STUDIES OF VASCULARISED ALLOGRAFT AND XENOGRAFT
REJECTION PATHWAYS

BY

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Doctor of Philosophy with the faculty of Medicine.

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ABSTRACT

The role of indirect T cell allorecognition in transplantation has been investigated. Peptides from the alpha helical regions of class I MHC have been used in a rat kidney graft model to study the effect of priming to indirect T cell allorecognition on the efficacy of cyclosporin A immunosuppression. The work in this thesis demonstrates that priming with peptide to indirect T cell allorecognition does not influence graft function or survival under cyclosporin A therapy. However, cyclosporin A could not suppress the early antibody response seen to the grafts in the primed rats.

Multiple blood transfusions given prior to grafting can lead to a prolongation of graft survival. To try to elucidate the mechanisms of this process LEW recipients were primed to indirect T cell allorecognition by immunisation with a DA class I peptide before or during multiple blood transfusions. These peptide immunisation studies suggest that multiple blood transfusion suppresses the T helper pathway.

The other main area of interest has been the study of the mechanisms of xenograft rejection, where indirect recognition could play a major role in the rejection process. In this thesis I have used various strategies to induce prolongation of survival of the concordant mouse-to-rat vascularised heterotopic heart graft model. Therapies including cyclosporin A, CTLA4-Ig and splenectomy were used. The rejection response between these closely related species was very vigorous. Marked prolongation of survival was seen when all three of the therapies were combined. Most of the recipients did not reject their grafts whilst under treatment. Interestingly nude rats also rejected their grafts in a similar time span to controls. Pathology of the grafts at the time of rejection indicated a mild infiltrate that was negative for T cell markers and contained polymorphonuclear cells. Rat antibodies to mice were detected at day 3 (the day of rejection), indicating a humoral rejection response. Recipients with grafts that survived past day 18 post transplant demonstrated a massive cellular infiltration of the graft at the time of rejection and no rat anti-mouse antibodies. These studies suggest that once the initial antibody-mediated phase has been overcome, cellular mechanisms might play a dominant role in the rejection response of these vascularised concordant cardiac xenografts.
ACKNOWLEDGMENTS

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<tr>
<td>ADCC</td>
<td>antibody dependant cell mediated cytotoxicity</td>
</tr>
<tr>
<td>ALG</td>
<td>anti-lymphocyte globulin</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATG</td>
<td>anti-thymocyte globulin</td>
</tr>
<tr>
<td>ATXBM</td>
<td>athymic irradiated bone marrow reconstituted</td>
</tr>
<tr>
<td>β₂m</td>
<td>β2-microglobulin</td>
</tr>
<tr>
<td>BQR</td>
<td>brequinar sodium</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CAD</td>
<td>accelerated coronary artery disease</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation antigen</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T cell</td>
</tr>
<tr>
<td>CTLA</td>
<td>cytolytic T lymphocyte associated antigen</td>
</tr>
<tr>
<td>CVF</td>
<td>cobra venom factor</td>
</tr>
<tr>
<td>DAF</td>
<td>decay acelerating factor</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSG</td>
<td>deoxyspergualin</td>
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DTH delayed type hypersensitivity
ELAM endothelial leucocyte adhesion molecule
ER endoplasmic reticulum
$F(ab')_2$ bivalent fragment of the antigen binding portion of immunoglobulin produced by pepsin cleavage
FCA Freunds complete adjuvant
FIA Freunds incomplete adjuvant
FITC fluorescein isothiocyanate
H and E haematoxylin and eosin
HAR hyperacute rejection
HLA human leucocyte antigen
HSA heat stable antigen
HUVEC human umbilical vein endothelial cells
ICAM intracellular adhesion molecule
IDC interstitial dendritic cell
IFN interferon
Ig immunoglobulin
li invariant chain
IL interleukin
IL-R interleukin receptor
kD kilodalton
LFA leucocyte function associated antigen
LPS lipopolysaccharide
$\mu$Ci microcurie
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mab</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>MCP</td>
<td>membrane cofactor protein</td>
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<td>2-ME</td>
<td>2-mercaptoethanol</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MM</td>
<td>mycophenolate mofetil</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>Myr</td>
<td>million years</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
</tr>
<tr>
<td>PRA</td>
<td>panel reactive antibodies</td>
</tr>
<tr>
<td>PVC</td>
<td>polyvinyl chloride</td>
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<tr>
<td>RAM</td>
<td>immunoadsorbent purified rabbit F(ab')$_2$ anti-mouse F(ab')$_2$</td>
</tr>
<tr>
<td>RAR</td>
<td>immunoadsorbent purified rabbit F(ab')$_2$ anti-rat F(ab')$_2$</td>
</tr>
<tr>
<td>RATG</td>
<td>rat anti-thymocyte globulin</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen presentation</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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TGN  
trans-Golgi network

Ti  
antigen specific portion of TCR

TL  
thymus leukaemia antigen

TNF  
tumour necrosis factor

UKTSSA  
United Kingdom Transplant Support Service Authority

VCAM  
vascular cell adhesion molecule

VEC  
vascular endothelial cell

VLA  
very late activation antigen

v/v  
volume/volume

w/v  
weight/volume
CHAPTER 1:
THE MAJOR HISTOCOMPATIBILITY COMPLEX

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CHAPTER 1:
THE MAJOR HISTOCOMPATIBILITY COMPLEX

CLASS I MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) MOLECULES

A map of the class I MHC region of human, mouse and rat can be seen in figure 1.1.

1.1 Mouse MHC class I genes and products

The mouse MHC is located on chromosome 17, the number of loci encoding classical molecules is relatively few, generally two or three, however these immunologically predominant class I loci represent only a fraction of the class I genes. Non classical class I genes constitute the majority of MHC class I loci. They are differentiated from classical class I molecules by limited polymorphism and lower cell surface expression. These non classical class I molecules still have a role to play in antigen presentation. Classical class I molecules bind a broad range of peptides whereas non classical class I molecules tend to bind a far narrower range of peptides.

1.1.1 Mouse classical class I genes and products

Mouse classical class I genes are highly polymorphic and are expressed on virtually all cell types. There are three subregions termed H-2K, D and L. There are considerable variations in the number of genes found for the different mouse haplotypes from between 25 to 40. BALB/c and C57BL/10 mice have two class I genes in the H-2K region, but have only one detectable H-2K product. However even though C57BL/10
Map of human (HLA), mouse (H-2) and rat (RT.1) MHC class I regions.

Classical class I genes are represented by the shaded blocks.
mice have only one gene in the H-2D region, BALB/c mice have five genes (including one from the H-2L region), with only two detectable products, H-2D and H-2L.

1.1.2 Mouse non classical class I genes and products

These genes are difficult to detect using classical techniques as their products are not expressed at high levels in most tissues. Many of these appear to be pseudogenes and in most cases no function has been ascribed to them. There are additional genes that are more closely related to class I genes than to any other family but are not linked to the MHC. These include CD1, the low affinity IgG Fc receptor of murine neonatal intestine and zinc α2 globulin. The number of non classical class I genes identified varies considerably depending on the species. They range from about a dozen in humans to thousands in the pygmy mouse. There are different reports describing the number of genes in the laboratory mouse ranging from 40 to 60 (Stroynowski 1990).

The first non classical class I molecule to be described was the thymus leukaemia antigen (TL) by Old et al in 1963. The second was not identified until 10 years later when Qa-1 and Qa-2 were characterised (Flaherty et al. 1976). Approximately 40-50% of non classical genes have an open reading frame and are expressed at the transcripational level, the remainder are unexpressed pseudogenes. The majority of genes are encoded within the Q, T, and M regions.

H-2M

There are 8 genes in the M locus, four of which have open reading frames H-2M1, H-2M2, H-2M3a formerly Hmt, and H-2M5. The other four genes H-2M4,6,7 and 8 contain premature stop codons and/or frame shift mutations. No mRNA has been
detected for \textit{H-2M5} and \textit{H-2M1} suggesting that these genes are either not expressed or that their expression is extremely limited (Wang and Lindahl 1993). It has been suggested that H-2M2 is expressed in the thymus, mRNA has also recently been found at very low levels in a variety of cells and in embryos as early as 12 days (Brorson \textit{et al} 1989). It potentially encodes a membrane bound glycoprotein of 315 amino acids. Six alleles of H-2M3 have been described. H-2m3a has been widely studied as the best example of a non classical class I antigen presenting molecule. It was first identified as restricting presentation of a mitochondrially encoded peptide derived from the amino-terminus of NADH dehydrogenase subunit 1 gene (ND1) (Loveland \textit{et al} 1990).

\textbf{H-2Q}

The Q region is composed of at least ten genes, of which \textit{Q4} and the Qa-2-encoding genes (depending on the mouse haplotype) \textit{Q5-Q9} are expressed in a wide variety of tissues. Q2, Q5 and Q10 are more tissue specific (Stroynowski 1990). The Q region also encodes Qb-1. Qb-1, Qa-2 and Q10 can all be detected as secreted proteins, they have been shown to bind \(\beta_2m\) and computer modelling suggests they have the potential to bind antigenic peptides. Qb-1 protein is secreted in T lymphocytes, however more recently high levels have also been detected on the cell surface of fibroblasts using immunoflorescence techniques (Day and Frelinger 1991). It is possible that the different forms have different functions. Q10 protein has a molecular weight of 40 kD and is known to associate with \(\beta_2m\) it is expressed on lymphocytes of all lineages and on primitive hematopoietic cells. It can be synthesised as a secreted form (sQa-2) or a cell
surface form that is attached to the membrane via a glycosylphosphatidylinositol anchor. Qa-2 is nonpolymorphic.

H-2T
There are at least 20 genes in this region in three clusters. Transcripts of 8 genes have been found, four of which are expressed with a tissue distribution similar to classical class I antigens. The thymus leukaemia antigen encoded by T3b and T18pd was the first discovered non classical class I molecule. TL is expressed on small intestinal epithelial cells of all strains of mice (Wu et al 1991) and is thought to play a role in T cell development in the thymus. Studies by Morita et al (1994) using transgenic mice that express TL in most tissues has shown that it can serve as a transplantation antigen.

1.2 Human MHC class I genes and products
The genetics and molecular biology of the MHC has been intensively studied due to its importance in the immune process. The human MHC is located on chromosome 6. The class I region has been shown to contain many genes with more being identified. Southern blot analysis of genomic DNA with a class I gene probe revealed 18 cross-hybridising restriction fragments, all of which have been cloned and characterised. Analysis revealed six HLA expressed genes and 12 HLA-related sequences that are pseudogenes or fragments of genes (Geraghty et al 1992a). An additional 19th sequence has been described in the A11,Bw60 haplotype.
Of the expressed genes so far described three encode the classical class I proteins HLA-A, B and C. Three more encode non classical class I proteins HLA-E, HLA-F and HLA-G.

1.2.1 Human classical class I genes and products
The HLA-A, B and C genes encode the classical transplantation antigens. They reside within about 1.5 million bases of DNA. HLA-A is the most telomeric and HLA-B the most centromeric locus.

1.2.2 Human non classical class I genes and products
The majority of human class I genes are non classical showing limited polymorphism and lower cell surface expression. These non classical genes include HLA-E, F and G, which are not homologous to the murine H-2Q,T and M regions. Cloning and physical mapping of the non classical class I region between HLA-E to HLA-F has been achieved with the use of yeast artificial chromosomes (YACS).

HLA E
The gene encoding HLA-E is localised between the HLA-C and HLA-A loci (Geraghty et al 1992b). HLA-E is highly transcribed in most tissues.

HLA-F
HLA-F is located at the 3' end of the MHC region, transcripts were detected in large quantities in fetal liver (Houlihan et al 1992) suggesting a specialised function in hematopoiesis or fetal development.
HLA-G

HLA-G is located between HLA-E and HLA-F and was first discovered by Geraghty et al in 1987. The protein is expressed in two forms, membrane bound and soluble both of which are associated with β2m. It has been suggested that it may have a role in the maternal-fetal immune interaction (Loke and King 1991).

A new locus has recently been described, termed HLA-X. This region is located to the left of HLA-B and consists of a family of 5 related genes which are distantly homologous to class I chains (Bahram et al 1994).

1.2.3 Human class I pseudogenes and gene fragments

Four full length HLA class I pseudogenes have been described by Geraghty et al (1992c) using a cosmid library from the cell line LCL 721. These pseudogenes termed HLA-54 (also termed HLA-H), HLA-59 (also termed HLA-J), HLA-70 and HLA-92 have the class I exon-intron structure as well as class I homology in the 5’ and 3’ flanking regions. The sequences were found to contain interruptions in the open reading frame which confirmed that they were pseudogenes. Four truncated genes have also been identified HLA-75, HLA-80, HLA-90 and HLA-16 and four gene fragments HLA-17, HLA-21, HLA-30 and HLA-81 (reviewed by Geraghty 1993).
1.3 Rat MHC class I genes and products

The rat MHC is located on chromosome 20.

1.3.1 Rat classical class I genes and products

Classical class I products are encoded by genes in the RT1.A region. These classical class I molecules have a wide tissue distribution and function as restriction elements for T cell mediated responses. They are analogous to the murine H-2K molecules.

1.3.2 Rat non classical class I genes and products

The non classical class I molecules are encoded by genes in the RT1.C, RT1.E and RT1.G regions. These molecules show a more restricted tissue distribution and a lower level of expression than classical class I molecules. Many non classical class I genes have been cloned in the rat, Jameson et al (1992) cloned 61 non classical class I genes from the PVG-RT1av1 haplotype, many of which appear to be located in the RT1.C region. Only seven genes were shown to have expressible products on the cell surface. Cross hybridisation with mouse Tla specific probes found no Tla homologues in the rat suggesting this group to be largely absent from the rat. An exception to this finding is the RT1.BM1 gene which shows a significant relationship with the BALB/c Tla gene (Parker et al 1991. A soluble non classical class I molecules has also been described (Spencer and Fabre 1987) the gene for this molecule has been mapped to cluster 2 (Jameson et al 1992). Wonigeit and Hänisch (1991) demonstrated the presence of three distinct cell surface molecules encoded by class I genes located to the right of the RT1.B/D region in the RT11 haplotype. These genes included the RT1.C genes and two new genes termed RT1.L and RT1.M. Non classical class I loci can also be found in the classical RT1.A region these are the RT1.F and Pa (pregnancy associated) loci.
1.4 Biochemistry of MHC class I molecules

Class I molecules are composed of a glycosylated polypeptide heavy chain of 45kD, non covalently associated with β2 microglobulin (β2m) 12kD light chain (fig 1.2). The heavy chain consists of five regions, three extracellular domains α1, α2 and α3 each approximately 90 amino acids long and encoded by separate exons, a transmembrane region and a cytoplasmic domain. The α2 and α3 domains both have intrachain disulphide bonds enclosing loops of 63 and 86 amino acids respectively. The α3 domain and β2m are relatively conserved and show amino acid sequence homology to immunoglobulin constant domains. The polymorphic α1 and α2 domains show no significant sequence homology to immunoglobulin constant or variable domains. In both human and mouse, heavy chains have an N-glycosylated asparagine residue at position 86 in the α1 domain. The major papain cleavage site is between α3 and the transmembrane region with an additional minor cleavage site between the second and third domains. The transmembrane region consists of about 25 hydrophobic uncharged residues. The hydrophilic cytoplasmic tail is about 30 (human) to 40 (mouse) residues and consists of approximately 50% polar amino acids, particularly serine.

β2m is a non-polymorphic protein in humans, although it is dimorphic in mice. It shows a high degree of sequence homology among species implying evolutionary conservation.
Figure 1.2

Diagrammatical representation of MHC class I molecules in association with $\beta_2m$.

(S-S denotes di-sulphide bridge).
1.5 Assembly and intracellular transport of MHC class I molecules

MHC class I heavy chain and β2m are synthesized in the endoplasmic reticulum (ER) where they assemble into a heterodimer that is unstable at physiological temperature, but is stabilized by exposure to peptides (Townsend et al 1990). Heavy chain (H chain), β2m and peptide assemble in a ratio of 1:1:1 (Silver et al 1991). However it is still unclear whether H chain forms a loose association with β2m which is then stabilized by peptides occupying the binding site, or peptides could stabilize the conformational change in H chain which then associates with β2m. Class I H chain exists in two conformational forms, prior to formation of the heterotrimer. Class I H chain is associated with a 88kD protein (p88) that appears to have a chaperone like function similar to that of immunoglobulin heavy chain binding protein (BiP), efficient dissociation of p88 requires both the binding of β2m and peptide (Degen et al 1992) (fig 1.3). These MHC class I peptides have been found to be of a restricted length from 8-11 amino acids. Both Falk et al (1991) and Schumacher et al (1991) found that class I molecules preferentially bound peptides of 9aa. These peptides are predominately generated from cytoplasmic proteins probably by the action of multicatalytic proteinase complexes or proteasome and are transported into the ER. The mechanism by which this is achieved is thought to be by the use of peptide transporters. Livingstone et al (1989) noticed that in the rat the alloantigenic structure of the classical class I locus could be changed significantly by the action of a gene located elsewhere in the rat MHC, this lead to the definition of a new locus that mapped in the class II region between RT1.A and RT1.D. This locus appeared in two allelic forms corresponding to the presence or absence of a dominant transacting modifier called class I modifier or cim. Other groups working with mutant cells that neither assemble stable class I
Figure 1.3

Model of assembly and intracellular transport of MHC class I molecules.

Peptides are transported into the endoplasmic reticulum (ER) by the TAP transporter.

MHC class I molecules are assembled in the ER from class I heavy chain and β2m, following binding of the peptide the p88 chaperone dissociates and the MHC class I molecule is released from the ER. The MHC class I molecules are transported through the Golgi to the cell surface.
molecules nor present intracellular antigens have located a defect in their peptide transport which has been localized to a large deletion in the MHC class II region. Two genes have been identified for human T2 cells called RING4 and RING11 (Spies et al 1990) which associate to form a complex (Kelly et al 1992). In the mouse RMA-S cells have been utilized to identify Ham-1 and Ham-2 genes (Monaco et al 1990). Both of these MHC-linked genes are required for class I antigen processing in the form of a heterodimer (Attaya et al 1992). To avoid confusion these genes have been given the same nomenclature regardless of the species and have been termed TAPI and TAP2 for transporter associated with antigen presentation. TAP molecules may also have a role in the assembly of class I molecules. Ortmann et al (1994) demonstrated that in human B-cell lines class I/β2m dimers in the ER are physically associated with TAP molecules rather than calnexin. This suggests that calnexin mediates dimerization and the subsequent binding of the dimers to TAP molecules facilitates their association with TAP transported peptides. It is possible that other mechanisms exist for peptide import into the ER. Henderson et al (1992) demonstrated proteolysis of signal peptide domains in the ER. After release from the ER, MHC class I molecules are transported through the Golgi apparatus and transgolgi reticulum to the cell surface (Neefjes and Ploegh 1988). At the cell surface empty class I molecules can appear, suggesting that the supply of peptides is limited (Ortiz-Navarette and Hammerling 1991). However these are unstable at physiological temperature and are rapidly degraded unless stabilized by peptides (Neefjes et al 1992). Where peptides are bound with only a low affinity release of peptide results in heterodimers and finally free class I heavy chains. The class I heavy chain can again associate with β2m and peptide from the medium. The half life of peptides is very short as they are very sensitive to degradation by
serum and cell derived proteases thus avoiding sensitisation of innocent bystanders for lysine by CTL.

Peptides presented by class I molecules are usually generated from proteins in the nucleus or cytoplasm. The degradation of these proteins needs to be halted at the peptide level without further degradation to amino acids. The mechanism by which this is achieved is poorly understood. Closely linked to the TAP1 and TAP2 genes in the class II region of the MHC are genes for the LMP2 and LMP7 proteins which resemble components of proteases. This lead to the assumption that they were involved in peptide processing for MHC molecules. However Arnold et al (1992) have shown that the presence of LMP2 and LMP7 are not essential for antigen presentation. This suggests that the ubiquitin-dependant proteolytic pathway which has been implicated in the production of these antigenic peptides (Michalek et al 1993) has a role to play in the degradation of these antigens.

1.5.1 Peptide binding in MHC class I molecules

The elucidation of the structure of the HLA-A2 antigen using X-ray crystallography by Bjorkman et al in 1987 showed the structure of the peptide binding site. The peptide binding site is formed from the α1 and α2 domains supported by a β-pleated sheet floor containing eight strands and bounded by two α helices, one from α1 and one from α2. β2m makes contact with the β sheet floor of the α1, α2 peptide binding region. The peptide binding region was found to contain a set of pockets on detailed analysis (Garrett et al 1989), some of these pockets were found to extend deep between the floor and the helical walls of the binding domain. These pockets have been designated A through to F. The predominant length of peptides associated with
most class I molecules so far analysed is nine residues, these length preferences could be due to selectivity in the peptide binding site. Crystallographic analysis show that the amino and carboxy terminal ends of class I associated peptides participate in an extensive set of hydrogen bonds to conserved regions that are located in pockets A and F at opposite ends of the binding site (Zhang et al 1992). This shows a preferred orientation of peptides with the amino terminal end in pocket A and the carboxy terminal end in pocket F. It has been suggested that these interactions provide a major part of the energy for peptide binding so defining an optimum length for peptide binding. However there appears to be an unusual dominance of 8 residue peptides in the H-2K^b haplotype. This could be due to the fact that the central region of the binding cleft is flatter than in most class I molecules, which allows peptides to adopt a more extended conformation and shortens the distance between the termini (Fremont et al 1992). However between 20% and 40% of the individual sequences analysed from several class I molecules are longer than 9 residues. A mechanism to explain this difference in length could be a kink in the centre of the peptide, an alternative would be that the carboxy terminal end of longer peptides extends out of the binding site and that a more internal residue acts as a carboxy terminal anchor (Hahn et al 1992). An alternative method for size selectivity is thought to be due to proteolytic trimming of class I associated peptides down to a protected core (Falk et al 1990) although this hypothesis does not have wide support.

The description of peptide characteristics important for binding has been largely based on identification of common structural features or motifs. Pockets B, C and F are largely responsible for the motifs observed for most class I molecules, however other pockets also exert a strong influence on binding specificity (Englehard et al 1994).
CLASS II MHC MOLECULES

A map of the class II MHC region of human, mouse and rat can be seen in figure 1.4.

1.6 Mouse MHC Class II genes and products

Mice were originally thought to express two class II molecules, I-A a heterodimer of the $\alpha$A and $\beta$A gene products which is homologous to $HLA-DQ$ and I-E a heterodimer of $\alpha$E and $\beta$E gene products which is homologous to $HLA-DR$. Original studies also revealed the presence of $\beta$A2 and $\beta$Aps genes in the I-A region. $\beta$A2 now called $Pb$ is a pseudogene, homologous to $HLA-DP$ although no $\alpha$ chain gene has been found. $\beta$A2 now known as $Ob$, which is homologous to $HLA-DO$, is much less polymorphic than the $\beta$A and $\beta$E genes but has been shown to produce a protein product (Karlsson et al 1991). This H-2Ob chain pairs with an $\alpha$ chain from the $Oa$ gene (homologous to $HLA-DN$) (Karlsson and Peterson 1992) and is expressed as a heterodimer at the cell surface. It shows more limited tissue distribution than H-2A molecules being absent on macrophages and dendritic cells, suggesting that its function is different to that of the classical class II molecules. Two further non classical mouse class II genes have been described, $H-2Ma$ and $H-2Mb$ (Cho et al 1991) which are homologous to $HLA-DM$ and are transcribed in normal tissues.
Figure 1.4

Map of the human (HLA), mouse (H-2), and rat (RT1) MHC class II regions.

(adapted from Campbell and Trowsdale 1993, Monaco et al 1993 and Deverson et al 1994)
1.7 Human MHC class II genes and products

The map of the class II region has been defined using a combination of techniques including recombination analysis, cosmid walking and pulse field electrophoresis. Seven \( \alpha \) genes and 11 \( \beta \) genes have been characterized so far. This region is divided into six families, HLA-DP, DN, DM, DO, DQ and DR. The DP, DQ, and DR subregions contain at least one \( \alpha \) and one \( \beta \) gene, encoding one \( \alpha \) and one \( \beta \) class II protein chain which forms a heterodimer. The DM subregion was identified as a class II gene in 1991 by Kelly et al from the previously discovered \textit{RING6} and \textit{RING7} genes. These genes are different from the typical class II genes suggesting that they may originate from a time prior to the divergence of the main class II genes (Radley et al 1994). They encode an \( \alpha \) and \( \beta \) chain respectively that forms a heterodimer with restricted peptide binding capacity. HLA-DM appears to act as a chaperone in the peptide loading stage of class II folding (Morris et al 1994). \textit{DN} codes for an \( \alpha \) chain only and \textit{DO} only codes for a \( \beta \) chain only.

In the DR region there are four expressed \textit{DRB} genes \textit{DRB1, 3, 4} and 5 and five pseudogenes \textit{DRB2, 6, 7, 8} and 9. The organisation of the \( \beta \) chain genes is not identical for all the haplotypes. The DR1 group (DR1, 10, 103 haplotypes) contains three genes \textit{DRB1, 6} and 9. DR51 group (DR15,16) contains the \textit{DRB1, 6, 5} and 9 genes. Whereas the DR52 group (DR3,11,12,12,14,1403,1404) consists of the \textit{DRB1, 2, 3} and 9 genes. The DR53 group (DR4,7 and 9 haplotypes) contains five genes \textit{DRB1, 7,8, 4} and 9. The DR8 haplotype contains the \textit{DRB1} gene and the \textit{DRB9} gene (reviewed by Andersson et al 1994).
1.8 Rat MHC class II genes and products

The first biochemical evidence for the presence of class II molecules in the rat was presented by Shinohara et al in 1977. Serological characterisation of these molecules followed (Sachs et al 1977). Two subregions were defined termed RT1.B and RT1.D homologous to murine I-A and I-E respectively (Fukumoto et al 1982) and HLA-DQ and HLA-DR respectively. These regions were each found to contain a cluster of 3 genes, an expressible α gene and β gene with an additional β gene. Restriction-fragment-length polymorphism analysis revealed the presence of another region termed RT1.H which mapped between the RT1.A and RT1.B regions and was found to be HLA-DP like (Watters et al 1987) although the Hβ gene has little homology to the DPβ gene. Studies by Fujii et al (1991) suggest that functional RT1.H is not expressed on the cell surface.

1.9 Biochemistry of MHC class II molecules

In mouse, rat and human, class II molecules are heterodimers consisting of an α chain of 33-35kD associated with a β chain of 26-29 kD (fig 1.5). Each chain consists of a heavily glycosylated extracellular region, containing 2 domains α1, α2 and β1, β2 a hydrophobic transmembrane region and a short carboxy cytoplasmic tail. The two N terminals α1 and β1 show no sequence homology to immunoglobulin domains. However the membrane proximal domains α2 and β2 possess structural characteristics of the immunoglobulin domains (Kappes and Strominger 1988). The β1 domain is 95aa long with cysteine residues at positions 15 and 79 which are disulphide linked, forming a disulphide loop of 64aa. The α1 domain is only 85-88aa long and lacks cysteine residues and therefore can not form a disulphide bond. The α chain bears 2 N
Figure 1.5

Diagrammatical representation of MHC class II molecule.

(S-S denotes disulphide bridge).
linked glycans, one complex and one high mannose, the β chain however contains just one complex carbohydrate. This accounts for much of the difference in molecular weight of the class II molecules. The second domains of both chains are each 95aa long and have strong sequence homology to immunoglobulin constant regions. Both chains bear a 56aa disulphide loop from residue 107 to 163 in α2 and from residue 117 to 173 in β2. Following the second domain is a hydrophobic region of 11-13aa rich in glutamic acid and proline in the α chain or serine in the β chain that connects the extracellular domains with the transmembrane hydrophobic region. The hydrophobic region ends with a cluster of positively charged residues that interact with the negatively charged phospholipids of the membrane thus anchoring the chains in the membrane. These residues are followed by a short proteolytically sensitive cytoplasmic tail of variable length containing many charged and hydrophobic residues. Class II molecules are associated with a third chain, a type II transmembrane protein known as the invariant chain. The predominant form of invariant chain in humans is p33 (also known as p31) although additional p35 (also known as p33), p41 and p43 forms exist (Strubin et al 1986).

1.10 Assembly and intracellular transport of MHC class II molecules

MHC class II molecules are assembled in the ER from α and β chains and the invariant chain (fig 1.6). The invariant chain and class II molecules associate soon after their assembly. The α, β and invariant chains occur in equimolar amounts forming a nine sub-unit transmembrane protein that contains three αβ dimers in association with an invariant chain trimer (Roche et al 1991). Lamb and Cresswell (1992) showed that this
complex is formed by the sequential addition of $\alpha\beta$ dimers to pre-existing invariant chain trimers. Calnexin is thought to play a key role in the assembly of this nonamer (Anderson and Cresswell 1994). The p35 and p43 forms of the invariant chain exert a strong ER retention signal which is lost following association of $\alpha$ and $\beta$ subunits (Machmer and Cresswell 1984), thereby facilitating movement of the complete nonamer out of the ER. The assembled $\alpha\beta$I complex then traverses the Golgi and is segregated from the normal constitutive transport pathway to the cell surface in the trans-Golgi reticulum or trans-Golgi network (TGN) (Peters et al 1991). However some $\alpha\beta$I complexes may go directly to the cell surface (Roche et al 1993). It has been suggested that the $\alpha\beta$I complex enters the endocytic pathway from the TGN at the early endosome and then progresses through the pathway to late endosomes or prelysosomes (Romagnoli et al 1993). In order for peptide binding and expression on the cell surface, class II molecules must first dissociate from the invariant chain. This is thought to be achieved by proteolysis of the invariant chain by cathepsin B and D (Cresswell 1994). The exact route that class II $\alpha\beta$ dimers follow to the cell surface is as yet undefined. It has been suggested that if dissociation occurs in a late endosome or prelysosome, the $\alpha\beta$ dimer may shuttle back to the TGN, enter the constitutive transport pathway, and thus be transported to the cell surface.

The role of the invariant chain when it was first discovered was unclear, as early experiments showed that the invariant chain was not vital to class II assembly as class II expression could occur in the absence of the invariant chain (Sekaly et al 1986). However later experiments demonstrated that it was involved in assembly and transport (Anderson et al 1992). Purified class II $\alpha\beta$I complexes are unable to bind peptides, suggesting that the invariant chain may prevent class II molecules from
Figure 1.6

Model of assembly and intracellular transport of MHC class II molecules.

MHC class II molecules are assembled in the endoplasmic reticulum (ER) from an α and β chain and the invariant chain (Ii), forming a nine subunit transmembrane protein which traverses the Golgi. The αβIi complex enters the endocytic pathway from the trans-Golgi network (TGN) where peptide binding occurs following dissociation of the Ii.
associating with inappropriate peptides early in biosynthesis (Roche and Cresswell 1990). Treatment of these complexes with low SDS, releases αβ dimers that are capable of binding antigenic peptides. Binding of these peptides stabilises the αβ dimer against SDS induced denaturation (Stern and Wiley 1992).

Proteins internalised by antigen presenting cells are broken down into peptides in the endosomal/lysosomal system before binding to class II molecules prior to their expression on the cell surface. The exact mechanism by which this is achieved is unclear. Reagents such as chloroquine which neutralise the acidic endosomal/lysosomal system have been shown to prevent antigen processing (Zeigler and Unanue 1982). Whether protein antigens are degraded in the endosome or lysosome still needs to be elucidated but the pathway used may depend on the antigen in question. The majority of peptides that bind to class II are self peptides derived from cell surface proteins (Rudensky et al 1991), these would include class II subunits. The αβI-complex remains intracellular for 1 to 3 hours before the appearance of αβ dimers on the cell surface.

1.10.1 Peptide binding in MHC class II molecules

The peptide binding site of class II molecules consists of eight strands of anti-parallel β pleated sheet with two anti-parallel α helical regions overlaying them. There is a deep cleft between the α helices which accommodates the bound peptide. Four of the β strands and one helical region are derived from each subunit. The precise mechanisms by which peptides are broken down in the endosomal/lysosomal system prior to binding to MHC class II molecules is unclear. The antigens presented by class II molecules are usually classified as exogenous, however the great majority of
peptides identified to date originate from membrane bound proteins or those resident in endosomes or lysosomes (Rudensky et al 1991). Those that originate from a truly exogenous source are low in number and come from proteins that are present in very high concentrations in the extra cellular environment, such as serum albumin, or for which there are membrane bound receptors specialised for their uptake (Englehard 1994). Peptide length is more variable and of a longer length than those for class I. Most of the peptides so far defined have 12-19 residues although lengths vary from 10 to 30 residues (Chicz et al 1993). This lack of size constraint was explained following the elucidation of the X-ray crystal structure of the human class II molecule HLA-DR1 (Brown et al 1993) as the peptide binding site is open at both ends, unlike the class I peptide binding site, allowing for overhang of the peptide. The shape of the excess electron density representing the bound peptide is more linear than for class I and is consistent with an overall peptide contact length of 15 residues in a fully extended conformation. The variation in length may be due to proteolytic cleavage either before or after the peptide binds which is tolerated because the binding interactions involve the central part of the peptide rather than its ends.

1.11 Tissue distribution of MHC class I and class II molecules

1.11.1 Classical class I molecules

It was originally thought that the distribution of class I MHC was ubiquitous to all tissues. An extensive study by Daar et al in 1984 on fresh human tissue snap frozen, using immunoperoxidase staining techniques, revealed that class I distribution was not ubiquitous. Class I molecules were seen to be absent from neurones, myocardial cells,
the acinar epithelium of the exocrine pancreas, the acinar epithelium of the parotid gland, the endothelium of the cornea, the chorionic villous trophoblast of the placenta and mature spermatozoa in the lumen of the epididymis. It was also found to be detectable only in very small amounts on hepatocytes with some staining class I negative and others weakly positive. Class I molecules are also absent from human red blood cells (RBC) although they are present on rat and mouse RBC.

(The tissue distribution of non classical class I molecules has already been discussed in the relevant section)

1.11.2 Class II molecules

Class II antigens are more restricted in their distribution than class I antigens. They are expressed by many cells in the haematopoetic system predominantly B cells, dendritic cells and the Langerhans cells of the skin and by some parenchymal cells (T cells express class II molecules when activated). Interestingly there are species differences in the expression of class II molecules. Vascular endothelial cells are class II negative in the rat but positive in human and many other species. Kupffer cells of the liver are class II negative in the rat but class II positive in humans. Epithelial cells of the proximal convoluted tubules of the rat are positive (Hart and Fabre 1981) as they are in most but not all human kidneys. Studies on human organs have revealed the presence of class II molecules on the epithelium of the small intestine, trachea, epiglottis, tongue and tonsils. Interestingly human brain and the villous trophoblast do not appear to express class I or class II molecules which suggests that these tissues are immunologically privileged.
CHAPTER 2:

IMMUNE RECOGNITION

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CHAPTER 2:

IMMUNE RECOGNITION

2.1 Introduction

Immune recognition requires the recognition of antigen by host cells. There are two types of antigen recognition, recognition of whole antigen in its native form and recognition of antigen in a degraded form presented in association with MHC molecules. The first is utilised by B cells whose surface immunoglobulins act as antigen receptors and the second by T cells whose T cell receptors recognise antigen in a processed form in association with MHC molecules.

2.1.1 Antigen recognition by B cells

B cells have immunoglobulins on their cell surface which act as antigen receptors, recognising determinants on intact native antigens. These antigen receptors are expressed by mature B cells. Recognition of antigen by surface immunoglobulins is a prerequisite for B cell activation. These activated B cells can then function as antigen presenting cells (APC), the antigen is taken up processed and presented by the B cell on the cell surface in association with MHC class II molecules. B cells can function as APC's for helper T cells (Rock et al 1984). This collaboration is important for the generation of an antibody response. T lymphocytes produce lymphokines which cause the activated B cell to differentiate and become antibody secreting plasma cells.
2.2 Antigen recognition by T cells

During an immune response the initial activation of the quiescent T cell is mediated by the interaction of the T cell receptor (TCR) with the antigenic peptide present in the antigen binding groove of a self MHC molecule or a foreign MHC molecule present on an antigen presenting cell. However it is now known that additional signals are also required for T cell activation. The original two signal model for lymphocyte activation was first described by Bretscher and Cohn in 1970 (reviewed by Bretscher 1992). In the absence of these second signals T cell anergy may occur (Schwartz 1990).

2.2.1 The T-cell receptor

Structure of the TCR

The TCR complex consists of a Ti heterodimer noncovalently associated with CD3 and a ζ containing homodimer or heterodimer (fig 2.1). The most common TCR is formed from one α and one β chain although there is a γδ form. The Ti chains are highly polymorphic and are structurally unique for each clone of T cells. They are immunoglobulin-like structures possessing constant and variable domains and a short cytoplasmic tail of 5 aa. The α/β chains are linked by a single disulphide bond. The CD3 complex consists of three chains γ, δ and ε, these chains are non polymorphic transmembrane proteins that are also immunoglobulin like. They have cytoplasmic domains ranging from 40 to 80 aa. The Ti/CD3 complex is noncovalently associated with a ζ dimer. The ζ chain is a transmembrane protein with a 9aa extracellular domain, a single transmembrane domain and cytoplasmic domain of 113 aa (Weissman et al 1988). It exists primarily as a homodimer although a small percentage of TCRs
Figure 2.1

Diagrammatical representation of the T cell receptor complex

(S-S denotes di-sulphide bridge)
contain a heterodimer of ζ chain complexed to a η chain (an alternatively spliced product of the ζ chain).

Expression of the TCR
The Ti heterodimer exists in two forms an α/β and γ/δ form. The α/β heterodimer is the most abundant form, being expressed on >95% of peripheral T cells and receptor expressing thymocytes. It appears to be the most important for mediating MHC restricted recognition, although the γ/δ form has been shown to recognise both class I and class II MHC (Matis et al 1989). Gamma/delta T cells were first discovered following isolation of cDNA clones (Brenner et al 1986). They appear earlier than α/β TCR in ontogeny. These T cells are present on a small percentage of peripheral T cells (1-10%). Each of the four TCR polypeptides are similar to one another each have V, J and C regions, β and δ also have D regions. TCR genes are first rearranged and expressed in the thymus during the earliest stages of T cell differentiation. TCR β, γ and δ chain genes are rearranged and transcribed first followed by the α chain. However α/β and γ/δ T cells are from two distinct lineages (Winoto and Baltimore 1989). The appearance of the γ/δ heterodimer precedes α/β expression by 1 or 2 days in fetal ontogeny (Chien et al 1987).

Diversity of the TCR
The TCR is formed from the rearrangement of the germline genes V, D and J connected to a constant region. The variability of the TCR is generated by the association of different V, D and J genes and by variation in the exact point of joining giving rise to local amino acid diversity at the junctions. Additional nucleotides not
encoded by either gene segment can be added at the junctions joining gene segments during rearrangement giving rise to further diversity.

Contact of the TCR with peptide and the MHC

The α/β, γ/δ chains of the TCR bind to the peptide/MHC complex (Dembic et al 1986). The V-(D)-J junctional region of the TCR (equivalent to the CDR3 region of immunoglobulin) is principally responsible for peptide contact, whereas the CDR1 and CDR2 regions contact the α helices of the MHC (Davies and Bjorkman 1988). MHC-TCR contacts are ‘fluid’ in that they can be made in a variety of ways with the same TCR and MHC, the final configuration appears to be significantly influenced by the peptide (Jorgensen et al 1992). Activation of the TCR by ligand binding of the Ti heterodimer leads to signal transduction via the CD3 ε chain (Letourner and Klausner 1992) and the ζ chains (Sussman et al 1988). However it is now known that T cell activation is not an on or off event. Studies by Evavold and Allen (1991) among others have shown the TCR to selectively trigger effector functions. The TCR was found to focus most intently on one residue of the peptide in the MHC binding groove, termed the primary T cell contact residue, other critical contact residues were termed secondary contact residues. Alterations to the primary contact residue results in little or no interaction with the TCR. Alterations in the secondary contact residues of the peptide can lead to either a partial agonistic response where, although incapable of inducing clonal proliferation, T cell functional responses such as T\textsubscript{H}1-mediated cytolysis and T\textsubscript{H}2-mediated help can still be elicited (Evavold et al 1993). Non-conservative alterations to secondary contact residues results in an antagonistic response whereby no identifiable signals are delivered to the T cell. However, when
this altered peptide analogue is presented to the T cell along with stimulatory peptides it causes a dose dependent inhibition of T cell proliferation (Ostrov et al 1993). The differences in these responses are due to changes in signaling events. Partial agonistic responses result in no signal transduction through the ζ chains whereas antagonistic responses show no signal transduction through the ζ chains and only partial activation of the CD3 complex (Evavold et al 1993).

2.2.2 CD4 and CD8 co-receptors
The CD4 and CD8 molecules are known to facilitate both cell adhesion and TCR signal transduction, forming a ternary complex that comprises the TCR and MHC molecules. MHC class I and class II molecules interact with the extracellular domains of CD4 and CD8 respectively. CD4 binds to the β2 domain of MHC class II (Cammarota et al 1992), whereas CD8 binds to the α3 segment of MHC class I (Salter et al 1990). Thus CD4 and CD8 could co-aggregate with the TCR complex as a consequence of binding distinct sites on the same MHC molecule involved in antigen presentation (Kupfer et al 1987). The cytoplasmic domains of both CD4 and CD8 have been shown to be physically associated with the src-related protein tyrosine kinase p56^ck which is involved in signal transduction (Rudd et al 1988). The significance of the association of CD4 with lck has been demonstrated with the use of a CD4 dependent, antigen specific, murine T cell that lacked endogenous CD4 or CD8. Restoration of TCR function occurred only after the introduction of CD4 molecules that were capable of associating with lck (Glaichenhaus et al 1992). CD45 may also play a role in TCR signal transduction (Pingel and Thomas 1989).
2.3 Additional molecules involved in T cell activation

There are many molecules present on the surface of T cells which, with their corresponding ligands on antigen presenting cells, may play a role in providing the additional signals required for T cell activation (fig 2.2). Prior to the cognate interaction of the TCR with the MHC-peptide complex, non-cognate adhesion needs to occur between the T cell and antigen presenting cell. This is facilitated by receptor-ligand pairs such as endothelial leukocyte adhesion molecule (ELAM)-1 and E selectin (Shimizu et al 1991).

2.3.1 Co-stimulation

Following cognate interaction of the TCR, additional signals are required for T cell activation, this is termed co-stimulation. Candidate molecules include the B7 family and its ligands CD28 and CTLA4, the adhesion molecules lymphocyte function-associated antigen (LFA)-3 which binds to CD2 receptor on T cells, intercellular adhesion molecule (ICAM)-1 and ICAM-2 with its receptor LFA-1, vascular cell adhesion molecule (VCAM)1 which binds to VLA-4 on T cells and heat stable antigen (HSA). Other molecules include CD40 and its ligand CD40L. Of the molecules studied to date the CD28-B7 receptor-ligand pair appears to be the most important. However these other receptor-ligand pairs may also have a role to play in co-stimulation.
Figure 2.2

Cell-cell contact involving TCR interaction with the MHC plus peptide (●), and accessory molecules involved in co-stimulation.
LFA-3
LFA-3 is a widely distributed adhesion molecule that binds to the CD2 receptor on T cells. It was thought to play a role in co-stimulation following studies that showed anti-CD2 mabs capable of stimulating T cell proliferation (Meurer et al 1984).

ICAM-1
Transfection of human ICAM-1 cDNA into MHC class II transfected fibroblasts was shown to enhance MHC class II restricted T-cell proliferative responses (Altmann et al 1989). ICAM-1 has been shown to be capable of delivering a transmembrane signal by Rothlein et al (1994).

VCAM-1
Studies by van Seventer et al (1991) among others has shown VCAM-1 to be a co-stimulatory molecule, antibodies to VLA-4 receptor have been shown to block the effects of VCAM-1 on T cell proliferation. HSA is a widely distributed and highly glycosylated molecule found on different types of APC, blocking of HSA with mabs led to T cell unresponsiveness (Liu et al 1992).

CD40
CD40 is a surface glycoprotein of 45-50kD. It is expressed on follicular dendritic cells and B cells among others. It’s ligand CD40L is expressed on activated but not resting T cells. When this CD40-CD40L interaction is inhibited, B cells are unable to proliferate or produce immunoglobulin in response to T cell signals (Spriggs et al 1992).
2.3.2 Co-stimulation via B7-CD28

CD28

CD28 is a cell surface glycoprotein composed of two identical disulphide linked 44 kD subunits that are encoded by an immunoglobulin like gene. This molecule is expressed by all human CD4+ T cells and approximately half of CD8+ T cells. In the mouse CD28 is expressed on all CD4+ T cells and CD8+ T cells. Evidence that CD28 was involved in signalling events distinct from that of the TCR complex was demonstrated when CD28 in conjunction with phorbol myristate acetate (PMA) induced cyclosporin independent T cell proliferation and interleukin 2 production (Thompson et al 1989). Further experiments by Harding et al (1992) and others showed that prevention of CD28 activation with anti-CD28 mabs during the activation of a T cell clone by antigen primed APC's, resulted in the failure of the clone to respond to subsequent antigenic challenge. The identification of a ligand for CD28 was demonstrated by Linsley et al (1990) who used antibodies to B7 to block the adhesion of B cells to Chinese hamster ovary cells transfected with CD28. Further work showed that cells transfected with B7 could provide co-stimulatory signals to antigen or mitogen activated T cells. B7 is a cell surface glycoprotein with a single immunoglobulin v-like and a single immunoglobulin C like domain.

CTLA4

An alternative ligand for B7 was demonstrated with the use of CTLA4-Ig. CTLA4-Ig is a fusion protein combining by genetic techniques the extracellular domain of human
CTLA4 and the constant region of human IgG1 (Linsley et al 1992a). This fusion protein was found to bind to B7 with a 20-fold higher affinity than CD28. CTLA4 (cytolytic T lymphocyte associated antigen) is a homologue of CD28 but follows a different distribution pattern being expressed only on T cells after activation. The relationship between CD28, CTLA4 and their ligands have recently been shown to be more complex than originally thought, with the elucidation of the B7 receptor family.

The B7 receptor family

Freeman et al (1993a) reported the isolation of a gene for a non-B7 receptor identified by the GL1 mab that bound CTLA4. It appeared significantly earlier on the cell surface than B7 following activation. This receptor was termed B7-2 and the original B7 molecule B7-1. Studies by others e.g. Hathcock et al (1993) also revealed the presence of an additional ligand for CTLA4. Azuma et al (1993) generated a monoclonal antibody IT2 that reacted with a 70kD glycoprotein termed B70. IT2 substantially inhibited the binding of CTLA4-Ig to B cell lines. Comparison of the nucleotide sequences of B70 and B7-2 revealed them to be the same gene. Additional ligands may also exist, Boussiotis et al (1993) proposed the existence of a third ligand termed B7-3 identified by the anti BB-1 mab.

Model for the interaction of CD28 and B7

A model for the interaction of the CD28 and B7 families on T and B cells suggests that the binding of native antigen to surface Ig on resting B cells induces rapid expression of B7-2 which is the principal ligand for CD28. This interaction would coincide with the presentation of processed antigen to the T cell and is sufficient for non proliferative
responses such as cytokine production. At this time the expression of CTLA4 (the principal ligand for B7-1, now termed CD80) is minimal. After the first 72 hours CTLA4 expression is maximal as is CD80 expression, this leads to preferential signalling through the CTLA4 pathway. The role of this additional pathway is unclear. CTLA4 has a higher affinity for CD80 and B7-2. It is therefore possible that CTLA4 may terminate CD28 mediated signal transduction by competition for shared ligands (Ronchese et al 1994). Alternatively it may amplify the response by stabilising and prolonging the CD28 mediated signals (Linsley et al 1992b).

In an immune response it is important for T cells and B cells to ‘communicate’ with one another. The CD40-CD40L interaction mentioned above enables a B cell to respond to an activated T cell whereas the CD28-CD80 interaction enables a T cell to respond to an activated B cell. CD80 expression may therefore coincide with CD40L expression on T cells. It is now known that cells other than professional antigen presenting cells such as macrophages and dendritic cells can also present antigen. Professional APCs constitutively express MHC class II molecules on their surface as well as adhesion molecules. Non-professional APCs acquire MHC class II antigens when activated. T cells fall into this latter category (Barnaba et al 1994). Activated T cells express MHC class II molecules as well as molecules involved in co-stimulation. Recent evidence has shown that activated T cells can also express CD80 (Azuma et al 1993) which was formerly described only on professional APCs and activated B cells. It was found to be preferentially expressed on activated T cells infiltrating the tissues but not on tissues in the circulation. It is thought that these T-APCs may function as negative APCs inducing anergy (Pilcher and Wyss-Coray 1994).
**In vivo studies using CTLA4-Ig**

The CTLA4-Ig fusion protein has been used in a number of *in vivo* systems. Lenschow *et al* (1992) used CTLA4-Ig to prolong survival of human pancreatic islets placed under the kidney capsules of diabetic mice. This indicated that donor-specific tolerance had occurred in the presence of CTLA4-Ig. CTLA4-Ig has also been shown to diminish the antibody response of mice to sheep red blood cells and keyhole limpet haemocyanin, indicating that it is a potent suppressor of primary antibody responses *in vivo*. However in these experiments large doses of CTLA4-Ig were required to diminish secondary antibody responses. Gimmi *et al* (1993) showed that in an antigen specific model antigen presentation by MHC class II molecules in the absence of B7 costimulation results in human T cell anergy. However when antigen is presented by MHC class II molecules on cells that also express B7, optimal T cell proliferation and cytokine production are induced.

### 2.4 Allorecognition

T cells recognise antigens as processed peptides presented by self MHC molecules on the surface of antigen presenting cells. This form of antigen recognition, where the peptide is processed and presented to the T cell in the context of self MHC on antigen presenting cells is termed indirect presentation in transplantation. This is the usual route for T cell recognition in an immune response. However the frequency of T cells reactive against a foreign MHC haplotype is 1 to 10% of the total population, although these alloreactive T cells belong to the same lineage as antigen-specific T cells (Matis
et al 1987). Two hypotheses have been proposed to account for the high frequency of alloreactive T cells. Firstly, Matzinger and Bevan (1977) proposed that alloreactive T cells recognised allo-MHC plus x, where x represents an array of endogenous molecules. A single type of allogeneic MHC molecule can therefore give rise to multiple distinct specificities by forming binary complexes with an array of endogenous molecules (now known to be peptides). Each of these peptide-MHC complexes could then stimulate a separate population of T cells, thereby accounting for the high precursor frequency of alloreactive T cells. The second hypothesis proposed by Bevan (1984) suggests that recognition of peptides is not important and the attention of the alloreactive T cells is focused on the allo-MHC molecule. Thus all the foreign MHC molecules (of the appropriate category) on the allostimulator cell can act as ligands leading to a high determinant density.

2.4.1 Contribution of MHC bound peptides to alloreognition

One of the first experiments to suggest that peptides bound by the MHC were also involved in allore cognition, demonstrated that incubation of class II presenting cells with exogenous antigen reduced the ability of alloreactive cells to recognise class I molecules. This would occur if these high concentrations of exogenous antigen inhibited presentation of a different peptide required for allore cognition (Eckels et al 1988). Demonstration of the importance of peptide in allore cognition became easier with the identification of mutant cell lines e.g. T2 and RMA-S defective in the processing and transport of endogenous peptides. Heath et al (1991) used a derivative of one of these lines transfected with the K\textsuperscript{b} gene (T2-K\textsuperscript{b}) that expressed relatively normal levels of K\textsuperscript{b} on its surface that were devoid of peptide. K\textsuperscript{b} specific
clones were used to react with these transfectants, however many of the clones failed to recognise the empty molecules. If these cell lines were exposed to cellular peptides that had been obtained either through CNBR cleavage of cytoplasmic proteins or through acid extraction of processed peptides from whole cell lysates or purified K^b molecules, the CTL clones that failed to recognise the empty class I molecules were then able to recognise them. These studies among others suggest that for many alloreactive T cells the peptide in the antigen binding groove of class I or class II molecules makes a critical contribution to alloreognition.

2.5 Indirect alloreognition

The interaction of the TCR directly with the allo-MHC molecule is termed direct presentation. This pathway of presentation does not require self APCs and was considered to be the primary route for T cell activation in transplant rejection. However alloantigens (including allo-MHC molecules) shed by the graft can be recognised via the indirect pathway. It is likely that allo-MHC molecules would make up the bulk of the shed alloantigens. Studies have shown the existence of the indirect pathway for alloreognition. However the importance of this process in the mechanisms of rejection still remains unclear. Liu et al (1993) showed that in in vitro studies, T cells involved in indirect recognition are 100 fold less frequent than T cells participating in the direct recognition pathway.
2.5.1 *In vivo* studies of indirect recognition

*In vivo* evidence of indirect presentation in a rejection response was shown by Sherwood *et al* (1986). Recipient CBA mice were sensitised by transfer of T cell depleted adherent splenocytes from primary host CBA mice, immunised with BALB/c cells. These recipients rejected BALB/c skin grafts in an accelerated fashion. Sensitisation could only have occurred through indirect recognition of allopeptides presented by the syngeneic splenocytes, which had been transferred to the recipients. Evidence of indirect presentation *in vivo* has also been demonstrated. BALB/c and SJL mice were sensitised to B10 mice by either infusion of spleen cells or a skin graft. The T cells from the sensitised mice were shown to proliferate to synthetic peptides derived from the polymorphic regions of α and β chains of class II molecules presented by host APC Benichou *et al* (1992). Studies by Dalchau *et al* (1992) from our laboratory demonstrated that priming to indirect allorecognition could be achieved by immunisation with pure denatured DA class I heavy chain and class II α and β chains. DA skin grafts to immunised LEW recipients were rejected in an accelerated fashion, these immunised LEW rats produced alloantibodies to each of the denatured chains but not to intact molecules on the cell surface. This excludes antibody formed prior to grafting from having a role in this accelerated rejection response. Further studies by Fangmann *et al* (1992a) from our laboratory demonstrated that administration of a rat RT1-A<sup>av1</sup> allo-peptide to LEW, induced accelerated rejection of DA skin grafts and increased kinetics of antibody production to the intact RT1-A<sup>av1</sup> molecule. These studies indicate that indirect recognition can play a role in the effector mechanisms of allograft rejection.
CHAPTER 3:
MECHANISMS OF ALLOGRAFT REJECTION

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3.1 Introduction

The mechanisms involved in the rejection response are many and varied, with a myriad of complex interactions between cells. These complex immunological processes have not been fully elucidated.

There are three types of rejection: **Hyperacute rejection** (HAR) which results in almost immediate graft destruction, within minutes or hours of transplantation; **acute rejection** which has a rapid onset and occurs usually within the first 6-12 months after grafting; and **chronic rejection** which results in a slow decline of graft function and can occur months or years post-transplant. Each of these are caused by different mechanisms. Acute rejection is predominantly mediated by cellular immune responses, although antibodies may play a part. Hyperacute rejection involves a pre-existing humoral response. The mechanisms involved in chronic rejection are unclear.

3.2 Acute rejection

3.2.1 Initiation of rejection

**Role of dendritic cells in the initiation of rejection**

The activation of unprimed T cells by allogeneic dendritic cells is thought to be an important route for the initiation of rejection. Dendritic cells (DC) were first defined by Steinman *et al* (1973) in lymphoid tissues. They were shown to be potent stimulators of unprimed allogeneic T cells in mixed lymphocyte reactions (Klinkert *et al* 1982).
Dendritic cells were subsequently shown to be resident in virtually all connective tissues of the body, and these were called interstitial dendritic cells (Hart and Fabre 1981). Mouse interstitial dendritic cells were found to be positive for CD45, MHC class II, CD11b, F4/80 antigen and CD44 markers (Austyn et al 1994) and B7 (Larsen et al 1992). Dendritic cells have been shown to be the critical sensitising elements following transplantation of vascularised grafts. Removal of allogeneic interstitial dendritic cells (also termed passenger leucocytes) by ‘parking’ a kidney in a tolerant host while the resident donor dendritic cells are replaced by recipient dendritic cells before retransplantation into an immunocompetent host syngeneic to the first host leads to prolonged survival (Batchelor et al 1979). Repopulation of the host with donor dendritic cells was found to lead to rejection of the graft (Lechler and Batchelor 1982).

Role of vascular endothelium in the initiation of rejection

Class II+ human endothelial cells can cause direct allostimulation of resting CD4+ and CD8+ T cells (Savage et al 1993) and can act as antigen presenting cells. Cultured human umbilical vein endothelial cells (HUVEC) do not constitutively express class II molecules but these can be induced by γIFN. However, quiescent HUVEC have been shown to be able to cause direct allostimulation of CD8+ helper T cells (Adams et al 1994). This T cell stimulation does not require co-stimulation through the CD28-CD80 pathway (Page et al 1994) although the T cell ligands CD2, LFA-1 and CD44, via interactions with the endothelial cell ligands LFA-3 and ICAM-1, were shown to be important (Westphal et al 1993).
Activation of T cells can also occur by indirect recognition, alloantigens shed from the graft can be processed and presented in association with MHC on recipient antigen presenting cells.

3.2.2 Responding T cells

CD4+ or CD8+ phenotype confers MHC restriction. CD8+ T cells recognise endogenously derived antigen in association with MHC class I molecules, whereas CD4+ T cells recognise exogenously derived antigens in association with MHC class II molecules. However these definitions have recently become less clear. CD4+ T cells function mostly as helper/inducer T cells, mediating their effects with the release of cytokines including IL-2 thereby providing help for the generation of antibody and the development of cytotoxic T cells. CD8+ T cells are described as cytotoxic cells, these cells secrete mainly γIFN and TNFβ, and are unable to provide help for the generation of antibody. Cytotoxic T cells are capable of causing lysis of target cells by the release of granzymes and perforins. However there is significant functional overlap between the two phenotypes, class I restricted CD8+ T cells may release certain lymphokines and conversely T cells that are cytotoxic towards class II MHC are found within the CD4+ helper phenotype.

Role of CD4 and CD8 subsets in rejection

T cells play a pivotal role in the rejection response. However, there has been much controversy over the roles played by CD4+ and CD8+ T cells in the mechanisms of allograft rejection. Early studies indicated a major role for CD8+ T cells in mediating allograft rejection as specific cytotoxic T cells could be readily isolated from rejecting
organ grafts (Strom et al 1975). However it was later shown by Loveland et al (1981) that ATXBM mice (mice rendered T cell deficient by thymectomy, lethal irradiation and bone marrow restoration) were capable of rejecting skin allografts following reconstitution with CD4+ T cells but not CD8+ T cells. The role of cytotoxic cells was not excluded by these experiments as CD8+ cells were found in the rejecting skin grafts, and the possibility of CD4+ cytotoxic cells could not be ruled out. The use of the nude rat has made the study of this question easier. Nude rats are athymic, although they possess some γ/δ T cells and possibly CD8+ NK cells (Bolton et al 1989). Nude rats are unable to reject skin and vascularised organ allografts. Nude rats injected intravenously with T cells are able to reject their allografts. If separated populations of T cells are used, purified CD4+ T cells are capable of eliciting a rejection response whereas purified CD8+ T cells are not (Bolton et al 1989). This implies that CD8+ T cells are dependent on CD4+ T cell help for their activation and proliferation. However the rejection of class I disparate skin allografts is initiated only if CD8+ T cells are included in the inoculum (Bell et al 1990). In order to overcome the problem of residual CD8+ T cells in ATXBM and nude rats, monoclonal antibodies (mabs) to either of the subsets can be administered following transplantation. In the rat, survival of class I disparate vascularised organ grafts can be extended with mabs to CD4+ T cells but not CD8+ T cells, (Gracie et al 1990) this is also the case for fully mismatched allografts (Wood et al 1991). Thus it appears that CD4+ T cells are necessary and sufficient for mediating the rejection response whereas CD8+ T cells are not essential and by themselves insufficient to mediate rejection. However in sensitised T cell populations, CD8+ cells are less dependent on CD4+ T cells for activation (Cobbold and Waldmann 1986).
3.2.3 Effector mechanisms

Role of CD4+ subsets in the rejection response

Once activated CD4+ T cells, by cytokine release, help or activate CD8+ T cells, NK cells, macrophages, B cells and cause a DTH type reaction which alters the endothelium of the graft to recruit nonspecific inflammatory cells. The trafficking of cells to and adhesion in the area of inflammation are mediated by adhesion molecule-ligand pairs. Some leukocytes undergo proliferation within the graft particularly the lymphocytes specific for antigens in the graft. Others such as macrophages undergo activation and immobilisation in response to the products of activated T cells. Graft inflammation is characterised by changes in vascular flow and permeability and the influx of leukocytes to the area of injury, classic signs of inflammation i.e. redness, oedema and loss of function are present in an acutely rejecting graft.

In 1986 Mossman et al proposed a sub division of mouse CD4+ T cell clones into Th0, Th1 and Th2 subsets based on differences in their pattern of cytokine production. However human T cell clones proved difficult to classify into these subsets until studies by Romagnani et al (1991) demonstrated the existence of CD4+ T cell clones that exhibited Th1 or Th2 like profiles in the tissues or peripheral blood of patients in different disease states. Th0 cells produce IL-2, IL-4, γIFN and lymphotoxin, Th1 cells produce IL-2, γIFN and lymphotoxin, and Th2 cells IL-4, IL-5, IL-6 and IL-10 (Mossman 1991). Naive T cells produce large amounts of IL-2 when first stimulated with antigen in the presence of APCs, continuing stimulation evokes production of IL-4 and γIFN (Th0). If the primary stimulation is accompanied by exogenous IL-4, the CD4+ T cells commit to a Th2 cytokine profile. However, the presence of anti-IL-4
mabs in the culture promotes expression of the Th1 phenotype. These and other studies demonstrate that naive T cells are not precommitted to a particular phenotype, but that the Th phenotype varies with the microenvironment. The release of cytokines from the different phenotypes into the microenvironment may cross regulate one another, so that activation of the Th1 phenotype may inhibit differentiation into the Th2 phenotype and vice-versa. γIFN inhibits the synthesis of cytokines by Th2 but not Th1 cells whereas IL-10 inhibits the synthesis of Th1 but not Th2 cells.

Activation of the Th1 phenotype is strongly associated with cell mediated immunity whereas the Th2 phenotype is often associated with humoral immunity.

Role of cytokines in the rejection response
Cunningham et al (1994) investigated the expression of cytokines in endomyocardial biopsies and found no one cytokine to be a predictor of rejection. However IL-2, IL-4 and IL-10 were found to be the cytokines most commonly associated with histopathological evidence of rejection while IL-1β, TNFα and TNFβ were also common where there was no evidence of rejection. Dallman et al (1991) using a murine heterotopic cardiac model demonstrated the expression of IL-1α and IL-3 in syngeneic and allogeneic grafts whereas IL-2, IL-4 and TNFβ were found only in allografts and IL-1β, IL-5, IL-6 and γIFN were present in the normal murine heart. Thus IL-2 appears to be expressed exclusively in allogeneic grafts. Takeuchi et al (1992) demonstrated enhanced levels of the Th2 cytokines IL-4 and IL-10 in mice tolerant to their cardiac allografts with a reduction in the levels of Th1 cytokines IL-2 and γIFN.
**CTL responses**

Activated CD8+ cytotoxic cells can cause direct lysis of target cells. CTL secrete soluble lytic proteins (granzymes and perforins) from specialised secretory granules leading to the lysis and nuclear degradation of the target cell. The specificity of killing by these granules is maintained by granule release at the point of membrane contact between the CTL and its target thereby preventing cells that are not recognised by the TCR from being lysed (reviewed by Griffiths 1995).

**Humoral rejection responses**

The predominant immunological mechanism in acute rejection is the cellular response discussed above. T cells activated during the rejection response produce cytokines that provide help for B cells enabling activated B cells to produce antibodies to the graft. Antibody responses have been shown to HLA antigens (Sucicu-Foca *et al* 1991) and non HLA antigens including antigens expressed on endothelial, monocytes and epithelial cell lines (Al -Hussein *et al* 1995). It is unclear whether these antibodies play an active role in damaging the graft although their presence has been correlated with rejection. In contrast anti-HLA donor reactive antibodies have been associated with an increase in the severity of rejection episodes (Smith *et al* 1992).

**MHC expression and induction**

Activation of T cells, by interaction of the TCR with MHC, indicates that MHC molecules play a central role in the immune response.
The level of expression of MHC molecules on a cell is not a fixed characteristic but can be influenced by a number of factors. Increase in expression of MHC molecules increases the grafts capacity to present foreign antigens to the T lymphocyte system. MHC class I molecules are expressed by most nucleated cells, whereas MHC class II molecules have a more restricted tissue distribution, mainly on antigen presenting cells including IDC, and vascular endothelial cells (although vascular endothelial cells of the rat are class II-).

Increased levels of MHC expression have been noted following transplantation. Experimental transplantation of isografts i.e. in the complete absence of rejection, results in a mild increase in class I expression in the graft but no increase in class II expression, with the exception of the Kupffer cells of the liver where isografts show class II induction (Settaf et al. 1988).

During allograft rejection expression of MHC class I molecules increases so that class I negative cells become positive and weakly class I positive cells become strongly positive. Milton and Fabre (1985) reported a 10-fold increase in class I expression in rat cardiac allografts by day 5 of rejection, as a result of induction of class I antigens on myocardial cells and other cells in the heart.

Induction of class II MHC molecules is more complex than that for class I. Kidney and pancreas show abundant induction of class II molecules on the parenchymal and vascular endothelial cells although the glomerulus of the kidney (Milton et al. 1986) and the islets of Langerhans in the pancreas are resistant to class II induction during a rejection response (Steiniger et al. 1985). Induction of class II on myocardial cells is variable and may be related to the vigour of the rejection response, by day 5 of rejection both endothelial and myocardial cells strongly express class II molecules.
Class II MHC antigens in livers are normally restricted to isolated class II positive cells found mainly in the portal triad and only very occasionally in the hepatic lobule. These probably represent interstitial dendritic cells. Interestingly hepatocytes have a poor capacity for the expression of class II MHC molecules during the rejection response in orthotopic liver grafts. However Icard et al (1990) demonstrated that MHC class II induction occurs by day 5 in auxiliary DA-to-PVG liver grafts.

Induction and upregulation of MHC expression by cytokines probably plays an important role in the rejection response. α IFN from a wide variety of leucocytes and β IFN from fibroblasts are able to induce class I MHC molecules (Fellous et al 1982), whereas γIFN is a potent inducer of both class I and class II MHC molecules (Morhen et al 1984). Gamma IFN expression has been shown by RT-PCR in human renal allograft fine-needle aspirates to occur in active acute rejection preceding clinical acute rejection (Nast et al 1994).

Upregulation of MHC class II expression has also been demonstrated in kidney and liver transplants during CMV infection (Lautenschlager et al 1990). It has been suggested that this upregulation is mediated by γIFN produced by activated lymphocytes during viral infection. However a direct mechanism of upregulation by the virus may also exist. Ustinov et al (1994) demonstrated CMV infection of kidney and liver parenchyma resulting in induction of MHC class II molecules in the absence of lymphocytic infiltration into the organ.

The expression of class II MHC on the cell surface does not necessarily confer the ability to activate T cells. The expression of other co-stimulatory and adhesion ligands
also plays a role. Class II+ vascular endothelial cells have the capacity to activate naïve T cells as discussed in 3.2.1. However, class II+ renal tubular epithelial cells fail to activate T cells. Cultured renal tubular epithelial cells express MHC class I, class II and the adhesion molecules ICAM-1, VCA-1 and LFA-3 when stimulated with γIFN, however B7 is not expressed (Wilson et al 1995). The absence of a co-stimulatory signal could explain the observations that mixed culture of allogeneic renal cells with lymphocytes results in hyporesponsiveness of the lymphocytes to the donor alloantigen (Kirby et al 1993).

Increased expression and induction of MHC class I molecules in the grafts increases the susceptibility of these cells to the host's cytotoxic T cell response.

3.3 Chronic rejection

Although the introduction of improved immunosuppressive therapies has lead to an increase in graft survival, unfortunately long term graft survival past 1 year has not significantly improved with 3 year graft survival reported from 78% to 56% in cardiac recipients. The histopathological changes seen in chronic rejection tend to be dominated by fibrosis, and vascular changes consisting of concentric fibrointimal thickening. Kidney transplants show glomerulopathy, liver transplants demonstrate vanishing bile duct syndrome, lung transplants show obliterative bronchiolitis and heart transplants accelerated coronary artery disease (CAD).

Actuarial graft survival is significantly reduced in patients with clinical and histological evidence of rejection. Ferguson et al 1992 demonstrated that kidneys that have never experienced acute rejection episodes are unlikely to develop chronic rejection. Knight
et al (1991) reported 51% and 25% 1 year and 3 year graft survival rates respectively in kidney transplant recipients with chronic rejection compared with 98% and 86% in a matched cohort of controls.

In cardiac transplantation, CAD affects 6% of patients at 1-year post transplant rising to 17% at three years (Rose 1993). Circulating donor-specific alloantibodies have been correlated with poor long term survival (Suciu-Foca et al 1991). However there is no clear evidence that the anti-HLA antibodies produced by patients following transplantation are associated with CAD. Dunn et al (1992) have demonstrated in CAD patients the presence of anti-endothelial antibodies which, in the majority of cases, are induced following transplantation. These IgM antibodies were found by immunofluorescent staining techniques to be specific for antigens on the endothelial cell surface of large vessels and not the microvasculature. This correlates with the distribution of CAD to the epicardial and larger myocardial vessels but not the myocardial capillaries.

The incidence of chronic rejection in liver transplants varies between 4% and 17% and has been suggested to be associated with HLA mismatching, CMV infections (O'Grady et al 1988) and in some cases pretransplant donor specific antibodies (Gordon et al 1986). In lung transplants bronchiolitis obliterans is associated with a history of acute rejection episodes.

Heidenreich et al (1994) suggested that chronic rejection could be divided into two subtypes, a subtype with high immune activation and a subtype with low immune activation. They found that patients with high αTNF responses to LPS stimulation of their monocytes in vitro showed a significantly elevated progression to transplant
failure during the one year study period compared to patients with low αTNF responses to LPS stimulation of monocytes, this group also suggested that monocytes were preactivated in chronic rejection. Using a rat model of chronic rejection LEW (RT1\(^{b}\)) to F-344 (RT1\(^{a}\)) Orloff et al (1995) demonstrated that in recipient animals where tolerance had been induced the occurrence of chronic rejection was significantly reduced. Interestingly in these animals cytokine levels were diminished including IL-2, IL-4 and γIFN suggesting that these cytokines have a role in chronic rejection. This was also shown by the use of anti-γIFN antibodies in a murine cardiac model leading to a reduced incidence of obstructive coronary arterial lesions (Russell et al 1994).

Tullius et al (1994) used a rat model of chronic rejection and found that if the kidney was retransplanted back into the donor (thereby removing any further immunological insult) up to 12 weeks post transplant, the damage caused by chronic rejection was reversible, if however they were retransplanted after 12 weeks the damage was irreversible. This suggests that there are alloantigen independent factors involved in the later stages of chronic rejection.

3.4 Hyperacute allograft rejection

The mechanisms of hyperacute rejection will be discussed in detail in chapter 4. Although rare in the clinical setting, because of preventive cross-matching procedures, hyperacute rejection in allografts is said to occur if rejection takes place within 24 hours of transplantation. In clinical transplantation hyperacute rejection in renal allografts is avoided by matching for ABO blood groups and cross matching of the prospective donor. The patients serum is incubated with donor lymphocytes in the
presence of complement. Lysis of the cells results in a positive crossmatch and is a contraindication for the transplant. Hyperacute rejection occurs in approximately 0.5% of cardiac transplants as prospective crossmatching is difficult to perform due to the shorter ischaemic times necessary for hearts (Rose 1993). In cardiac allografts it is associated with a large infiltrate of polymorphonuclear granulocytes with damage to the vascular endothelium and deposits of IgG, IgM and the complement components C1 and C3.

3.4.1 Role of antibodies in hyperacute allograft rejection

In cardiac transplantation patients with a high PRA (panel reactive antibodies) are more likely to have a positive crossmatch from 4.5% with a PRA of 0 to 10% to 75% with a PRA of 51% to 100%. However a positive crossmatch does not necessarily result in hyperacute rejection although long term actuarial graft survival is reduced (Rose 1993). Positive crossmatch against donor T cells by patient IgG antibodies was shown to be predictive of early graft failure which suggests that the heart undergoes rapid rejection in the presence of IgG antibodies. Non-lymphocytotoxic antibodies have also been reported to have a role in hyperacute rejection. Hyperacute rejection has been reported in patients with a negative crossmatch. Immunohistology of these grafts revealed IgG, IgM and complement deposits on the endothelial cell surface. These antibodies could be against HLA class I or class II which are expressed on endothelial cells or some other surface antigen (Trento et al 1988). Preformed lymphocytotoxic antibodies have also been shown to have a deleterious effect on graft survival. In liver transplantation 'hyperacute rejection' is rare although there is a higher
incidence of rejection within one month of transplantation with a positive cross match (Ogura et al 1994).

3.5 Strategies for immunosuppression

3.5.1 Immunosuppressive Drugs

The first immunosuppressive drug available for transplantation was 6-mercaptopurine. This drug, from which azathioprine was derived, was shown to suppress both primary and secondary antibody production (Schwartz and Damesk 1959). Azathioprine has been used extensively in clinical transplantation in conjunction with corticosteroids or cyclosporin.

Immunosuppressive drugs act at different levels; azathioprine, mizorbine, mycophenolate mofetil, cyclophosphamide and brequinar sodium inhibit DNA synthesis and so act at the level of proliferation. Rapamycin and leflunomide inhibit the action of cytokines notably IL-2. Cyclosporin and FK506 inhibit IL-2 gene transcription and deoxyspergualin inhibits monocyte/macrophage function.

Corticosteroids cause lysis of immature cortical thymocytes as well as certain T cell lines by activating endogenous nucleases that cleave DNA. However, corticosteroids do not lyse mature medullary thymocytes or mature T cells isolated from blood or peripheral lymphoid organs. Corticosteroids also act by blocking cytokine gene transcription in and cytokine secretion from mononuclear phagocytes.
Cyclosporin A

Unlike the earlier immunosuppressive drugs used in transplantation, CsA selectively inhibits adaptive immune responses. The major action of CsA is to inhibit transcription of the IL-2 gene. CsA binds to cyclophilin, the resulting complex binds to calcineurin and inhibits its function. The absence of IL-2 results in a failure of T cells to mount an effective immune response, as IL-2 is central to this immune response. The advent of CsA into clinical transplantation in the early 1980’s opened up a new era in the field of transplantation, improving survival and diminishing the impact of HLA mismatching and pretransplantation blood transfusion. Unfortunately one of the side effects of CsA is nephrotoxicity.

FK506

Although exhibiting a very similar molecular action to that of cyclosporin, FK506 is effective in smaller doses. FK506 binds to the cytoplasmic enzyme peptidylprolyl isomerase termed FKBP. The FK506-FKBP complex binds to calcineurin inhibiting its function. The blockade of signal transduction mediated by calcineurin results in a failure of the IL-2 gene transcription and therefore in inhibition of IL-2 production. In contrast to CsA, FK506 suppresses both activation and proliferation, but not differentiation of B lymphocytes by blocking B cell division in G1 of the cell cycle. (Wicker et al 1990).
Rapamycin

Rapamycin is chemically very similar to FK506. It acts on the IL-2R, blocking T cell responses to IL-2 and other cytokines (Kahan et al 1991).

Mycophenolate Mofetil

Mycophenolate Mofetil (RS61443) (MM) inhibits de novo purine biosynthesis. T and B cells are more dependent on de novo purine synthesis than other cells. Therefore MM has a T and B cell selective anti proliferative effect and has been shown to inhibit the rejection of many types of grafts, e.g. vascularised organ allografts in rats (e.g. Morris et al 1990). It has been used clinically where it has been shown to reverse OKT3 resistant rejection episodes (Sollinger et al 1992).

3.5.2 Use of antibodies in transplantation

The first antibodies used in transplantation were polyclonal, rabbit anti-lymphocyte globulin (ALG). These antibodies are used extensively for the treatment of rejection in allograft recipients. With the advent of hybridoma technology, monoclonal antibodies have been developed against various targets. The most widely used of these is OKT3 a murine mab against CD3. OKT3 is a depleting antibody, the CD3+ cells being opsonised and removed from the circulation. However, its main mode of action is likely to be blocking of antigen recognition. The use of OKT3 in place of ALG leads to a reduction in acute rejection episodes and improved allograft function. In experimental systems anti-CD4 mab has been used to induce donor unresponsiveness in vascularised and non vascularised transplants in rodents although the efficiency depends on the haplotypes used. Anti-CD4 mab react with the CD4 T cell surface
antigen (CD4+ T cells represent 55-60% of peripheral T cells). Anti-CD4 therapy has been shown to be effective at suppressing acute rejection in long term surviving renal transplant recipients (Reinke et al 1991). Human studies using OKT4A show that it acts by modulating T cell function rather than by depleting the cells (Wee et al 1992). Other antibodies undergoing clinical trials or experimental studies include anti-CD8, anti-TCR αβ, anti-IL-2R, anti-TNFα, anti-LFA-1 and anti-ICAM-1 among others (reviewed by Masroor et al 1994).

3.5.3 The role of blood transfusion in immunosuppression

In 1973 Opelz demonstrated that blood transfusion prior to transplantation was associated with improved graft survival. At this time the immunosuppressive drugs used were azathioprine and steroids. In one multicenter survey, one year graft survival was 87% in patients who had had blood transfusion and only 23% in those that had not. As graft survival has improved with the advent of improved surgical technique and more effective immunosuppression, in particular the use of cyclosporin, the beneficial effects of pretransplant blood transfusions have become controversial. Lundgren et al (1986) have shown that there was no significant improvement in graft survival following pretransplant blood transfusion when CsA was used. The mechanisms for the beneficial effect of blood transfusion are unknown. However blood transfusions can also have a sensitising effect.

Experimental studies have looked at the effect of different components of the blood on the blood transfusion effect. Fabre and Morris (1972) demonstrated that transfusion of serum prior to transplantation had no effect on graft survival. Westra et al (1988), have shown that mononuclear blood leukocytes are more proficient at prolonging graft
survival than red blood cells. Leukocytes have also been shown to play an important role in the immunosuppressive effect (Busch et al 1993). An intact spleen is required to achieve improved allograft survival in mice transfused with donor-specific blood. Thus a mechanism for the favorable effect of blood transfusion may be the generation of splenic suppressor cells in response to transfusion (Shelby et al 1984). Mottram et al (1990) demonstrated the induction of CD4+ T suppressor cells following blood transfusion.

Blood transfusion at day -1 prior to rat heterotopic heart transplantation is insufficient to prolong survival, however when in combination with CsA, graft survival can be prolonged as long as CsA is commenced on the day of blood transfusion in the ACI to LEW rat strain (Brunson et al 1991). The administration of immunosuppression at the time of blood transfusion has the potential advantage of minimising antibody response and thereby sensitisation to the blood transfusion. (van Twuyer et al 1991). A combination of random blood transfusion with anti-CD4 mab has been shown to induce donor specific unresponsiveness which could be extended by random blood transfusion alone (Bushell et al 1994).

3.6 Transplantation Tolerance

The long term acceptance of a graft in the absence of continuous immunosuppression is known as tolerance. Unfortunately, although this state has been achieved in experimental systems, it has so far been unobtainable in clinical transplantation. Many experimental regimens for tolerance induction would not be suitable for clinical applications, although they aid in our understanding of the mechanisms involved.
3.7.1 Central tolerance

Mechanisms of central tolerance operate within the thymus. Self tolerance is the process whereby T cells undergo negative selection to remove T cells that recognise self antigens. The cortical epithelial cells appear to be critical in positive selection (Lo 1986), whereas bone marrow derived medullary dendritic cells appear to be important for negative selection. Specific recognition of peptides by TCR may also be involved in positive selection (Ashton-Rickardt et al 1993).

The original experimental studies of tolerance involved neonatal tolerance, whereby the presence of donor transplantation antigens prior to the development of a mature immune system enables developing T cells to recognise donor antigens as if they were self (Billingham et al 1953). Unfortunately neonatal tolerance has little clinical application. Central tolerance to donor antigens can also be achieved by donor bone marrow reconstitution following lethal whole body irradiation. The donor bone marrow cells repopulate the recipient thymus with donor dendritic cells and induce tolerance to donor antigens in the same way as self tolerance is achieved in normal animals. An alternative route is by injection of donor cells directly into the thymus. Initial studies with intrathymic injection used pancreatic islet cells following elimination of recipient mature T cells with ATG (Posselt et al 1990) although intrathymic injection of lymphoid cells has also been used. One of the disadvantages of this procedure for clinical application is that the thymus of adults is involuted and it is uncertain whether it still functions.
3.7.2 Peripheral tolerance

The T cell is instrumental in the immune response, thus ‘switching off’ the T cells is an important goal in attaining peripheral tolerance. T cell anergy is the phenomenon whereby activation of T cells via the TCR but in the absence of a co-stimulatory signal leads to inactivation of that T cell (as discussed in detail in chapter 2). This can be achieved by blocking co-stimulatory ligands by the use of antibodies, by the introduction of non-professional antigen presenting cells which do not possess the co-stimulatory molecules and by manipulation of the cytokine profile. T suppressor cells were originally thought to represent a separate lineage of T cells. However, it is now thought that these effects are mediated by manipulation of cytokine profiles, as T cells can be down regulated by the action of IL-4 and IL-10 which down regulate T_H1 generation whereas γIFN may down regulate T_H2 generation. A model proposed by Lombardi et al (1994) suggests that antigen-specific T cells that have been rendered non-responsive suppress other T cells that have the same specificity in a passive manner through competition for ligand and for cytokines such as IL-2.
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CHAPTER 4:

XENOTRANSPLANTATION

4.1 Introduction

Xenotransplantation is the transplantation of organs or tissues from one species to another. The current interest in xenotransplantation stems from the shortage of donor organs currently available, as transplantation techniques have improved more recipients have become eligible for transplantation resulting in long waiting lists. At the end of 1994, there were 4,970 patients waiting for a kidney transplant with 320 on the waiting list for a heart transplant (data kindly supplied by UKTSSA).

4.1.1 Historical perspective on clinical xenotransplantation

The concept of xenotransplantation is not a new one with early experimental attempts by Ullman in 1902 (reported in 1914) and early clinical attempts using vascularised grafts by Jaboulay (1906) which were unsuccessful. A resurgence of interest occurred in the 1960’s when Reemtsma transplanted 12 patients with chimpanzee kidneys, one of which survived for 9 months when the patient died with a functioning graft. (Reemtsma et al 1964, Reemtsma 1969). Starzl et al (1964) transplanted 6 patients with baboon kidneys with a maximum survival of 2 months. Other organs were not attempted until the early 1970’s when Starzl transplanted 3 chimpanzee livers Starzl et al (1974) with one surviving for 14 days, and Barnard et al (1977) transplanted a baboon and chimpanzee heart heterotopically which only supported the patients for a few hours. These early attempts were in the pre cyclosporine A (CsA) era and it is not
known whether the immunosuppressive strategies currently available would have extended graft survival.

The first clinical transplant with the use of CsA was a heart transplant from a baboon to a neonate which lasted for 4 weeks (Bailey et al 1985). Unfortunately baboon's have a similar blood group system to humans and in this case the ABO match was incompatible. The most recent clinical xenotransplant's have been performed by Starzl using baboon livers. Xenograft survival was reported as 70 days in one case and 22 days in the other. Recipients were splenectomised, received FK506, prednisolone, cyclophosphamide and prostaglandin E therapy. Death in each case was due to infection with suboptimal liver graft function (Starzl et al 1995).

4.1.2 Concordant and discordant xenografts

The use of animals as organ donors raises many ethical issues including animal rights and the potential transmission of infections from the donor animal to the patient, especially with regard to the use of nonhuman primates. Chimpanzee and most other nonhuman primates are in short supply (although baboon's are plentiful in certain parts of the world) are slow to breed and may be infected with important pathogens for man. The alternatives are to turn to animals other than primates, one candidate for these studies is the pig.

Pigs are already used as a food source, breed well and are of a comparable size to humans. The major disadvantage of using the pig is that it is a discordant species. In 1970 Calne coined the phrases discordant and concordant to distinguish between the rejection mechanisms seen in experimental xenotransplantation. Transplants between distantly related species i.e. pig-to-primate resulted in a hyperacute rejection (HAR)
response with graft destruction in a few minutes to hours, (with the exception of chicken-to-guinea pig (Braidley et al 1995)). This response was said to be due to the presence of preformed natural antibodies to the graft and was termed discordant. Natural preformed antibodies are not present at birth but develop within a few weeks or months as soon as the gastrointestinal tract of the neonate is colonised with microorganisms. Xenografts of pig hearts to neonate baboon's have been shown to be free of HAR due to the low levels of natural preformed antibodies present (Kaplon et al 1995). Survival of grafts between closely related species i.e. chimpanzee-to-man and hamster-to-rat survived for days, this rejection response was not thought to involve preformed natural antibody and was termed concordant. The distinction between these terms is now less clear and will be discussed in the following pages.

4.2 Hyperacute rejection

Hyperacute rejection is the rejection response which occurs immediately after revascularisation of a graft, often resulting in graft destruction within a few minutes. The pathological picture of HAR is one of haemorrhage, oedema and thrombosis, resulting from changes to the endothelial cells which line the lumen of blood vessels. There is some controversy as to whether these endothelial cells are 'activated' Platt et al (1990a) or damaged, studies by McKenzie et al (1968) demonstrated that porcine endothelial cells were sensitive to lysis by human serum. Endothelial cells normally provide a barrier for the egress of proteins and cells from blood vessels, they maintain an anticoagulant environment intravascularly and are non-adherent for platelets and leucocytes. Disruption of endothelial cells leads to a markedly increased vascular
permeability, loss of heparin sulphate and thrombomodulin which leads to loss of anticoagulant activity and the surface expression of P-selectin which promotes adhesion of neutrophils and monocytes along with the complement component iC3b to the endothelial cell surface. HAR can be initiated by the specific binding of xenoreactive antibodies to the blood vessels of the donor organ (Perper and Najarian 1966), binding of these antibodies activates complement which mediates endothelial cell changes leading to a cascade of events culminating in haemorrhage, oedema and thrombosis. The presence of these antibodies at the time of transplantation indicates that they are preformed natural antibodies.

Another mechanism for the initiation of HAR is that of the alternative pathway of complement. In the guinea pig-to-rat model the occurrence of HAR does not depend on natural antibody binding (Miyagawa et al 1988). It is also possible that a complement independent pathway exists, whereby natural antibody binds to it's target allowing the Fc portion to bind to Fc receptors on cytotoxic cells (probably macrophage-type) in an antibody dependent cytotoxic cell (ADCC) response (Galili 1993).

4.2.1 Role of preformed natural antibody in hyperacute rejection

IgM preformed natural antibody appears to be the most important antibody in initiating HAR. Platt et al (1991) has shown that whereas both IgM and IgG natural antibodies are capable of binding porcine endothelium, it is the binding of IgM which activates complement. The targets of these natural antibodies have recently become clearer. Platt et al (1990b) and others demonstrated that human xenoreactive antibodies recognised a triad of glycoproteins on the surface of pig endothelial cells with
molecular weights of 115kD, 125kD and 135kD. In 1984 Galili et al. reported that large amounts of natural antibody reacted against the carbohydrate Galα(1,3)Gal. Subsequent studies by many groups have shown that Galα(1,3)Gal is an important epitope in xenograft rejection. The majority of the haemagglutinating and cytotoxic antibodies in human serum could be absorbed out by using Galα(1,6)Glu coupled to Sepharose (Sandrin et al. 1993). Cooper et al. (1993) demonstrated that by infusing Galα(1,3)GalβGlu hyperacute rejection could be delayed. Although the Galα(1,3)Gal epitope has been shown to be important in xenograft rejection the molecules on which it resides are still unclear. Studies by Sandrin and McKenzie (1994) demonstrated at least 25 cell surface glycoproteins on endothelial cells that carry the epitope. The synthesis of complex carbohydrates involves the action of glycosyltransferases. One such glycosyltransferase, α1,3 galactosyltransferase is responsible for making this epitope. Thus removal of this transferase enzyme would prevent these molecules from becoming galactosylated. One approach to achieve this would be to suppress the expression of α1,3 galactosyltransferase by genetic manipulation. The gene for pig α(1,3)GT has been mapped to chromosome 1 (Strahan et al. 1995). The lack of expression of this gene in humans, apes and Old World primates results in the absence of the epitope and thus the absence of antibodies against this structure in other mammals (Larsen et al. 1990).

Other techniques to control the humoral response include splenectomy (Cooper et al. 1988) and plasmapheresis. Plasmapheresis is a nonselective technique for antibody removal. It has been used in allografts for antibody removal in human ABO incompatible kidney grafts (Alexandre et al. 1987) and has been used in conjunction with splenectomy and immunosuppression to prolong porcine renal xenografts in
baboons to 22 days (Alexandre et al 1989). A more selective approach is to use specific immunoadsorbent columns for the removal of IgG and IgM. This approach has been shown to be effective for the removal of anti-pig antibody from human plasma (Leventhal et al 1992a). The most selective method of antibody removal is extracorporeal perfusion of a donor organ prior to transplantation (Cooper et al 1988), although this approach can lead to activation of the complement and coagulation cascades. An alternative approach is the depletion of xenoreactive antibody, administration of anti-\(\mu\) monoclonal antibodies has been shown to deplete circulating xenoantibodies in rats without immune complex disease (Latinne et al 1994).

4.2.2 Role of complement in hyperacute rejection

Complement can be activated by two pathways the classical and alternative. The C3 component plays a major role in both pathways. The classical pathway is activated by the interaction of antibody with C1 which then splits C4 into C4a and C4b. C2 then binds C4b leading to C4b2a. This C3 convertase cleaves C3 into C3a and C3b. C4b2a3b activates C5 to assemble the membrane attack complex (MAC) with the components C5 to C9. MAC forms transmembrane channels which displace lipid molecules and other constituents, thus disrupting the phospholipid bilayer of target cells leading to cell lysis. The alternative pathway does not require antibody for activation but can be activated by certain molecular structures such as lipopolysaccharide. Although the alternative pathway is continually activated it is kept at a low level by the action of factor H and factor I. The C3 convertase in this pathway is C3b,Bb. Factor I in association with factor H cleaves C3b into iC3b which can no
longer form the C3 convertase with factor B. Host cells have the membrane proteins decay accelerating factor (DAF) and membrane cofactor protein (MCP) that have functions similar to those of factor H. These proteins actively prevent the formation of the C3 convertases of both pathways thus preventing lysis of the host cells by MAC. The complement cascade also produces anaphylatoxins, chemotactic factors and facilitates opsonisation leading to an inflammatory response. The complement receptors CR1 and CR3 also promote adhesion of leucocytes to the endothelial cell surface.

Alternative approaches to control HAR rejection lie in the manipulation of the complement cascade. The most effective agent is cobra venom factor (CVF) which acts by causing exhaustion of the complement cascade. CVF has been used in a variety of studies to prolong discordant xenograft survival (Leventhal et al 1993). The action of complement is enhanced in the xenograft situation by the failure of DAF and MCP in the donor organ to inhibit the activation of the recipient complement, as these regulators have been shown to be inefficient across this xenogeneic barrier (Atkinson et al 1991). Thus the incorporation of recipient DAF or MCP into the vascular endothelium using transgenic techniques could facilitate a marked inhibition of complement activity (Oglesby et al 1991).

4.2.3 Rodent models of discordant xenotransplantation

The guinea pig-to-rat model has been widely used as a model for discordant xenotransplantation. Hyperacute rejection of guinea pig-to-rat cardiac xenografts occurs in minutes with vascular rejection, interstitial haemorrhage, and oedema (Miyagawa et al 1988). This response was mediated by activation of the alternative
pathway of complement (Miyagawa et al 1988) and was not prevented by the depletion of B cells and preformed natural antibody (Baldwin et al 1995). However complement depletion with CVF markedly prolongs xenograft survival for several days (Leventhal et al 1993). The rat has preformed natural antibodies to guinea pig (Leventhal et al 1992a), although not of primary importance several studies have demonstrated a role for preformed natural antibodies in the rejection response. Therapies using deoxyspergualin (DSG), splenectomy and plasma exchange have been shown to reduce the titres of circulating anti-guinea pig antibodies which resulted in a marginal prolongation of graft survival although graft survival was still less than an hour (Leventhal et al 1992b).

4.3 Concordant xenotransplantation

Until the problems of hyperacute rejection are solved the cellular response to a discordant xenograft cannot be addressed, much experimental xenotransplantation is therefore aimed at addressing the cellular response using concordant models.

4.3.1 The hamster-to-rat model of concordant xenotransplantation

The hamster-to-rat concordant vascularised organ model has been the most extensively studied in xenotransplantation. The majority of studies have used cardiac transplantation. Rejection times are rapid (mean survival 2-4 days in untreated recipients (Homan et al 1981)) in comparison to rodent allografts and can not be controlled by conventional immunosuppression. Although extensive long term graft
survival was achieved by Knetchle et al (1987) using total lymphoid irradiation and cyclosporin, these results have not been confirmed by others.

**Therapies to modulate rejection of hamster-to-rat cardiac xenografts**

Long term survival has been difficult to achieve using non toxic levels of immunosuppression. Immunosuppressive agents that suppress T cell functions, such as CsA, have been found to be ineffective e.g. Van den Bogaerde et al (1991) with a few exceptions (Homan et al 1981). Studies using T cell deficient rats as recipients demonstrated a graft survival time comparable to that of immunocompetent recipients (Lim et al 1990) indicating that rejection could occur in the absence of T cell function. This suggested that antibodies played a role in this rejection response. The presence of antibodies at the time of rejection was confirmed by many groups e.g. Cranmer et al (1992) these antibodies appear to be T cell independent. It is unclear whether the antibodies that mediate this rejection are preformed, as rat anti-hamster antibodies are barely (Van den Bogaerde et al 1991) or not detectable (Monden et al 1989) prior to transplantation. However antibody titres begin to rise from day 2 (Suzuki et al 1993) with a dramatic increase at day 3 to 4 (Murase et al 1993) coinciding with rejection. These antibodies mediate their damage by activating the classical pathway of complement. Recipients receiving CVF and CsA survived up to day 49 although the grafts were rejected during therapy (Van den Bogarede et al 1991). The spleen is known to be important in the antibody response to antigens, studies involving splenectomy of the recipient result in an increased graft survival of a few days (Suzuki et al 1993). When this regimen is combined with other T cell immunosuppressive agents graft survival is prolonged further although rejection often occurs during
treatment, preceded by a large rise in antibody titre (Monden et al 1989). The timing of splenectomy is also important, Araneda et al (1992) demonstrated splenectomy at day 2 after transplantation to be more beneficial than splenectomy at day 0 or day 1, postulating that post transplant splenectomy eliminates sensitising cells which had migrated to the spleen.

Use of antiproliferative drugs e.g. DSG results in increased graft survival (Suzuki et al 1993). The use of combinations of other antiproliferative drugs such as brequinar sodium (BQR) and mycophenolate mofetil (MM) increased survival further (Murase et al 1993). However the addition of CsA to these drug cocktails resulted in extended survival, Fujino et al (1994) combined MM and BQR with CsA to achieve survival of 40 days albeit with a high mortality. In this study rejection occurred without a significant rise in antibody titre suggesting that these grafts were rejected by cellular mechanisms. Combinations of irradiation and CsA (Steinbruchel et al 1993) or DSG (Aqaisi et al 1992) have also led to extended graft survival.

Xenograft survival in all of the studies mentioned has been very heterogeneous considering that inbred strains have been used, with graft survival in the same experimental group varying from no effect to long term survival. These variations make it difficult to evaluate the efficacy of the immunosuppressive protocols used.

Morphology of hamster-to-rat cardiac xenografts

Histological analysis of rejecting hearts in the hamster to rat model show haemorrhage, myocardial necrosis (Homan et al 1981), IgM deposits (Monden et al 1989) and in some cases cellular infiltrates of mainly granulocytes (Nielsen et al 1992). In animals that rejected their grafts after a prolonged period, mononuclear cell infiltrates were
observed (Marchman et al 1992). Sakakibara et al (1990) have suggested that the majority of these infiltrating cells are not conventional T cells.

Liver graft survival in the hamster to rat model is twice as long as cardiac survival. Although high anti donor antibody titres have been demonstrated (Murase et al 1993) these grafts appear to survive this initial antibody response and undergo cellular infiltration.

4.3.2 The mouse-to-rat and rat-to-mouse models of concordant xenotransplantation

The mouse-to-rat and rat-to-mouse models have been studied less intensely. The mouse-to-rat model would appear to be more closely related than the hamster-to-rat model and thus more concordant allowing the investigation of the cellular aspects of xenograft rejection. The majority of these studies utilise the primarily vascularised heterotopic cardiac xenograft model. The phylogenetic relationship between these species shows that the divergence between rats and hamsters is around 19.1 and 16.5 million years (Myr) and that between rats and mice is around 12 and 8 Myr (Chaline et al 1994). Rejection times between mouse-to-rat vascularised organ grafts are rapid with rejection in about 3 days (e.g. Makowa et al 1990). Therapies using T cell immunosuppressive agents only e.g. CsA have no effect on graft survival (e.g. Tufveson et al 1990). As in the hamster-to-rat model, splenectomy improves graft survival by a few days (Gannedahl et al 1990) which can be further extended by a few days if these therapies are combined (Tufveson et al 1990). Monotherapy of DSG (Pruitt et al 1991) or FK506 (Makowka et al 1990) has no effect on cardiac survival although FK506 prolongs liver graft survival in this model (Pan et al 1992). Therapies combining splenectomy and DSG have prolonged graft survival up to a mean of 17.8
days (Gannehadl et al 1990). LEW rats have no detectable haemagglutinating antibody titres to mouse RBC, although lymphocytotoxic IgM antibodies to spleen cells are present (Gannehadl et al 1994) which increase at the time of rejection. Histological analysis of rejecting hearts show a mononuclear cell infiltrate (Tufveson et al 1990). Interestingly graft survival of rat-to-mouse cardiac xenografts are easier to achieve, although the length of prolongation appears to be recipient strain specific (Burdick et al 1979). Therapies using anti-thymocyte globulin (ATG) can prolong graft survival in this model up to 106 days (Sakakibara et al 1989). CsA monotherapy is capable of increasing graft survival to 14 days (Sugimoto et al 1985). Histology of these grafts show a diffuse mononuclear infiltrate at the time of rejection (Corry and Kelly 1975). The mechanisms whereby mouse-to-rat cardiac xenografts are rejected more rapidly than rat-to-mouse cardiac xenografts is unclear.

4.4 Xenotransplantation of non-vascularised tissues

4.4.1 Xenotransplantation of pancreatic islets

Xenotransplantation of non-vascularised tissues is at an advantage in that the problems of haemorrage and thrombosis associated with hyperacute rejection of vascularised grafts do not apply. Thus the prospect of clinical xeno islet transplantation using the pig as a donor may be easier to achieve than in organ xenotransplantation. Islet xenografting was first performed in the last century (Williams 1894) using minced sheep pancreas. Recent clinical xenografting of porcine islets to 8 patients showed islet survival in 4 patients with 1 patient demonstrating a low level of function up to 300 days although the patient required exogenous insulin (Groth et al 1993).
Experimental transplantation of islets has shown that discordant islet grafts are rejected more quickly than concordant islet grafts. Gray 1995 (personal communication) has demonstrated that pig islets exposed to human serum containing antibody, complement and neutrophils are hyperacutely rejected within a few hours. Transplantation of human islets placed under the renal capsule of mice are rejected in 3.7 days with no treatment (Tze et al 1990) whereas transplantation of hamster islets to the renal capsule of mice are rejected in 8.9 days with no treatment (Lu et al 1995). However the purity of the islet preparation can have a bearing on the result Falqui et al (1991). The site of islet transplantation can also affect survival. Sites for islet transplantation include the renal subcapsular space (Tze et al 1990), liver (Jaegar et al 1995), spleen (Kaufmann et al 1990), thymus (Goss et al 1994) and testis (Bellgrau et al 1990). The testis has the advantage of being an immunologically privileged site. There is some debate over which is the best site with Goss et al (1994) favouring the thymus and Jaegar et al (1995) the renal subcapsular site.

Manipulation of islets prior to transplantation

Encapsulation of islets in for instance polylysine-alginate membranes has been used to 'protect' transplanted islets from immunological attack (O'Shea and Sun 1986). In the dog-to-mouse model, encapsulation of islets increased their survival from a mean of 0.6 days to 37.8 days (Aomatsu et al 1995). Culture of the islets prior to transplant has also been shown to extend survival. Culturing of islets at 37°C prolongs graft survival which is more pronounced if they are cultured at 22°C (Jaegar et al 1994).
Immunosuppression of recipient

Cyclosporin A is a poor suppressor of islet xenograft rejection (Teraska et al 1986) although it is effective if the islets are transplanted to the testis (Bellgrau et al 1990). Anti-lymphocyte serum (ALS) has been used to some effect especially when the islets are precultured (Lacy et al 1980). The most promising immunosuppressive protocols to date involve the use of DSG. DSG has been shown to extend survival in some studies (Lu et al 1995) but not others (Wennberg et al 1995). However when used in combination with other therapies e.g. microencapsulation (Aomatsu et al 1995), rat anti-thymocyte globulin (RATG) (Thomas et al 1995) or anti-CD4 mab (Lu et al 1995) prolongation of islet survival is enhanced.

Mechanisms of rejection of islet xenografts

The mechanism by which islets are rejected is unclear. Islet grafts are non-vascularised so that unlike a vascularised graft the processes of HAR e.g. haemorrhage and thrombosis would not be apparent. Studies using the pig-to-rat model where rats have preformed natural antibody to pig demonstrated no visible deposition of IgM on the islet cells (Satake et al 1994) suggesting that preformed natural antibody do not contribute during the early phases of rejection. It would appear therefore that cellular mechanisms play an important role in the rejection of islet xenografts. Purified islet cells are not believed to be conventional antigen presenting cells (Markmann et al 1989) (although there are dendritic cells in the islets) thus the major pathway of T cell activation would be by the indirect route. Studies utilising anti-CD4 mab have indicated that CD4+ T cells have a major role to play in this rejection response (Gill
Histological analysis of pig islets transplanted to mice reveal an intense cellular infiltrate consisting predominantly of CD4+ cells, macrophages and eosinophils (Simeonovic et al 1990). Recent evidence has begun to suggest that the CD4+ cells seen are macrophages. Using the pig-to-rat model the majority of the cellular infiltrate was shown to express both F4/80 (macrophage cell phenotype) and CD4 markers, although cells of NK phenotype were also found (Korsgen et al 1995). If this effector cell is a macrophage it would explain why DSG is so effective as this agent has anti-macrophage activity (Thomas 1991). The macrophage has also been implicated in the primary non-function of islet grafts (Marquet et al 1995). Adhesion molecules have also been shown to have a role in islet rejection. Treatment with anti-mouse LFA-1 was found to extend rat-to-mouse islet xenograft survival from 10 days to 53 days (Ohta et al 1995).

4.4.2 Xenotransplantation of skin

Unlike vascularised organs there is a delay of 5-7 days before the graft becomes vascularised making it resistant to antibody mediated vascular rejection (Jooste et al 1981). The survival of mouse to rat skin grafts is around 5 days (Thomas et al 1991a). Skin graft survival is easier to obtain than vascularised graft survival. Hamster-to-rat skin graft survival can be prolonged with CsA monotherapy for over a hundred days (Bouwman et al 1991) indicating a cell mediated mechanism of rejection.

4.5 In vitro studies of the cellular interactions in xenogeneic combinations
There has been much debate as to whether cellular interactions are the same in xenogeneic systems as for allogeneic systems. Early *in vitro* studies demonstrated the responding cells to be the same as that for an allogeneic response namely T cells (Newlands *et al* 1975). However conflicting reports suggest that this response is either weaker in xenogeneic systems (Wilson *et al* 1970) or similar (Swain *et al* 1983). Many studies indicate that the targets for this response are MHC antigens, in particular the polymorphic regions of the MHC (Swain *et al* 1983). The majority of these *in vitro* assays use lymphocytes or splenocytes as targets. Haisch *et al* (1990), developed an *in vitro* assay using vascular endothelial cells (VEC) as targets and demonstrated that the VEC gave a stronger stimulation than splenocytes. This study also demonstrated that human anti-mouse responses gave a higher proliferation than rat anti-mouse responses. Can T lymphocytes recognise xenogeneic MHC directly, without a requirement for antigen processing and presentation? This question is of fundamental importance. If direct recognition is not possible then the immune response would be like that for any nominal antigen. The consensus so far indicates that indirect recognition requiring CD4+ T helper cells and responder APC (Moses *et al* 1990) is the major pathway for responses against disparate responders e.g. human anti mouse (Lucas *et al* 1990), mouse anti human (Yoshizawaka and Yano 1984) but that direct recognition can take place between closely related combinations e.g. rat anti-mouse (van den Bogaerde *et al* 1990). However Swain *et al* (1983) demonstrated that human T cells depleted of non T cell populations could proliferate to porcine MHC class II in a MLR. This was confirmed by Yamada *et al* (1995) who also demonstrated that the indirect pathway could also take place. These studies suggest that elements required for cell-cell interactions appear intact across this xenogeneic barrier. However defects in the cell-
cell interactions inhibiting direct recognition in the murine/human combination include an inefficiency of the α3 domain of human class I to interact with murine CD8 (Kalinke et al 1990) and inefficient interaction of accessory molecules, the LFA-2/LFA-3 interaction has been shown not to function in human anti-mouse responses (Barbosa et al 1986). Alter and Bach (1990) demonstrated that there was no deficiency in the T cell repertoire as addition of cytokines enabled human T cells to proliferate to murine xenoantigens. An important consideration in assessing these studies is that the responder population must be devoid of APC.

4.6 Physiology of xenotransplantation

Once the immunological problems have been overcome the restraints on xenotransplantation will be of a physiological nature. The pig appears to be the donor of choice for clinical xenotransplantation. Its breeding capacity makes it an ideally suited candidate for the production of transgenic colonies.

The pig can harbour bacterial, viral, fungal, protozoal and helminth organisms, some of which could lead to infection in man if transferred with the transplanted organ. The ideal solution to this problem would be the use of germ free or gnotobiotic pigs. However this would prove expensive and become impractical after 3 to 4 months due to their increase in size. The ability of pig organs to function in man also needs to be addressed. This would not be as important for organs that have only a mechanical role e.g. the heart, as for organs such as the liver involving a large number of biochemical interactions.
CHAPTER 5:

MATERIALS AND METHODS

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CHAPTER 5:
MATERIALS AND METHODS

5.1 Experimental animals

5.1.1 Rats

Inbred DA (RT1<sup>av</sup>), LEW (RT1<sup>1</sup>), PVG (RT1<sup>6</sup>), and (DAxLEW)F1 hybrid rats were obtained from Harlan UK Ltd, Oxon, UK. Randomly bred athymic nude rats HAN: RNU-rnu were also obtained from this source. All rats were adult males under 6 months of age.

5.1.2 Mice

Inbred BALB/c (H-2<sup>d</sup>) mice were purchased from Harlan UK Ltd, Oxon, UK. All mice were adult females under 4 months of age.

5.2 Transplantation

All microsurgery was performed by myself with the aid of an assistant where appropriate. All surgery was performed using inhalational anaesthetic of halothane, N<sub>2</sub>O and O<sub>2</sub>.

5.2.1 Orthotopic kidney transplantation in rats

Donor surgery

A midline abdominal incision was performed. The left kidney was cleared and the left renal artery and left renal vein dissected free. The ureter was cut approximately 1.5 cm away from the kidney. Once the recipient was prepared the donor was heparinised by
injection with 200 units of heparin in 1ml of saline into the inferior vena cava (IVC). After two minutes the aorta was clamped above the left renal artery and the renal artery and vein cut close to the aorta and IVC respectively. The kidney was removed and placed into cold saline. The aortic clamp was removed and the donor exsanguinated.

**Recipient surgery**

A midline abdominal incision was performed. The left kidney was cleared and the left renal artery and left renal vein dissected free. The renal artery and renal vein were clamped close to the aorta and IVC respectively using Scoville Lewis Clips (Aesculap Downs, London, UK). The vessels were cut close to the kidney, the ureter was cut as it enters the hilum and the kidney removed. The vessels were flushed with saline. The donor kidney was placed in the orthotopic position and the donor renal artery anastomosed end-to-end to the recipient renal artery with 10/0 nylon (Ethicon W2850) (A.C. Daniels, London, UK) using interrupted sutures. The donor renal vein was anastomosed to the recipient renal vein in the same way. The vascular clamps were removed and the kidney perfused with blood. The donor ureter was cut 0.5cm away from the kidney and anastomosed end-to-end to the recipient ureter using interrupted sutures. The kidney was tied down at the upper pole to prevent twisting of the vessels and the rat sewn up.

At day 7 the recipient's right kidney was removed, the graft was biopsied, and the biopsy placed in formalin for histological studies. Thereafter, graft function was monitored by serial blood estimations of urea levels using a kit obtained from
Boehringer Mannheim (Sussex, UK). Normal serum urea is between 50 to 60mg/100ml.

5.2.2 Heterotopic heart transplantation from mouse-to-rat

Donor mouse surgery

The donor mouse was heparinised by injection of 100 units of heparin into the IVC. After 2 minutes the chest was opened and the right and left superior vena cavae (SVC) ligated. The aorta was cut at the begining of the aortic arch and the heart was perfused with ice-cold saline via the IVC. The pulmonary artery was cut at the bifurcation. All the remaining vessels were tied off with one ligature, the heart was removed and placed in ice-cold saline.

Recipient rat surgery

The rat recipient was prepared as for kidney transplantation with the renal artery and vein clamped close to the aorta and IVC. The ureter was ligated and the left kidney was removed. The mouse heart was placed in the abdomen, protected by gauze and kept moist with cold saline. The mouse aorta was anastomosed end-to-end to the left renal artery using interrupted sutures and the mouse pulmonary artery was anastomosed end-to-end to the left renal vein. The heart starts to beat as soon as the clamps are removed. Blood flow through the heart in this heterotopic position is from the left renal artery into the mouse aorta and into the coronary artery system from where it drains back into the right atria and is expelled into the renal vein via the right ventricle. In this
system the left side of the heart is redundant. Rejection was taken as the day on which the heart ceased to beat, determined by daily palpation through the abdomen.

5.2.3 Heterotopic heart transplantation in rats

The surgery is similar to heart transplantation from mouse-to-rat as described in section 5.2.2 above, except that either the right subclavian artery or the right common carotid artery was anastomosed to the left renal artery and the right pulmonary artery was anastomosed to the left renal vein.

5.2.4 Harvesting of tissues for analysis

Rats were killed in a CO$_2$ chamber either on the day of rejection or at a predetermined date.

Samples for histology were placed in 10% v/v formalin in normal saline and processed by the Pathology department.

Samples for immunohistological analysis were mounted on chucks with O.C.T. compound (BDH Leics UK) and snap frozen in liquid nitrogen within 15 minutes of death.

5.2.5 Collection of serum samples for urea estimations and antibody tests

Blood samples were taken via the tail vein at pre-determined points following transplantation. These were left to clot at room temperature, ringed and placed at 4°C for the clot to constrict, then centrifuged at 1000g for 10min at 4°C. The serum was collected and stored at -35°C in aliquots to avoid repeated freezing/thawing of the
samples. Serum samples for alloantibody tests were heat inactivated in a water bath for 30 min at 56° C prior to use.

5.3 Immunosuppression

5.3.1 Splenectomy

Splenectomy was performed during the cardiac xenograft surgery by dissection and ligation of the splenic vessels close to the spleen. The spleen was then removed.

5.3.2 Cyclosporin A

Cyclosporin A powder was a kind gift from Sandoz Ltd, Basel. CsA was dissolved at either 10mg/ml or 20mg/ml in olive oil by heating to 60°C, stored at room temperature in the dark and used within two weeks of preparation. It was administered orally by gavage.

5.3.3 CTLA4-Ig

CTLA4-Ig was a kind gift from Bristol-Myers Squibb (Seattle, USA). CTLA4-Ig was diluted in saline to 1mg/ml on receipt. It was stored in 1ml aliquots at -35°C. Just prior to use an aliquot was thawed, diluted to 200μg/ml in saline and 1ml injected into the tail vein of the rat. Once thawed the preparation was kept at +4°C for a maximum of 48 hours.
5.4 Peptides

Peptide 1 and 3 were synthesised by Cambridge Research Biochemicals (Cambridge, UK). They were stored dessicated in a powdered form at -35°C, dissolved in water using sonication to assist solution and further purified on a Sephadex G-10 column. They were then freeze-dried and reconstituted in 0.15M NaCl at 1mg/ml. The aliquots were stored at -35°C.

Peptide 1 is a 24mer corresponding to amino acids 57 to 80 of the α helical region of the α1 domain of the DA RT1.A class 1 molecule.

Peptide 3 is a 22mer from the α2 domain of the same molecule, amino acids 143 to 164.

An irrelevant peptide P32 was used as a control in the proliferation assays. This peptide was derived from the mouse H-2Aβ class II molecule.

Peptide 1: Pro-Glu-Tyr-Glu-Gln-Thr-Arg-Ile-Ala-Lys-Glu-Trp-Glu-Gln-Ile-Tyr-Arg-Val-Leu-Arg-Thr


5.4.1 Peptide immunisations

LEW rats were immunised with unconjugated peptide emulsified in Freunds complete adjuvant (FCA) (ICN-Flow, Bucks, UK). LEW rats immunised with two different
peptides were injected with unconjugated peptide emulsified in an equal volume of FCA, 100 μg of peptide in 200μl of emulsion. Each peptide was injected into one hind footpad. LEW rats immunised with one peptide were injected with unconjugated peptide diluted 1:2 with saline, emulsified in an equal volume of FCA. 50μg of peptide in 200μl of emulsion was injected into each hind footpad. Where appropriate, boosts were given 4 weeks after the initial immunisation in Freund's incomplete adjuvant (FIA) (ICN-Flow, Bucks, UK). Control animals were immunised with saline emulsified in an equal volume of Freund's adjuvant.

5.5 Blood transfusions

DA rats were exsanginated into a syringe containing 200 units of heparin in 0.8ml saline. The fresh DA blood was diluted to twice its volume in normal saline and 1ml injected into the tail vein of LEW rats at twice weekly intervals as appropriate. Control rats were injected with 1ml saline.

5.6 Buffers and solutions

All commonly used chemicals were obtained from either BDH, Leics, UK or Sigma, Dorset, UK unless otherwise stated.

5.6.1 Phosphate buffered saline (PBS)

Dulbecco's A (Unipath Ltd, Hamps, UK) formulation was bought in tablet form and dissolved in distilled H₂O.

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5.6.2 Tris buffered saline (TBS)

0.05M tris, 0.15M NaCl, 0.02% NaN₃ pH 7.5 at 25°C

5.6.3 Normal saline

9g NaCl per litre in distilled water (0.9%w/v)

5.6.4 Bovine serum albumin (BSA)

10%w/v was prepared in PBS. 30g bovine serum albumin fraction V (Miles Scientific Slough, UK) was added to 300ml PBS. Once dissolved this was dialysed twice against 5 litres of saline and once against PBS at 4°C and stored in 10ml aliquots at -20°C.

5.6.5 Trypan blue

A stock solution of 1% w/v in distilled water was diluted 1/10 in PBS prior to use. An equal volume was added to the cell suspension before counting on a haemocytometer.

5.7 Targets for allo and xeno antibody assays

5.7.1 Red blood cells (RBC)

Rats were exsanguinated from the aorta into a syringe containing 200 units of heparin in 0.8ml normal saline. Mice were exsanguinated by cardiac puncture into a heparinised tube. The red blood cells were washed twice in normal saline and twice in PBS at 1000g for 10min at 4°C. The supernatant and buffy coat were removed each time. The RBC were counted using a haemocytometer. For indirect radioactive binding assays, the final pellet was resuspended in 0.5% BSA/PBS to a concentration of 10⁹/ml
and stored on ice. For haemolysis assays the pellet was resuspended in 0.5% BSA/PBS to $2.5 \times 10^6$/ml and kept at room temperature.

5.7.2 Spleen cell suspensions

Spleens were removed, minced in PBS and pushed through a sieve with a syringe plunger. The suspension was allowed to settle for 5 min, the suspension was washed in PBS by centrifugation at 400g for 10 min at 4°C and allowed to settle for another 5 min before being washed again in PBS. The cells were counted on a haemocytometer and the viability assessed by trypan blue exclusion. The resulting single cell suspension was resuspended to $10^9$/ml nucleated cells in 0.5% BSA/PBS.

5.7.3 Mouse heart homogenate

400 BALB/c mouse hearts without atria were obtained individually frozen from Harlan UK Ltd (Oxon, UK). Thawed tissues were minced in PBS and passed through a metal sieve before homogenisation using a mechanical homogeniser. The homogenate was centrifuged at 110,000g for 1 hr at 4°C. The pellets were pooled, resuspended in PBS and homogenised again prior to centrifugation as before. The pellets were re-homogenised as above in PBS, resuspended to 25% v/v in PBS and stored at -35°C. Protein content using the Lowry method (described in section 5.11.0) was estimated to be 40 mg protein/ml in 25% heart homogenate. Homogenates to be used as targets in the indirect radioactive binding assays were suspended to 2% v/v in 0.5% BSA/PBS.
5.7.4 Platelets

Blood was collected into a tube containing 1.5% EDTA in 0.42% NaCl, 100μl of EDTA/NaCl per ml of blood. Blood was diluted 1:3 in normal saline, centrifuged at 200g for 20min at 4°C. The platelet rich supernatant was transferred to another tube and centrifuged again at 200g for 20 min at 4°C. The pellet was discarded and the supernatant centrifuged again at 200g for 20 min at 4°C. The supernatant was then centrifuged at 2000g for 15min 4°C to pellet the platelets. The resulting supernatant was discarded and the pellet washed again as above in saline before being resuspended in 0.5% BSA (dialysed against calcium and magnesium free PBS (Gibco, Paisley, Scotland)) in calcium and magnesium free PBS at 5x10^8/ml for indirect radioactive binding assays and 4x10^7/ml for flow cytometry. (Only plastic tubes, pipettes etc were used).

5.8 Antibody Assays

5.8.1 Indirect radioactive binding assays for allo and xeno antibody studies

All procedures were performed on ice or at 4°C.

Tripling dilutions of sera were made in 0.5% BSA/PBS. Duplicates of 25μl of heat inactivated sera were incubated with 25μl of target cells for 1 hour in LP3 tubes (Luckham Ltd, Sussex, UK). Unbound antibody was removed by washing twice with 1.6ml of 0.1% BSA/PBS and centrifugation at 1000g for 10 min. Bound antibody was detected by incubation with 100μl of ^125^I RAR (Immunoadsorbent purified rabbit F(ab')^2 anti-rat F(ab')^2 had been previously prepared in this laboratory, essentially as described by Dalchau and Fabre (1979) and was iodinated using the chloramine T.
method) at 300,000 cpm per tube for 1 hour. The unbound \(^{125}\text{I}\) labelled antibody was washed off by washing twice as above. The pellets were resuspended in 0.6ml PBS, transferred to fresh LP3 tubes and the bound radioactivity measured using a LKB Minigamma spline counter.

When using rat sera against mouse targets the \(^{125}\text{I}\) RAR was adsorbed with 5% heat inactivated normal mouse serum to remove non-specific activity to mouse targets.

5.8.2 Indirect radioactive binding assay to determine rat MHC haplotypes

All procedures were performed on ice or at 4°C.

Rats to be typed were bled from the tail vein, 0.5ml blood was collected into a tube containing 25 units of heparin in 1ml saline. The RBC were washed four times, twice in saline and twice in PBS at 1000g for 10 min at 4°C removing the buffy coat and upper layer of RBC each time. The cells were resuspended to 1ml in 0.5%BSA/PBS.

25µl of blood was incubated for 1 hour on ice in LP3 tubes with an equal volume of one of the following antibodies. OX27 (Serotec, Oxon, UK) which detects a polymorphic RT1.A determinant present on PVG but not LEW rats (Jefferies et al 1985) and NDS60 which detects a polymorphic RT1.A determinant specific for LEW rats (Tellides 1988)(a gift from Dr. M. Dallman, Nuffield Department of Surgery, Oxford UK) both antibodies were in the form of culture supernatant and were used undiluted. The cells were washed twice in 0.1% BSA/PBS by centrifugation at 1000g for 10 min. 100µl of \(^{125}\text{I}\) RAM (Immunoabsorbent purified rabbit F(ab')\(^2\) anti-mouse F(ab')\(^2\) previously prepared in this laboratory, essentially as described by Dalchau and Fabre (1979) and iodinated using the chloramine T method) at 300,000 cpm per tube were added to the cell pellets of the second wash and incubated for 1 hour on ice. The
cells were washed twice as above. The pellets were resuspended in 0.6ml PBS, transferred to fresh LP3 tubes and the bound radioactivity measured using a LKB Minigamma spline counter.

5.8.3 Indirect radioactive binding assay for anti-peptide antibody studies

All procedures were performed on ice or at 4°C.

96 well round bottomed PVC Plates (Dynatech, Sussex, UK) were coated with 25μl peptide at 100μg/ml or saline as a control and incubated overnight. The peptide or saline was removed and the plates incubated with 5% BSA/PBS for 30 minutes to block remaining protein binding sites. The plates were washed three times in 0.1% BSA/PBS and incubated with 25μl of sera diluted in 0.5% BSA/PBS in duplicate for 1 hour. The sera was removed and the plates washed as before. 50μl $^{125}$I RAR in 0.5%BSA/PBS was added at 300,000 cpm per well and incubated for 1 hour at 4°C. The plates were washed three times as before, the wells cut out and placed in LP3 tubes and the bound radioactivity measured in a gamma counter.

5.8.4 Haemolysis assays

25μl of mouse blood containing $2.5\times10^8$RBC/ml (prepared as in section 5.7.1) in 0.5% BSA/PBS was placed into 96 well round bottomed tissue culture plates (Sterilin, Staffs, UK). 25μl of rat sera in doubling dilutions from neat to 1/128 in 0.5% BSA/PBS were added and the plate was incubated for 1 hour at room temperature. Degree of lysis was assessed by careful observation of the plate. Then 25μl of guinea pig complement (Serotec, Oxford, UK) was added following the initial incubation and the plates observed after an hour.
5.8.5 Flow cytometry

Platelets were prepared as described in section 5.7.4. 50μl of platelets at 4x10^7/ml were incubated with 50μl of rat sera at various dilutions for 1 hour on ice. Platelets were washed twice by centrifugation at 2000g 15min at 4°C in calcium and magnesium free PBS. 50μl FITC-RAR (prepared in our laboratory, essentially as described by Dalchau and Fabre 1979) at 50μg/ml and blocked with 20% normal mouse serum for 1 hour before use, was added to the platelet pellet. The pellet was vortexed and incubated for 1 hour on ice. The pellet was washed twice as above and resuspended in 2% formalin/saline to await analysis on a Becton/Dickinson flow cytometer.

5.9 Proliferation Assays

Cervical, para-aortic and popliteal lymph nodes were removed from rats under aseptic conditions. The nodes were minced through a sieve in RPMI 1640 with HEPES (Gibco BRL, Paisley, Scotland). The suspension was allowed to settle for 5 min, the suspension was washed twice by centrifugation at 400g for 10min at 4°C. The viability was assessed using trypan blue exclusion, and was always greater than 70% . The cells were resuspended at 2x10^5/ml in RPMI 1640 medium without HEPES supplemented with 5% normal rat serum, 2mM glutamine, 100units/ml penicillin, 100μg/ml streptomycin (ICN-Flow, Oxon UK) and 5x10^-5M 2-mercaptoethanol (BDH, Leics UK).

100μl of lymph node cells at 2x10^5/ml were incubated in 96 well round bottomed tissue culture plates. Cultures were stimulated with specific or irrelevant peptides at
20μg/ml, 10μg/ml and 1μg/ml, culture medium alone (negative control) or concanavalin A (Sigma, Dorset, UK) (positive control) at 10μg/ml. Incubations were at 37°C in 5% CO₂ in air. Twenty four hours prior to harvesting 1μCi of ³H thymidine (Amersham, Berks, UK) in 20μl culture medium was added to each well. Cells were harvested using a semi-automatic cell harvester. Filters were dried at 56°C for three hours or overnight at room temperature and counted using Opticsint Hisafe III scintillation fluid (LKB/Pharmacia Herts, UK) and incorporated radioactivity measured on a scintillation counter.

5.10 Immunohistology

5.10.1 Monoclonal antibodies against rat antigens

MN4-91-6 : (IgG1 isotype) is directed against a polymorphic determinant of RT1.A class I antigens. It is positive for DA, SHR rat strains and negative for PVG, LEW, WAG, BN, AS2 and M520 rat strains (Milton and Fabre 1985).

F16-4-4 : (IgG1 isotype) is directed against a monomorphic determinant of class I RT1.A antigens. It is positive for all rat strains tested to date (Hart and Fabre 1981).

MRC OX6 : (IgG1 isotype) is directed against a monomorphic determinant of RT1.B class II antigens. It is positive for all rat strains tested to date (Fukumoto et al 1985).

MRC OX8 : (IgG1 isotype) is directed against the rat CD8 molecule which is expressed predominantly on cytotoxic T cells (Brideau et al 1980) and is also expressed on rat NK cells.
**W3/25** : (IgG1 isotype) is directed against the rat CD4 molecule which is expressed predominantly on helper T cells (Williams et al 1977) and is also expressed on macrophages.

**MRC OX1** : (IgG1 isotype) is directed against the rat leucocyte common antigen CD45 which is expressed on all leucocytes (Sunderland et al 1979).

**MRC OX19** : (IgG1 isotype) is directed against the CD5 molecule which is expressed on thymocytes and peripheral T cells (Dallman et al 1982).

**R73** : (IgG1 isotype) is directed against the α/β TCR on T cells (Hunig and Bevan 1982).

**BMAC-5** : (IgG1 isotype) is directed against a rat determinant expressed on macrophages (Spencer and Fabre 1990).

**NDS61** : (IgG1 isotype) (Serotec, Oxon, UK) is directed against CD25 (IL-2 receptor) (Tellides et al 1987).

The W3/25, MRC OX1, OX6, OX8, OX19 and R73 cell lines were a kind gift from Dr. A. F. Williams, MRC Celluar Immunology Unit, Oxon, UK.

### 5.10.2 Monoclonal antibodies against mouse antigens

All monoclonal antibodies used were mouse anti-mouse antibodies.

**MCA 180** : (IgG2a subclass) (Serotec, Oxon, UK) is directed against I-E^k mouse MHC class II (Hammerling et al 1979).

**MCA 648** : (IgG2a subclass) (Serotec, Oxon, UK) is directed against H-2K^d mouse MHC class I (Arnold et al 1985).
5.10.3 Monoclonal antibodies against human antigens

F15-42-1 is directed against human Thy-1 and was used as a control (McKenzie and Fabre 1981).

5.10.4 Cryostat sections

Cryostat sections of 5μm were cut on to gelatinised slides and freeze dried overnight. They were then fixed in acetone for 10 min at room temperature and stored at -20°C or used immediately.

5.10.5 Immunoperoxidase staining

To stain the sections 50μl of the appropriate monoclonal antibody at saturating concentrations (usually 1/100 dilution) was added in 0.5% BSA/PBS. The slides were incubated in a humidified atmosphere for 30 min at room temperature. The slides were washed three times in TBS. 50μl of horse-radish peroxidase labelled rabbit anti-mouse immunoglobulin (Dako Bucks, UK) at a 1/7 dilution in 0.5% BSA/PBS (containing 20% heat inactivated normal rat serum to block any cross reactivity of the rabbit anti-mouse immunoglobulin with rat immunoglobulin), was added and incubated for 30 min at room temperature. The slides were washed with warm TBS and incubated with 50μl warm substrate (Diaminobenzidine tablets were dissolved in 5ml distilled water by vortexing (Sigma, Dorset, UK)) for 6 to 8 min until the brown colour had developed. The sections were washed again in TBS and counterstained with Harris’s haematoxylin (BDH, Leics, UK), dehydrated in graded alcohols and mounted in DPX (BDH, Leics UK).
5.11 Lowry estimation of protein content for heart homogenate

Solution A: 2ml of 2% CuSO₄.5H₂O, 2ml of 4% NaKTartrate and 96ml of 3% anhydrous Na₂CO₃ (Sigma Dorset, UK) were added together in the above order and allowed to stand at room temperature for an hour.

Crystalline BSA standard (1mg/ml in distilled H₂O) was diluted in 1M NaOH to give 200, 160, 120, 80 and 40mg/ml. 200μl of each dilution was transferred to LP3 tubes in duplicate. Doubling dilutions of the heart homogenate from 1/50 to 1/400 were made in 1M NaOH as above. 1ml of solution A was added to each tube and vortexed. 10 minutes later 100μl of 50% Folin and Ciocalteau's Phenol reagent (Sigma Dorset, UK) (made up in distilled water just before use) was added and the tubes immediately vortexed. 30 min later the tubes were vortexed and the absorbance read at 640nm against 1M NaOH in a spectrophotometer (Ultraspec III Pharmacia Herts. UK). A standard curve of the optical density against the protein concentration was plotted for the standard and the unknown sample protein calculated.
CHAPTER 6:

INDIRECT T CELL ALLORECOGNITION

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CHAPTER 6:
INDIRECT T CELL ALLORECOGNITION

6.1 Introduction

The recognition of intact foreign MHC molecules on the cells of the graft by recipient T cells (direct allorecognition) (Matzinger and Bevan 1977, Lechler et al 1990) has for a long time been considered the major pathway of T cell activation in transplantation. In recent years the usual method for T cell activation against nominal antigens whereby T cells recognise antigen that has been taken up, processed and presented in association with self MHC molecules on the surface of self APC has begun to be considered as a mechanism that might be important in transplantation. This pathway is now termed indirect allorecognition to distinguish it from the direct allorecognition pathway.

In order for indirect recognition to take place donor MHC needs to be taken up, processed and presented in recipient class II molecules which need to be recognisable by recipient TCR. The involvement of recipient class II molecules was demonstrated by studies in our laboratory where rat kidney allograft rejection was suppressed by the use of recipient specific monoclonal antibodies to class II molecules (Priestley et al 1992). The source of antigen for indirect allorecognition will be that which is shed from the graft.

These antigens are exogenous antigens and so would be taken up, processed and presented in association with MHC class II molecules to the TCR of CD4+ T helper cells. Since the quantity of antigen present would be important it is likely than class I MHC antigens are more likely to be important than class II MHC antigens as there are
more class I molecules present in a graft than class II molecules. Although it is possible for T cell recognition to occur with all antigens, the major polymorphic antigens on an allograft are likely to be the MHC molecules. The original studies from this laboratory involved the denatured heavy chains from class I and class II MHC molecules and later peptides derived from the class I heavy chain. This has important considerations when looking at rejection responses in xenografts as in these cases there would be many more molecules that are likely to elicit an immune response. Indirect recognition is also likely to play a more important role in xenograft responses where there is incompatibility between cells involved in direct recognition for example adhesion molecules, co-stimulatory molecules and cytokines.

Studies by Dalchau et al (1992) in our laboratory demonstrated that priming to indirect allorecognition could be achieved by immunisation of LEW rats with pure denatured DA class I heavy chains and class II α and β chains. Immunised rats produced alloantibodies to each of the denatured chains but not to intact molecules on the cell surface. DA skin grafts on these animals showed an accelerated rejection response. Antibody production to intact DA MHC molecules was not present prior to transplantation in this model, excluding a role for antibody formed prior to grafting in this accelerated rejection response. Fangmann et al (1992) in our laboratory continued these studies using DA class I peptides. Peptide 1 from the α helical region of the first domain, peptide 3 from the α helical region of the second domain and peptide 2 from the β sheet of the second domain. Immunisation of LEW recipients with the peptides 1 and 3 prior to DA skin grafting resulted in an accelerated rejection response. These studies demonstrated that priming for indirect allorecognition of donor MHC antigens can contribute to the effector mechanisms of skin allograft rejection. However
when CsA was administered the accelerated rejection seen in the skin grafts was lost. In clinical transplantation immunosuppressive agents such as CsA are in routine use, I therefore wished to determine whether priming for indirect allorecognition would influence the effectiveness of CsA immunosuppression for kidney allograft rejection and to examine the influence of CsA on the T helper response for antibody production following priming to indirect recognition. LEW rats were immunised with peptides 1 and 3 from the class I heavy chain of DA rats in Freund's adjuvant prior to receiving a DA kidney allograft and a course of CsA therapy.
6.2 Materials and Methods

6.2.1 Experimental animals

Inbred DA (RT1<sup>av</sup>) and LEW (RT1<sup>i</sup>) rats were purchased from Harlan UK Ltd (Oxon, UK). All rats were adult males.

6.2.2 Peptide immunisations

The RT1.A<sup>av</sup> classical class I peptides termed peptide 1 and peptide 3 described in section 5.4.0 were used. LEW rats received 100µg of free peptide emulsified in Freund's complete adjuvant subcutaneously into each hind footpad. Rats were boosted four weeks later with the same dose of peptide emulsified in Freund's incomplete adjuvant. Peptide 1 was administered to the left footpad and peptide 3 to the right footpad.

6.2.3 Left orthotopic kidney transplantation

Donor DA kidneys were transplanted into LEW recipients using the technique described in section 5.2.1. Total ischaemic times were in the range of 25 to 30 minutes. A nephrectomy was performed at day 7 at which time the graft was biopsied for histological studies. Recipients were bled from the tail at day 0, 7, 10 and weekly thereafter for assessment of graft function by urea analysis as described in section 5.2.1.
6.2.4 Cyclosporin A

Cyclosporin A at 10mg/ml was prepared as in section 5.3.2 and administered daily from day 0 to day 14 post transplant by gavage.

6.2.5 Assays for alloantibody response to intact DA class I molecules

Indirect radioactive binding assays (described in section 5.8.1) were performed on sera taken at day 0, 7 and 14 days post transplant using DA fresh red blood cells (prepared as in section 5.7.1) as a source of intact DA class I molecules.

6.2.6 Assays for antibody response to peptide

Indirect radioactive binding assays using PVC plates coated with peptide 1, peptide 3 and saline as described in section 5.8.3 were used on sera taken on the day of peptide boost, two weeks post boost and day of transplant.
6.3 Results

6.3.1 Graft function and survival

The survival of DA to LEW kidney allografts is summarised in table 6.1. Survival of untreated rats is equal to or less than 14 days with blood urea levels greater than 300mg/100ml at day 10 post graft (normal blood urea is <60 mg/100ml). The survival of LEW recipients immunised with the DA class I peptides in Freunds adjuvant or saline in Freunds adjuvant prior to receiving a DA kidney graft is shown in table 6.1. Immunisation with DA class I peptides in Freunds adjuvant had no obvious effect on graft function and survival in comparison with control rats immunised with saline in Freunds adjuvant.

Survival of the 5 peptide immunised rats ranged from 42 to 77 days after grafting with blood ureas at day 10 from 82 to 163mg/100ml. Survival of the 5 LEW rats immunised with saline in Freunds adjuvant ranged from 56 to 105 days after grafting with blood urea levels from 62 to 118 mg/100ml. These results demonstrate that conventional doses of CsA were able to suppress any effect that priming to indirect allore cognition had on the rejection response to the kidney grafts.

6.3.2 Histology

Histological analysis of biopsy samples taken at day 7 post transplant showed no difference between the two groups (data not shown).
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Table 6.1
Survival and blood urea levels of DA kidney allografts in LEW recipients
^ Untreated LEW recipients of DA kidney allografts.
# LEW recipients were immunised with saline in Freunds adjuvant and were given a DA kidney allograft four weeks after the booster.
~ LEW recipients were immunised with peptides 1 and 3 of the DA RT1.A class I molecule in Freunds adjuvant and were given a DA kidney allograft four weeks after the booster.
* CsA was administered at 10mg/kg daily by gavage for 14 days from the day of transplant.
ϕ p=0.116 between survival data of peptide and saline immunised groups (Mann Whitney U Test)
ND = not done
6.3.3 Anti-peptide antibody responses following immunisation with peptides 1 and 3

The generation of an antibody response following immunisation with peptide is a good indicator of peptide interaction with class II MHC and subsequent recognition by TCR of peptide in association with MHC molecules. Antibody responses were assessed using the plate binding assay. Sera were analysed at day of boost i.e. 4 weeks after the primary immunisation and 2 weeks post boost i.e. week 6 following primary immunisation. This time point enables a secondary antibody response to be studied. Assays were performed for each rat in the control and experimental groups against peptide 1, peptide 3 and saline as a control. The response for two animals to peptide 1 is shown in figure 6.1a. At week 4, LEW rats immunised with peptide 1 and 3 showed an antibody response although one had only a weak response. By week 6 the two peptide immunised animals showed strong anti-peptide 1 antibody responses indicating a secondary antibody response had been made. LEW rats immunised with saline in Freunds adjuvant showed no response to peptide 1 at week 4 or 6. The response to peptide 3 (fig 6.1b) was similar to that made to peptide 1 but with both peptide immunised rats showing a good antibody response at week 4 which is accentuated at week 6. Rats immunised with saline in Freunds adjuvant showed no antibody response to peptide 3. None of the sera showed any binding to the saline coated plate (fig 6.2) indicating that antibody responses to peptides 1 and 3 were not due to non-specific binding of antibodies to the PVC plates. These assays were performed for each of the rats in the peptide immunised and saline immunised groups and representative assays are shown. The provision of T cell help for B cells requires recognition by the helper T cell receptor of antigenic peptides on the class II molecules of the B cells (Noelle
Figure 6.1
Antibody response to (a) peptide 1 and (b) peptide 3 of the DA class I molecule.
LEW rats were immunised with peptides 1 and 3 of the DA RT1.A class I molecule in Freunds adjuvant or with Freunds adjuvant only and boosted four weeks later. Sera were taken from a peptide immunised rat at week 4 (■) and week 6 (□), and from another peptide immunised rat at week 4 (●) and week 6 (○). Sera were taken from a control rat immunised with saline at week 4 (●) and week 6 (○), and from another control rat at week 4 (▼) and week 6 (▼). The sera were tested for binding to the peptide on PVC plates.
CPM indicates $^{125}$I RAR bound per assay.
Figure 6.2
Control for nonspecific background binding.

LEW rats were immunised with peptides 1 and 3 of the DA RT1.A class I molecule in Freunds adjuvant or with saline in Freunds adjuvant and boosted four weeks later. Sera were taken from a peptide immunised rat at week 4 (■) and week 6 (○), and from another peptide immunised rat at week 4 (●) and week 6 (○). Sera were taken from a control rat immunised with saline at week 4 (●) and week 6 (○), and from another control rat at week 4 (▼) and week 6 (▼). Sera were tested for binding to the PVC plate coated with saline.

CPM indicates $^{125}$I RAR bound per assay.
and Snow 1990). These antibody responses to the peptide therefore indicate that 
priming to indirect allore cognition has occurred

6.3.4 Alloantibody response to intact DA class I molecules

Priming to indirect allore cognition primes T helper cells for antibody production
(Dalchau et al 1992). It was of interest to determine if CsA therapy affects antibody 
production to the graft in peptide immunised recipients. LEW rats were immunised 
with either peptides 1 and 3 in Freunds adjuvant or saline in Freunds adjuvant. Four 
weeks following the boost they received a DA kidney allograft with CsA therapy at 
10mg/kg for 14 days which commenced at the time of transplantation. Alloantibody 
responses to intact class I prior to and following kidney transplantation were studied. 
In these assays DA RBC were used as a source of intact DA class I molecules.

The alloantibody response at day 0, 7 and 14 to intact DA class I molecules can be 
seen in figure 6.3. At day 0 prior to kidney transplantation there is no detectable 
antibody responses to intact class I in either the peptide immunised rats or rats 
immunised with saline in Freunds adjuvant. At day 7 a strong antibody response is seen 
in the peptide immunised rats which falls substantially by day 14. Although the saline 
immunised rats have a weaker antibody response at day 7 in comparison with the 
peptide immunised rats, this level of antibody response is still reduced by day 14. 
Similar results were obtained for all rats and representative titrations are shown.
Figure 6.3

Kinetics of antibody responses to intact class I MHC molecules after kidney grafting. LEW rats were immunised with peptides 1 and 3 of the DA RT1.A class I molecule in Freunds adjuvant or with saline in Freunds adjuvant and were given a DA kidney allograft four weeks after the booster injection. CsA was given orally, daily for 14 days after the kidney graft. Sera were taken from a peptide immunised rat at the time of grafting (●), at day 7 (■) and at day 14 (□) after kidney grafting and from another peptide immunised rat at the same times, day 0 (△), day 7 (●) and day 14 (○). A control rat immunised with saline in Freund adjuvant was bled at day 7 (▲) and day 14 (△) after grafting. The sera were tested for binding to DA RBC.

CPM indicates $^{125}$I RAR bound per assay.
The alloantibody response at day 7 to intact DA class I molecules can be seen in figure 6.4. Sera from 5 rats from each group were titrated on DA RBC. The control rats immunised with saline in Freund's adjuvant only had barely detectable antibody responses to the graft at day 7. The peptide immunised rats however all had high levels of antibody to the intact class I antigen. This demonstrates that priming of the T helper compartment resulted in an antibody response that was poorly suppressed by CsA.
Figure 6.4

Alloantibody responses to intact class I MHC molecules at day 7 after kidney grafting. LEW rats were immunised with peptides 1 and 3 of the DA RT1.A class I molecule in Freund's adjuvant (●) or with saline in Freund's adjuvant (○) and were given a DA kidney allograft four weeks after the booster injection. CsA was given orally, daily for 14 days after the kidney graft. Sera taken at day 7 were tested for binding to DA RBC, and the results for individual rats are given. (▲) indicates normal rat serum. CPM indicates ¹²⁵I RAR bound per assay.
6.4 Discussion

The role of indirect and direct recognition in the rejection response is poorly understood. The routes of activation for these pathways are likely to be different and the state of sensitisation after any form of exposure to foreign MHC antigens, for example blood transfusions, is likely to vary. The contribution of indirect recognition to the effector mechanisms of allograft rejection is unclear, although it is likely to have a role some time after primary exposure to antigens e.g. in the late stages of primary rejection responses and in chronic rejection. Earlier studies in our laboratory have demonstrated that priming to indirect allorecognition results in an accelerated rejection response to skin grafts (Dalchau et al 1992a, Fangmann et al 1992). This accelerated rejection response is abrogated when skin grafting is performed under CsA therapy (Dalchau et al 1992b), demonstrating that priming to indirect recognition can be suppressed by conventional doses of CsA. Administration of CsA for 10-14 days daily by gavage is sufficient to prolong survival of kidney allografts for greater than 100 days at doses as low as 5mg/kg and is capable of suppressing alloantibody responses to the graft (Priestley et al 1989).

In my study I wished to extend the work performed using the skin graft model to determine whether priming to indirect allorecognition would influence the effectiveness of CsA in abrogating the rejection response in a vascularised kidney allograft model. Accelerated rejection responses are difficult to determine in the kidney graft model due to the short rejection times of 10 to 14 days following a nephrectomy at day 7 in unmodified recipients. Therefore groups receiving peptide immunisation prior to kidney grafting in the absence of CsA were not performed, and the study was restricted to comparisons of groups given CsA. Graft function and survival in LEW rats
immunised with the DA class I peptides prior to a DA kidney allograft under CsA therapy were similar to those of control LEW rats immunised with saline in Freund's adjuvant. This demonstrates that the action of CsA in abrogating DA kidney allograft rejection is not influenced by priming to indirect allorecognition. Thus in rat kidney allograft rejection, the effector mechanisms dependent upon indirect T cell recognition are susceptible to CsA suppression. However CsA therapy was unable to suppress the antibody response following kidney allografting in recipients primed to indirect allorecognition, indicating that once clonal expansion of T cells involved in indirect T cell help has occurred this pathway of T help for B cells is poorly suppressed by CsA. This has important clinical implications as CsA is virtually in universal use as an immunosuppressive agent in clinical transplantation. Failure of CsA to suppress the alloantibody response following a vascularised organ allograft in recipients primed to the indirect recognition pathway, although of no consequence in the rat where antibody does not necessarily have a deleterious affect (Fabre and Morris 1974) has potential important implications in the clinic especially for acute vascular rejection (Jeanett et al 1970) and chronic rejection (Suciu-Foca et al 1991) where antibodies have been implicated in the rejection response. Alloantibodies to intact class I were absent in all groups prior to transplantation at day 0. This demonstrates that antibodies to the DA class I peptides do not react with the intact DA class I molecules, therefore at the time of grafting the primed animals would have primed helper T cells and virgin B cells with regard to the native class I molecule. The titre of alloantibody to intact DA class I fell substantially between day 7 and day 14 in both groups. It is interesting that in the rats whose helper T cells have been primed, CsA is unable to suppress the antibody response at day 7 although it appears to begin to have an effect by day 14. Although
this reduction could be due to the kinetics of the response it is unlikely, as Fangmann et al (1992) demonstrated an increase in antibody response rising from day 7 and increasing to day 14 following skin grafting of peptide immunised rats.
CHAPTER 7:
INDIRECT T CELL ALLORECOGNITION AND THE MECHANISMS OF
IMMUNOSUPPRESSION BY DONOR BLOOD TRANSFUSION

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CHAPTER 7:
INDIRECT T CELL ALLORECOGNITION AND THE MECHANISMS OF IMMUNOSUPPRESSION BY DONOR BLOOD TRANSFUSION

7.1 Introduction
In 1973 Opelz published his clinical study that demonstrated the beneficial effects of pre-graft blood transfusions on graft survival. Blood transfusions have been used by several groups to achieve long term survival of organ allografts in rodent models. The immunosuppressive effects of blood transfusion have also been seen after burns and surgery where a higher incidence of infection is noted following blood transfusion (Galandiuk et al 1990). It is now known that the formed elements of the blood are important (Fabre and Morris 1972), (El-Malik et al 1984). The blood transfusion effect has been demonstrated in rodents using RBC (Wood et al 1985), platelets (Hibberd and Scott 1983) and leucocytes (Cranston et al 1987). However the blood transfusion effect appears to be strain specific (Soulilou et al 1984) and cannot be induced in every strain combination. The mechanisms of this blood transfusion effect are poorly understood, and have not previously been considered in terms of direct and indirect T cell recognition. In order to clarify the mechanisms behind the blood transfusion effect we wished to study the influence of priming to indirect allorecognition using MHC class I peptides. As we wished to study the effect of DA class I peptides on the blood transfusion effect it was necessary to use the DA-to-LEW strain combination as LEW rats have been shown to react to specific RT1.A class I peptides of the DA strain (Fangmann et al 1992a). Earlier studies giving a DA blood transfusion to LEW rats, previously immunised with a DA class I peptide in Freunds adjuvant or saline in
Freunds adjuvant, seven days prior to a (DAxLEW) F1 kidney graft resulted in no prolongation of survival even in the saline immunised group. This indicates that a single donor specific transfusion (DST) in this strain combination was insufficient to induce tolerance. This is in contrast to the studies by Wood et al (1985) where long term survival was achieved and Fabre and Morris (1972) where marginal prolongation was achieved in this strain combination. However immunisation in adjuvant was not performed in these studies. Multiple blood transfusions i.e. repeated exposure to the donor blood given over a period of several weeks has been shown to be more effective (Fabre and Morris 1972). Therefore in this model multiple blood transfusions will be used. The timing of multiple blood transfusions is important as some investigators have found them to be ineffective (Bushell et al 1994).

In this study the influence of priming to indirect allorecognition on the blood transfusion effect prior to and during multiple blood transfusion was addressed.

LEW rats were immunised with either the DA class I peptide or saline in Freunds adjuvant either prior to or during the course of DA multiple blood transfusions. Twice weekly blood transfusions of 0.5ml DA blood in 0.5ml saline were administered via the tail vein for periods of up to 12 weeks. Antibody responses were studied to peptide 1 and intact DA class I molecules throughout the time course. T cell proliferation to the DA class I peptide was also studied.
7.2 Materials and Methods

7.2.1 Experimental animals
Inbred DA (RT1<sup>Av</sup>) and LEW (RT1<sup>L</sup>) rats were purchased from Harlan UK Ltd (Oxon, UK). All rats were adult males.

7.2.2 Peptide immunisations
The RT1.A<sup>Av</sup> classical class I peptide termed peptide 1 described in section 5.4.0 was used. LEW rats received 50μg of unconjugated peptide emulsified in Freund's complete adjuvant subcutaneously into each hind footpad as described in section 5.4.1. Rats were boosted four weeks later with the same dose of peptide emulsified in Freund's incomplete adjuvant. Control rats received an equal volume of saline emulsified in Freund's adjuvant.

7.2.3 Blood transfusions
LEW rats received twice weekly injections of 0.5ml of fresh heparinised DA blood in an equal volume of saline by i.v. injection into the tail vein as described in section 5.5.0. Control rats received 1ml of saline.

7.2.4 Assays for alloantibody response to intact class I molecules
Indirect radioactive binding assays (described in section 5.8.1) were performed on sera taken as stated in the experimental groups (7.3). DA fresh red blood cells were used as a source of intact DA class I molecules prepared as in section 5.7.1.
7.2.5 Assays for antibody response to peptide

Indirect radioactive binding assays using PVC plates coated with peptide 1 as described in section 5.8.3 were used on sera taken as stated in the experimental groups (7.3).

7.2.6 T cell proliferation assays

T cell proliferation assays were performed 10 days following peptide boost as described in section 5.9.0. Cervical, popliteal and paraaortic lymph nodes were removed aseptically and dispersed into a single cell suspension plated out at $2 \times 10^6$/ml in culture medium. Peptide 1, 3 and an irrelevant peptide, peptide 32 from the β chain of mouse class II (H-2Aβ) were added at 20μg, 10μg and 1μg. Con A at 10μg/ml was used a positive control. 24 hours before harvesting 1μCi in 20μl culture medium was added to the plates. Plates were harvested at day 4 and day 5. Cell bound radioactivity was measured using a rack beta counter.
7.3 Experimental Groups

7.3.1 Peptide immunisation during multiple blood transfusions

**Group 1** 5 LEW rats were injected i.v. twice weekly with 0.5ml DA blood in an equal volume of saline for up to 12 weeks. At week 6 they received 50μg of peptide 1 emulsified in Freunds complete adjuvant to each hind footpad and were boosted at week 10 in Freunds incomplete adjuvant. They were bled for serum as shown below.

| tail bleed |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| week      | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | d10 post boost |
| peptide 1 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| boost     |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

Start twice weekly blood transfusion

End twice weekly blood transfusion

**Group 2** (Control for the effects of peptide immunisation *per se*). 5 LEW rats were injected twice weekly with 1ml of saline for up to 12 weeks. At week 6 they received 50μg of peptide 1 emulsified in Freunds complete adjuvant to each hind footpad and were boosted at week 10 in Freunds incomplete adjuvant. They were bled for serum as shown below.

| tail bleed |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| week      | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | d10 post boost |
| peptide 1 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| boost     |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

Start twice weekly saline transfusion

End twice weekly saline transfusion
**Group 3** (Control for the effects of Freund’s adjuvant). 5 LEW rats were injected twice weekly with 0.5ml DA blood in an equal volume of saline for up to 12 weeks. At week 6 they received saline emulsified in Freund’s complete adjuvant to each hind footpad and were boosted at week 10 in Freund’s incomplete adjuvant. They were bled for serum as shown below.

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\[ \text{start twice weekly blood transfusion} \quad \text{end twice weekly blood transfusion} \]

**7.3.2 Peptide immunisations prior to multiple blood transfusions**

**Group 4** 5 LEW rats were immunised with peptide 1 in Freund’s complete adjuvant at week 0. At week 4 they received twice weekly injections of 0.5ml DA blood in an equal volume of saline until week 10. They were bled for serum as shown below.

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<td>3</td>
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<td>8</td>
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<td>10</td>
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\[ \text{start twice weekly blood transfusion} \quad \text{end twice weekly blood transfusion} \]
Group 5 (Control for the effects of Freunds adjuvant). 5 LEW rats were immunised with saline emulsified in an equal volume of Freunds complete adjuvant at week 0. At week 4 they received twice weekly injections of 0.5ml DA blood in an equal volume of saline until week 10. They were bled for serum as shown below.

| tail bleed | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ | ↓ |
| week | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 14 |
| saline | ↑ | | | | | | | | | | | |
| start twice weekly blood transfusion | | | | | | | | | | | | end twice weekly blood transfusion |
7.4 Results

7.4.1 T cell proliferation assays

T cell proliferation assays were performed on rats in group 1 and group 2 at day 10 post boost. This was to see if the multiple blood transfusions suppressed the indirect T cell response to the peptide.

T cell proliferation was seen to the immunising peptide, peptide 1 in a dose dependent manner with 20μg giving the highest response followed by 10μg and 1μg. T cell proliferation at day 4 was higher than day 5. T cell proliferation was seen in both the blood transfused and saline transfused rats immunised with peptide 1. Each assay compared one blood transfused and one saline transfused rat. In three out of the 4 assays blood transfused peptide immunised rats demonstrated a lower T cell proliferation to peptide 1 than saline transfused peptide immunised rats seen in fig 7.1. In the remaining assay T cell proliferation was approximately the same for blood and saline transfused rats. These results suggest that there may be a partial suppression of T cell proliferation to peptide 1 in blood transfused LEW rats.

T cell proliferation was not seen in any of the rats to the DA class I peptide, peptide 3 or to the irrelevant peptide, peptide 32. This suggests that DA blood transfusion did not sensitise to indirect T cell recognition.

7.4.2 Antibody responses to peptide of rats immunised with peptide during blood transfusion

Antibody responses to peptide 1 were performed using the plate binding assay in groups 1-3. This was to check for any functional sensitisation or suppression of the T
Figure 7.1

T cell proliferative responses to DA class I peptides, following peptide immunisation during blood transfusion.

LEW rats were immunised with peptide 1 in Freund's complete adjuvant at week 6 and boosted four weeks later with peptide 1 in Freund's incomplete adjuvant. Cervical, mesenteric and para-aortic lymph node cells were removed 10 days following the boost in LEW rats receiving (a) twice weekly DA blood transfusions of 0.5ml DA blood in an equal volume of saline i.v. via the tail vein from week 0 or (b) twice weekly injections of 1ml saline i.v. via the tail vein from week 0. Three out of 4 pairs of rats studied demonstrated the profile shown here. Each result shown is the mean response with standard error bars, of triplicate wells containing 2x10^5 cells and stimulated with the concentration of peptide shown at day 4 (solid bars) and day 5 (open bars). med indicates medium alone, con A indicates concanavalin A at 10μg/ml as a positive control.

CPM refers to mean of triplicate counts of ^3H thymidine incorporated into the cells.
CPM x10^{-3}

peptide concentration ug/ml

peptide concentration ug/ml
helper responses to the peptide, although an effect on B cells responding to the peptide could not be ruled out.

Group 1: Blood transfused LEW rats immunised with peptide 1 showed no anti-peptide antibody responses to peptide 1 until after peptide immunisation at week 6. There was a weak response seven days following the peptide immunisation. By week 10 (4 weeks after immunisation) this antibody response was very strong. This antibody response was elevated further following the boost at week 10 (fig 7.2a). Four out of five rats studied followed this pattern. One of the five rats studied failed to demonstrate any anti-peptide antibodies following peptide immunisation (fig 7.3a). Of particular interest, this rat also failed to follow the normal pattern of antibody production to intact DA class I as discussed later in section 7.4.4.

Group 2: Saline transfused LEW rats immunised with peptide 1 followed a similar pattern of anti-peptide response. Anti-peptide antibody response was not demonstrated until 7 days after peptide immunisation. A strong antibody response was seen at week 10 which was elevated after the boost (fig 7.2b).

Group 3: None of the blood transfused rats immunised with saline in Freund's adjuvant showed any antibody response to peptide 1 (fig 7.3b).

Thus in only one rat in five did there appear to be any suppression of either the T helper response or (less likely) the B cell responses to the peptide following blood transfusion. The antibody response shown in fig 7.2a is a primary type response, this demonstrates that blood transfusions do not sensitize to antibody production to the peptide. Therefore there was no priming of T helper or B cells to the peptide by the blood transfusions.
Figure 7.2

Kinetics of antibody response of LEW rats, immunised with peptide 1 during multiple saline or blood transfusions, to the DA class I MHC peptide, peptide 1.

(a) Twice weekly injections of 0.5ml DA blood in an equal volume of saline i.v. via the tail vein or (b) twice weekly injections of 1ml of saline i.v. via the tail vein were administered. Blood or saline transfusions commenced at week 0. At week 6 LEW rats were immunised with 50μg of peptide 1 in Freunds complete adjuvant to each hind footpad and were boosted at week 10 in Freunds incomplete adjuvant. Sera were taken at week 0 (●), week 1 (□), week 2 (△), week 4 (○), week 6 (▼), week 7 (■), week 8 (▲), week 10 (●) and 10 days after the boost (▼). All rats in each group followed a similar profile and a representative rat for each group is shown. Target cells were DA RBC at 10^9/ml.

CPM indicates ^125^I RAR bound.
Figure 7.3

Kinetics of antibody response of LEW rats, immunised with peptide 1 during multiple blood transfusions, to the DA class I MHC peptide, peptide 1.

Twice weekly injections of 0.5ml DA blood in an equal volume of saline i.v. via the tail vein were administered. Blood transfusions commenced at week 0. At week 6 LEW rats were immunised with (a) 50μg of peptide 1 or (b) saline in Freunds complete adjuvant to each hind footpad and were boosted at week 10 in Freunds incomplete adjuvant. Sera were taken at week 0 (◇), week 1 (□), week 2 (△), week 4 (○), week 6 (▼), week 7 (■), week 8 (▲), week 10: (●) and 10 days after the boost (▼). Fig 3a The LEW rat which did not demonstrate any immunosuppressive effects with multiple blood transfusion, is shown. All saline immunised rats (b) followed a similar profile and a representative rat is shown. Target cells were DA RBC at 10^9/ml. CPM indicates ^125^I RAR bound.
CPM BOUND

$10^{-3}$

SERUM DILUTION

147
7.4.3 Antibody responses to peptide in rats immunised with peptide prior to blood transfusion

Group 4: LEW rats were immunised with peptide 1 prior to multiple blood transfusion. A weak anti-peptide 1 antibody response was demonstrated at week 2 following the peptide immunisation at week 0 (fig 7.4a). A strong antibody response was seen at week 4 which remained at a similar level throughout the course of the blood transfusion. By week 11 (one week after cessation of blood transfusion) the antibody response began to decline, decreasing further by week 14, four weeks after cessation of blood transfusion. This pattern was seen in all rats studied in this group.

Group 5: LEW rats were immunised with saline in Freund's adjuvant prior to multiple blood transfusion. No antibody responses were seen to peptide 1 in this group of rats (fig 7.4b).

Antibody responses to plates coated with saline were negative for rats in all five groups indicating that non-specific binding had not occurred.

The blood transfusions did not boost the response to the peptide, as did secondary peptide immunisation. This shows that blood transfusion neither primes nor stimulates antibody responses to the peptide.

7.4.4 Alloantibody responses to intact DA class I in rats immunised with peptide during multiple blood transfusion

Group 3: The alloantibody responses of LEW rats to intact DA class I molecules following twice weekly blood transfusion of DA blood for 10 weeks and immunisation of saline in Freund's adjuvant at week 6 and week 10 are shown in figure 7.5. At week 0 prior to treatment no antibody response is seen, at week 1 (after 2 blood
Figure 7.4

Kinetics of antibody response of LEW rats, immunised with peptide 1 prior to multiple blood transfusions, to the DA class I MHC peptide, peptide 1.

Twice weekly injections of 0.5ml DA blood in an equal volume of saline i.v. via the tail vein were administered. LEW rats were immunised with either 50µg of peptide 1 (a) or saline (b) in Freunds complete adjuvant to each hind footpad. Immunisations were at week 0, blood transfusions commenced at week 4 and continued to week 10. Sera were taken at week 0 (◇), week 2 (□), week 4 (△), week 5 (●), week 6 (▼), week 8 (■), week 10 (▲), week 11 (○) and week 14 (▼). All rats in each group followed a similar profile and a representative rat for each group is shown. Target cells were DA RBC at 10⁹/ml.

CPM indicates ¹²⁵I RAR bound.
Figure 7.5

Kinetics of antibody response of LEW rats, immunised with saline during multiple blood transfusions, to intact DA class I MHC molecules.

Twice weekly injections of 0.5ml DA blood in an equal volume of saline i.v. via the tail vein were administered. Blood transfusions commenced at week 0. At week 6 LEW rats were immunised with saline in an equal volume of Freunds complete adjuvant to each hind footpad and were boosted at week 10 in Freunds incomplete adjuvant. Sera were taken at week 0 (○), week 1 (□), week 2 (▲), week 4 (○), week 6 (▼), week 7 (■), week 8 (▲), week 10 (●) and 10 days after the boost (▼). All rats studied followed a similar profile and one representative rat is shown. Target cells were DA RBC at 10^9/ml.

CPM indicates ^125^I RAR bound.
transfusions) a strong antibody response is seen to intact DA class 1. This antibody response begins to decline by week 2 and gradually reduces down to background levels by week 10 even though twice weekly blood transfusions are taking place. Immunisation with saline in Freund's adjuvant at week 6 and week 10 has no bearing on the antibody response which continues to decline.

Group 1: The antibody responses to intact DA class I molecules following twice weekly blood transfusions of DA blood for 10 weeks and immunisation with a DA class 1 peptide in adjuvant at week 6 and week 10 are shown in figure 7.6. A strong antibody response is seen at week 1 which starts to drop off during the course of repeated blood transfusions decreasing in strength weekly down to week 6 as in group 3. However one week after peptide immunisation (week 7) the antibody response begins to rise increasing by week 8 (two weeks after the initial peptide immunisation) to the same levels as that seen at week 1 in one of the five rats studied or greater in three of the five rats studied. The antibody response remains elevated following continuing blood transfusions. Ten days following the peptide boost at week 10 in one of the five rats an increased antibody response to intact DA class I was seen, in one rat antibody levels remained the same, in three rats antibody levels were slightly decreased following the boost. Twice weekly blood transfusions were continued throughout the course. One out of the five animals did not follow the pattern of suppression of the antibody response, in this case the antibody response continued to rise from week 1 reaching a maximum at week 6 it then began to slowly decline from week 6 week 7, 8, 10 and 12 (fig 7.7). Either the blood transfusion effect still occurred but at a much slower rate or the peptide immunisation caused a reduction in the antibody response. Interestingly this rat did not demonstrate any
Figure 7.6

Kinetics of antibody response of LEW rats, immunised with peptide 1 during multiple blood transfusions, to intact DA class I MHC molecules.

Twice weekly injections of 0.5ml DA blood in an equal volume of saline i.v. via the tail vein were administered. Blood transfusions commenced at week 0. At week 6 LEW rats were immunised with 50μg of peptide 1 in Freunds complete adjuvant to each hind footpad and were boosted at week 10 in Freunds incomplete adjuvant. Sera were taken at week 0 (◇), week 1 (□), week 2 (▲), week 4 (○), week 6 (▼), week 7 (■), week 8 (▲), week 10 (●) and 10 days after the boost (▼). With one exception all rats studied followed a similar profile and a representative rat is shown. Target cells were DA RBC at 10^9/ml.

CPM indicates 125I RAR bound.
Figure 7.7

Kinetics of antibody response to intact DA class I molecules of the LEW rat that showed no immunosuppressive effect with multiple blood transfusions.

Twice weekly injections of 0.5ml DA blood in an equal volume of saline i.v. via the tail vein were administered. Blood transfusions commenced at week 0. At week 6 the LEW rat was immunised with 50μg of peptide 1 in Freund's complete adjuvant to each hindfootpad and was boosted at week 10 in Freund's incomplete adjuvant. Sera were taken at week 0 (○), week 1 (□), week 2 (△), week 4 (○), week 6 (▼), week 7 (■), week 8 (▲), week 10 (●) and 10 days after the boost (▼). Target cells were DA RBC at 10⁹/ml.

CPM indicates ¹²⁵I RAR bound.
anti-peptide 1 antibodies following immunisation with peptide 1 as discussed in section 7.4.2.

Group 2: LEW rats transfused with saline immunised with peptide 1 in Freund's adjuvant. Alloantibody response to intact DA class I was not demonstrated by any of the rats in this group (fig 7.8).

These assays show that priming the T helper responses by immunisation with peptide, reversed the effect of the blood transfusions in suppressing the antibody response, thus turning an immunosuppressive effect in to a sensitising effect. This demonstrates that blood transfusion suppresses T helper responses.

7.4.5 Alloantibody responses to intact DA class I in rats immunised with peptide prior to multiple blood transfusion

Group 4: LEW rats were immunised with peptide 1 prior to multiple blood transfusion. As expected, there were no antibody responses to intact DA class I following peptide 1 immunisation and prior to blood transfusion (fig 7.9a). At week 5 one week after blood transfusions commence an antibody response is seen to intact DA class I, this antibody response increases with time during the blood transfusion course to a maximal level at week 10. Following cessation of blood transfusion the antibody response begins to decline at week 11 (week 1 after cessation of treatment) and decreases further by week 14. All rats studied in this group followed the same pattern. This demonstrates that the immunosuppressive effect of blood transfusion is reversed in T helper primed rats.

Group 5: LEW rats immunised with saline in Freund's adjuvant prior to blood transfusion. Antibody responses to intact DA class I were not seen prior to blood
Figure 7.8

Kinetics of antibody response of LEW rats, immunised with peptide 1 during multiple saline transfusions, to intact DA class I MHC molecules.

Twice weekly injections of 1ml of saline i.v. via the tail vein were administered. Saline transfusions commenced at week 0. At week 6 LEW rats were immunised with 50μg of peptide 1 in Freunds complete adjuvant to each hind footpad and were boosted at week 10 in Freunds incomplete adjuvant. Sera were taken at week 0 (○), week 1 (□), week 2 (△), week 4 (○), week 6 (▼), week 7 (■), week 8 (▲), week 10 (●) and 10 days after the boost (▼). All rats studied followed a similar profile and a representative rat is shown. Target cells were DA RBC at 10⁹/ml.

CPM indicates ¹²⁵ I RAR bound.
Figure 7.9

Kinetics of antibody response of LEW rats, immunised with peptide 1 prior to multiple blood transfusions, to intact DA class I MHC molecules.

Twice weekly injections of 0.5ml DA blood in an equal volume of saline i.v. via the tail vein were administered. LEW rats were immunised with (a) 50µg of peptide 1 or (b) saline in Freunds complete adjuvant to each hindfootpad. Immunisations were at week 0, blood transfusions commenced at week 4 and continued to week 10. Sera were taken at week 0 (◇), week 2 (□), week 4 (▲), week 5 (●), week 6 (▼), week 8 (■), week 10 (▲), week 11 (○) and week 14 (▼). All rats in each group followed a similar profile and a representative rat for each group is shown. Target cells were DA RBC at 10⁹/ml.

CPM indicates ¹²⁵I RAR bound.
transfusion (fig 7.9b). A strong antibody response was seen at week 5 (one week after start of blood transfusion). This antibody response was seen to decline down to background levels by week 10 where it remained after cessation of blood transfusion at week 10 and week 14. All rats studied in this group followed the same pattern. Thus FCA does not influence the normal pattern of the antibody response to intact DA class I following blood transfusion.
7.5 Discussion

Results from this study show multiple allogeneic blood transfusions have an immunosuppressive effect on the antibody response to the challenging antigen. Antibody responses to intact DA class I molecules of LEW rats receiving twice weekly DA blood transfusions follow a pattern of high initial antibody response at week 1 which gradually declines with time down to background levels by week 6 throughout the course of the transfusions and remains low in spite of 4 additional weeks of twice weekly blood transfusion. Immunisation with saline in Freunds adjuvant either prior to or during the multiple blood transfusions does not influence this profile. However this immunosuppressive effect can be abrogated by immunisation with a DA class I peptide, peptide 1 in Freunds adjuvant. Immunisation with peptide 1 during blood transfusions at week 6 breaks this immunosuppressive effect reversing the decline of antibody to intact DA class I. Following peptide priming, the antibody to intact class I rises from week 6, completely altering the pattern of antibody response.

Peptide immunisation prior to commencement of multiple blood transfusion leads to a high antibody response to intact DA class I which is not influenced by the multiple blood transfusions. All rats immunised with peptide 1 with one exception demonstrated a strong antibody response to peptide 1 by week 4 following immunisation. This demonstrates that the T helper cells were primed for antibody production to the peptide. These helper T cells for the antibody response to the peptide are also involved as helper T cells for the antibody response to intact class I molecules. Thus priming T helper cells results in an abrogation of the immunosuppression associated with the blood transfusion effect.
The antibody response to the immunising peptide, peptide 1 was not increased following multiple blood transfusions. It is possible that the B lymphocytes of the graft are exposed primarily to whole MHC molecules during blood transfusion rather than degraded or denatured MHC molecules. Interestingly the rat which did not develop the normal suppression pattern to multiple blood transfusions also demonstrated no anti-peptide antibody response to the immunising peptide indicating that the T helper cells were not primed by the peptide immunisation.

This was further elucidated by the results from the groups of animals immunised for the T cell proliferation assays. The T cell proliferation assays indicate that there may be a reduction in T cell proliferation to peptide 1 in LEW rats immunised with peptide 1 receiving multiple blood transfusions in comparison with T cell proliferation of LEW rats immunised with peptide 1 receiving saline transfusions. T cell proliferation requires CD4+ T helper cells, if the T helper cell pathway is suppressed a reduction in T cell proliferation would be evident.

The results of this study demonstrate that multiple blood transfusions of DA blood suppress the antibody response of LEW recipients to intact DA class I molecules on the surface of DA RBC. Diminishing the response with time down to background levels by week 6. Immunisation of DA class I peptides either prior to or during blood transfusion breaks this suppression, resulting in an elevated antibody response. Earlier work (discussed in chapter 6) has demonstrated that immunisation of DA class I peptides primes the T helper cells. Thus priming of T helper cells prevents the immunosuppressive effect of the blood transfusions. As this priming breaks the immunosuppressive effect of these multiple blood transfusions this demonstrates that the blood transfusion effect suppresses the T helper pathway.
Other studies have demonstrated an immunological involvement in the mechanisms of the blood transfusion effect. The mechanism of the blood transfusion effect has been attributed to the induction of suppressor cells. Mottram et al (1990) demonstrated the importance of CD4+ T cells in adoptive transfer studies of spleen cells from mice receiving a donor specific blood transfusion and cardiac allograft surviving more than 21 days. These cells given prior to cardiac grafting in naive hosts prolonged survival to >40 days however when CD4+ cells were removed from the cell suspension graft survival was not enhanced. Interestingly the presence of the cardiac allograft was found to be an important factor in the maintenance of cell mediated suppression. The persistence of antigen appears to be important for the maintenance of in vivo tolerance (Ramsdell and Fowlkes 1992). Our multiple blood transfusion protocol of twice weekly injection provides a sustained antigenic challenge. The immunosuppressive effect seen in our studies is not immediate, taking up to 6 weeks for the antibody response to be reduced down to background levels. In the clinic van Twuvyer et al (1990) demonstrated a reduction in CTL precursor frequency following blood transfusion between donor-recipient pairs bearing one HLA DR and class I match. This reduction occurred 4 to 16 weeks after the transfusion and was only apparent where there was a match between the donor and recipient. However in the rodent model the blood transfusion effect has been shown by many studies to be effective across a full MHC mismatch and to be donor specific (e.g. Brunson et al 1991) although certain strain combinations fair better than others. If blood transfusion is given in conjunction with anti-CD4 mab (Bushell et al 1994) random blood transfusion was also shown to
be effective at prolonging murine cardiac allograft survival although this could be due to cross reactivity between TCR and MHC haplotype.

The MHC appears to play an important part of the blood transfusion response. All the blood components that have been shown to elicit the transfusion response have MHC molecules on their surface. Sumimoto and Kamada (1990) showed that MHC molecules played an active role in producing the immunosuppressive effect of the blood transfusion response. Immunisation of MHC antigens alone have also been shown to have an immunosuppressive effect and prolong graft survival. (e.g. Nisco et al 1995).

This study has helped to elucidate the mechanisms of the blood transfusion effect by demonstrating, with the use of peptide immunisation to prime T helper cells, that the T helper compartment plays a role in the mechanisms of the blood transfusion effect.
## CHAPTER 8:
THE MOUSE-TO-RAT CONCORDANT MODEL OF XENOGRAFT REJECTION

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CHAPTER 8:
THE MOUSE-TO-RAT CONCORDANT MODEL OF XENOGRRAFT
REJECTION

8.1 Introduction

Xenotransplantation is a potential solution to the problem of donor organ shortage which would reduce the number of patient deaths on waiting lists for heart and other vital organ grafts, and improve the quality of life of those waiting for kidney grafts. Transplants between distantly related species, e.g. pig-to-man, where preformed natural antibodies to the donor are present, results in a hyperacute rejection response with graft destruction within a few minutes to hours. This is termed a discordant combination. Transplants between closely related species e.g. chimpanzee-to-man do not undergo hyperacute rejection responses and are termed concordant combinations. For clinical transplantation, the donor species of choice is the pig, in spite of the problem of hyperacute rejection. This is mainly on grounds of availability and ethical acceptance. The intensity of the hyperacute rejection response makes it impossible to study any other concomitant or later immune response to discordant xenografts. The use of concordant xenografts allows the study of these other responses. The hamster-to-rat concordant xenograft model has been studied in great detail although long term tolerance has still not been achieved (reviewed by Leventhal 1994). Prolonged survival can be achieved with a cocktail of immunosuppressive drugs, but this results in a high mortality rate as a consequence of the drug contents. This study has utilised the mouse-to-rat model which although more technically demanding than the hamster-to-rat model involves more closely related species. The aim was to understand the mechanisms of rejection in this model and prevent them using non-toxic
immunosuppressive regimens, including the use of a new immunosuppressive agent CTLA4-Ig developed by Bristol-Myers Squibb. This preparation is formed by the fusion of the extracellular domain of human CTLA4 (cytolytic T lymphocyte associated antigen) with the constant region of human IgG1 by genetic manipulation techniques (Linsley et al 1991). CTLA4-Ig binds to B7-1 and B7-2 thereby blocking the binding of CD28/CTLA4 to B7-1/B7-2. CD28/CTLA4 to B7-1/B7-2 forms an important co-stimulatory pathway, the blocking of which can lead to T cell anergy. CTLA4-Ig has been shown to be effective in an allograft model, although long term tolerance has not been achieved. In the vascularised heterotopic cardiac model, a 7 day course of CTLA4-Ig at 0.05mg/day starting from the day of transplant gave a survival range of 18 to 40 days in the BN-to-LEW rat model. A similar survival curve was demonstrated when the dose was increased to 0.5mg/day (Turka et al 1992). Administration of CTLA4-Ig commencing during rejection (Baliga et al 1994), or given to the donor (Pearson et al 1994) has no effect on graft survival. Histological studies show that CTLA4-Ig does not inhibit lymphocytic infiltration of the graft, as demonstrated in lungs (Matsumura et al 1995), kidney (Sayegh et al 1995) and heart (Turka et al 1992). In contrast to vascularised organ allografts, CTLA4-Ig was able to induce long term survival of human pancreatic islets placed under the kidney capsules of mice using a low dose of 0.05mg on alternate days for 14 days (Lenschow et al 1992).

The mechanisms of xenograft rejection in concordant xenografts has not yet been elucidated. Indirect recognition may play a significant role in these mechanisms of xenograft rejection. The array of foreign peptides available for presentation on self class II molecules would be greater than for allografts. In widely disparate species
combinations the direct cell-to-cell interaction required for direct recognition may not be possible e.g. due to differences in ligand-receptor pairs and cytokines. *In vitro* studies suggest that disparate species combinations are unable to utilise the direct recognition pathway and require self APC. Lucas *et al* (1990) demonstrated that human anti-mouse responses utilised the indirect recognition pathway. However direct recognition can take place between closely related species combinations e.g. rat anti-mouse (van den Bogaerde 1990). Interestingly human anti-pig responses can utilise the direct recognition pathway (Swain *et al* 1983) as well as the indirect recognition pathway (Yamada *et al* 1995). The role of indirect recognition in *in vivo* studies of xenograft rejection is unclear.

In this study primarily vascularised heterotopic cardiac xenografts have been performed from BALB/c mice donors to LEW rat recipients using microsurgical techniques. Recipients received various immunosuppressive therapies. Graft survival, antibody responses and immunohistology have been studied.
8.2 Materials and Methods

8.2.1 Experimental animals
Inbred BALB/c (H-2d) mice, LEW (RT1b), DA (RT1"t"), PVG (RT1c) and randomly bred athymic nude HAN/RNU-nu rats were purchased from Harlan UK Ltd (Oxon, UK). BALB/c mice were adult females. All rats were adult males.

8.2.2 Heterotopic cardiac xenotransplantation
BALB/c mouse hearts were transplanted into LEW, DA, PVG or nude recipients as described in section 5.2.2. Total ischaemia times were in the range of 38 to 60 minutes. Heart beats were monitored daily by palpation through the abdominal wall under light anaesthesia.

8.2.3 Heterotopic cardiac allotransplantation
LEW (RT1b) hearts were transplanted to nude recipients as described in section 5.2.3. Total ischaemia times were in the range 53 to 62 minutes. Heart beats were monitored by daily palpation through the abdominal wall.

8.2.4 Splenectomy
Splenectomy was performed during the transplantation procedure by ligation of the splenic vessels and removal of the spleen as described in section 5.3.1.
8.2.5 CTLA4-Ig

CTLA4-Ig kindly supplied by (Bristol-Myers Squibb) was prepared as in section 5.3.3 and administered at 200µg/ml in a 1ml aliquot i.v. via the tail vein. CTLA4-Ig was administered just prior to surgery on day 0 and continued until the day of harvest or day 10 post transplant.

8.2.6 Cyclosporin A

Cyclosporin A (kindly supplied by Sandoz) was prepared as in section 5.3.2 and administered daily at 10mg/kg or 20mg/kg by gavage from day 0 to the day of harvest or day 14 post transplant.

8.2.7 Histology

Mouse hearts were harvested on days 1, 2, 3, 6 and 7 post transplant and on the day of rejection. In each case one section was fixed in formalin for H&E histology and another was snap frozen in liquid nitrogen for immunohistology.

8.2.8 Immunohistology

Mouse heart sections were stained as described in section 5.10.5 using the following monoclonal antibodies (described in detail in sections 5.10.1, 2 and 3) for mouse MHC class I (MCA 180), mouse MHC class II (MCA 648), rat IL-2R (NDS61), rat CD4 (W3/25), rat CD8 (OX8), rat TCR (R73), rat CD5 (OX19) rat leucocyte common antigen CD45 (OX1), rat macrophages (BMAC 5), rat MHC class I (F16-4-4), rat MHC class II (OX6) and human thy 1 (F15-4-2-11) as a control.
8.2.9 Assays for antibody response to BALB/c mouse targets

Indirect radioactive binding assays (described in section 5.8.2) were performed on sera taken at day 0, 3, 4, 7, 14 and day of rejection using either RBC at $10^9$/ml, spleen cells at $10^9$/ml, heart homogenate at 2%v/v and platelets at $5 \times 10^8$/ml as described in sections 5.7.1, 2, 3 and 4.

8.2.10 Haemolysis assays

These assays were performed as described in section 5.8.4 using sera taken on days 0, 3, 7, 14 and the day of rejection with BALB/c RBC as targets.

8.2.11 Flow cytometry

Flow cytometry was performed as described in section 5.8.5 using sera taken on day 0, day 3 and day 7 using BALB/c platelets as targets.

8.2.12 Indirect radioactive binding assay to determine rat MHC haplotypes

These assays were performed as described in section 5.8.2 using RBC taken from nude, PVG and LEW rats, and incubated with monoclonal antibodies OX27 and NDS60 to determine MHC class I haplotype of the nude rats.
8.3 Results

8.3.1 Graft survival of BALB/c mouse hearts transplanted to rat recipients

Graft rejection of BALB/c mouse hearts transplanted heterotopically to LEW rat recipients is very rapid. Grafts were monitored by daily palpation of the abdomen, and rejection was taken as the day when the heart beat ceased. Graft survival in this model was usually three days, which is comparable to that seen in the hamster-to-rat combination. Monotherapy of the T cell immunosuppressive agent CsA at 10mg/kg or 20mg/kg administered daily by gavage had no effect on graft survival. Monotherapy with CTLA4-Ig at 200μg/day which inhibits the CD28/CTLA4 to B7-1/B7-2 co-stimulatory pathway was also ineffective at prolonging graft survival. Splenectomy of the recipient at the time of transplant prolonged graft survival by 1 to 2 days (table 8.1). The spleen was demonstrated to be important in this rejection response as combinations of these therapies without splenectomy did not prolong survival. Treatment with a combination of CTLA4-Ig and CsA at 10mg/kg did not prolong survival even reducing it to 2 days in some cases. However, the combination of CsA with splenectomy led to a prolongation of 2 to 4 days with one animal continuing to day 21. This combination was more effective than CTLA4-Ig and splenectomy where survival was prolonged by 1 to 3 days. The combination of CTLA4-Ig and CsA however, when combined with splenectomy resulted in extended graft survival in this mouse to rat heterotopic cardiac xenograft model (6, 8, 10, 18, 20, 20, 24, 24, 30) (table 8.2).

Interestingly graft survival in nude recipients was similar although more rapid than the euthymic LEW controls with the majority of grafts rejected at day 2. However when
Table 8.1
Graft survival of BALB/c mouse heterotopic cardiac xenografts to LEW recipients receiving monotherapy treatment of CsA, CTLA4-Ig or splenectomy.

* CsA was administered at 10mg/kg or 20mg/kg in olive oil daily by gavage from the time of transplant.
# Splenectomy was performed at the time of transplant
~ CTLA4-Ig was diluted in saline and administered at 200μg/ml daily by i.v. injection of 1ml into the tail vein from the time of transplant.
ϕ p<0.0002 compared to untreated controls (Mann-Whitney U Test)
Table 8.2
Graft survival of BALB/c mouse heterotopic cardiac xenografts to LEW recipients receiving combined treatment of CsA, CTLA4-Ig and splenectomy.

* CsA was administered at 10mg/kg in olive oil daily by gavage from the time of transplant until day 14 post transplant.

# Splenectomy was performed at the time of transplant

~ CTLA4-Ig was diluted in saline and administered at 200μg/ml daily by i.v. injection of 1ml into the tail vein from the time of transplant until day 10 post transplant.

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*p = 0.012 compared to splenectomy alone (see tab 8.1)
θ *p = 0.426 compared to splenectomy alone (see tab 8.1)
ψ p<0.002 compared to untreated controls (see tab 8.1)
σ p<0.002 compared to splenectomy (see tab 8.1)
Ω p = 0.0006 compared to CTLA4-Ig and splenectomy
(p values were calculated using Mann-Whitney U Test)
DA or PVG rats are used as recipients; graft survival is also around day 2 (table 8.3). As a control nude rats were transplanted with LEW cardiac allografts which were not rejected. By typing using haplotype specific monoclonal antibodies the RT1.A class I antigens of these nude rats were shown to be RT1\textsuperscript{e}.

8.3.2 Antibody responses to mouse targets in recipients of BALB/c heterotopic cardiac xenografts.

In normal LEW rats there appears to be some antibody binding to spleen cells and heart homogenate but none to RBC (fig 8.1). It is difficult to determine whether this binding is due to natural preformed antibody in the rat to the mouse or if it is non-specific binding to the target cells. In recipients not receiving CsA, an antibody response is seen to all targets at week 1 which increases by week 2 and continues to increase at week 4. The greatest response was seen to RBC targets followed by spleen cells and then heart homogenate. CsA abrogates this response reducing the antibody levels at week 2 and week 4 to that seen in a normal rat. Interestingly, at week 1, CsA is capable of suppressing the antibody response to heart homogenate and spleen cells but there is a small but definite response to RBC. This assay system was repeated for 3 sets of rats and the results were similar in each case. Titrations from one set of rats can be seen in figure 8.1. In these studies the rejected mouse heart was left in situ.

It is interesting that splenectomy has no effect on the antibody response to mouse spleen cells, even though it gives slight but significant prolongation of graft survival. Conversely CsA at 10mg/kg powerfully suppresses this antibody response, but has no effect on graft survival. CTLA4-Ig partially suppresses this strong antibody response
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<td>of LEW allografts</td>
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Table 8.3

Graft survival of BALB/c mouse heterotopic cardiac xenografts in different rat recipient strains and LEW cardiac allografts in nude recipients.

\( \psi p = 0.008 \) compared to LEW controls (Mann-Whitney U Test)
Figure 8.1a,b

Serum antibody responses of recipients of BALB/c heterotopic cardiac xenografts to mouse RBC, spleen cells and heart homogenate at day 0 and week 1 post transplant using indirect radioactive binding assays.

Antibody response to BALB/c mouse RBC at $10^9$/ml (●), spleen cells at $10^8$/ml (■) and heart homogenate at 2%v/v (●) in LEW recipients of BALB/c heterotopic cardiac xenograft at (a) day 0 prior to transplantation and (b) at week 1 after grafting in recipients receiving either CsA at 10mg/kg/day (solid lines) or no treatment (dashed lines).

CPM indicates $^{125}\text{I}$ RAR bound.
a

CPM BOUND
\( \times 10^{-3} \)

N 1/3 1/9 1/27 1/81 1/243

b

SERUM DILUTION
Figure S.1c,d

Serum antibody responses of recipients of BALB/c heterotopic cardiac xenografts to mouse RBC, spleen cells and heart homogenate at week 2 and week 4 post transplant using indirect radioactive binding assays.

Antibody response to BALB/c mouse RBC at $10^9$/ml (▲), spleen cells at $10^8$/ml (■) and heart homogenate at 2%v/v (●) in LEW recipients of BALB/c heterotopic cardiac xenograft at (c) week 2 and (d) week 4 after grafting in recipients receiving either CsA at 10mg/kg/day (solid lines) or no treatment (dashed lines).

CPM indicates $^{125}$I RAR bound.
We detected no antibody response to mouse spleen cells in the nude recipients, even though these recipients rejected their grafts at a similar tempo to untreated controls (fig 8.2b). Combination of the therapies without CsA did not effectively reduce antibody levels beyond that seen with individual therapies. The combination of all three therapies reduced the antibody response to background levels (fig 8.2c). The antibody response of recipients receiving CsA, CTLA4-Ig and splenectomy remained at background levels at all times even to the day of rejection as shown in figure 8.2d by two rats that rejected their grafts at day 18 and at day 20.

Antibody levels to mouse RBC using haemolysis assays are given in figure 8.3. This assay system was tested as it might be more sensitive than binding assays. At the time of grafting there is no detectable antibody to mouse RBC. However by day 3 (which is the day of rejection), an antibody response is detected and increases by day 7. CsA at 10mg/kg is unable to suppress this response at day 3, but has some effect at day 7. CsA is not as effective in reducing the antibodies detected by RBC targets as for spleen cell targets, confirming the results seen with the indirect radioactive binding assay (fig 8.1b). CsA at 20mg/kg reduced the response at day 3 to background levels even though this treatment fails to prolong graft survival. Splenectomy failed to influence the antibody response to mouse RBC. CTLA4-Ig also had no effect on this antibody response at day 3 and day 7.

Recipients receiving triple therapy of CsA, CTLA4-Ig and splenectomy had no detectable antibodies to mouse RBC at day 7, day 14 and the time of rejection (day 20 for the graft shown in fig 8.3). One animal on the triple therapy rejected at day 6 with no detectable antibody to mouse RBC.
Figure 8.2a,b

Serum antibody responses of recipients of BALB/c heterotopic cardiac xenografts to mouse spleen cells at day 7 using indirect radioactive binding assays.

Antibody response to BALB/c spleen cells at $10^9$/ml (a) in LEW recipients of BALB/c heterotopic cardiac xenograft at day 7 post transplant. The rats received monotherapy of CsA at 10mg/kg/day (▼), CTLA4-Ig at 200μg/day (♦), or splenectomy (▲), or were untreated (■). Background level is given by recipient serum at day 0 prior to transplant (●). (b) Nude recipients at day 7. Two individual nude rats are shown (▼) and (○). (●) represents normal LEW prior to transplant.

CPM indicates $^{125}$I RAR bound.
Figure 8.2c,d

Serum antibody responses of recipients of BALB/c heterotopic cardiac xenografts to mouse spleen cells following immunosuppressive therapy using indirect radioactive binding assays.

Antibody response to BALB/c spleen cells at $10^9$/ml in LEW recipients of BALB/c heterotopic cardiac xenograft receiving combined therapies at day 7 post transplant (c) CTLA4-Ig and splenectomy (▼), CTLA4-Ig and CsA (▲), CsA and splenectomy (●) and CTLA4-Ig, CsA and splenectomy (Δ). Background levels are indicated by recipient serum at day 0 prior to transplant (●). (d) At day 14 post transplant and day of rejection, in rats receiving triple therapy of CsA, CTLA4-Ig and splenectomy. Two individual rats are shown at day 14 (◇) and day of rejection at day 18 (□) and day 14 (Δ) and day of rejection at day 20 (▼). Background levels are indicated by normal LEW serum at day 0 prior to transplant (●).

CPM indicates $^{125}$I RAR bound.
Figure 8.3

Serum antibody response to BALB/c mouse RBC at 10⁶ cells/well in recipients of BALB/c heteropic cardiac xenografts using haemolysis assays.
PVG and DA recipients demonstrated a strong antibody response at day 7 which appears stronger than the antibody response from the LEW recipient at day 7.

No antibody to mouse RBC was demonstrated in the sera of nude recipients at any time after grafting, even though they rejected their grafts at day 2/3.

Assays were performed with and without guinea pig complement. Interestingly the addition of exogenous guinea pig complement was not necessary to demonstrate lysis. The presence or absence of guinea pig complement did not influence the results. The results shown in fig 8.3 are for incubation of the rat sera with mouse RBC, without the addition of guinea pig complement. These assays were repeated for three sets of rats each giving a similar profile.

Antibody production to mouse platelets is given in figure 8.4

In these indirect radioactive binding assays an increase in antibody response to mouse platelets is seen from day 0 to day 3, with a marginal increase from day 3 to day 4. A strong antibody response is seen at day 7. This assay was repeated for five rats all of which demonstrated a similar profile. Two individual rats are shown in figure 8.4.

Antibody levels to mouse platelets were also studied using flow cytometry (fig 8.5). The histograms show an increase in fluorescence from the negative medium control to sera at day 0, then a marginal increase in fluorescence intensity from day 0 to day 3 with a strong increase in fluorescence intensity at day 7 indicating that the antibody response in this rat is higher at day 3 than at day 0 with a strong response at day 7. This assay was repeated for three individual rats all of which gave similar profiles.
Figure 8.4

Kinetics of antibody responses of recipients of BALB/c heterotopic cardiac xenografts to mouse platelets using indirect radioactive binding assays.

Antibody response to BALB/c mouse platelets at $5 \times 10^9/ml$ in LEW recipients of BALB/c heterotopic cardiac xenografts at day 0 prior to transplantation (○), day 3 (□), day 4 (Δ) and day 7 (○) in two individual rats (a) and (b).

CPM indicates $^{125}\text{I}$ RAR bound.
Figure 8.5

Antibody response to BALB/c mouse platelets at $4 \times 10^7$/ml in LEW recipients of BALB/c heterotopic cardiac xenografts using flow cytometry (a) 0.5%BSA/PBS medium control, (b) at day 0 prior to transplantation, (c) day 3, and (d) day 7.
8.3.3 Histopathology and immunohistology of mouse hearts transplanted to LEW recipients

The histology of the hearts at various times after grafting, together with immunohistological identification of leucocyte infiltrates and graft antigen expression were studied, as these might help to elucidate the mechanisms of rejection.

At day 1 following transplantation the myocardial cells and large blood vessels are completely normal. A diffuse capillary haemorrhage is seen in many areas with myocyte damage and local infiltration of polymorphonuclear leucocytes (fig 8.6a). This infiltrate contains CD45+ cells (fig 8.6c) but is negative for the rat T cell markers CD4 (fig 8.6d), CD8, IL-2R, TCR and CD5 (not shown). The occasional macrophage (fig 8.6e) and class II +ve cell (fig 8.6f) was also seen.

By day 2 there was patchy myocyte necrosis with a more pronounced leucocyte infiltrate, still containing cells with a polymorphonuclear morphology.

On day 3 (the day of rejection), large areas of myocardial necrosis with interstitial haemorrhage can be seen (fig 8.7a) with medial necrosis in many arterioles. In some areas the myocardial cells have maintained their structure, but without nuclei (fig 8.7b). The leucocytic infiltrate in the substance of the heart contains many polymorphonuclear leucocytes (fig 8.7b) staining positive for CD45 (fig 8.7c). Very few cells stain positive for T cell markers CD5 (fig 8.7d) CD4, IL-2R, TCR (not shown, although CD8 staining was more prominent (fig 8.7e). The occasional class I+ cell (fig 8.7f), class II+ cell and macrophage (not shown), was also seen. However in the epicardial areas of the heart (where there is access by leucocytes from the surrounding rat connective tissue) there was a dense mononuclear leucocyte infiltrate,
Figure 8.6

Histology and immunohistology of BALB/c mouse heart transplanted to LEW rats at day 1 post transplant.

a) H+E section of a normal mouse heart.

b) H+E section of graft at day 1 showing a diffuse capillary haemorrhage with a local infiltration of polymorphonuclear leucocytes.

c) Immunoperoxidase staining of graft at day 1, stained with OX1 mab to CD45.
Figure 8.6

Immunohistology of BALB/c mouse heart transplanted to LEW rats at day 1 post transplant.

d) Immunoperoxidase staining of graft at day 1, staining negative with W3/25 mab to CD4.

e) Immunoperoxidase staining of graft at day 1, showing occasional positive staining for macrophages with BMAC-5 mab.

f) Immunoperoxidase staining of graft at day 1, showing occasional rat class II positive cells stained with OX6 mab.
Figure 8.7

Histology and immunohistology of BALB/c mouse heart transplanted to LEW rats at day 3 post transplant.

a) H+E staining showing interstitial haemorrhage.

b) H+E section of graft at day 3, showing a leucocytic infiltrate containing polymorphonuclear leucocytes (indicated by arrows).

c) Immunoperoxidase staining of graft at day 3, stained with OX1 mab to CD45.
Figure 8.7

Immunohistology of BALB/c mouse heart transplanted to LEW rats at day 3 post transplant.

d) Immunoperoxidase staining of graft at day 3 staining negative with OX19 mab to CD5.

e) Immunoperoxidase staining of graft at day 3, showing occasional positive staining for CD8 with OX8 mab.

f) Immunoperoxidase staining of graft at day 3, showing occasional rat class I positive cells stained with F16-4-4 mab.
containing cells positive for the T cell markers CD5 (fig 8.8a), CD4, CD8, IL-2R, TCR (not shown) plus macrophages (fig 8.8b), class II (fig 8.8c) and class I positive cells (not shown).

Three days after rejection (day 6) there are large areas of complete necrosis of the myocardium with all blood vessels destroyed (fig 8.9a). Myocardial cells without nuclei are seen in patches. The leucocyte infiltrate is very similar to that of day 3.

Therapy with CsA at 10mg/kg or 20mg/kg did not alter the histopathology or immunohistology of the grafts.

The recipients receiving CTLA4-Ig, CsA and splenectomy which rejected their grafts at day 6, 8, 10, 10 demonstrated a mild polymorphonuclear leucocyte infiltrate with haemorrhage, similar to that of the hearts that rejected on day 3 (fig 8.9b). However grafts that survived for 18 days or more showed a massive mononuclear infiltrate (fig 8.9c). Therefore treatment with a combination of CTLA4-Ig, CsA and splenectomy does not alter the histological picture of rejection unless prolonged graft survival past day 18 is achieved.

Expression and induction of mouse MHC class I and class II antigens during the rejection response are shown in figure 8.10. It was not possible to stain for mouse MHC antigens in normal mouse heart with the mouse monoclonal antibodies available to us. There were high levels of background staining with the peroxidase labelled anti-mouse immunoglobulin second reagent, due to mouse immunoglobulins in the connective tissues of the heart. However by day 1 after grafting, background staining was no longer a problem as mouse immunoglobulins had diffused out of the heart (fig 8.10a). At day 1 post transplantation there was scattered class II positive cells in the connective tissues (fig 8.10b), presumably interstitial dendritic cells. There was a
curious but strong class II staining of the blood vessels (fig 8.10c). Weak staining of class I molecules in the connective tissues was also apparent at day 1 (fig 8.10d). By day 3, the day of rejection, the vessels were still strongly class II positive. There was obvious and substantial induction of class I (fig 8.10e) and class II MHC (fig 8.10f) antigens in the connective tissues of the rejecting hearts. However, neither class I nor class II MHC induction was obvious on the cardiac myocytes. At day 6 there was substantial amounts of class I and class II in areas of surviving tissue.
Figure 8.8

Immunohistology of BALB/c mouse heart transplanted to LEW rats at day 3 post transplant.

a) Immunoperoxidase staining of the epicardial areas of the heart at day 3, showing positive staining with OX19 mab to CD5.

b) Immunoperoxidase staining of the epicardial areas of the heart at day 3, showing positive staining for macrophages with BMAC-5 mab.

c) Immunoperoxidase staining of the epicardial areas of the heart at day 3, showing positive staining of rat class II positive cells with OX6 mab.
Figure 8.9

Histology of BALB/c mouse heart transplanted to LEW rats.

a) H+E section of a rejected heart at day 6 post transplant showing myocardial necrosis and an obliterated artery.

b) H+E staining of mouse heart at the time of rejection (day 8) in a recipient receiving therapy of CTLA4-Ig, CsA and splenectomy, showing mild leucocyte infiltration.

c) H+E staining of mouse heart at the time of rejection (day 18) in a recipient receiving therapy of CTLA4-Ig, CsA and splenectomy, showing massive mononuclear infiltration.
Figure 8.10

Expression of mouse class I and class II MHC molecules in BALB/c mouse hearts transplanted to LEW rats.

a) Immunoperoxidase staining of graft at day 1. Control staining with F15-42-1 mab to human thy 1.

b) Immunoperoxidase staining of graft at day 1, stained with MCA 648 to mouse MHC class II, showing scattered class II+ cells.

c) Immunoperoxidase staining of graft at day 1, stained with MCA 648 to mouse MHC class II, showing class II + blood vessel.
Figure 8.10

Expression of mouse class I and class II MHC molecules in BALB/c mouse hearts transplanted to LEW rats.

d) Immunoperoxidase staining of graft at day 1, showing weak staining of MCA 160 to mouse class I MHC in connective tissues.

e) Immunoperoxidase staining of graft at day 3, stained with MCA 160 to mouse MHC class I, showing class I staining of connective tissues.

f) Immunoperoxidase staining of graft at day 3, stained with MCA 648 to mouse MHC class II, showing class II staining of connective tissues.
8.4 Discussion

This study demonstrates that the mouse-to-rat primarily vascularised heterotopic cardiac xenograft model is a difficult model of concordant xenotransplantation. The rejection time in untreated controls is very rapid at 3 days. This is comparable to that seen in the hamster-to-rat model, even though these species are more distantly related. Rodent models of cardiac allografting and xenografting utilise the heterotopic method. In this situation the blood flow through the heart is different from that in an orthotopic heart transplant. In the heterotopic heart, blood flows into the coronary arteries from the aorta and drains into the right atria where it is expelled into the pulmonary vein via the right ventricle. Thus the left ventricle is redundant. Therefore these cardiac grafts do not precisely mirror the situation in an orthotopic setting.

Monotherapy with the T cell immunosuppressive agent CsA (which inhibits IL-2 production) at 10mg/kg/day or 20mg/kg/day has no effect on graft survival. These results confirm those seen by others (Gannedahl et al 1990). Monotherapy with the T cell immunosuppressive agent CTLA4-Ig has no effect on graft survival, in spite of the impressive effects of CTLA4-Ig on human-to-mouse pancreatic islet xenograft survival (Lenschow et al 1992). CTLA4-Ig acts by inhibiting co-stimulation through the CD28/CTLA4 to B7-1/B7-2 pathway. This pathway is CsA resistant (Hess et al 1991) and thus the combination of CTLA4-Ig and CsA would seem likely to be a powerful T cell immunosuppressant. Although CTLA4-Ig augments the immunosuppressive effect of CsA in the allograft situation (Perico et al 1995), in our xenograft model CsA and CTLA4-Ig failed to extend xenograft survival. Thus immunosuppression using T cell immunosuppressive agents only has no effect on graft survival.
Splenectomy at the time of transplantation prolonged survival by 1 to 2 days, and by 2-4 days when combined with CsA. Graft survival in our study was substantially prolonged only when a combination of all 3 therapies was used. When CTLA4-Ig and CsA were combined with splenectomy, seven out of eleven animals did not reject their grafts whilst under therapy. However the half life of human CTLA4-Ig in a rodent model is reported to be 2.8 days for one 0.5mg dose. (Turka et al 1992). Although we did not check CTLA4-Ig levels it is possible that active levels of CTLA4-Ig were present during rejection. It is also possible that co-stimulatory pathways other than CD28/CTLA4 to B7-1/B7-2 may be active in rats, as in allograft experiments rejection often occurs in the presence of detectable levels of serum CTLA4-Ig (Lin et al 1993) and no long term tolerance has been achieved. This is not thought to be due to repopulation of T cells from the thymus as thymectomy prior to transplantation does not alter allograft survival (Turka et al 1992).

We used human CTLA4-Ig in these experiments, murine CTLA4-Ig may have been more effective as demonstrated by Wallace et al (1995) but this was not available to us. However Blazar et al (1995) demonstrated no difference between the two species of CTLA4-Ig in a graft-versus-host disease model. The survival data in this study show that mouse-to-rat heterotopic cardiac xenografts can only be significantly prolonged when recipients receive CTLA4-Ig, CsA and splenectomy. The novel immunosuppressive agent CTLA4-Ig is only of benefit in prolonging graft survival when used as a component of an immunosuppressive cocktail.

Nude recipients of mouse cardiac xenografts rejected their grafts at a similar tempo to control recipients. These nude rats did not reject LEW allografts. Although the nude
rats were outbred, typing with monoclonal antibodies revealed their MHC class I haplotype to be RT1\(^e\) and not RT1\(^I\) (data not shown).

The spleen is known to be important for the antibody response to antigens. The timing of splenectomy may be important when splenectomy is combined with CsA. In the hamster-to-rat model Araneda et al. (1992) demonstrated that graft survival was more beneficial when splenectomy was performed 2 days after transplantation in rats treated with CsA than if it was performed on day 1 or day 3. Interestingly splenectomy of nude animals in the hamster-to-rat model leads to a prolongation of survival (Thomas et al. 1991). This reinforces the role of the spleen in this rejection response and suggests that any antibodies present would be T cell independent antibodies. Interestingly the B cells of the marginal zone of the spleen are known to be important in T cell independent antibody production.

The importance of the spleen in prolonging survival of these mouse-to-rat heterotopic cardiac xenografts suggests a humoral mechanism in this rejection response. We studied the rat anti-mouse antibody response to various mouse antigens before and after transplantation in animals treated with the different immunosuppressive regimens.

Following transplantation rats produce a strong antibody response to mouse RBC, spleen and heart homogenate at days 7, 14 and 28 following transplantation. Interestingly this antibody response to the graft is markedly suppressed by the administration of CsA, even though CsA therapy does not prolong graft survival. No antibodies were detected to spleen cells and heart homogenate. However a weak antibody response was detected to RBC at day 7. The specificities of these antibody responses are not known, there may be many different target antigens or there may be antigenic determinants that are shared by all of these cell types.
Using spleen cells as targets, CTLA4-Ig also reduced the antibody response at day 7, but was not as effective as CsA. This suggests that the strong antibody response seen at day 7 is T cell dependent. Interestingly although splenectomy was the only monotherapy to increase graft survival it had no effect on antibody production detected by spleen cell targets at day 7. Therapies including CsA suppressed the antibody response but did not prolong graft survival without the addition of splenectomy. This suggests that the antibody response detected at day 7 is not an essential component of the rejection response.

Combined therapy of CTLA4-Ig, CsA and splenectomy where prolonged survival was achieved completely suppressed the antibody response. The antibody level remained at background levels throughout the course studied even at the time of rejection in these extended survivors. This was also seen using mouse RBC targets in haemolysis assays.

At day 3 (day of rejection) an antibody response can be seen to mouse platelets in untreated recipients using flow cytometry and indirect radioactive binding assays. A similar profile of antibody responses is seen using mouse RBC as targets in a haemolysis assay. At day 3 following transplantation an antibody response is seen to mouse RBC in untreated rats. This response is not reduced by CsA at 10mg/kg/day. However, it is suppressed by CsA at 20mg/kg/day even though these grafts are rejected at day 3. CTLA4-Ig has no effect on this response at day 3. The combination of CsA at 10mg/kg/day and CTLA4-Ig also has little effect on this antibody response at day 3 which is the time of rejection. This suggests that only some of these antibody responses at day 3 are T cell dependent.

Nude recipients of mouse cardiac xenografts, which reject at the same tempo as untreated recipients have no detectable antibodies to mouse RBC in the haemolysis
assays, or to mouse spleen cells in the indirect radioactive binding assays. Thus either T cell independent antibodies are not present or were not detected by these assay systems. This suggests that alternative mechanisms may be operating or that antibodies are at a level too low to be detected in these systems. Possible alternatives include NK cells and antibody dependent cell mediated cytotoxicity, where low levels of antibody can play a role. Studies by Thomas et al (1991) demonstrated that rejection of skin allografts and xenografts were delayed in Beige mice recipients. These mice have defective NK and Killer cell function.

Our studies demonstrated an antibody response by the rat to the mouse heart xenograft at day 3 following transplantation. This response is probably too early for a primary antibody response. It was important therefore to determine whether preformed natural antibody to mouse were present prior to transplantation. In the haemolysis assays, there was no detectable rat antibody to mouse RBC prior to transplantation, even though an antibody response is seen at day 3. However in the indirect radioactive binding assays, although there was no detectable antibody binding to mouse RBC in normal rats, binding was seen to mouse spleen cells and mouse heart homogenate. This could be due to a low level of natural antibody to mouse, but it could also be due to non-specific background binding of rat immunoglobulins to these targets. Flow cytometry using mouse platelets also demonstrated a brighter staining by normal rat serum in comparison with the medium control. However, although this suggests that preformed natural antibody are present in the rat to mouse platelets, it is impossible to exclude the possibility of non-specific background binding of rat immunoglobulins. Studies by Gannedahl et al (1990) in this model demonstrated no detectable antibodies to mouse RBC prior to grafting, although a low level of natural antibodies to spleen
cells were seen. Absorption studies to try to remove any preformed antibodies would not have been practical due to the low levels of antibodies present.

Although natural preformed antibodies to mouse appear not to be present to any great extent in the rat, there is the possibility that there are primed B cells, ready to elicit a second set humoral response. This is of particular interest since CTLA4-Ig can block primary antibody responses, but is less effective at blocking secondary antibody responses (Linsley et al 1992). CTLA4-Ig is also less effective at blocking Th2 responses, than Th1 cytokines in particular IL-10 (Lu et al 1995). Th2 cells are more involved in humoral immunity.

Very little is known about the histology of the mouse-to-rat heterotopic cardiac xenografts. Tufveson et al (1990) reported that the intimas were infiltrated with cells of a macrophage morphology, with mononuclear infiltrates in the pericardium and some lymphocyte infiltrates. Gannedahl et al (1994) reported a moderate lymphocytic infiltration. There appear to have been no studies using immunohistology to demonstrate specific cell types in the infiltrate of these rejecting mouse hearts. In this study the histology of these grafts in untreated recipients show a mild cellular infiltration consisting of polymorphonuclear leucocytes which stain positive for CD45. There are no T cells or macrophages in the infiltrate. At the time of rejection i.e. day 3 the picture is similar except that there are occasional cells with T cell markers, mainly CD8+ cells. Occasional macrophage and class I and class II positive cells are also seen. This is interesting as rat NK cells stain positive for CD8 and class I, (Bolton et al 1989) so that NK cells may be present in small numbers in these rejecting cardiac xenografts. Studies in the hamster-to-rat model show a similar profile at rejection with
a diffuse infiltration of neutrophilic granulocytes stained positive for MRC OX41 and OX42 with only a few scattered T cells (Nielsen et al. 1993).

This histology suggests a humoral mechanism of rejection and demonstrates that T cells do not have a major role in the rejection response in grafts where prolongation of survival is not achieved. However recipients receiving triple therapy, where the grafts survived longer than day 18, presented a different picture on histological examination. In these cases a massive mononuclear cell infiltration was seen throughout the graft suggesting a cellular mechanism of rejection.

Levels of expression of mouse MHC class I and class II molecules were increased in the mouse heart xenografts. Strong staining of blood vessels was observed by class II with scattered class II positive cells in the connective tissue of the mouse heart at day 1. The level of expression of class II in the connective tissues was increased by day 3 (the day of rejection). Weak staining with class I was seen at day 1 with an increased expression on day 3 in the connective tissues. At day 6 there were substantial amounts of class I and class II in areas where there were dense infiltrates, these presumably represent surviving and perhaps proliferating mouse connective tissue cells with upregulated levels of MHC antigen expression.

The mechanisms of rejection of BALB/c mouse hearts by rat recipients remains unclear. The rapid rejection in untreated recipients, along with the difficulty in prolonging survival without the use of splenectomy and the lack of a substantial mononuclear infiltrate at the time of rejection, suggest a humoral mechanism is involved in this response. However we were unable to correlate antibody levels with rejection, as nude recipients and those treated with CsA at 20mg/kg/day rejected their
grafts at day 3 with no detectable antibodies to mouse RBC. It is possible that the assay systems used were not sensitive enough or used inappropriate targets to detect these antibodies. Rejection mechanisms such as NK cells or ADCC could play a role in this rejection mechanism as nude rats possess NK cells. If the early rejection mechanisms can be overcome, graft rejection follows a more cellular pattern which is more difficult to suppress than that seen in allografts. This suggests that different mechanisms are involved in the rejection response to xenografts. It is likely that indirect recognition plays an important role in these rejection responses. Xenogeneic tissues would provide a plethora of foreign peptides available for presentation by self APC.
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DISCUSSION

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DISCUSSION

The work in this thesis addresses the influence of indirect T cell recognition in various situations in transplantation.

The role of indirect recognition in the mechanism of rejection has not been fully elucidated. This thesis demonstrated that when rats are primed to indirect allorecognition by immunisation with peptide, the immunosuppressive agent CsA was unable to suppress the antibody response to the graft, although priming to indirect allorecognition had no effect on graft function and survival whilst under CsA therapy. This has potentially important clinical implications. Although antibody to donor antigens do not necessarily have a deleterious effect in the rat (e.g. Fabre and Morris 1974) in the clinical situation antibody may play a role in acute vascular rejection (Jeanett et al 1970) and chronic rejection (Suciu-Foca et al 1991).

The mechanisms whereby pre-graft blood transfusion has a beneficial effect on graft survival is poorly understood. This study demonstrates that the T helper pathway probably plays a crucial role in the immunosuppression of the blood transfusion effect. Activation of the T helper pathway by priming to indirect allorecognition prior to multiple blood transfusion suppressed the immunosuppressive effect of blood transfusion. Priming to indirect allorecognition during multiple blood transfusion reversed the suppression. Thus the blood transfusions suppressed the T helper pathway for antibody production.
The mechanisms of rejection in xenotransplantation are difficult to study in discordant species combinations. The work in this thesis used the mouse-to-rat heterotopic cardiac xenograft model of concordant xenotransplantation which is a closer species combination than the hamster-to-rat model. Interestingly the rejection was very rapid between these strains even though they are closely related with rejection at day 3. Use of CsA, CTLA4-Ig and splenectomy when used alone failed to prolong xenograft survival. CTLA4-Ig is an immunosuppressive agent which acts by blockade of the CD28/CTLA4 to B7-1/B7-2 co-stimulatory signal. CTLA4-Ig has been used in allograft systems to effectively suppress the rejection response (e.g. Turka et al 1992). The early rejection response appeared to be humoral. Histology and immunohistology of the grafts at day 3 showed a mild infiltrate consisting of polymorphonuclear cells. This infiltrate was positive for CD45 but negative for T cell markers. Rat antibodies to mouse were detectable in most cases at day 3 (day of rejection). However antibody response did not correlate with rejection in all cases. Nude rats and rats treated with CsA at 20mg/kg rejected their grafts at day 2/3 with no detectable antibody response to mouse targets. It is possible that other mechanisms such as NK cells or ADCC could be involved in this rejection response. Marked prolongation of cardiac xenograft survival was achieved only when CsA, CTLA4-Ig and splenectomy were combined. In this group of rats, recipients that survived past day 18 demonstrated a more cellular pattern of rejection, with a massive mononuclear leucocytic infiltrate into the graft at the time of rejection and no concomitant antibody response to mouse targets. This study demonstrates that xenograft rejection is more difficult to control than in allograft systems where CTLA4-Ig and CsA are capable of suppressing the immune response and suggests that different mechanisms are involved. It is likely that indirect
recognition will play a more important role in the rejection response of xenografts, even if the direct recognition pathway can still function as in the rat-to-mouse pathway (van den Bogaedre et al 1990) as there will be many more foreign peptides available for presentation on self APC. Virtually every protein in the mouse heart will carry substantial sequence differences from the homologous rat proteins.
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APPENDIX I

PUBLICATIONS ARISING FROM THIS THESIS

PUBLISHED

Sawyer, G.J. Dalchau, R. and Fabre, J.W.
Indirect T cell allorecognition: a cyclosporin A resistant pathway for T cell help for antibody production to donor MHC antigens. 1993 Transplantation Immunology; vol 1: 77-81

Sawyer, G.J. Gustafsson, K. and Fabre, J.W.

IN PREPARATION

Sawyer, G.J. and Fabre, J.W.
The influence of indirect T cell allorecognition on the immunosuppressive effects of multiple blood transfusions.

Sawyer, G.J. and Fabre, J.W.
Characterisation of the mouse-to-rat heart xenograft model: a difficult concordant combination.