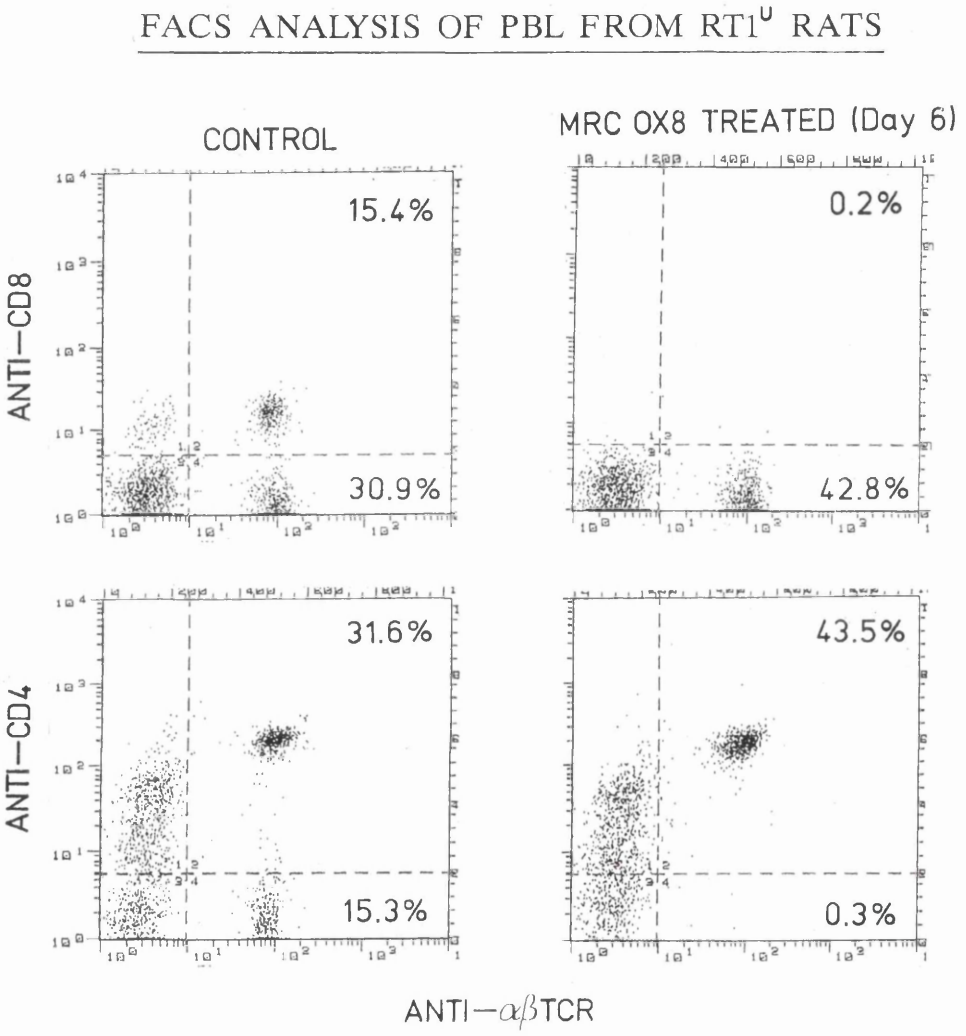
4:3.1 FACS analysis in OX8 treated group.

The result of the FACS analysis in the rats which had received OX8 is shown in Figure 4.1.

The mAb R73 was used to label T cells, while the CD8 molecule was labelled by OX8.

In the control animal, the normal distribution of cells in a FACS result can be seen. Following anti-CD8 mAb treatment, there has been an obvious and complete depletion of $CD8^+$ cells, including both T cells and non-T cells alike. Furthermore, it can be clearly seen that all the remaining T cells in these rats are $CD4^+$, and these have not been affected by the anti-CD8 depletion therapy. This finding demonstrates that OX8 mAb treatment has resulted in true depletion of the $CD8^+$ cell population, and not simply modulation of the surface antigen.

Figure 4.1 FACS analysis of peripheral blood leukocytes in anti-CD8 treated animals and controls.



* Controls were treated with OX21

** There has been complete depletion of the $CD8^{+}$ cells following OX8 mAb treatment.

*** Treatment with OX21 (anti-human C3) has had no effect on the PBLs.

4:3.2 Immunoperoxidase histology in OX8 treated group.

This technique is described in detail in Chapter 2.

Figure 4.2 shows peroxidase-stained sections of day 5 heart grafts taken from an unmodified, rejecting heart, and a rejecting heart from an anti-CD8 treated animal, respectively. Once again, it is clear that treatment with OX8 has resulted in the complete depletion of $CD8^+$ cells from the graft, confirming the FACS result of elimination of $CD8^+$ cells from the peripheral blood and lymph nodes following mAb administration.

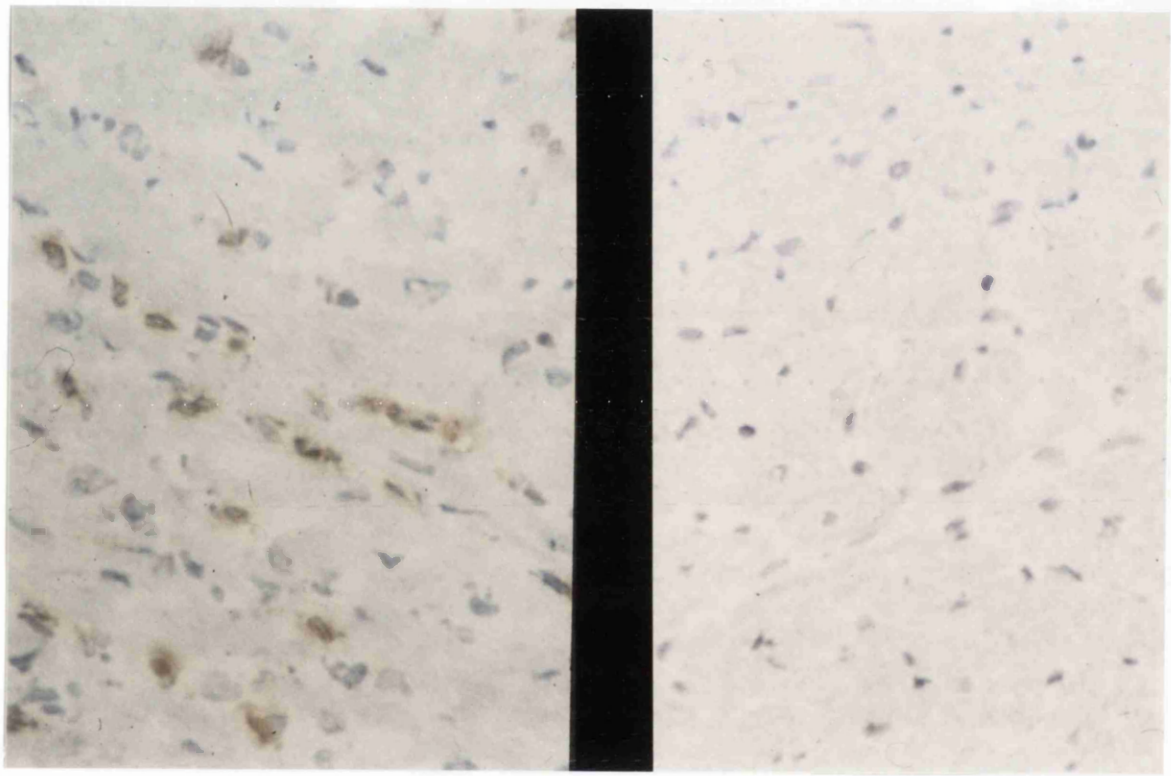
4:3.3 Generation of cytotoxic T cells.

To assess the effect of anti-CD8 mAb treatment on the function of CTLs in the recipient animals, cytotoxic T cells were generated, *in vitro*, from RT1u lymph node cells of both control (OX21), and OX8 treated animals, by co-culture for 5 days with irradiated RT1a stimulators. The cytotoxicity of the cells was then determined by measuring the % specific release when the cells were combined with ^{51}Cr labelled R8 lymphoblasts.

Figure 4.3 shows that there was a high level of specific cytotoxicity against the donor-strain lymphoblasts in the control group, and that this activity was completely abrogated in the animals which had received anti-CD8 treatment.

Taken in combination, the results of the FACS analysis; the immunoperoxidase histology, and the *in vitro* cytotoxicity assay, have clearly demonstrated that OX8 mAb treatment is extremely effective in, both phenotypically and functionally, depleting $CD8^+$ cells from recipient animals.

Figure 4.2 Immunohistological assessment of $CD8^+$ T cell depletion by anti-CD8 mAb.



a) Unmodified control R8 ---- $RT1^u$ (day 5)

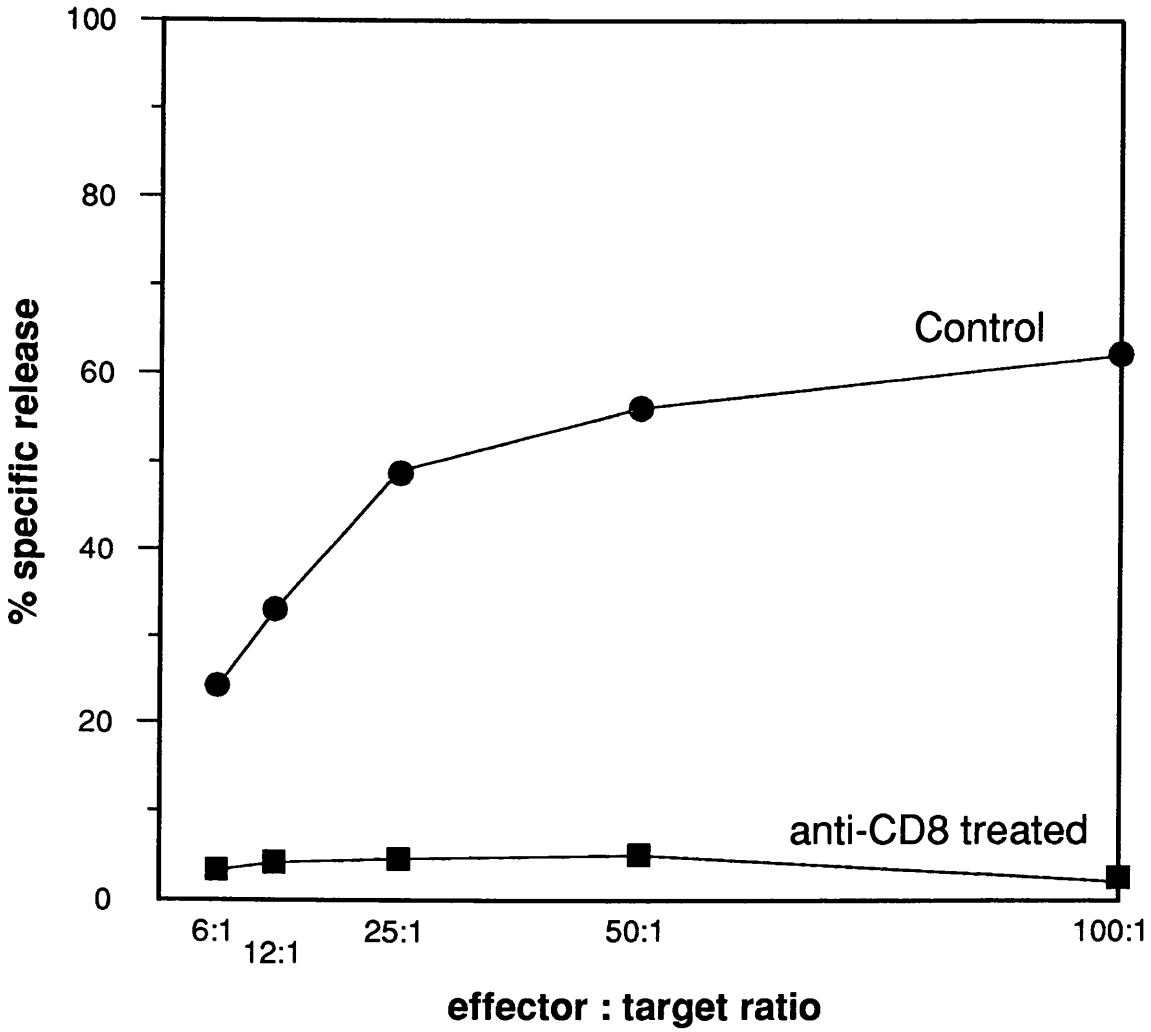
b) R8 ---- $RT1^u$ + OX8 (day 5)

* Both sections have been stained by an indirect immunoperoxidase technique for $CD8^+$ cells

* The $CD8^+$ cells seen in the unmodified control rejecting graft have been completely removed by the OX8 treatment.

The class I disparate graft in b) is being rejected in the absence of $CD8^+$ cells.

Figure 4.3 The effect of in vivo anti-CD8 mAb treatment on generation of class I specific cytotoxic T cells in vitro.



* Cytotoxic T cells were generated from RT1u lymph node cells (control or anti-CD8 treated) by culture for 5 days with irradiated RT1a stimulators. Cytotoxicity was determined against 51Cr labelled R8 lymphoblasts.

** The control mAb was OX21.

***** Treatment with OX8 has prevented the generation of class I-specific cytotoxic T cells.**

4:4 Discussion.

The results of the experiments have shown that, in this strain combination, an isolated class I disparate heart graft has been rejected, with first-set kinetics, in the complete absence of $CD8^+$ T cells.

This directly contradicts the findings of Lowry et al (1985) who concluded that the cytotoxic, $CD8^+$, T cell was crucial in the rejection of a heart graft bearing such an MHC incompatibility, albeit in a different strain combination [irradiated (PVGxWF)F1 recipients of irradiated PVG-RT1r1 grafts]. In addition, it brings into question the commonly held belief that the CTL, more than any other, is the predominant effector mechanism in organ allograft rejection.

Unless the recent hypothesis of an anti-CD8 mAb resistant, $CD8^+$ CTL, put forward by Rosenberg et al (1991) is to be believed, it is apparent from these results that it is perfectly possible for a class I disparate graft to be rejected without any contribution from the CTL subset. It obviously cannot be assumed from these results, that the CTL does not play a significant role in the rejection of other allografts, with different MHC disparities, but it can be stated, with some certainty, that, in this model, an allograft is being rejected by some mechanism other than by cytotoxic T cells.

The fact that this particular class I disparate graft has been rejected, without $CD8^+$ CTLs, raises two important questions:

1. How is the foreign class I MHC recognised by the recipient, given the MHC restrictions of $CD4^+$ and $CD8^+$ T cells?

2. What is the effector mechanism which is initiating the rejection of this particular graft?

There is increasing evidence to suggest that foreign, class I MHC antigen can be recognised by $CD4^+$ T cells following processing of the antigen and presentation to the T cell in the context of self class II MHC (Golding & Singer, 1984; Rock, Barnes, Germain et al, 1983; Cheng, Madrigal, Parham, 1990). This explanation would account for the recognition of this graft as foreign, if the class I MHC antigens, which by convention are only recognised by $CD8^+$ T cells, are being processed and presented to $CD4^+$ T cells, in the context of self class II.

As will become apparent when the results of experimental depletion of $CD4^+$ T cells are examined, it appears that there is a direct correlation between the production of specific alloantibody against a graft, and the acute rejection of that graft. The possibility that this alloantibody could be the initiator of the immunological rejection response mounted against a class I disparate graft will be explored later in this thesis.

CHAPTER FIVE

The role of $CD4^+$ T cells and of specific, cytotoxic alloantibody in the rejection of an MHC class I disparate graft

5:1 The $CD4^+$ T cell in the rejection of a class I disparate graft.

Having established, in both the kidney and heart transplant models in the rat, that complete depletion of the $CD8^+$ T cell subset by OX8 mAb treatment, had no effect on the survival time of a class I disparate graft, attention was turned to the contribution of the $CD4^+$ T cell subset to the rejection of a heterotopic heart graft with the same MHC antigen incompatibility.

Since the work of Loveland & McKenzie and Dallman & Mason, both of whom described graft rejection in irradiated animal models following the administration of $CD8^-$, but not $CD4^-$ T cells, the crucial role of the $CD4^+$ T cell in allograft rejection has been increasingly emphasised. In major reviews of the current literature both Mason & Morris (1986) and Hall (1991) stress the importance of T cell "help" for the activation and initiation of several mechanisms, both cellular and humoral, which may contribute to the rejection of various allografts, in both the experimental and clinical setting. This help comes in the form of lymphokines which are predominantly, but not exclusively, secreted by $CD4^+$ T cells (Gajewski, Scott, Schell et al, 1989). The lymphokines Il-2,3,4,5,6 & 10 are all produced, for the most part, by $CD4^+$ T cells in the human and the rat, although it has been shown that in some strains of mouse $CD8^+$ T cells produce substantial amounts of Il-2 (Sprent et al, 1986; Mizuochi et al, 1986), which may account for the apparent importance of the $CD8^+$ T cell in rejection experiments involving these particular strains.

Activated $CD8^+$ T cells can secrete both IFN-γ and TNF-β. However, the bulk of the evidence suggests that in most situations $CD8^+$ T cells are dependent upon $CD4^+$ T cells to become activated. Further subdivision of the $CD4^+$ T cell subset has been suggested on the basis of lymphokine production. Two types of

murine, $CD4^+$ T cell clones have been developed with different lymphokine signatures:

T_H1, which produces Il-2, TNF-β and IFN-γ but not Il-4,5 or 10, and

T_H2, which produces Il-4,5 and 10, but not Il-2, TNF-β or IFN-γ (Mossmann, Cherwinski, Bond et al, 1986).

It has been postulated that these different subgroups of T_H cells may provide help for different effector mechanisms. It has also been shown that each T_H subgroup has the ability to influence the growth and function of the other and, if the hypothesis above is correct, thereby influence the type of immune response which results (Fiorentino, Bond & Mossmann, 1989; Gajewski, Joyce & Fitch, 1989). This is, however, an area which requires further investigation and clarification.

In an attempt to assess the relevance of the $CD4^+$ T cell to the rejection of a class I allograft, *in vivo* depletion of $CD4^+$ cells by monoclonal antibodies has been employed.

5:2 Effect of anti-CD4 mAb treatment on graft survival.

As with the previous experiment looking at the role of $CD8^+$ T cells, the strain of the donor rat was PVG R8 while the recipient was the high responder PVG RT1u.

Heterotopic cardiac transplants were performed and the recipient rats were treated with the mixture of OX35 and OX38, both anti-CD4 monoclonal antibodies, as follows:

OX35 (3mg/ml)

1.0ml on the day of transplant(D.0)
0.5ml on D.1
0.5ml on D.3
0.5ml on D.6
0.5ml on D.9
0.5ml on D.12

OX38 (5mg/ml)

1.0ml on D.0
0.5ml on D.1
0.5ml on D.3
0.5ml on D.6
0.5ml on D.9
0.5ml on D.12

In contrast to the rats treated with anti-CD8 mAb, which rejected their grafts rapidly, there was a marked prolongation in the graft survival time following treatment with the anti-CD4 cocktail of OX35 and OX38. Of the 8 rats which received anti-CD4 mAb, 2 recipients rejected their grafts with only a few days delay at 12 and 14 days (5-7 days longer than unmodified rejection or in the anti-CD8 treated group), but the remaining 6 all had significantly prolonged graft survival times of 34, 57 & 73 days, and 3 rats which were sacrificed with functioning cardiac allografts 250 days following transplantation (Fig. 5.1.).

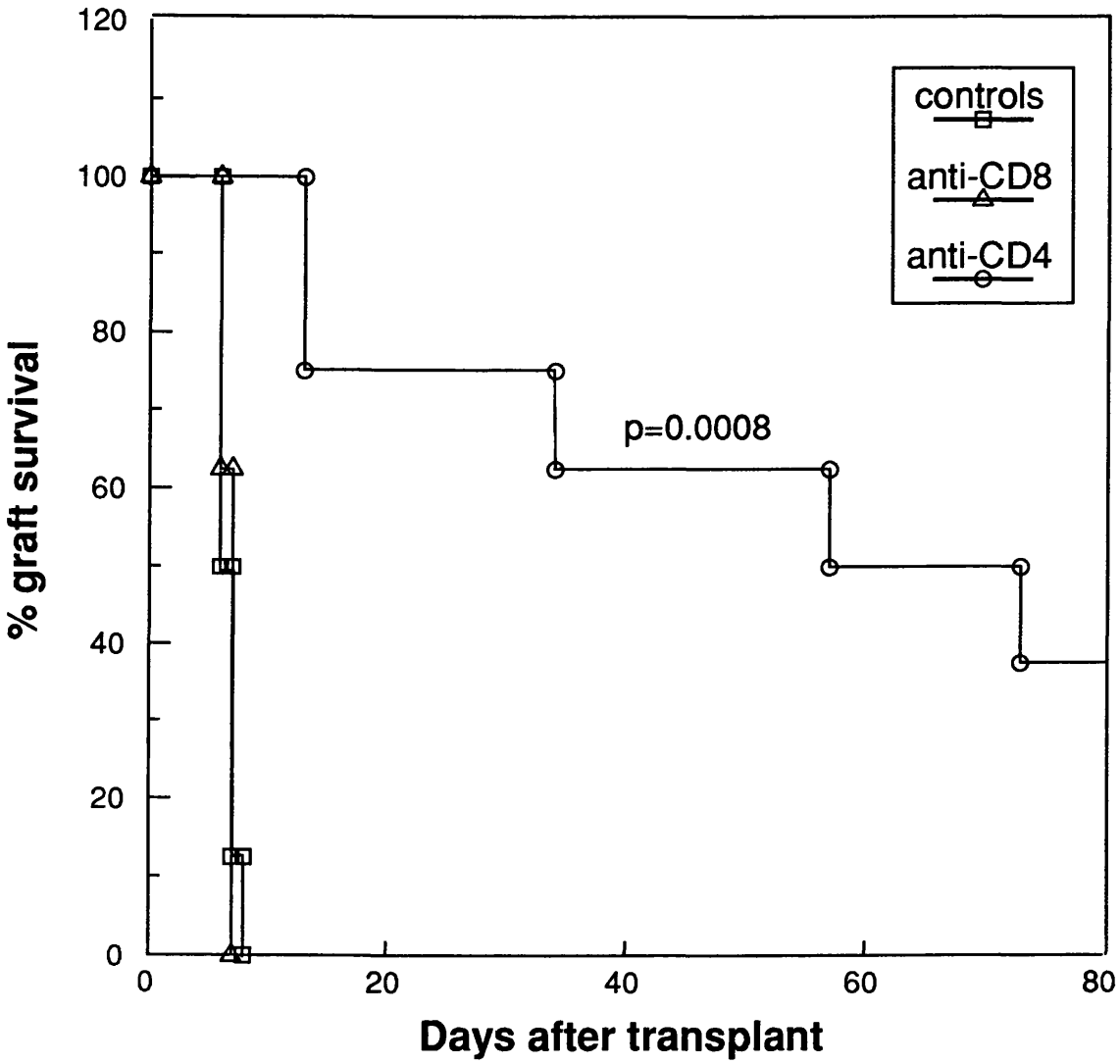
It is clear from these results, which have been consistently reproduced in subsequent experiments, that treatment with anti-CD4 monoclonal antibodies is an effective method of producing prolonged survival of a class I disparate graft in this "high responder" strain combination. This adds weight to the argument for a central role in allograft rejection for the $CD4^+$ T cell, but it does not, in itself, provide any direct evidence as to the effector mechanism, or mechanisms, which are mediating the destruction of these allografts, other than to suggest that these mechanisms are under the control of $CD4^+$ T cells. Equally, it does not exclude an

Table 3. Graft Survival Time (GST) in unmodified rejection; anti-CD8 treatment,and anti-CD4 treatment.

Strain combination	mAb	GST (days)	Mean GST (days)
R8→RT1u	-----	6,6,6,6,7,7,7,8	6.8
R8→RT1u	OX8	6,6,6,7,7,7,7,7	6.8
R8→RT1u	OX35/38	12,14,34,57,73,>200, >200,>200	98.7

Mean graft survival time of this class I disparate heart graft has been significantly prolonged by targeting CD4+ T cells with mAb (p=0.0008)...but not by targeting CD8+ cells

Figure 5.1 Ability of mAb treatment to prevent rejection of a class I disparate heart graft.



- anti-CD8 treatment resulted in the same GST as controls.

- anti-CD4 treatment significantly prolonged GST.
Three hearts in this group were still beating 200 days post transplant when the animals were sacrificed.

important role for $CD8^+$ cells although clearly they are unable to cause rejection of class I disparate grafts autonomously.

5:3 FACS analysis following anti-CD4 treatment.

To assess the effectiveness of the mixture of OX35 & OX38 in depleting $CD4^+$ T cells, lymph node cells (LNC) from treated rats were submitted to 2 colour FACS analysis, and the results are shown in Figure 5.2. As before, the results from the treated rats are compared with unmodified control animals.

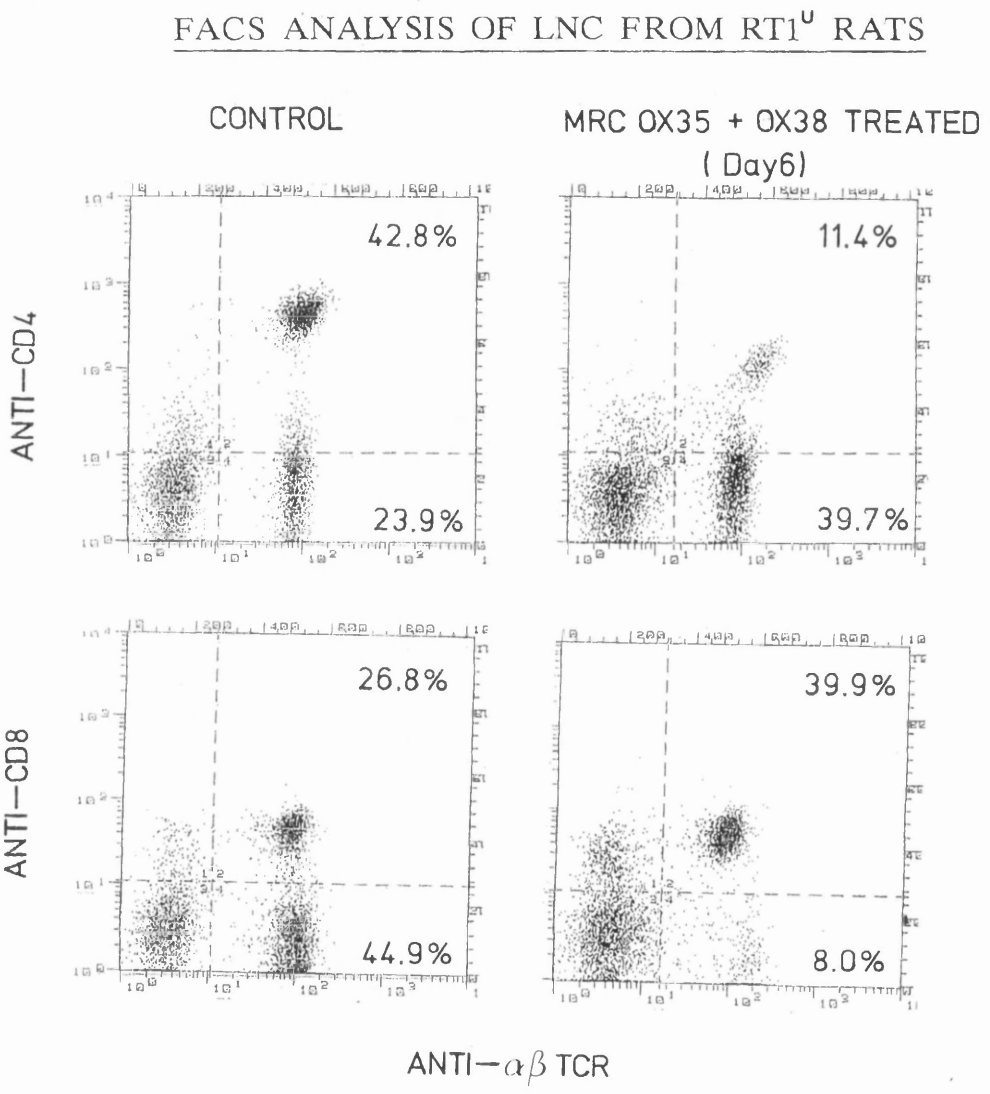
Knowing that treatment of these rats with this combination of monoclonal antibodies had been so effective in prolonging graft survival, it is perhaps surprising to find in the FACS analysis, that although there has certainly been a reduction in the number of $CD4^+$ cells following treatment with OX35 & OX38, the numerical depletion has been nothing like as complete as that of the $CD8^+$ cells by mAb OX8.

Of greater interest than simple numerical depletion, however, is the effect of the anti-CD4 mAb treatment on the functional capability of the $CD4^+$ T cell to provide help for the mediation of effector mechanisms of rejection.

5:4 Anti-CD4 treatment and alloantibody.

As it had already been demonstrated that this class I disparate graft was being rejected in the complete absence of $CD8^+$ CTLs, and that OX8 treatment had had no effect on the high levels of alloantibody which was generated, specifically, against the cardiac graft, it was of great interest to determine whether the prolonged graft survival obtained with anti-CD4 treatment was associated with any effect on the level of specific alloantibody produced by the recipient against its graft.

Figure 5.2 FACS analysis of lymph node cells from anti-CD4 treated animals and controls.



* Controls were treated with OX21

** OX35/38 mAb treatment has depleted, but not eliminated, the $CD4^+$ T cell population compared with the OX21 treated controls. This $CD4^+$ cell depletion is associated with significant prolongation of graft survival time.

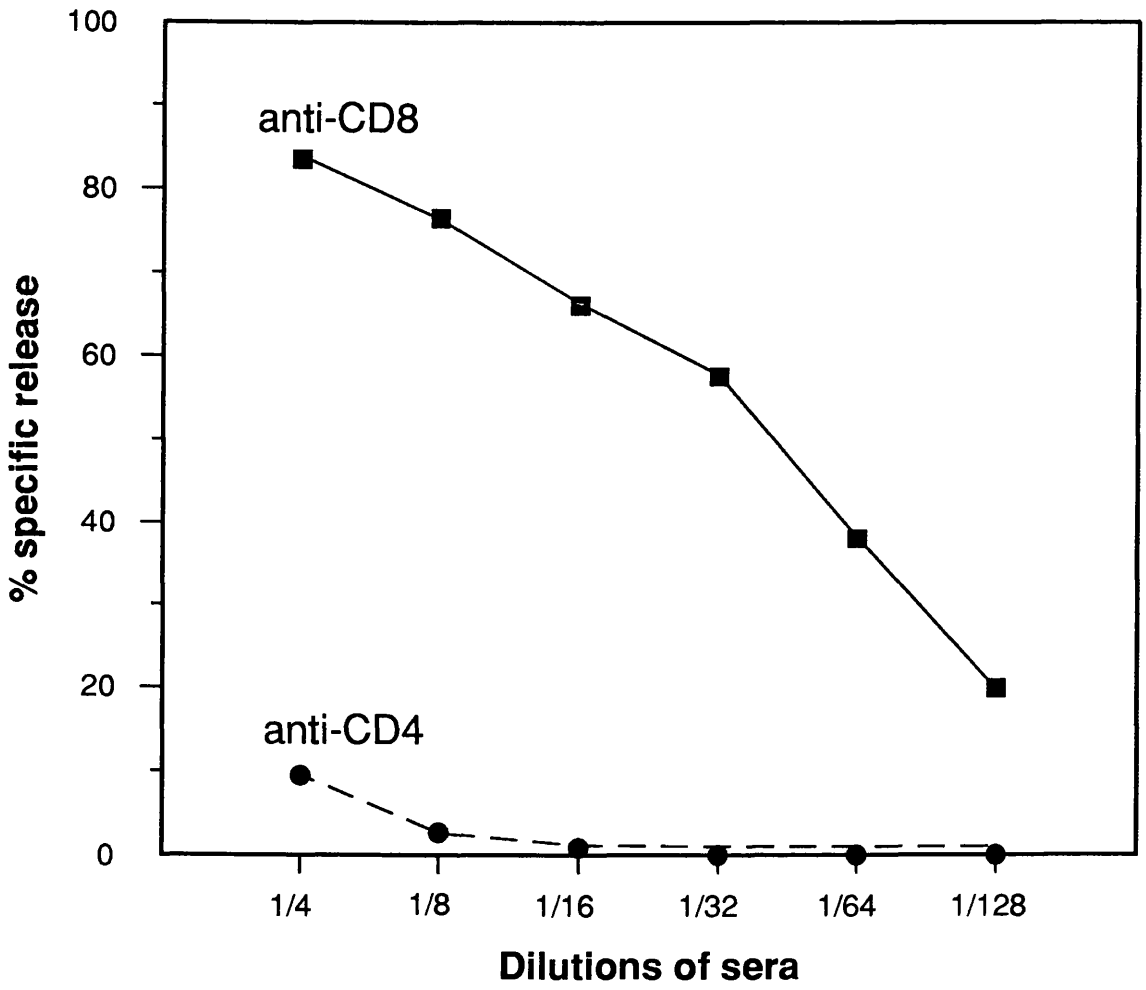
As was the case with both the unmodified rats and those treated with anti-CD8 mAb, the serum levels of specific cytotoxic alloantibody in graft recipient serum were measured by ^{51}Cr release from labelled lymphoblasts, in the presence of guinea pig complement. The results are shown in Figure 5.3.

The results shown are of day 7 sera, in all groups. There was a consistently high level of specific alloantibody production in the unmodified group of animals, all of whom rejected their grafts with first set kinetics. Similarly, there was a high level of alloantibody in the sera of those animals which had been treated with anti-CD8 mAb and had rejected their grafts in the proven absence of $CD8^+$ CTLs. In complete contrast, however, in those animals who had been treated with anti-CD4 mAb, and had not acutely rejected their grafts, it was found that the specific cytotoxic alloantibody response had been completely abrogated.

This interesting finding tends to suggest that specific alloantibody may well be acting as the crucial effector mechanism in the rejection of the class I disparate cardiac allograft in this rat model, as those animals in which prolonged graft survival was achieved were those in which the alloantibody production had been prevented.

The combination of the fact that anti-CD4 treatment resulted in prolonged graft survival, and that the alloantibody response was completely abrogated, suggest that the fact that $CD4^+$ cells were not completely depleted (as shown by FACS analysis) is not of great significance, and the likelihood is that the mAb treatment has functionally, if not numerically, depleted the $CD4^+$ T cell population.

Figure 5.3 The effect of in vivo monoclonal antibody treatment on cytotoxic alloantibody response (day 7) to a class I disparate graft.



* RT1u sera were assayed for specific cytotoxicity against 51Cr labelled R8 lymphoblasts in the presense of guinea pig complement.

**** Depletion of CD4+ cells by mAb has eliminated the specific cytotoxic alloantibody response against a class I disparate heart graft.**

5:5 Restoration of rejection by serum transfer.

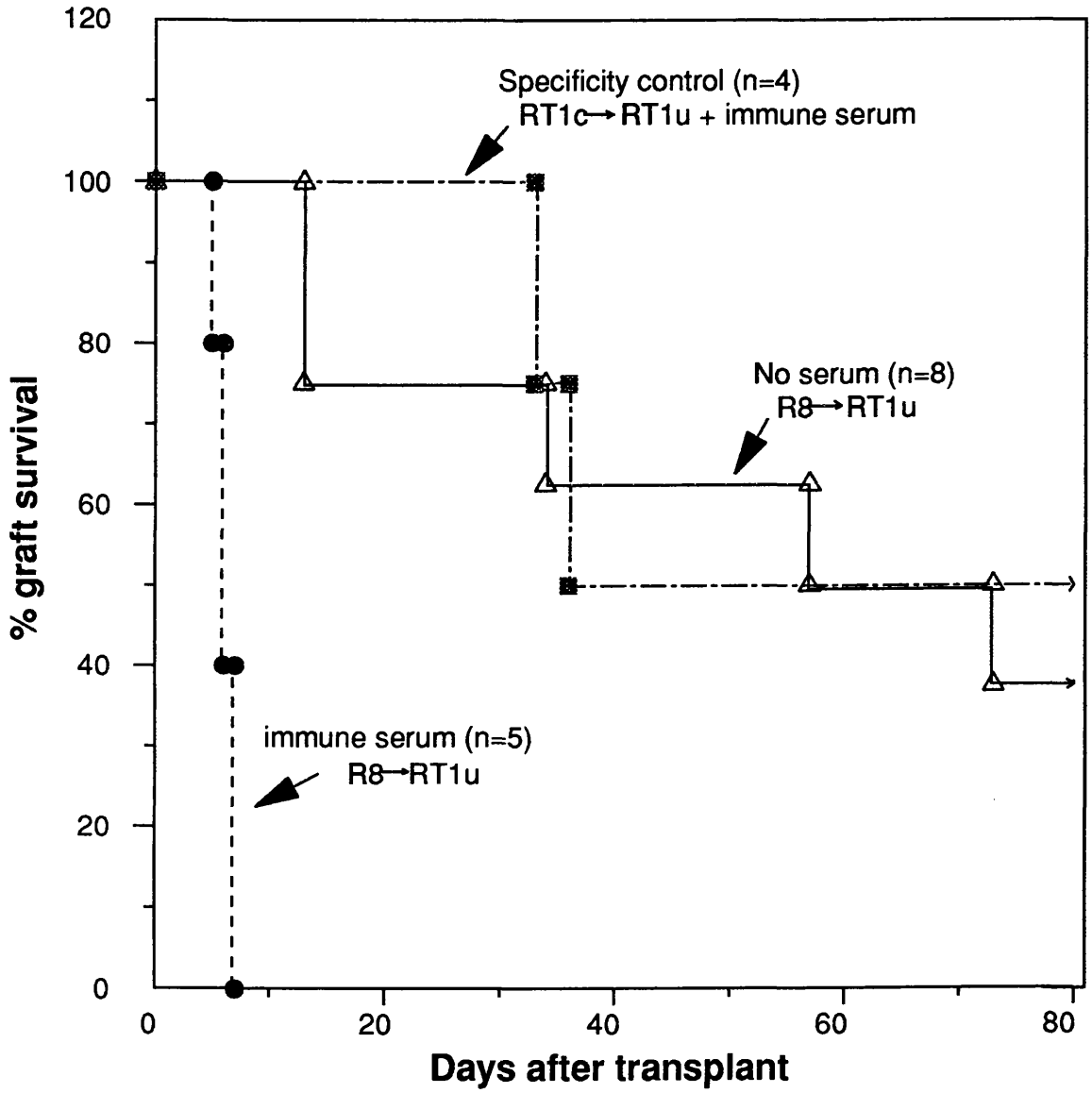
To further the hypothesis, that specific alloantibody effects the acute rejection of a cardiac allograft, it was decided to see whether it was possible to restore the ability to reject such a graft to an animal which would not normally do so, by the administration of immune serum.

To this end, a group of 9 RT1u rats were transplanted. Five of these received an R8 cardiac graft and the other 4 an RT1c heart. As before, they were all treated with the anti-CD4 mAbs OX35 & OX38 from the day of transplantation.

These animals were then given three intravenous injections of day 10 rejecting serum, on days 3,4 & 5 post-transplant. This time scale was chosen to mimic the appearance of specific alloantibody in the serum of an unmodified rejecting animal which occurs from day 3 onwards. The day 10 rejecting serum was obtained by pooling the sera from several RT1u rats which had rejected R8 hearts and were sacrificed on day 10 post-transplant. The serum was heat inactivated in a water bath at 56^{o}C for 30 minutes and then stored at minus 21^{o}C until required. The effect of the passive transfer of this immune serum is represented in Figure 5.4.

It was found that those animals which had received an RT1c heart, anti-CD4 treatment, and had then been reconstituted with the rejecting serum, all had significant prolongation of graft survival time (33,36,>175,>175 days), as had been seen with anti-CD4 treatment alone (12,14,34,57,73,>200,>200,>200). In striking contrast, however, those animals which had been given R8 hearts and treatment with anti-CD4 mAb and had then been reconstituted with day 10 rejecting serum (raised against an R8 graft), all rejected their allografts with first-set kinetics (5,6,6,7 & 7 days) [p=0.004, Mann-Whitney].

Figure 5.4 Ability of passively transferred immune serum to restore rejection in anti-CD4 treated recipients.



** Treatment with three intra-venous injections of immune serum on days 3,4 & 5 post-transplant completely restored the ability of a anti-CD4 treated animal to specifically reject an R8 heart graft.

*** p=0.004

The conclusion to be taken from these results is that passive transfer of immune serum is sufficient to restore rejection to an anti-CD4 mAb treated animal.

5:6 Conclusions from anti-CD4 mAb experiments.

In the previous chapter it was shown that this particular class I disparate allograft was being acutely rejected without any significant contribution from $CD8^+$ cytotoxic T cells. This work has been expanded to examine the role of the $CD4^+$ T cell and it has been shown that partial numerical depletion of these cells, by *in vivo* administration of OX35 & OX38 monoclonal antibodies, results in prolonged graft survival, and that this failure to reject the allograft correlated with a failure to produce the specific cytotoxic alloantibody response seen in unmodified, and in anti-CD8 treated, animals.

Shizuru et al published similar findings when they examined the effect of pre-transplant treatment with OX38 alone, on the survival of fully MHC disparate heart grafts in the rat. Following pre-treatment of the rat, which ended on the day of transplantation, all rats treated with OX38 had prolonged survival of their grafts for >175 days. In addition they reported that rats treated in this way failed to develop an antibody reaction against the OX38 mAb itself. They went on to give a second heart graft to those anti-CD4 treated animals which had not rejected their first grafts and found that these animals showed donor-specific unresponsiveness to the second graft, but not to a third party graft which was rejected rapidly. This effect even persisted when the second graft was given >90 days following removal of the first graft, during which time the recipient was without any alloantigenic stimulation, and they conclude that the anti-CD4 pre-treatment

depletes thymically processed, alloantigen specific, $CD4^+$ T cells and that new emerging thymic migrants are rendered unresponsive or anergic following encounter with the alloantigen (Shizuru, Seydel, Flavin et al, 1990).

Finally, it has been convincingly demonstrated that the passive transfer of immune serum can specifically restore rejection to an anti-CD4 treated animal.

The conclusion from the above is that it is the $CD4^+$ T cell which is crucial to the regulation of the rejection of this class I disparate cardiac allograft and that it is sufficient, with or without the presence of $CD8^+$ T cells, to mediate rejection in this model.

Furthermore, the results of this work suggest that the $CD4^+$ T cell is acting by providing cytokine "help" for the production of specific, cytotoxic alloantibody against the transplanted graft.

The question remains, however, whether this specific alloantibody is the true effector mechanism of the rejection response, presumably by mediating complement dependent cytotoxicity (CDC), or whether it simply produces its effect by interactions with cellular effector components by way of an antibody dependent cellular cytotoxicity reaction (ADCC). Earlier observations in this laboratory, that graft infiltrating cells in class I disparate kidney allografts, both rejecting and not rejecting, lacked any donor-specific cytotoxicity, but that the rejecting animal generated high levels of donor-specific alloantibody, stimulated the interest in alloantibody as a potential effector mechanism in <u>acute</u> allograft rejection (Gracie et al, 1990). In this more recent work with the cardiac graft model, it has been possible to demonstrate the ability of rejecting serum to cause lysis, *in vitro*, of cultured layers of donor strain, cardiac endothelial cells, by CDC as described in Chapter 3 (3:3). In this experiment the recipient rats own serum was the only source of complement. To extend this work to the *in vivo* animal

model it was decided to examine the effect of complement depletion of the recipient, on the ability to restore rejection to an anti-CD4 treated animal by passive transfer of heat-inactivated, day 10 rejecting serum. Without complement depletion such an animal will reject its graft acutely and if , as we are suggesting, the effector mechanism is CDC following activation by alloantibody, it might be expected that complement depletion would result in prolonged graft survival, in this model.

As a means of depleting serum complement in the graft recipients intramuscular injections of cobra venom factor (CoVF) were used, which results in a significant, but transient, reduction of complement activity through inactivation of C3, and subsequent complement components. Multiple injections of CoVF produce a prolonged depletion of C3, but after about 6 days of treatment the C3 inactivator complex is eliminated more rapidly than before, as a result of an immune response against the CoVF itself (Cochrane, Muller-Eberhard & Aikin, 1970). Treatment with CoVF has been shown to abrogate hyperacute rejection of cardiac allografts in rats (Whittum & Lindquist, 1977; Forbes, Pinto-Blonde & Guttmann, 1978), and Forbes et al also demonstrated that the usual morphological features of hyperacute rejection, namely, platelet aggregation, intravascular fibrin, endothelial destruction, neutrophil infiltration, and myocardial necrosis were absent in those animals whose complement was depleted prior to transplantation. They also found that rat C3 could not be detected bound to the allograft, although IgG was frequently detected, and they concluded that complement activation by graft-bound alloantibody was a critical effector mechanism of hyperacute rejection in that particular model.

There is little similar published work concerning acute allograft rejection, however, where the role of alloantibody is much less generally accepted.

5:7 Complement depletion by cobra venom factor.

Earlier in this thesis it has been shown that, in the strain combination of R8 to RT1u, an RT1u recipient of an R8 heart which has been treated with anti-CD4 mAbs, will not reject that heart graft. In addition, it has been demonstrated that passive transfer of day 10 rejecting serum to such animals completely restores their ability to reject such a graft, with similar kinetics to those of an unmodified, acute rejection.

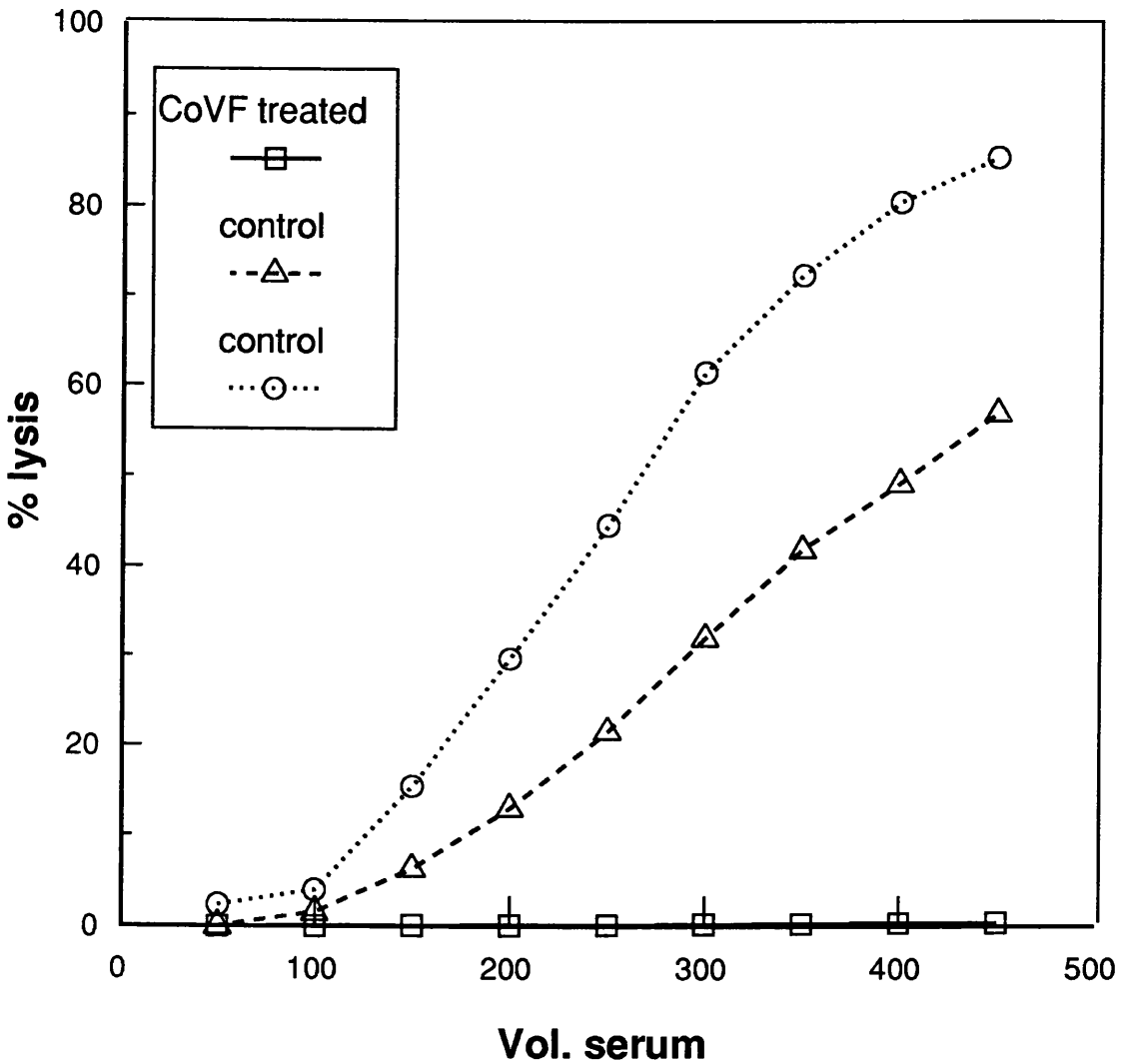
It was next decided to test the hypothesis that alloantibody was initiating the rejection response in these animals by CDC.

To this end, four RT1u recipients were pre-treated, on the day prior to transplantation, with a 0.2ml, intramuscular injection of cobra venom factor, and this was repeated every second day, up to and including day 10 post-operatively. Figure 5.5 shows the effect on serum complement levels in the RT1u rat of this CoVF regime.

On the day after the first dose of CoVF the RT1u rats were given an R8 heart graft and were started on the usual anti-CD4 treatment regime. Each rat was then given 3 consecutive, daily intravenous injections of 1ml of day 10 rejecting serum, and the hearts were monitored in the usual fashion, by daily palpation through the abdominal wall.

The finding was of a short, but statistically significant, increase in the graft survival time in the CoVF treated group ($p=0.017$). Following depletion of serum complement, the mean graft survival time in those animals reconstituted with rejecting serum was 11.2 days (11,11,11,12), compared with a mean graft survival time of 6.2 days (5,6,6,7,7) in those animals with an intact complement system (Figure 5.6). This result suggests that the detrimental effect of the passively

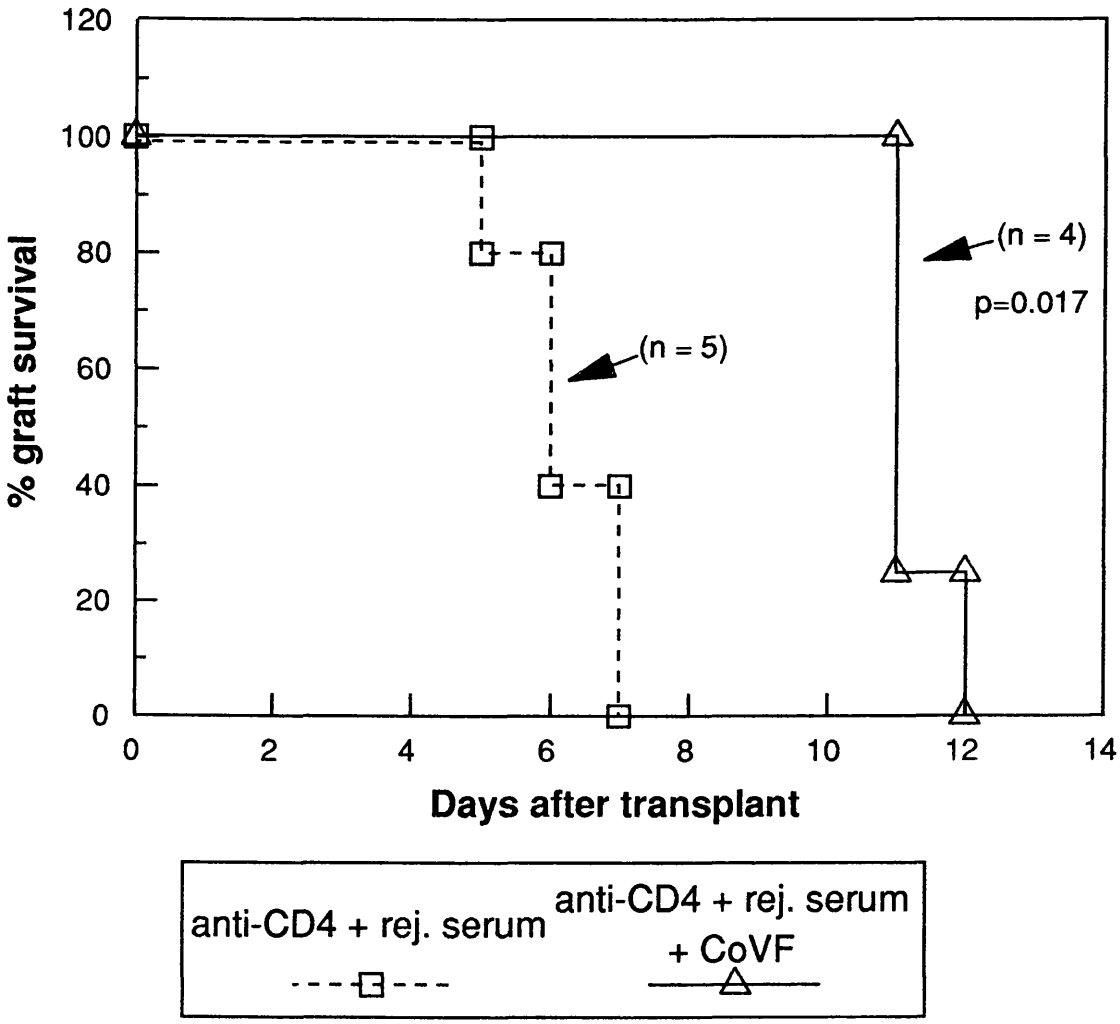
Figure 5.5 Complement depletion by Cobra Venom Factor (CoVF).



* The ability of CoVF to deplete serum complement was assessed.

**** Treated animals were given 0.2ml CoVF by intramuscular injection on the day before analysis. This resulted in the complete erradication of the complement dependent cytotoxicity of the control serum.**

Figure 5.6 Effect of complement depletion by Cobra Venom Factor on the ability of passive transfer of rejecting serum to restore rejection to anti-CD4 treated animals



- rejecting serum was given by intravenous injection on days 3,4 & 5 post-transplant.

- CoVF was given by intramuscular injection on day -1 and every 48 hours thereafter.

transferred rejecting serum is, indeed, as a result of alloantibody mediated CDC, as it is prevented, albeit temporarily, by the depletion of serum complement. A likely explanation for the fact that rejection is restored around day 11 or 12 is that administration of CoVF becomes ineffective in depleting serum complement as it is known that an immune response is mounted against the CoVF-C3 complex after about 6 days of administration and it becomes progressively more difficult to maintain complement depletion with repeated injections of CoVF (Cochrane et al, 1970). It would be of interest, to complete the picture, to know what the effect of treating with anti-CD4 mAb and CoVF would be, without the addition of the rejecting serum. This experiment has not been performed.

CHAPTER 6

General Summary, Conclusions and Discussion.

6:1 The need for further study of allograft rejection.

The aim of this thesis has been to examine the cellular and humoral events taking place, *in vivo*, in the rejection of a cardiac allograft in the rat.

The work has been undertaken with a view to increasing our understanding of the complex interactions at play in acute allograft rejection, which remains the greatest, and most commonly encountered challenge in clinical transplantation. The "pot of gold" at the end of this particular rainbow is the development of a highly specific, and hopefully much less toxic, form of immunosuppression as a result of targeting those effector mechanisms which we know to be crucial in any one rejection response.

Over recent years, research into the mechanisms of allograft rejection has resulted in the introduction of increasingly effective treatment regimens for the prevention of organ graft rejection, such as cyclosporin, FK506 and OKT3, but it remains the case that all of the current forms of immunosuppression are associated with significant, and in some cases life-threatening, side-effects. Given the chronic shortage of all types of human organs for transplantation, and the ever-spiralling cost of long-term hospital and community support for patients with end-stage organ failure, of whatever type (Green, 1988), it is imperative that those organs which are made available for transplant should have the best possible chance of long-term survival in their recipient.

This clinical end-point validates the continuing experimental search to elucidate the cause of acute allograft rejection.

As has been explained earlier in this thesis, the fact that all clinical transplant recipients are receiving immunosuppression to prevent the rejection of their grafts makes it impossible to study the mechanisms of unmodified allograft

rejection in the human model. Fortunately, there are considerable similarities in the immune response to an allograft in different species and it is therefore in animal models of acute rejection that the bulk of current research in this field is taking place.

As a result of previous work in this laboratory (Gracie et al, 1990), which challenged the widely held concept of a crucial role for the $CD8^+$ CTL in the rejection of an isolated class I MHC disparate graft (Lowry et al, 1985), and which suggested a possible role for alloantibody in the rejection of a class I disparate renal allograft, it was decided to investigate the effector mechanisms in the rejection of an isolated class I MHC disparate, heterotopic, cardiac allograft in the rat.

6:2 Allorecognition.

The MHC restrictions of $CD4^+$ and $CD8^+$ T cells, which recognise class II and class I MHC respectively, are generally accepted.

As such, it was surprising to find, in both renal and cardiac transplantation between rat strains differing only at the classical class I locus, that the graft was being rejected in the complete absence of $CD8^+$ CTLs, as conventional thinking on antigen presentation and recognition would have it that the foreign, or non-self, class I MHC could only be detected by $CD8^+$ T cells. One would, therefore, have expected that removal of these effector cells would result in the long-term survival of such a graft. That this was not the case, in this experimental model, suggests one of two possibilities:

1) The depletion of $CD8^+$ T cells, by the mAb OX8, has not been complete, and there are some $CD8^+$ cells remaining which are able to recognise the class I disparity of the transplanted allograft, and to subsequently mount a response against it.

This work has shown, by the combination of immunohistology, two-colour FACS analysis and *in vitro* cytotoxicity assays, that treatment with MRC OX8, in this rat model, has resulted in complete elimination, both phenotypically and functionally, of $CD8^+$ T cells and it can be confidently stated that these class I disparate grafts are being rejected in the absence of $CD8^+$ cells.

It has recently been suggested that the mAb treatment, itself, activates a unique population of $CD8^+$, OX8-resistant CTL precursors which, with the assistance of T_H cells and in the presence of some non-MHC, minor antigen mismatch, are responsible for the rejection of a class I disparate skin graft in mice (Rosenberg et al, 1991). In the model of rat heart allograft used in this thesis however, no such cells were found and no CTL activity could be demonstrated, but it is true to say that the donor and recipients were fully matched for minor antigens, which Rosenberg implies would prevent the activation of the precursor cells by the anti-CD8 mAb. This is an area which requires closer inspection before any valid conclusions can be drawn.

2) $CD8^+$ T cells are not required for the recognition and rejection of a class I disparate graft.

If this hypothesis is to be believed, some alternative to the accepted mechanisms of MHC recognition and allograft rejection must be proposed.

Conventional thinking regarding allorecognition believes that the direct recognition of foreign MHC antigens by the TCR results in the activation and proliferation of T lymphocytes, as seen in the mixed lymphocyte reaction, *in vitro* (Bach & Hirschorn, 1964).

As was mentioned briefly in chapter 4, there is increasing evidence to support the interesting concept of an "indirect pathway" of alloantigen recognition by T cells (Rock et al, 1983; Golding & Singer, 1984; Cheng et al, 1990; Lechler et al, 1990). By this mechanism it is suggested that MHC class I antigens can be processed by antigen presenting cells and that peptide fragments derived from the allogeneic MHC may then be presented to $CD4^+$ T cells in the protein presentation groove of self, MHC class II molecules. It has recently been convincingly demonstrated by Dalchau and co-workers that the injection of purified peptide fragments of MHC molecules, derived from a DA rat, into an allogeneic Lewis recipient results in the production of alloantibodies against the peptide fragments (but not the intact MHC), suggesting that indirect recognition had occurred. When these immunised Lewis rats were subsequently given a DA skin graft it was rejected in accelerated fashion. The authors conclude that indirect allorecognition can play an important role in the effector mechanisms of allograft rejection especially in the priming of T_H cells (Dalchau, Fangmann & Fabre, 1992; Fangmann, Dalchau & Fabre, 1992).

This alternative theory of antigen presentation and recognition is appealing as an explanation of how a class I disparate graft, such as has been studied in this thesis, can be recognised as foreign by a recipient which is completely devoid of $CD8^+$ T cells.

It is our contention that MHC class I molecules are shed from the graft in large quantities following transplantation, and that this antigenic material is then

processed, by APCs centrally in the graft recipient, and is then presented as peptide fragments, to the recipient's $CD4^+$ T cells in the groove of self, class II MHC molecules. The result of this process will be the activation of the $CD4^+$ T cells and, by the release of various cytokines, the initiation of the effector mechanisms leading to the specific rejection of the graft.

Alternatively, it has also been proposed that the activation of the $CD4^+$ T cells may occur in the graft itself, following recognition of MHC disparities as presented by APCs of donor origin. The initial argument against such a possibility, in our class I disparate, $CD8^+$ T cell depleted model, was the belief that class II MHC molecules always presented peptides derived from the degradation of exogenously derived proteins, and would therefore, in this model, be identical to recipient class II MHC, and would not be perceived as foreign by the recipient $CD4^+$ T cells.

It has now been demonstrated, however, that peptides derived from **endogenously** synthesized proteins may be presented by class II MHC molecules (Jin, Shih, Berkower, 1988; Jacobson, Sekaly, Jacobson et al, 1989), and, moreover, that endogenous peptide derived from self class I MHC may be presented in the peptide binding groove of self class II MHC molecules (Chen et al, 1990).

It is conceivable, therefore, that $CD4^+$ T cells could be activated locally, within the graft, by recognition of donor class I MHC peptide fragments being presented in the groove of donor class II MHC molecules, on the surface of donor APCs.

The more conventional theory of central activation, in draining lymph nodes or recipient spleen, following antigen processing and presentation by recipient APCs remains, in my view, the more plausible.

Whether the $CD4^+$ T cell activation takes place in the recipient lymphoid system or within the graft itself, the concept of an indirect pathway of antigen recognition, as outlined above, could account for the finding of the recognition of a class I MHC disparate allograft as foreign by a recipient devoid of $CD8^+$ T cells. It does not, however, give us any information as to the effector mechanisms which bring about the subsequent destruction of the allograft, other than that they are initiated by the influence of activated $CD4^+$ T cells, and that $CD8^+$ T cells are not required.

6:3 Effector mechanisms of acute allograft rejection.

Whatever the final pathway of acute allograft rejection might be, it is widely recognised that the $CD4^+$ T cell plays a crucial role in recruiting, and directing the different arms of the immune response against an allograft (Steinmuller, 1985; Mason & Morris, 1986; Hall, 1991). This control is mediated by the release of various lymphokines by the $CD4^+$ T cell. The number and nature of these molecules, and their effects on cellular and humoral components of the immune response, are currently the subject of intense research, in the field of prevention of allograft rejection, as new techniques for their identification and assessment, such as polymerase chain reaction (PCR) and in situ hybridization, are developed.

Other than to acknowledge the importance of these molecules as a product of activated T cells, no further attempt to explore their role in allograft rejection has been made in this thesis, which has concentrated on the effector mechanisms which bring about the destruction of a rejected graft, albeit as a result of cytokine release by activated $CD4^+$ T cells.

As has already been stated, the demonstration of the rejection of a class I MHC disparate graft in an animal devoid of $CD8^+$ cells has called into question the belief that the $CD8^+$ CTL was essential in this system (Lowry et al, 1985).

We have consistently shown that such a graft can be acutely rejected in the complete absence of $CD8^+$ T cells, following *in vivo* depletion of such cells by mouse anti-rat monoclonal antibodies.

Subsequently, it has been demonstrated that similar depletion of $CD4^+$ T cells, by *in vivo* administration of anti-CD4 monoclonal antibodies, resulted in the long-term survival of a class I disparate graft, despite the continuing presence of $CD8^+$ T cells in the graft recipients.

The conclusion from this work is that the $CD4^+$ T cell is, indeed, crucial in the initiation of the acute rejection response, in this model, but the $CD8^+$ T cell is not required.

It is important, however, not to completely disregard the $CD8^+$ T cell, as it was also found that when the allograft recipient is treated with both anti-CD4 and anti-CD8 mAbs, the heart grafts were rejected with first-set kinetics, in the absence of an alloantibody response. This finding may suggest that $CD8^+$ cells may have a suppressor effect on those $CD4^+$ cells which remain following depletion with OX35/OX38 mAb treatment, and that removal of these cells by OX8 mAb therapy allows the remaining $CD4^+$ T cells to initiate an effective cellular rejection response.

What, then, is the effector mechanism of the rejection response which results in the destruction of a class I disparate heart graft, following the depletion of $CD8^+$ T cells, and which is abrogated by the depletion of $CD4^+$ T cells by anti-CD4 mAb treatment?

The various types of immune responses which may, individually or collectively, be implicated in allograft rejection were summarised in Chapter 1 of this thesis (1:9.3).

Influenced by previous work in this laboratory (Gracie et al, 1990), which demonstrated a strong, specific alloantibody response against a class I disparate renal allograft, the concept of alloantibody as an effector mechanism of acute allograft rejection has been pursued further.

As with the renal allograft model (and using the same rat strain combinations), we found that both unmodified heart graft rejection, and rejection following $CD8^+$ cell depletion, occurred in the presence of a pronounced, and specific, alloantibody response. In the subsequent experiments involving depletion of $CD4^+$ T cells, with resulting long-term graft survival, the specific alloantibody response had been prevented. It, therefore, seemed an attractive prospect that the alloantibody had been the effector mechanism responsible for the rejection of the grafts in the anti-CD8 treated animals. Were this to be the case, the passive transfer of specific, hyperimmune serum, to a graft recipient which has been treated with anti-CD4 mAb, should restore that animals' ability to reject its class I disparate graft. This was, indeed, the finding of the experiment detailed in Chapter 5 (5:5), where passive transfer of hyperimmune serum, given intravenously on days 3,4 & 5 post-transplant, resulted in the restoration of first-set graft rejection to a $CD4^+$ T cell, depleted group of rats.

It has previously been shown that a rat cardiac allograft can be acutely rejected in the absence of an alloantibody response (Lowry & Forbes, 1984), and this is in keeping with our finding of graft rejection in those animals which had been treated with both anti-CD4 and anti-CD8 mAbs and which had no demonstrable alloantibody present at the time of rejection, but it cannot be

concluded from this that alloantibody is incapable of producing rejection in different circumstances. The fact that administration of hyperimmune serum is able to restore rejection to an animal which will otherwise not reject a heart graft, and which has had its alloantibody production prevented, is strong evidence to suggest that specific alloantibody has the ability to act as an effector of acute rejection, in this particular model of a cardiac allograft with a class I MHC antigen disparity.

6:4 Mechanism of tissue destruction.

The ideal form of effective immunosuppression should intervene in the immune response to prevent any form of tissue damage from occurring in the graft.

In order to establish whether a cellular or a humoral phenomenon is a potential effector mechanism of rejection, it is essential to demonstrate its' capacity to cause actual cellular damage to cell types found within the graft. The various types of known immunological injury were summarised in Chapter 1 of this work, and include: cytotoxic T cells; delayed-type hypersensitivity reaction; antibody dependent, cellular cytotoxicity & antibody mediated, complement dependent cytotoxicity.

The conclusion of this thesis, that alloantibody **is** capable of acting as the effector mechanism in the rejection of a class I disparate cardiac allograft, is strengthened by the demonstration of the ability of immune serum to specifically lyse neonatal cardiac endothelial cells, of donor origin, cultured *in vitro*. This cell destruction can only be as a result of complement dependent cytotoxicity. In addition, it has been shown here that depletion of serum complement *in vivo*, by intramuscular injections of cobra venom factor, is capable of delaying the

rejection of a graft by a $CD4^+$ T cell depleted animal which has been reconstituted with day 10 rejecting serum, supporting the argument that alloantibody is causing rejection by mediating a complement-dependent cytotoxic reaction.

6:5 Conclusion

It is not my contention that alloantibody, alone, should be put forward as the major effector mechanism in acute allograft rejection, but rather that it should be recognised as a potentially important effector in this type of graft rejection. This has not been the case up until now, as it has generally been accepted that the importance of alloantibody lies in the, relatively uncommon, clinical situation of hyperacute rejection, where pre-existing alloantibody causes the immediate rejection of a vascularised allograft.

If specific antibody, produced as a result of exposure to foreign MHC antigens, following allograft transplantation, is capable of causing the acute rejection of that graft then it presents another possible target for specific immunosuppression therapies, with potential therapeutic applications.

Whilst it is true that the results described in this thesis were produced in a very highly controlled, and artificial environment, it is becoming ever-more apparent that the rejection of an allograft is a multi-factorial phenomenon, involving many potential effector mechanisms with the ability to adapt their response to deal with whatever challenge they are presented with.

The $CD4^+$ T cell is increasingly seen as the linch-pin upon which the entire process of graft rejection depends.

REFERENCES

Adams, D.H., Wang, L., Hubscher, S.G. & Neuberger, J.M. (1989) Hepatic endothelial cells: Targets in liver allograft rejection? Transplantation, **47**: 479-482.

Aherne, W.A. & Dunnill, M.S. (1982) In Morphology; **33**; London; Arnold.

Austyn, J.M. & Larsen, C.P. (1990) Migration patterns of dendritic leukocytes. Implications for transplantation. Transplantation, **49**: 1-7.

Bach, F.H. & Hirschorn, K. (1964) Lymphocyte interaction: a potential histocombatibility test in vitro. Science, **143**: 813-814.

Bach, F.H., Bach, M.L. & Sondel, P.M. (1976) Differential function of major histocompatibility complex antigens in T-lymphocyte activation. Nature, **259**: 273-281.

Baldwin, W.M., Westedt, M.L., van Gemert, G.W., Henny, F.C., Paul, L.C., Daha, M.R. & van Es, L.A. (1986) Association of rheumatoid factors in renal transplant recipients with cytomegalovirus and not with rejection. Transplantation, **43**: 658-662.

Baldwin, W.M., Pruitt, S.K. & Sanfilippo, F. (1991) Alloantibodies: Basic and clinical concepts. Transplantation Reviews, **5**: 100-119.

Baldwin, W.M., Rhoton, K. & Sanfilippo, F. (1991) IgM and IgG alloantibody production by splenocytes and deposition in rat renal allografts are decreased by donor-specific blood transfusion. Transplantation, **51**: 481-485.

Bank, I. & Chess, L. (1985) Perturbation of the T4 molecule transmits a negative signal to T cells. Journal of Experimental Medicine, **162**: 1294-1303.

Barclay, A.N. (1981) Different reticular elements in rat lymphoid tissue identified by localization of Ia, Thy-1 and MRC OX-2antigens. Immunology, **44**: 727-736.

Barger, B., Shroyer, T.W., Hudson, S.L., Deierhoi, M.H., Barber, W.H., Curtis, J.J., Julian, B.A., Luke, R.G. & Diethelm, A.G. (1989) Sucessful renal allografts in recipients with crossmatch-positive, dithioerythritol-treated negative sera. Race, transplant history, and HLA-DR1 phenotype. Transplantation, **47**: 240-245.

Barker, C.F. & Billingham, R.E. (1968) The role of afferent lymphatics in the rejection of skin homografts. Journal of Experimental Medicine, **128**: 197-221.

Barnard, C.N. (1967) The Operation. A human cardiac transplant: an interim report of a sucessful operation performed at Groote Schuur Hospital, Cape Town. South African Medical Journal, **41**: 1271-1274.

Basham, T., Smith, W., Larnier, L., Morhenn, V. & Merigan, T. (1984) Regulation of expression of class II major histocompatibility antigens on human peripheral blood monocytes and Langerhans cells by interferon. Human Immunology, **10**: 83-93.

Biddison, W.E., Rao, P.E., Talle, M.A., Goldstein, G. & Shaw, S. (1982) Possible involvement of the T4 molecule in T cell recognition of class II HLA antigens: evidence from studies of cytotoxic T lymphocytes specific for SB antigens. Journal of Experimental Medicine, **156**: 1065-1076.

Biddison, W.E. & Shaw, S. (1989) CD4 expression and function in HLA class II-specific T cells. Immunological Reviews, **109**: 5-15.

Billingham, R.E., Brent, L. & Medawar, P.B. (1956) Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. Philosophical Transactions of the Royal Society, **239**: 357-414.

Bishop, G.A., Waugh, J.A., Landers, D.V., Krensky, A.M. & Hall, B.M. (1989) Microvascular destruction in renal transplant rejection. Transplantation, **48**: 408-414.

Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. & Wiley, D.C. (1987) Structure of the human class I histocompatibility antigen, HLA-A2. Nature, **329**: 506-512.

Blankenhorn, E.P., Cecka, J.M., Frelinger, J., Gotze, D. & Hood, L. (1980) Structure of Ia antigens from the rat. Mouse alloantisera demonstrate at least two distinct molecular species. European Journal of Immunology, **10**: 145-151.

Blankenhorn, E.P., Symington, F.W. & Cramer, D.V. (1983) Biochemical characterisation of Ia antigens encoded by the RT1.B and RT1.D loci in the rat MHC. Immunogenetics, **17**: 475-484.

Bolton, E.M., Gracie, J.A., Briggs, J.D., Kampinga, J. & Bradley, J.A. (1989) Cellular requirments for renal allograft rejection in the athymic nude rat. Journal of Experimental Medicine, **169**: 1931-1946.

Borel, J.F., Feurer, C., Magnee, C. & Stahelin, H. (1977) Immunology, **32**: 1017-1025.

Bradley, J.A., Mason, D.W. & Morris, P.J. (1985) Evidence that rat renal allografts are rejected by cytotoxic T cells and not by nonspecific effectors. Transplantation, **39**: 169-175.

Brent, L., Brown, J. & Medawar, P.B. (1958) Skin transplantation immunity in relation to hypersensitivity. Lancet, **2**: 561-564.

Brunner, K.T., Mauel, J., Cerottini, J-C. & Chapius, B. (1968) Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogenic target cells *in vitro*: inhibition by isoantibody and by drugs. Immunology, **14**: 181-196.

Burdich, J.F. & Clow, L.W. (1986) Rejection of murine cardiac allografts. I. Relative roles of major and minor antigens. Transplantation, **42**: 67-72.

Busch, G.J., Reynolds, E.S., Galvanek, E.G., Braun, W.E. & Dammin, G.J. (1971) Human renal allografts. The role of vascular injury in early graft failure. Medicine, **50**: 29-83.

Butcher, G.W. & Howard, J.C. (1979) r7 and r8: two new recombinant RT1 haplotypes. Rat News Letters, **5**: 13.

Calne, R.Y., Alexander, G.P.J. & Murray, J.E. (1962) A study of the effects of drugs in prolonging survival of homologous renal transplants in dogs. Annals of the New York Academy of Sciences, **99**: 743-761.

Calne, R.Y., White, D.J.G., Thiru, S. et al (1978) Cyclosporin A in patients receiving renal allografts from cadaver donors. Lancet, **ii**: 1323-1327.

Calne, R.Y., White, D.J.G., Evans, D.B. et al (1981) Cyclosporin A in cadaveric organ transplantation. British Medical Journal, **282**: 934-936.

Cantrell, D.A., Robins, R.A. Brooks, C.G. & Baldwin, R.W. (1982) Phenotype of rat natural killer cells defined by monoclonal antibodies marking rat lymphocyte subsets. Immunology, **45**: 97-103.

Chassoux, D.M., Linares-Cruz, L.G., Bazin, H. & Stanislawski, M. (1988) K-cell mediated cytotoxicity induced with rat monoclonal antibodies: I. Antibodies of various isotypes differ in their ability to induce cytotoxicity mediated by rat and human effectors. Immunology, **65**: 623-628.

Chen, B.P., Madrigal, A. & Parham, P. (1990) Cytotoxic T cell recognition of an endogenous class I HLA peptide presented by a class II HLA molecule. Journal of Experimental Medicine, **172**: 779-788.

Cochrane, C.G., Muller-Eberhard, H.J. & Aikin, B.S. (1970) Depletion of plasma complement *in vivo* by a protein of cobra venom: its effect on various immunologic reactions. Journal of Immmunology,**105**: 55-69.

Corry, R.J., Winn, H.J. & Russell, P.S. (1973) Heart transplantation in congenic strains of mice. Transplantation Proceedings, **5**: 733-735.

Daar, A.S., Fuggle, S.V., Fabre, J.W., Ting, A. & Morris, P.J. (1984) The detailed distribution of MHC class II antigens in normal organs. Transplantation, **38**: 293-298.

Dalchau, R., Fangmann, J. & Fabre, J.W. (1992) Allorecognition of isolated, denatured chains of class I and class II major histocompatibility complex molecules. Evidence for an important role for indirect allorecognition in transplantation. European Journal of Immunology, **22**: 669-677.

Dallman, M.J. & Mason, D.W. (1982) Role of thymus-derived and thymus-independent cells in murine skin allograft rejection. Transplantation, **33**: 221-223.

Dallman, M.J., Mason, D.W. & Webb, M. (1982) The roles of host and donor cells in the rejection of skin allografts by T cell-deprived rats injected with syngeneic T cells. European Journal of Immunology, **12**: 511-518.

Dallman, M.J. & Mason, D.W. (1983) Induction of Ia antigens on murine epidermal cells during the rejection of skin allografts. Transplantation, **36**: 222-224.

Dausset, J., (1958) Iso-leuco-anticorps (Iso-leuko-antibodies). Acta Haematologica, **20**: 156-166.

de Waal, R.M.W., Bogman, M.J.J., Maass, C.N., Cornelissen, L.M.H., Tax, W.J.M. & Koene, R.A.P. (1983) Variable expression of Ia antigens on the vascular endothelium of mouse skin allografts. Nature, **303**: 426-429.

Dijkstra, C.D., Dopp, E.A., Joling, P. & Kraal, G. (1985) The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognised by monoclonal antibodies ED1, ED2 and ED3. Immunology, **54**: 589-599.

Doyle, C. & Strominger, J.L. (1987) Interactions between CD4 and class II MHC mediates cell adhesion. Nature, **330**: 256-259.

Dreyfuss, M., Harri, E., Hotmann, H., Kobel, H., Pache, W. & Tischerter, H. (1976) European Journal of Applied Microbiology, **3**: 125-133.

Fabre, J.W. & Morris, P.J. (1972) Experience with passive enhancement of renal allografts in a (DA x Lewis) F1 to Lewis strain combination. Transplantation, **13**: 604-609.

Fabre, J.W. & Morris, P.J. (1974) Passive enhancement of homozygous renal allografts in the rat. Transplantation, **18**: 429-435.

Fangmann, J., Dalchau, R. & Fabre, J.W. (1992) Rejection of skin allografts by indirect allorecognition of donor class I major histocompatibility complex peptides. Journal of Experimental Medicine, **175**: 1521-1529.

Fellous, M., Nir, U., Wallach, D., Merlin, G., Rubinstein, M. & Revel, M. (1982) Interferon-dependent induction of mRNA for the major histocompatibility antigens in human fibroblasts and lymphoblastoid cells. Proceedings of the National Academy of Sciences of the U.S.A., **79**: 3082-3086.

Ferry, B., Halttunen, J., Leszczynski, D., Schellekens, H., van der Meide, P.H. & Hayry, P. (1987) Impact of class II major histocompatibility complex antigen expression on the immunogenic potential of isolated rat vascular endothelial cells. Transplantation, **44**: 499-503.

Fiorentino, D.F., Bond, M.W. & Mossmann, T.R. (1989) Two types of mouse T helper cell: IV. T_h2 secrete a factor that inhibits cytokine production by T_h1 clones. Journal of Experimental Medicine, **170**: 2081-2095.

Forbes, R.D.C., Pinto-Blonde, M. & Guttmann, R.D. (1978) The effect of anticomplementary cobra venom factor on hyperacute rat cardiac allograft rejection. Laboratory Investigation, **39**: 463-470.

Forbes, R.D.C., Guttmann, R.D., Gomersall, M. & Hibbred, J. (1983) A controlled serial ultrastructural study of first-set cardiac allograft rejection in the rat: Evidence that the microvascular endothelium is the primary target of graft destruction. American Journal of Pathology, **111**: 184-196.

French, M.E. & Batchelor, J.R. (1969) Immunological enhancement of rat kidney grafts. Lancet, **2:** 1103-1106.

French, M.E. (1972) The early effects of alloantibody and complement on rat kidney allografts. Transplantation, **13**: 447-451.

Fukumoto, T., McMaster, W.R. & Williams, A.F. (1982) Mouse monoclonal antibodies against rat major histocompatibility antigens: two Ia antigens and expression of Ia and class I antigens in the rat thymus. European Journal of Immunology, **12**: 237-243.

Gajewski, T.F., Joyce, J. & Fitch, F.W. (1989) Antiproliferative effect of IFN-γ in immune regulation. III. Differential selection of T_H1 and T_H2 murine helper T lymphocyte clones using recombinant Il-2 and recombinant IFN-γ. Journal of Immunology, **143**: 15-22.

Gajewski, T.F., Scott, R., Schell, S.R., Nau, G. & Fitch, F.W. (1989) Regulation of T cell activation: differences among T cell subsets. Immunological Reviews, **111**: 79-110.

Gibson, T. & Medawar, P.B. (1943) The fate of skin homografts in man. Journal of Anatomy, **77**: 299-313.

Gillman, S.C., Rosenberg, J.S. & Feldman, J.D. (1982) Membrane phenotype of the rat cytotoxic T lymphocyte. Journal of Immunology, **129**: 1012-1016.

Golding, H. & Singer, A. (1984) Role of accessory cell processing and presentation of shed H-2 alloantigens in allospecific cytotoxic T lymphocyte responses. Journal of Immunology, **133**: 597-605.

Gorer, P.A., Lyman, S. & Snell, G.D. (1948) Studies on the genetic and antigenic basis of tumour transplantation. Proceedings of the Royal Society. **B, 135**: 499-505.

Govaerts, A. (1960) Cellular antibodies in kidney homotransplantation. Journal of Immunology, **85**: 516-522.

Gowans, J.L., McGregor, D.D., Cowen, D.M. & Ford, C.E. (1962) Initiation of immune responses by small lymphocytes. Nature, **196**: 651-655.

Gracie, J.A., Bolton, E.M., Porteous, C. & Bradley, J.A. (1990) T cell requirements for the rejection of renal allografts bearing an isolated class I MHC disparity. Journal of Experimental Medicine, **172**: 1547-1557.

Green, C. (1988) Recent progress in organ transplantation, Oxford: The Medicine Group (UK) Ltd.

Griem, P., Wallny, H-J., Falk, K., Rotzschke, O., Arnold, B., Schonrich, G., Hammerling, G. & Rammensee H.G. (1991) Uneven tissue distribution of minor histocompatibility proteins versus peptides is caused by MHC expression. Cell, **65**: 633-640.

Groenewegan, G., Buurman, W.A. & van der Linden, C.J. (1985) Lymphokine dependence of in vivo expression of MHC class II antigens by endothelium. Nature, **316**: 361-363.

Gunther, E., Stark, O. (1979) The major histocompatibility system of the rat. Transplantation Proceedings, **11**: 1550-1553.

Gurley, K.E., Lowry, R.P. & Forbes, R.D.C. (1983) Immune mechanisms in organ allograft rejection. II. T helper cells, Delayed-type hypersensitivity, and rejection or renal allografts. Transplantation, **36**: 401-405.

Guttmann, R.D. (1976) A genetic survey of rat cardiac allograft rejection in presensitised recipients. Transplantation, **22**: 583-588.

Guttmann, R.D., Lindquist, R.R. & Ockner, S.A. (1969) Renal transplantation in the inbred rat. IX. Heamopoietic origin of an immunogenic stimulus of rejection. Transplantation, **8**: 472-484.

Guttmann, R.D., Forbes, R.D.C., Fuks, A. & Hibberd, J. (1985) Rejection and prolongation of rat cardiac allografts across intra-major histocompatibility complex (MHC) and non-MHC differences using congenic lines: Evidence for decreased class I immunogenicity. Transplantation Proceedings, **17**: 1911-1913.

Hall, B.M., de Saxe, I. & Dorsch, S.E. (1983) The cellular basis of allograft rejection in vivo. III. Restoration of first-set rejection of heart grafts by T helper cells in irradiated rats. Transplantation, **36**: 700-705.

Hall, B.M., Gurley, K.E. & Dorsch, S.E. (1985) The possible role of cytotoxic T cells in the mediation of first-set allograft rejection. Transplantation, **40**: 336-339.

Hall, B.M., Pearce, N.W., Gurley, K.E. & Dorsch, S.E. (1990) Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporin: III. Further characterisation of the $CD4^+$ suppressor cell and its mechanism of action. Journal of Experimental Medicine, **171**: 141-157.

Hall, B.M. (1991) Cells mediating allograft rejection. Transplantation, **51**: 1141-1151.

Halloran, P.F. & Madrenas, J. (1990) Regulation of MHC transcription. Transplantation, **50**: 725-738.

Hart, D.N.J. & Fabre, J.W. (1981) Demonstration and characterisation of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. Journal of Experimental Medicine, **154**: 347-361.

Hayry, P., von Willebrand, E. & Soots, A. (1979) In situ mechanisms in rat kidney allograft rejection. III. Kinetics of the inflammatory response and generation of donor-directed killer cells. Scandanavian Journal of Immunology, **10**: 95-108.

Hirschberg, H., Evensen, S.A., Henriksen, T. & Thorsby, E. (1975) The human mixed lymphocyte-endothelium culture interaction Transplantation, **19**: 495-504.

Hirschberg, H., Scott, H. & Thornsby, E. (1981) Human endothelial cells can present antigen to sensitised T lymphocytes *in vitro*. Transplantation Proceedings, **13**: 100-102.

Hsiung, L-M., Barclay, A.N., Brandon, M.R., Sim, E. & Porter, R.R. (1982) Purification of human C3b inactivator by monoclonal antibody affinity chromatography. Biochemical Journal, **203**: 293-298.

Hume, D.M., Merril, J.P., Miller, B.F. & Thorn, G.W. (1955) Experience with renal homotransplantation in the human: report of nine cases. Journal of Clinical Investigation, **34**: 327-382.

Hunig, T., Wallny, H.J., Hartley, J.K., Lawetzky, A. & Trefenthaler, G. (1989) A monoclonal antibody to a constant determinant of the rat T cell antigen receptor that induces T cell activation. Differential reactivity with subsets of immature and mature T lymphocytes. Journal of Experimental Medicine, **169**: 73-86.

Jacobson, S., Sekaly, R.P., Jacobson, C.L., McFarland, H.F. & Long, E.O. (1989) HLA class II-restricted presentation of cytoplasmic measles virus antigens to cytotoxic T cells. Journal of Virology, **63**: 1756-1762.

Janeway, C.A., Rojo, J., Saizawa, K., Dianzani, U., Portoles, P., Tite, J., Haque, S. & Jones, B. (1989) The co-receptor function of murine CD4. Immunological Reviews, **109**: 77-92.

Jeffries, W.A., Green, J.R. & Williams, A.F. (1985) Authentic T helper CD4 (W3/25) antigen on rat peritoneal macrophages. Journal of Experimental Medicine, **162**: 117-127.

Jin, Y., Shih, J.W-K. & Berkower, I. (1988) Human T cell response to the surface antigen of hepatitis B virus (HBs Ag): endosomal and nonendosomal processing pathways are accessible to both endogenous and exogenous antigen. Journal of Experimental Medicine, **168**: 293-306.

Katz, S.M., Liebert, M., Gill, T.J., Kuntz, H.W., Cramer, D.V. & Guttman, R.D. (1983) The relative roles of MHC and non-MHC genes in heart and skin allograft survival. Transplantation, **36**: 96-101.

Kissmeyer-Nielsen, F., Olsen, S. Petersen, V.P. & Fjeldborg, O. (1966) Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibody against donor cells. Lancet, **2**: 662-665.

Klein, J. (1978) H-2 mutations: their genetics and effects on immune functions. Advances in Immunology, **26**: 55-146.

Klempnauer, J., Steiniger, B., Wonigeit, K. & Gunther, E. (1985) Genetics of heart allograft rejection in the rat. Transplantation Proceedings, **17**: 1897-1899.

Laboratory report, (1979) Rat News Letters, **5**: 30.

Lampert, I.A., Suitters, A.J. & Chisolm. P.M. (1981) Expression of Ia antigen on epidermal keratinocytes in graft-versus-host disease. Nature, **293**: 149-150.

Lechler, R.I. & Batchelor, J.R. (1982) Restoration of immunogenicity to passenger cell depleted kidney allografts by the addition of donor strain dendritic cells. Journal of Experimental Medicine, **155**: 31-41.

Lechler, R.I., Lombardi, G., Batchelor, J.R., Reinsmoen, N. & Bach, F.H. (1990) The molecular basis of alloreactivity. Immunology Today, **11**: 83-88.

Leszczynski, D., Ferry, B., Schellekens, H., van der Meide, P. & Hayry, P. (1986) Antagonistic effects of γ-interferon and steroids on tissue antigenicity. Journal of Experimental Medicine, **164**: 1470-1477.

Lobel, S.A. & Cramer, D.V. (1981) Demonstration of a new genetic locus in the major histocompatibility system of the rat. Immunogenetics, **13**: 465-473.

Lobo, P.I. (1981) Nature of autolymphocytotoxins present in heamodialysis patients. Transplantation, **32**: 233-237.

Loveland, B.E., Hogarth, P.M., Ceredig, R.H. & McKenzie, I.F.C. (1981) Cells mediating graft rejection in the mouse. I. Lyt-1 cells mediate skin graft rejection. Journal of Experimental Medicine, **153**: 1044-1057.

Loveland, B.E. & McKenzie, I.F.C. (1982) Cells mediating graft rejection in the mouse. II. The Ly phenotypes of cells producing tumour allograft rejection. Transplantation, **33**: 174-180.

Lowry, R.P. & Forbes, R.D.C. (1984) The non-essential role of a humoral antibody response in acute rat cardiac allograft rejection. Transplantation, **37**: 182-187.

Lowry, R.P., Forbes, R.D.C., Blackburn, J.H. & Marghesco, D.M. (1985) Immune mechanisms in organ allograft rejection. V. Pivotal role of the cytotoxic-suppressor T cell ubset in the rejection of heart grafts bearing isolated class I disparities in the inbred rat. Transplantation, **40**: 545-550.

McDowall, R.A.W., Batchelor, J.R. & French, M.E. (1973) Hyperacute rejection and enhancement of rabbit-kidney allografts. Lancet, **1**: 797-801.

McWhinnie, D.L., Thompson, J.F., Taylor, H.M., Chapman, J.R., Bolton, E.M., Carter, N.P., Wood, R.F.M. & Morris, P.J. (1986) Morphometric analysis of cellular infiltration assessed by monoclonal antibody labelling in sequential human renal allograft biopsies. Transplantation, **42**: 352-358.

Mason, D.W., Dallman, M. & Barclay, A.N. (1981) Graft-versus-host disease induces expression of Ia antigen in rat epidermal cells and gut epithelium. Nature, **293**: 150-151.

Mason, D.W., Pugh, C.W. & Webb, M. (1981) The rat mixed lymphocyte reaction: roles of a dendritic cell in intestinal lymph and T-cell subsets defined by monoclonal antibodies. Immunology, **44:** 75-87.

Mason, D.W., Arthur, R.P., Dallman, M.J., Green, J.R., Spickett, G.P. & Thomas, M.L. (1983) Functions of rat T-lymphocyte subsets isolated by means of monoclonal antibodies. Immunology Reviews, **74**: 57-82.

Mason, D.W. & Morris, P.J. (1986) Effector mechanisms in allograft rejection. Annual Reviews in Immunology, **4**: 119-145.

Mason, D.W. & Simmonds, S.J. (1988) The autonomy of $CD8^+$ T cells *in vitro* and *in vivo*. Immunology, **65**: 249-257.

Medawar, P.B. (1944) The behaviour and fate of skin autografts and skin homografts in rabbits. Journal of Anatomy, **78**: 176-199.

Medawar, P.B. (1945) A second study of the behaviour and fate of skin homografts in rabbits. Journal of Anatomy, **79**: 157-176.

Miller, J.F.A.P. (1962) Effects of neonatal thymectomy on the immunological responsiveness of the mouse. Proceedings of The Royal Society of London. Series B, **156**: 415-428.

Miller, J.F.A.P., Vadas, M.A., Whitelaw, A. & Gamble, J. (1976) Role of major histocompatibility complex gene products in delayed-type hypersensitivity. Proceedings of the National Academy of Sciences of the U.S.A., **73**: 2486-2490.

Milton, A.D. & Fabre, J.W. (1985) Massive induction of donor type class I and class II major histocompatibility antigens in rejecting cardiac allografts in the rat. Journal of Experimental Medicine, **161**: 98-112.

Milton, A.D., Spencer, S.C. & Fabre, J.W. (1986a) Detailed analysis and demonstration of differences in the kinetics of induction of class I and class II major histocompatibility antigens in rejecting cardiac and kidney allografts in the rat. Transplantation, **41**: 499-508.

Milton, A.D., Spencer, S.C. & Fabre, J.W. (1986b) The effect of cyclosporin on induction of donor class I and class II MHC antigen in heart and kidney allografts in the rat. Transplantation, **42**: 337-347.

Mintz, B. & Silvers, W.K. (1970) Histocompatibility antigens on melanoblasts and hair follicle cells. Cell-localized homograft rejection in allophenic skin grafts. Transplantation, **9**: 497-505.

Mizuochi, T., Ono, S., Malek, T.R. & Singer, A. (1986) Characterisation of two distinct primary T cell populations that secrete interleukin 2 upon recognition of class I or class II major histocompatibility antigens. Journal of Experimental Medicine, **163**: 603-619.

Mossmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. & Coffman, R.L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. Journal of Immunology, **136**: 2348-2357.

Oluwole, S.F., Tezuka, K., Wasfie, T. Stegall, M.D. Reemtsma, K. & Hardy, M.A. (1989) Humoral immunity in allograft rejection. The role of cytotoxic alloantibody in hyperacute rejection and enhancement of rat cardiac allografts. Transplantation, **48**: 751-755.

Ono, K. & Lindsey, E. (1969) Improved technique of heart transplantation in rats. Journal of Thoracic and Cardiovascular Surgery, **57**: 225-229.

Opelz, G., Sengar, D.P.S., Mickey, M.R. & Terasaki, P.I. (1973) Effect of blood transfusions subsequent kidney transplants. Transplantation Proceedings, **5**: 253-259.

Patel, R. & Terasaki, P.I. (1969) Significance of the positive crossmatch test in kidney transplantation. New England Journal of Medicine, **280**: 735-739.

Pederson, N.C. & Morris, B. (1970) The role of the lymphatic system in the rejection of homografts: A study of lymph from renal transplants. Journal of Experimental Medicine, **131**: 936-969.

Pedersen, N.C. & Morris, B. (1974) The role of humoral antibody in the rejection of primary renal allografts in sheep. Journal of Experimental Medicine, **140**: 619-630.

Petty, H.R., Francis, J.W. & Anderson, C.L. (1989) Cell surface distribution of Fc receptors II and III on living human neutrophils before and during antibody dependent cellular cytotoxicity. Journal of Cellular Physiology, **141**: 598-605.

Pober, J.S., Collins, T., Gimbrone, M.A., Cotran, R.S., Gitlin, J.D., Fiers, W., Clayberger, C., Krensky, A.M., Burakoff, S.J. & Reiss, C.S. (1983) Lymphocytes recognise human vascular endothelial and dermal fibroblast Ia antigens induced by recombinant immune interferon. Nature, **305**: 726-729.

Rock, K.L., Barnes, M.C., Germain, R.N. & Benacerraf, B. (1983) The role of Ia molecules in the activation of T lymphocytes. II. Ia-restricted recognition of allo K/D antigens is required for class I MHC-stimulated mixed lymphocyte responses. Journal of Immunology, **130**: 457-462.

Roopenian, D.C. (1992) What are minor histocompatibility loci? A new look at an old question. Immunology Today, **13**: 7-10.

Rosenberg, A.S., Mizuochi, T. & Singer, A. (1986) Analysis of T-cell subsets in rejection of Kb mutant skin allografts differing at class I MHC. Nature, **322**: 829-831.

Rosenberg, A.S., Mizuochi, T., Sharrow, S.O. & Singer, A. (1987) Phenotype, specificity and function of T cell subsets and T cell interactions involved in skin allograft rejection. Journal of Experimental Medicine, **165**: 1296-1315.

Rosenberg, A.S. & Singer, A. (1988) Evidence that the effector mechanism of skin allograft rejection is antigen-specific. Proceedings of the National Academy of Sciences, U.S.A. **85**: 7739-7742.

Rosenberg, A.S., Munitz, T.I., Maniero, T.G. & Singer, A. (1991) Cellular basis of skin allograft rejection across a class I major histocompatibility barrier in mice depleted of $CD8^+$ T cells *in vivo*. Journal of Experimental Medicine, **173**: 1463-1471.

Roser, B.J. (1989) Cellular mechanisms in neonatal and adult tolerance. Immunological Reviews, **107**: 179-202.

Rosoff, P.M., Burakoff, S.J. & Greenstein, J.L. (1987) The role of the L3T4 molecule in mitogen and antigen-activated signal transduction. Cell, **49**: 845-853.

Ruers, T.J.M., Buurman, W.A., van Boxtel, C.J., van der Linden, C.J. & Koostra, G. (1987) Immunohistological observations in rat kidney allografts after local steroid administration. Journal of Experimental Medicine, **166**: 1205-1220.

Ruud, C.E., Trevillyan, J.M., Dasgupta, J.V., Wong, L.L. & Schlossman, S.F. (1988) The CD4 antigen is complexed in detergent lysates to a protein tyrosine kinase (pp58) from human T lymphocytes. Proceedings of the National Academy of Sciences of the U.S.A., **85**: 5190-5194.

Shimonkevitz, R., Kappler, J., Marrack, P. & Grey, H. (1983) Antigen recognition by H-2 restricted T cells. I. Cell-free antigen processing. Journal of Experimental Medicine, **158**: 303-316.

Shizuru, J.A., Seydel, K.B., Flavin, T.F., Wu, A.P., Kong, C.C., Hoyt, E.G., Fujimoto, N., Billingham, M.E., Starnes, V.A. & Fathman, C.G. (1990) Induction of donor-specific unresponsiveness to cardiac allografts in rats by pretransplant anti-CD4 monoclonal antibody therapy. Transplantation, **50**: 366-373.

Shreiber, S.L. & Crabtree, G.R. (1992) The mechanisms of action of cyclosporin A and FK506. Immunology Today, **13**: 136-142.

Smith, L., Steinmetz, M. & Hood, L. (1986) In Handbook of Experimental Immunology; Ed Weir,D.M. (4th edition). **Chapter 87**: The major histocompatibility complex of the mouse. Oxford: Blackwell Scientific Publications.

Snell, G.D. (1957) The homograft reaction. Annual Reviews in Microbiology. **II**:439-458.

Sprent, J., Schaefer, M., Lo, D. & Korngold, R. (1986) Properties of purified T cell subsets. II *In vivo* responses to class I vs class II H-2 differences. Journal of Experimental Medicine, **163**: 998-1011.

Sprent,J., Schaefer, M., Lo, D. & Korngold, R. (1986) Functions of purified $L3T4^+$ and $Lyt\text{-}2^+$ cells *in vitro* and *in vivo*. Immunological Reveiws, **91**: 195-218.

Starzl, T.E., Iwatsuki, S., Van Thiel, D.H. Gartner, J.C., Zitelli, B.J., Malatack, J.J., Schade, R.R., Shaw Jr., B.W., Hakala, T.R., Rosenthal, J.T. & Porter, K.A. (1982) Evolution of liver transplantation. Hepatology, **2**: 614-636.

Starzl, T.E., Iwatsuki, S., Shaw, B.W. & Gordon, R.D. (1985) Orthotopic liver transplantation in 1984. Transplantation Proceedings, **17**: 250-258.

Starzl, T.E., Abu-Elmag, D., Tzakis, A., Fung, J.J., Porter, K.A. & Todo, S. (1991) Selected topics on FK506, with special references to rescue of extrahepatic whole organ grafts, transplantation of forbidden organs, side-effects mechanisms & practical pharmokietics. Transplantation Proceedings, **23**: 914-919.

Steiniger, B., Klempnauer, J. & Wonigeit, K. (1985) Altered distribution of class I and class II MHC antigens during acute pancreas allograft rejection in the rat. Transplantation, **40**: 234-239.

Steinman, R.M. & Witmer, M.D. (1978) Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. Proceedings of the National Academy of Sciences of the U.S.A., **75**: 5132-5136.

Steinmuller, D. & Tyler, J.D. (1983) Cross-priming reveals similar tissue-restricted CTL-defined alloantigens on mouse, rat and human epidermal cells. Transplantation Proceedings, **15**: 238-241.

Steinmuller, D. (1985) Which T cells mediate allograft rejection. Transplantation, **40**: 229-233.

Stock, W. & Gunther, E. (1982) Serologic and cellular characterisation of products of a new major histocompatibility region, RT1C, of the rat; possible homology to mouse H-2 Qa. Journal of Immunology, **128**: 1923-1928.

Stuart, F.P., Bastien, E., Holter, A., Fitch, F.W. & Elkins, W.L. (1971) Role of passenger leukocytes in the rejection of renal allografts. Transplantation Proceedings, **3**: 461-464.

Sunderland, C.A., McMaster, W.R. & Williams, A.F. (1979) Purification with monoclonal antibody of a predominant leukocyte-common antigen and glycoprotein from rat thymocytes. European Journal of Immunology, **9**: 155-159.

Takacs, L., Szende, B., Rot, A.S. & Diamanstein, T. (1985) Expression of MHC class II antigens on bile duct epithelium in experimental graft versus host disease. Clinical and Experimental Immunology, **60**: 449-456.

Veillette, A., Bookman, M.A., Horak, E.M. & Bolen, J.B. (1988) The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. Cell, **55**: 301-308.

von Willebrand, E. & Hayry, P. (1978) Composition and *in vitro* cytotoxicity of cellular infiltrates in rejecting human kidney allografts. Cellular Immunology, **41**: 358-372.

Voronoy, U. (1936) Siglo Med, **97**: 296

Wallny, H-J. & Rammensee, H-G. (1990) Identification of minor histocompatibility antigen as cell-derived peptide. Nature, **343**: 275-278.

Wang, G.X., Terashita, G.Y. & Terasaki, P.I. (1989) Platelet crossmatching for kidney transplants by flow cytometry. Transplantation, **48**: 959-961.

Whittum, J.A. & Lindquist, R.R. (1977) Mechanisms of cardiac allograft rejection in the inbred rat. The effect of complement depletion by cobra venom factor on hyperacute cardiac allograft rejection. Transplantation, **24**: 226-228.

Williams, G.M., Hume, D.M., Hudson, R.P., Morris, P.J., Kano, K. & Milgrom, F. (1968) "Hyperacute" renal-homograft rejection in man. New England Journal of Medicine, **279**: 611-618.

Williams, G.M. (1984). In Kidney Transplantation: Principles and Practice. Ed. Morris,P.J., **2nd edition**: pp 335-354. New York: Grune & Stratton.

Wong, G.H.W., Clark-Lewis, I., Harris, A.W. & Schrader, A.W. (1984) Effect of cloned interferon-α on expression of H-2 and Ia antigens on cell lines of hemopoetic, lymphoid, epithelial, fibroblastic and neuronal origin. European Journal of Immunology, **14**: 52-56.

Yacoub, M., Festenstein, H., Doyle, P. et al (1987) The influence of HLA matching in cardiac allograft recipients receiving cyclosporin and azathioprine. Transplantation Proceedings, **19**: 2487-2489.