INDIRECT T CELL ALLORECOGNITION OF THE RT1.A^{a} MHC CLASS I MOLECULE.

by

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ABSTRACT

The extent to which the indirect pathway of T cell recognition contributes to graft rejection remains to be clarified, and this thesis examines its role in the antibody-mediated rejection of allografts in the MHC class I disparate rat strain combination, PVG-R8 (RT1.A^aB/C^uD^u) to PVG-$RT1^u$ (RT1.A^uB/C^uD^u). A series of overlapping 15-mer allopeptides (P1-P18) derived from the donor A^a antigen, were used to map the immunogenic, dominant and sub-dominant epitopes of the A^a molecule.

Analysis of the alloantibody response mounted to individual allopeptides following their subcutaneous administration suggested that the 15-mer peptides P7 and P16, derived from the hypervariable regions of the α1 and α2 domains respectively, and the 24 amino acid α1 peptide were immunogenic. The dominant T cell epitope was characterised by examination of the *in vitro* T cell proliferative responses to individual allopeptides by LNC from $RT1^u$ animals immunised with A^a-bearing R8 allografts. Proliferation focused upon those peptides derived from the hypervariable region of the α1 domain, an area corresponding to P7 and P8.

Analysis of the alloantibody response to the intact A^a molecule following peptide priming demonstrated the presence of two additional sub-dominant T cell epitopes located within P1 and P9. These peptides derive from areas of the A^a molecule that are identical in amino acid sequence to the corresponding areas of the $RT1^u$ molecule, and are therefore, in effect, self-$RT1^u$ peptides. Priming with both dominant and sub-dominant epitopes accelerated the rejection of subsequent R8 cardiac allografts, suggesting that peptide priming is able to indirectly activate recipient T cells.

It was examined whether the dominant and sub-dominant T cell epitopes could be used to favourably modulate the immune response to the intact A^a molecule by intravenous administration of high doses of P7 and P1 to $RT1^u$ animals before challenge with an R8 blood transfusion. Downregulation of the cytotoxic and IgM alloantibody responses were observed and in addition, P7 was able to downregulate

the IgG2b response. Slight downregulation of the cytotoxic alloantibody response to an R8 cardiac allograft was seen following immunisation with P7, but the IgM and IgG2b responses were unaltered. Prolonged allograft survival was not observed.

These results suggest that the indirect response to an allogeneic MHC molecule may involve additional unexpected epitopes and consequently, that the success of peptide-based tolerogenic protocols requires a fuller understanding of this process.

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DECLARATION

I declare that the conceptual ideas and experimental design for the work presented in this thesis were conceived by the author, Miss Emma Lovegrove and Professor JA Bradley. The experimental procedures described in this thesis were undertaken solely by the author, with the exception of the rat cardiac allograft transplants, which were performed by Mr. Gavin Pettigrew.

I confirm that all of the work presented in this thesis is original and has not been submitted for another degree at this or any other University.

Some of the results described in Chapters 3 and 4 were presented orally at the British Transplantation Society's Annual meeting in November 1997:

"Analysis of Class I MHC Epitopes that Provide Cognate T Cell Help for Alloantibody-Mediated Graft Rejection."

E Lovegrove, G Pettigrew, EM Bolton, JA Bradley.

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ABBREVIATIONS

AICD	Antigen-Induced Cell Death
ALS	Anti-Lymphocyte Serum
APC	Antigen Presenting Cell
ARAM	Antigen Recognition Motifs
ATP	Adenosine Triphosphate
BCR	B Cell Receptor
β_2M	β_2-Microglobulin
BMC	Bone Marrow Cells
BSA	Bovine Serum Albumin
C	Constant Gene Segment
Ca	Calcium
CAV	Coronary Artery Vasculopathy
CD	Cluster of Differentiation
CDR	Complementarity Determining Region
CFA	Freund's Complete Adjuvant
CIITA	MHC Class II Transactivator Protein
CIIV	Class II-Containing Vesicle
CLIP	Class II-Associated Invariant Chain Peptide
cm	Centimetres
ConA	Concanavalin A
CO_2	Carbon Dioxide
cpm	Counts Per Minute
^{51}Cr	Chromium 51
CsA	Cyclosporine A
cTEC	Cortical Thymic Epithelial Cells
C-Terminal	Carboxy Terminal
C-Terminus	Carboxy Terminus
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen –4
^0C	Degrees Celsius

D	Diversity Gene Segment
DAG	1,2-sn-Diacylglycerol
DNA	Deoxyribonucleic Acid
DP	Double Positive T Cell
DST	Donor Specific Tolerance
DTH	Delayed Type Hypersensitivity
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme-Linked Immunosorbant Assay
ER	Endoplasmic Reticulum
ERp57	Endoplasmic Reticulum Protein 57
Fas	Fibrobalst Associated Anigen
FCS	Foetal Calf Serum
FITC	Fluorosine Isothiocyanate
FK506	Fujisawa K506
Fmoc	9-Fluorenylmethoxycarbonyl
g	Grams
G	Gauge
H	Immunoglobulin Heavy Chain
HA	Haemagglutinin
HBSS	Hanks Buffered Salt Solution
HBTU	O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
H + E Staining	Haematoxylin and Eosin Staining
HI	Heat Inactivated
HLA	Human Leukocyte Antigen
HPLC	High Pressure Liquid Chromatography
Hrs	Hours
HSC	Haematopoietic Stem Cell
Hsp	Heat Shock Protein
^{125}I	Iodine 125
Ia	Classical MHC Class I Genes
Ib	Non-Classical MHC Class I Genes
ICAM-1	Intercellular Cell Adhesion Molecule-1

IFN-γ	Interferon-γ
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG2b	Immunoglobulin G 2b
IgM	Immunoglobulin M
Ii	Invariant Chain
Il-2	Interleukin-2
Il-2R	Interleukin-2 Receptor
IP_3	1,4,5 Inositol Triphosphate
IT	Intrathymic
ITAM	Immunoreceptor Tyrosine-Based Activation Motifs
IVC	Inferior Vena Cava
J	Joining Gene Segment
kb	Kilobases
kDa	Kilo Dalton
kg	Kilograms
L	Immunoglobulin Light Chain
LDA	Limiting Dilution Analysis
LFA-1	Lymphocyte Function-Associated Antigen-1
LMP	Low Molecular Weight Protein
LNC	Lymph Node Cell
mAb	Monoclonal Antibody
MBq	Mega Becquerel
2-Me	2-Mercaptoethanol
MECL-2	Multicatalytic Endopeptidase Complex-Like 2
MHC	Major Histocompatibility Complex
MIIC	MHC Class II Compartment
MLR	Mixed Leukocyte Reaction
ml(s)	Millilitre(s)

mm	millimetre(s)
mM	Millimolar
MST	Median Survival Time
mTEC	Medullary Thymic Epithelial Cells
μCi	Micro Curie
μg	Microgram(s)
μl	Microlitre(s)
NaCl	Sodium Chloride
NF-AT	Nuclear Factor of Activated T Cells
NK	Natural Killer cell
NP	(viral) Nucleoprotein
N-Terminal	Amino Terminal
PA28	Proteasome Activator 28
PBA	PBS/0.2%BSA/0.1% sodium azide
PBS	Phosphate Buffered Saline
PIP_2	Phosphatidyl Inositol 4,5-Biphosphate
PKC	Protein Kinase C
PLCγ1	Phospholipase C Gamma 1 Isoform
PTK	Protein Tyrosine Kinase
PTPase	Protein Tyrosine Phosphatase
RAG	Recombinant Activator Gene
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
SH2	Src Homology 2
SP	Single Positive T Cell
SVC	Superior Vena Cava
TAP	Transporter Associated with Antigen Processing
TCR	T Cell Receptor
T_h	Helper T Cell
^{3}H-Thymidine	Tritiated Thymidine
TNF	Tumour Necrosis Factor

V	Variable Gene Segment
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very Late Antigen-4
ZAP	Zeta Chain-Associated Protein

AMINO ACID SYMBOLS

Amino Acid	Three Letter Symbol	One Letter Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

For Mum, Dad
and Gavin

CHAPTER 1:

INTRODUCTION

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CHAPTER ONE:

INTRODUCTION

1.1 THE MAJOR HISTOCOMPATIBILITY COMPLEX:

1.1.1 *Genetics*

The major histocompatibility locus was originally defined by the work of Gorer (Gorer 1937, Gorer 1938), through his studies on the rejection of tumours by inbred strains of laboratory mice. The region was later called H-2 (Gorer et al 1948). Since the discovery of H-2, a similar gene complex has been observed in all vertebrate species examined. It has been termed the Human Leukocyte Antigen (HLA) in humans (Dausset 1958), and RT1 in rats (Palm 1964). See Table 1.

The MHC consists of three main regions, class I, class II and class III. All three regions encode products involved in the immune response to protein antigen. Class I and class II MHC molecules are generally expressed as cell surface glycoproteins. Class III genes however encode soluble products such as C2, C4 and factor B, which are members of the complement cascade, (Porter 1985), TNF-α and -β (Spies et al 1986, Carroll et al 1987), and some of the heat shock protein 70 (hsp70) family (Sargent et al 1989)

The MHC is situated on different chromosomes in different species. In humans, it is found on chromosome 6, in mice, chromosome 17, and in rats, on chromosome 20 (Locker et al 1990, Helou et al 1998). The HLA and H-2 complexes are amongst the most definitively mapped regions of the human and mouse genomes respectively (Trowsdale 1995), and although the RT1 complex has not been as extensively mapped, much information about its genomic arrangement has been recently gathered (Gill et al 1997).

The genomic organisation of the HLA complex is similar to that seen in the majority of species studied, with the notable exception of mice and rats. The class II genes are found at the centromeric end of the complex on the short arm of chromosome 6,

SPECIES	MHC	CLASS I GENES	CLASS II GENES
HUMAN	HLA	A B C	DP DQ DR
MOUSE	H-2	K D L	I-A I-E
RAT	RT1	A C/E	B D

Table 1.1: The nomenclature of the MHC class I and class II genes in the human, mouse and rat.

followed by the class III genes, and the class I genes are located towards the telomeric end of the complex.

There are three major class II loci, HLA-DP, -DQ and -DR (Trowsdale et al 1991) Each of these loci code for at least one α and one β chain. The class II region also encompasses a cluster of genes associated with peptide processing and transport, including TAP (Spies et al 1990), tapasin (Herberg et al 1998) and LMP (Driscoll 1994). In addition, a non-classical class II gene, HLA-DM (Kelly et al 1991), involved in the loading of peptides onto class II molecules (Fling et al 1994) is present in this region.

The classical (Ia) class I loci in the HLA complex are known as HLA-A, -B and -C (Trowsdale et al 1991). HLA-A and -B are highly polymorphic, with over 20 HLA-A and 40 HLA-B alleles having been identified to date. HLA-C is far less polymorphic, with just over 10 alleles thus far identified, and is thought to be involved in NK cell specificity (Ciccone et al 1990). In addition to the classical class I genes, several non-classical (Ib) class I genes have also been identified; HLA-F, -G and -H. These have not been well defined, although it is known that HLA-G is expressed on the trophoblast at the maternal-foetal interface, and may be involved in immunity to the developing foetus (Ellis et al 1990).

The organisation of the murine histocompatibility complex is fundamentally different to that seen in humans, in that it appears to have undergone a major translocation event which has re-positioned the class II and class III loci between the

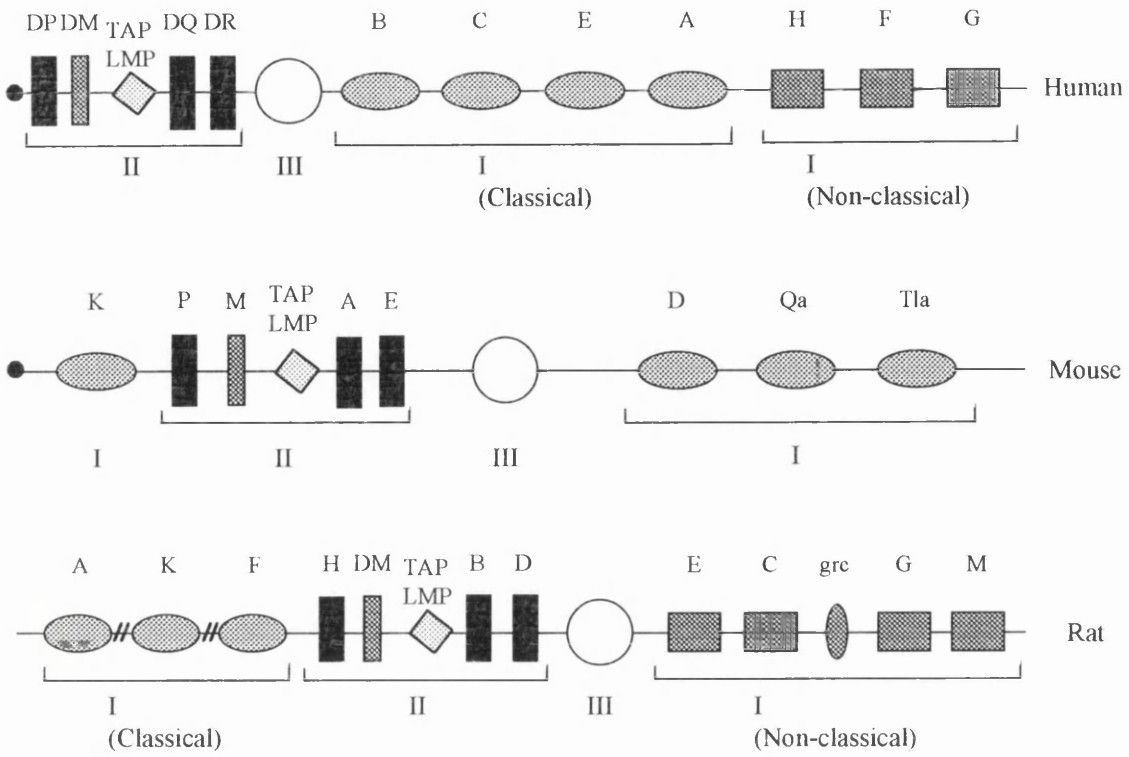

Figure 1.1: Genomic organisation of the human, mouse and rat MHC regions (maps are not to scale). The relative locations of the class I, class II and class III loci are illustrated, as well as other relevant genes, including those involved in antigen processing. Based upon Trowsdale, Immunogenetics, 41 (1): 1-17; 1995.

two major class I loci (see Figure 1.1). As the rat displays a similar MHC organisation to the mouse, this translocation event probably happened shortly before the evolutionary separation of the two species. Thus, in the mouse, the class II loci, I-A and I-E, and the class III loci separate the two classical class I loci, H-2K and H-2D/L. The murine class II region bears striking similarity to the human class II region, with I-A and I-E comparable to HLA-DQ and HLA-DR. Again, a cluster of transporter genes are found within the class II region, including H2-M, which is equivalent to the human HLA-DM (Cho et al 1991).

The two major rat class I loci, RT1.A and RT1.E, occupy two separate regions of the MHC, but only the RT1.A locus expresses antigens with classical functions. Between the two class I loci are the functional class II genes which encode RT1.B and RT1.D, while a third class II locus, RT1.H is not expressed. A similar

arrangement of RT1.DM (analogous to HLA-DM and H2-M), TAP (Carter et al 1994), tapasin (Herberg et al 1998) and LMP exists. The second class I region is situated to the right of the histocompatibility complex, and encodes the RT1.E, .C, .G and .M antigens. These antigens, although numerous (Jameson et al 1992), are classed as non-classical, in that they do not exhibit polymorphism, are not serologically detectable, do not bind peptide and do not elicit a cytotoxic T cell response. This view has however been recently challenged by the observation that certain rat non-classical antigens are, in fact, functional (Leong et al 1999). The RT1.A locus displays a more limited polymorphism than its equivalent antigens in mouse and human (Gill et al 1987), and it has been suggested that in the rat, the use of non-classical antigens may compensate for this.

1.1.2 *Structure and Function*

MHC class I and class II molecules are both heterodimeric transmembrane glycoproteins, whose primary function is the presentation of protein fragments to T cells. The products encoded by the MHC are members of the Ig-superfamily (Bjorkman et al 1987b, Williams et al 1988). However, fundamental differences in the structure and function of the two MHC sub-classes exist.

MHC class I (see Figure 1.2a) consists of two sub-units, a polymorphic α chain of 45kDa tethered to the cell membrane, and a non-covalently linked 12kDa molecule, β_2-microglobulin (β_2M), which is not attached to the membrane. The gene encoding β_2M displays limited polymorphism, and is on a different chromosome from the MHC (chromosome 15 in humans). The heavy chain consists of five domains; three globular extracellular domains, α1, α2, and α3, each of which are approximately 90 amino acids long, a transmembrane domain, and an intracellular domain. The proximal α3 domain and β_2M display the typical folding structure of Ig family members, whereas the distal α1 and α2 domains display a structure uniquely tailored to their function. Each comprises an extended α-helix, above a platform of four antiparallel β strands. The distal part of the class I molecule therefore forms a

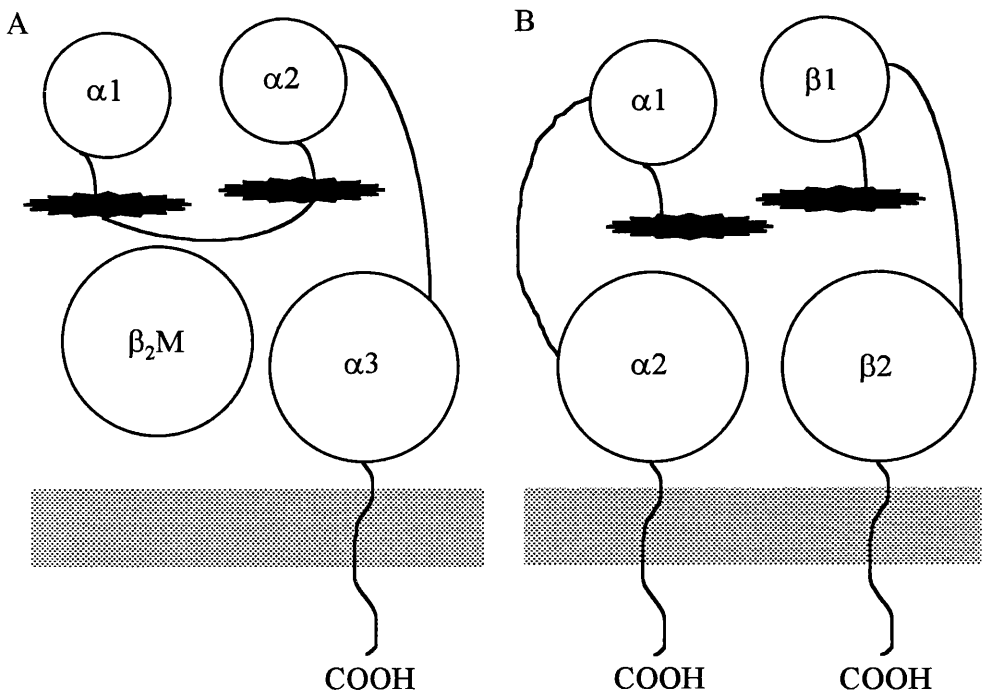

Figure 1.2: Schematic representation of A) MHC class I, and B) MHC class II. Adapted from Lechler et al, 1995.

groove, with the β-pleated sheets constituting the base of the groove, and the two α-helices forming the walls.

MHC class II molecules (Figure 1.2b) consists of two separate chains, an α chain (32kDa) and a β chain (28kDa), both encoded within the MHC class II region. Each chain has two extracellular domains (α1 and α2, and β1 and β2), a transmembrane domain, and an intracellular domain. Again, the two sub-units closest to the cell membrane (α2 and β2) display a typical Ig-like structure, and the two distal domains (α1 and β1) consist of an α-helix above a floor of β-pleated strands.

Prior to the elucidation of the fine structure of the class I MHC molecule, it was appreciated that MHC formed a complex with antigenic peptide (Babbitt et al 1985, Buus et al 1987), but the nature of this complex was unclear. Bjorkman's seminal work in elucidating the crystal structure of the HLA-A2 molecule (Bjorkman et al 1987b, Bjorkman et al 1987a) (see Figure 1.3) clearly demonstrated the presence of an antigen-binding cleft. Furthermore, an area of electron dense material was

identified within this cleft, and it is now known that this represents bound peptide. The crystal structures of human HLA-Aw68 (Garrett et al 1989) and HLA-B27 (Madden et al 1991), and mouse H-2K^b (Fremont et al 1992) and H-2D^b (Young et al 1994) class I molecules have been subsequently characterised, and a similar structure has been found in all cases. The comparison of MHC class I sequences has shown that the area which exhibits the greatest degree of polymorphism is that comprising the T cell receptor contact region/antigen-binding site (i.e. the α1 and α2 domains). This supports the hypothesis that MHC polymorphism exists to permit binding to as wide an array of peptides as possible.

The crystal structure of HLA-DR1 was published in 1993 (Brown et al 1993), and it was seen that the basic structures of class I and class II molecules are similar. They do, however, exhibit fundamental differences in their ability to bind and present peptide. MHC class I optimally binds peptides of 8-10 amino acids in length (Falk et al 1991). The groove itself is enclosed at either end, and peptide is seen to bind tightly to either end of the groove by means of both hydrogen bonds at conserved residues, and allele-specific pockets, created by amino acid variations, into which peptide side chains can slot. Peptides of slightly longer length have also been observed binding to MHC class I (Guo et al 1992). These peptides bind conserved residues at position number 2 and at the carboxy terminus and bulge outwards from the middle of the groove.

The antigen-binding cleft of MHC class II in contrast appears to be open-ended. This allows for greater flexibility in the length of class II-associated peptide, as the peptide can extend out of either end of the groove. The optimal length of peptides eluted from MHC class II is between 13 and 17 amino acids (Rudensky et al 1991, Chicz et al 1992). To compensate for the lack of binding sites at its ends, the class II groove contains binding points for peptide along its length. These binding points are conserved, resulting in a more stringent orientation of peptide within the groove.

For example, Stern and colleagues (Stern et al 1994) observed that a peptide from the influenza virus bound to HLA-DR1 was held in an extended conformation with a pronounced twist. Whilst five of the thirteen peptide side chains were embedded into

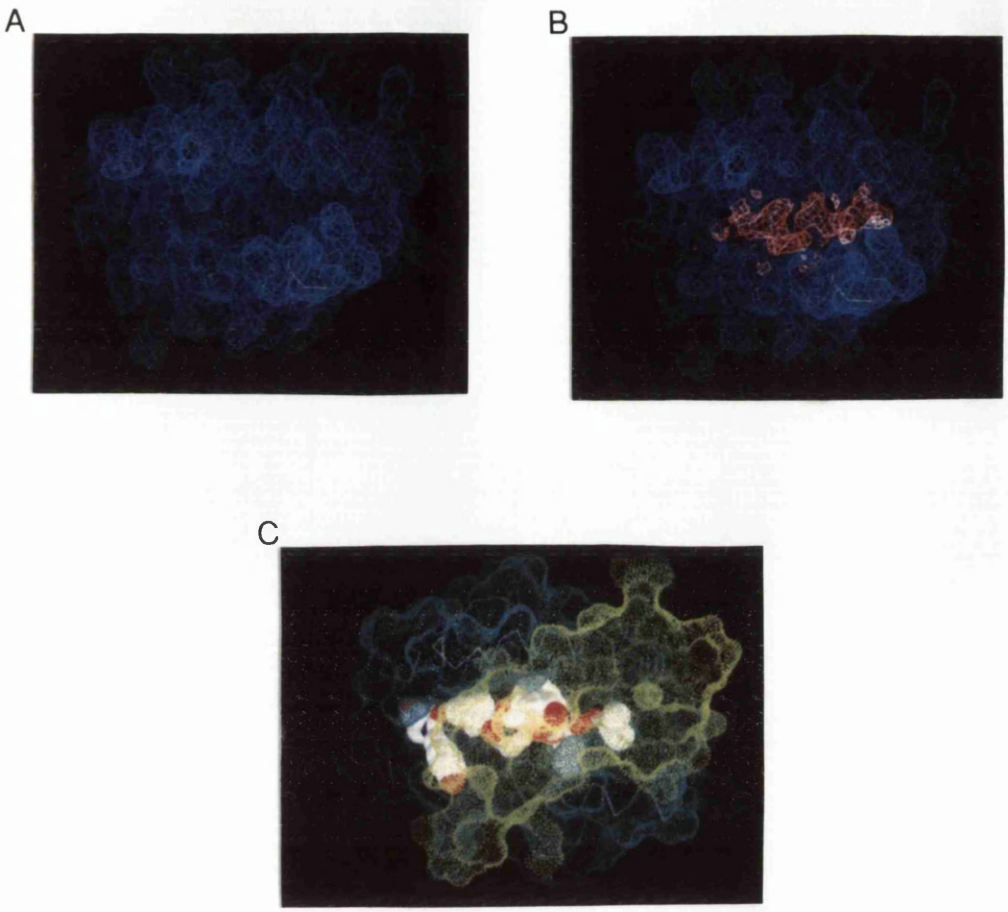

Figure 1.3: The crystal structure of the MHC class I molecule A) without bound peptide, B) with bound peptide and C) in association with the TCR. Pictures are taken from Bjorkman et al, 1987.

allele-specific pockets (thus accounting for the peptide specificity of HLA-DR1), twelve hydrogen bonds between conserved HLA-DR1 residues and the peptide main chain also contributed to the binding of the peptide.

1.2 ANTIGEN PROCESSING AND PRESENTATION:

1.2.1 *MHC Class I*

1.2.1.1 *Antigens are Processed Before Presentation by MHC Class I*

MHC class I molecules generally present protein antigen derived from endogenous sources (e.g. viral antigens) to CD8 T lymphocytes. It was originally thought that virus-specific CTL recognised intact viral glycoproteins expressed on the surface of infected cells. It was not until the mid 1980's that Townsend (Townsend et al 1985) suggested that CD8 T cells may in fact recognise degraded antigen in a manner similar to that previously described for CD4 T cells (Unanue 1984). Initial studies examined the response to viral nucleoprotein (NP); a non-glycosylated protein that accumulates in the nuclei of infected cells. Transfection of murine cells with a series of truncated viral NP genes showed that three distinct NP epitopes could be recognised by CTL. These results suggested the existence of a mechanism allowing for transport to and the presentation of proteins at the cell surface (Townsend et al 1985). Furthermore, the NP epitopes recognised by CTL could be defined by small synthetic peptides, implying that CTL recognise short stretches of protein (Townsend et al 1986b). A further study from the same group (Townsend et al 1986a) reported that CTL raised against the intact glycosylated transmembrane viral protein, haemagglutinin (HA) could respond to cells expressing HA devoid of its signal sequence, and vice versa.

Maryanski et al performed further studies to determine whether this method of viral protein recognition was also applicable to other antigens. Using murine cells transfected with the genes for HLA-A24 and HLA-CW3, the group generated CTL that were specific for human class I antigens. In addition to lysing HLA-transfected cells, these CTL were also able to lyse syngeneic HLA-negative cells in the presence

of synthetic HLA peptide (Maryanski et al 1986). This was the first demonstration that peptide could associate with intact MHC class I molecules in order to provoke a cytotoxic T cell response. Further proof that peptide does in fact associate with MHC class I was provided by Guillet et al (Guillet et al 1986). They demonstrated that the addition of non-related peptides could block the response of T cells to peptides derived from the bacteriophage λ cl protein, and concluded that the non-related peptides were competitively inhibiting the binding of the bacteriophage peptides to self-MHC.

The above studies all suggest that small stretches of modified protein antigen associate with MHC class I molecules within the cell before being transported to the cell surface for survey by CD8 T cells. As mentioned in section 1.1.2, Bjorkman's elucidation of the crystallographic structure of MHC, and in particular, the nature of the peptide binding cleft (Bjorkman et al 1987b, Bjorkman et al 1987a) confirmed the necessity of antigen processing prior to presentation.

Stable cell surface expression of MHC class I requires that all the subunits of the molecule are correctly assembled, which takes place in the ER under the care of chaperone molecules. Following assembly, class I-β_2M dimers associate with peptide. Peptide generation occurs in the cytosol, where endogenous proteins are degraded by the main cytosolic protease, the proteasome (Goldberg et al 1992). Following their generation, peptides are translocated into the ER by means of the ATP-dependent TAP transporter complex, where they associate with nascent MHC class I molecules.

Targeting of endogenous antigen to the proteasome is thought to be largely due to conjugation of multiple ubiquitin molecules to internal lysine residues of the protein. As well as providing a signal for proteasomal uptake, ubiquitination is thought to destabilise protein molecules, thereby rendering them more susceptible to degradation. It is unclear exactly how such destabilisation is achieved, although unfolding of the tertiary structure has been suggested (Hochstrasser 1996).

1.2.1.2 *The Proteasome*

The proteasome is a 20S multicatalytic proteinase complex consisting of four 7-membered rings stacked above one another to form a hollow cylindrical structure. Each ring is composed of either 7 α or 7 β subunits, and there are two rings of each type. They are arranged $\alpha_7\beta_7\beta_7\alpha_7$, with the inner β subunits containing the proteolytic sites, therefore suggesting that intact protein must pass down the centre of the cylinder for degradation to occur.

In the mammalian proteasome, three of the 7 β subunits are catalytically active, β1 (Y/δ), β2 (Z) and β5 (X/MB1) (Reviewed in (Uebel et al 1999). Upon IFN-γ stimulation, these subunits are replaced by LMP (low molecular weight protein) 2, multicatalytic endopeptidase complex-like 2 (MECL-1, LMP10) and LMP7 respectively (Gaczynska et al 1993, Hisamatsu et al 1996). The LMP2 and LMP7 subunits are encoded within the MHC itself (Driscoll 1994) and the role that they play in antigen processing has generated much interest. Although it was initially demonstrated that these two subunits were not critical for antigen processing (Momburg et al 1992), it has more recently been shown that a deficiency of these two genes, particularly LMP7, can result in the attenuated presentation of some protein antigens (Cerundolo et al 1995). Furthermore, it has been demonstrated that both subunits alter the specificity with which protein antigen is cleaved (Driscoll et al 1993, Gaczynska et al 1993): with the LMP7 gene increasing the capacity of the proteasome to cleave after basic or hydrophobic residues, and the LMP2 gene decreasing the capacity of the proteasome to cleave after acidic residues (Gaczynska et al 1994). As MHC class I molecules typically bind peptides with C-terminal hydrophobic residues, it may be that LMP2 and LMP7 induction confers physical advantages to processed peptide for MHC binding.

The proteasome in higher eukaryotes is associated with further regulatory accessory complexes, which appear to enhance its action. One such complex implicated in the generation of peptide for presentation to CTL is the IFN-γ-inducible molecule PA28 (Groettrup et al 1995). An 11S hexamer composed of α and β subunits, PA28

attaches to both ends of the proteasome and enhances peptide generation regardless of the presence of LMP2 and LMP7 (Groettrup et al 1995). Its mode of action firstly appears to increase the rate of protein cleavage after hydrophobic residues (Ustrell et al 1995) and secondly, exposes protein to two sites of protease activity concurrently (Dick et al 1996).

It has been generally assumed that peptides of an appropriate length for MHC class I association (i.e. ~9 amino acids) are generated by the proteasome. However, it has been recently demonstrated that only a small minority of processed peptides are in fact of the correct length for MHC binding (Kisselev et al 1998, Kisselev et al 1999). This suggests that further trimming may take place within the endoplasmic reticulum (ER), once peptide has been loaded onto class I.

It has been recently shown by Benham and colleagues (Benham et al 1998), that the inhibition of proteasomal action by lactocystin does not inhibit the assembly of three human class I alleles, HLA-A3, HLA-A11 and HLA-B35. In addition, *in vitro* loss of the proteasome complex is seen to be compensated for by the over-expression of another large proteolytic complex in some cells (Glas et al 1998). Therefore, the above studies suggest that under certain circumstances, peptide-loading of MHC class I molecules is free from the limitations imposed by proteasomal processing.

The proteasome may also process exogenous as well as endogenous antigens, therefore providing a mechanism by which MHC class I may present antigen from extracellular sources (Reviewed by (Rock 1996). This phenomenon is particularly prevalent in macrophages and dendritic cells (Bevan 1987), which appear to have a predisposition to transfer antigen from the class II-processing compartment (the endosome), into the cytosol for proteasomal lysis (Reviewed by (Watts 1997).

1.2.1.3 *The TAP Transporter*

Following antigen processing in the proteasome, peptides must be transported into the ER for association with nascent MHC class I-β_2M dimers. It has been demonstrated that an ATP-dependent heterodimeric transporter complex, known as the transporter associated with antigen processing (TAP), is responsible for peptide

translocation, and also for the final assembly of class I-peptide complexes (Neefjes et al 1993). The TAP transporter consists of two subunits, TAP1 and TAP2, both of which are encoded within the MHC class II region (Powis et al 1991). Both TAP1 and TAP2 span the ER membrane, and consist of a hydrophobic N-terminal region that bridges the membrane and a C-terminal hydrophilic region contained within the cytosol. The peptide-binding area has been mapped to the C-terminal end of the hydrophilic region, adjacent to the hydrophobic domain (Nijenhuis et al 1996a, Nijenhuis et al 1996b).

TAP displays a degree of specificity for the peptides it binds, both in amino acid sequence (Momburg et al 1994b) and length (Momburg et al 1994a). It has been demonstrated that in the mouse, TAP has a high affinity for peptides with a hydrophobic C-terminal amino acid residue, whereas the human TAP complex appears to be more permissive, binding peptides regardless of their C-terminal residue. The rat TAP transporter complex is unusual however, in that two functional TAP2 alleles have been identified, TAP2-A and TAP2-B (referred to as TAP-A and TAP-B) (Deverson et al 1990). These two alleles display profound differences in the peptides that they are able to transport. For example, in RT1.A^a-bearing hosts, which normally express TAP-A, the A^a antigen becomes immunogenic if it is instead linked to TAP-B (Livingstone et al 1989, Livingstone et al 1991, Powis et al 1992). Subsequent studies have demonstrated that the RT1.A^a molecule has a strong preference for binding peptides with hydrophilic C-terminal cysteine residues. These are only provided by the permissive TAP-A allele, with linkage to TAP-B resulting in a completely different spectrum of available peptides, which explains the resulting immunogenicity. No such functional TAP polymorphism has been seen in either the mouse or human (Schumacher et al 1994, Daniel et al 1997, Marusina et al 1997).

Genetic sequencing of the TAP-A and TAP-B alleles has found that they differ by just 25 amino acids, with the majority of the polymorphic residues located within the N-terminal two thirds of the TAP2 chain (Momburg et al 1996, Deverson et al 1998).

The TAP and RT1.A loci are physically very close, only 250kb apart. Consequently, they display linkage disequilibrium, which helps to ensure that the RT1.A^a allele is inherited with the TAP-A antigen. However, the stimulus for evolutionary persistence of TAP-B is unclear, as its substitution with TAP-A does not result in faulty class I peptide-loading (Joly et al 1998).

1.2.1.4 *Assembly of the MHC Class I-Peptide Complex*

Stable cell surface expression of MHC class I requires firstly that the heavy chain is associated with a β_2M subunit and secondly, that this complex is bound with high affinity to peptide. If attempts to form a stable complex are unsuccessful, then the class I molecule itself will undergo degradation (Hammond et al 1995). The formation of a functional class I-peptide complex requires the assistance of a series of ER-retained chaperone molecules. Their function is to retain the class I molecule in the ER and also to mediate the association of the individual components of the class I complex.

Nascent class I heavy chains are found in the ER in association with a transmembrane chaperone molecule known as calnexin, which is thought to play a role in the correct folding of the heavy chain (Jackson et al 1994). In humans, the subsequent binding of β_2M results in the exchange of calnexin for another homologous ER chaperone protein called calreticulin (Sadasivan et al 1996). In the mouse, the situation is slightly different, in that following β_2M-binding, calnexin is not replaced by calreticulin. ERp57, a thiol oxidoreductase, has recently been shown to bind the MHC molecule at around about the same time as calnexin or calreticulin. It is thought that this molecule might aid MHC class I folding (Hughes et al 1998, Lindquist et al 1998, Morrice et al 1998). The calreticulin-associated class I-β_2M dimer then binds to the TAP transporter, a process mediated by another chaperone molecule, tapasin. Tapasin is a 48kDa molecule (Ortmann et al 1997), encoded for within the MHC class II region (Herberg et al 1998). The precise role of tapasin in class I assembly is unclear, although it appears to stabilise empty class I molecules, bridge the binding of the calreticulin-associated class I-β_2M dimer to the TAP complex and increase the rate of peptide translocation of TAP. Up to four tapasin

molecules have been seen to bind to a single TAP complex (Ortmann et al 1997), a phenomenon that is believed to enhance the association of peptides and MHC molecules. Each MHC class I molecule samples several peptides, before selecting one to which it binds with sufficiently high affinity. Peptide binding signals the release of all auxiliary molecules from the class I complex, and the complex leaves the ER via the Golgi apparatus, for transport to the cell surface.

1.2.2 *MHC Class II*

The primary function of MHC class II molecules is presentation of exogenous antigens to CD4 T lymphocytes. Unlike MHC class I, which is expressed on all cell types, MHC class II is constitutively expressed on only a limited population of cells. It is ordinarily found on B cells, dendritic cells and macrophages, but is readily induced on a wide range of cells upon stimulation with IFN-γ and in particular, on vascular endothelial cells (Pober et al 1983). MHC class II gene transcription has recently been found to be regulated by the MHC class II transactivator protein (CIITA) (e.g. (Zhou et al 1995). This protein is also largely responsible for regulating expression of the invariant chain (Ii) and HLA-DM (Chang et al 1995, Kern et al 1995). Class II peptide complexes are largely formed *de novo* by the association of nascent class II dimers with peptide in endocytic compartments (Neefjes et al 1990). However, a separate pathway also exists, by which class II molecules can recycle from the cell surface and exchange bound peptide (Pinet et al 1995).

Following translation and dimerisation of the α and β MHC class II subunits in the ER, association with a third protein chain, known as the invariant chain (Ii), occurs. Binding of Ii to $\alpha\beta$ complexes is mediated by a small 25-residue section of the Ii chain, which is referred to as the class II-associated invariant chain peptide (CLIP). CLIP can be readily eluted from purified class II molecules, and crystallographic studies confirm that it binds in a manner similar to that of antigenic peptide (Ghosh et al 1995). The association of Ii with class II is thought to have a dual function. Firstly, the invariant chain provides the signal that targets class II molecules to the endocytic pathway and secondly, the occupation of the peptide-binding groove by

CLIP protects αβ complexes from binding endogenously derived peptide in the ER (Cresswell 1996).

Heterodimeric αβ complexes and Ii are retained in the ER by chaperone molecules until they are properly folded (e.g. (Hurtley et al 1989). Ii chains subsequently trimerize via their C-termini (residues 163-183) (Bijlmakers et al 1994, Bertolino et al 1995), resulting in the formation of nonomeric structures (Roche et al 1991), with the three αβ class II dimers arranged around the core of the Ii trimer. The nonomeric complexes enter the secretory pathway and are transported to the trans Golgi Network. The class II-Ii nonomers are then diverted to the endocytic pathway by means of di-leucine signals situated within the cytoplasmic N-termini of the Ii molecules (Bakke et al 1990, Lotteau et al 1990, Pieters et al 1993). The exact mechanism of endocytic targeting is unclear, but the trimeric Ii chain structure is known to be important (Arneson et al 1995).

1.2.2.1 *Antigen Processing*

Exogenous protein antigens are internalised by pinocytosis or receptor-mediated endocytosis and enter the endocytic system. There, they undergo degradation in the increasingly acidic environment of the early endosomes to form peptides of a suitable length for MHC class II-association. Antigen fragments are further degraded in late endosomes through the action of proteolytic enzymes including cathepsin D (van Noort et al 1994) and cathepsin B (Takahashi et al 1989).

1.2.2.2 *MHC Class II Peptide-Loading*

It is thought that nascent MHC class II molecules encounter degraded protein antigen in class II-enriched lysosome-like vesicles termed MHC class II compartments (MIIC), or class II-containing vesicles (CIIV) (Amigorena et al 1994). Before antigenic peptide is able to associate with MHC class II, the Ii must be removed from the peptide-binding groove.

Sequential processing of Ii begins at its luminal C-terminal end. As it is this region that is important for Ii oligomerisation (Bijlmakers et al 1994, Bertolino et al 1995),

cleavage presumably results in the dissociation of nonomeric complexes to trimeric αβ-Ii structures. Early proteolysis, by as yet unidentified proteases, leads to the formation of an Ii fragment of ~24kDa which remains associated with αβ dimers. Subsequent processing of this 24kDa fragment forms a smaller Ii fragment of ~10kDa, comprising the N-terminal region through to the C-terminus of CLIP. Cathepsin S has been implicated in the processing of this smaller 10kDa fragment to CLIP. Inhibition of cathepsin S in human B cells blocks further processing, thereby preventing CLIP generation and delaying cell surface expression of MHC class II-peptide complexes (Riese et al 1996).

Following proteolysis with cathepsin S, CLIP is exchanged for antigenic peptide. This process normally requires the help of the non-classical class II molecule, HLA-DM (DM), which acts as a chaperone (Cho et al 1991, Kelly et al 1991). It has been shown that mutant human cell lines unable to express DM, still express class II on their surface, but mainly in conjunction with CLIP. Similar results have recently been demonstrated *in vivo*, using mice deleted of H2-M, the murine DM homologue (Fung-Leung et al 1996, Martin et al 1996, Miyazaki et al 1996).

The exact means by which DM influences peptide exchange is unknown, although several overlapping mechanisms have been suggested. Firstly, isolated DM can accelerate the CLIP off-rate from the class II complex (Denzin et al 1995, Sherman et al 1995, Sloan et al 1995) and kinetic data have recently been provided for this enzyme-like function (Vogt et al 1996). Secondly, DM may perform a chaperone-like function, by binding to (Sanderson et al 1996) and stabilising (Sadegh-Nasseri et al 1992, Denzin et al 1996) empty class II dimers, that would otherwise be rapidly degraded in the acid environment of the endosome. Finally, DM may actively select peptide epitopes for class II-binding, by increasing the dissociation rates of putative peptides that bind to the class II dimer with insufficient affinity (Kropshofer et al 1996, Weber et al 1996). Under ordinary circumstances however, some class II-CLIP/low affinity peptide complexes do reach the cell surface. The physiological significance of this expression is uncertain, although such complexes may reflect a

low concentration of high affinity peptides within the endosomal pool, or may possibly act as a class II reservoir for the recycling pathway (Pinet et al 1995).

DM interacts efficiently with class II-CLIP complexes, but only weakly with stable class II peptide complexes (Vogt et al 1996) and therefore dissociates upon successful class II loading. This permits transport of the MHC class II-peptide complex to the cell surface, where it is inserted, as a dimer of dimers, into the cell membrane.

In conclusion, several concurrent mechanisms are involved in class II-restricted processing and presentation. These are dynamic processes and their relative activity may influence both the nature and sequence of presented peptides.

1.3 THE T CELL RESPONSE:

1.3.1 *The T Cell Receptor*

T cells recognise peptide, presented by MHC molecules, through the T cell receptor (TCR) (Figure 1.4) which is a heterodimeric transmembrane structure, consisting of α and β (αβ), or γ and δ (γδ) polypeptide chains. The α and β chains each have two Ig-like extracellular domains, a transmembrane domain and a short, cytoplasmic, C-terminal domain. The membrane-distal regions of the α and β chains display hypervariability in loops 2, 3 and 6, resulting from gene rearrangement during T cell development, and these loops form the complementarity determining regions (CDR) 1, CDR2 and CDR3. Upon engagement of an MHC-peptide complex, the CDR1 and CDR2 loops of the TCR α chain bind to the α helix of the α1 domain of MHC class I or class II (Hong et al 1992). Similarly, the corresponding loops of the β chain engage either the α2 domain of MHC class I, or the β1 domain of MHC class II. This leaves the two CDR3 regions to interact with the MHC-bound peptide (Garboczi et al 1996, Garcia et al 1996). TCR-peptide-MHC complexes have been defined to a high resolution (Garboczi et al 1996, Garcia et al 1998), and it is apparent that during binding, the TCR is orientated diagonally over the MHC

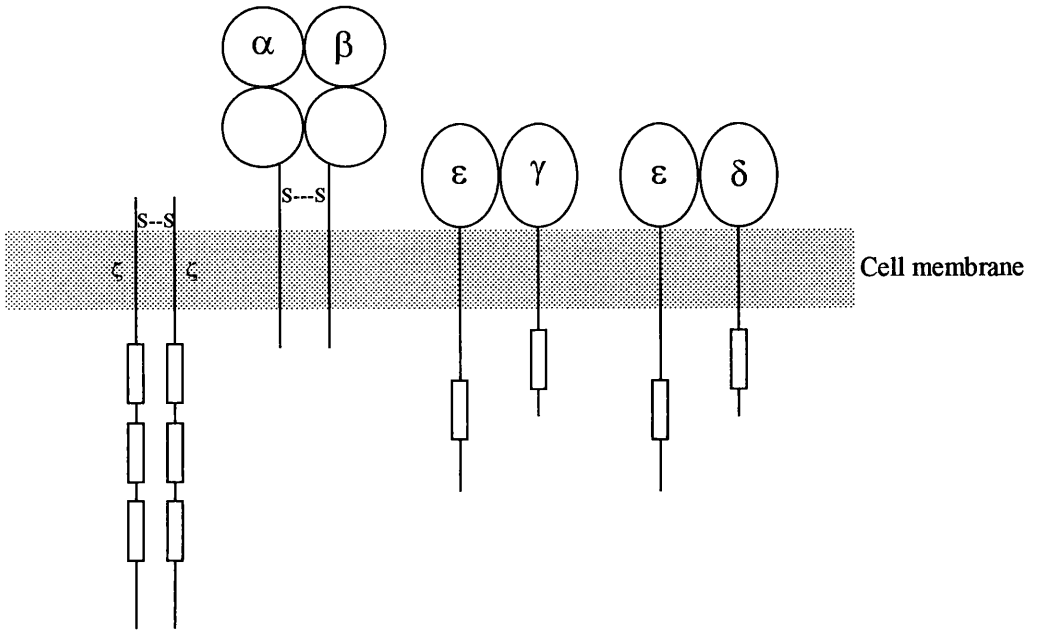

Figure 1.4: Schematic representation of the TCR complex. Rectangles denote ARAM motifs. Based upon Garcia and Teyton, 1998.

molecule (see Figure 1.3). The TCR α chain sits over the amino terminus of the peptide, and the β chain over the carboxy-terminus. It appears that there is a dominance of TCR-MHC contacts over TCR-peptide contacts, at a ratio of ~3:1. Although the γδ TCR complex is likely to have a structure analogous to that of the αβ complex, this has yet to be confirmed.

The αβ TCR itself does not possess intrinsic signalling functions, and instead relies upon the associated CD3, CD4/8 and CD45 cell surface complexes for signal transduction following TCR ligation. CD3 consists of two heterodimeric chains, γε and εδ, associated with a ζ chain, which exists either as a heterodimer (ζη or ζγ), or more commonly, a homodimer (ζζ) (Baniyash et al 1988). All the members of the CD3 complex, unlike the αβ TCR, have large cytoplasmic segments containing highly conserved 17 amino acid sequence repeats, known as antigen recognition motifs (ARAMs), or immunoreceptor tyrosine-based activation motifs (ITAMs) (Weiss 1993). Following TCR stimulation, ARAMs associate with cytosolic protein tyrosine kinases to enable intracellular signal transduction.

1.3.2 *Cell Adhesion Molecules*

Allograft rejection is often associated with a dense cellular infiltrate into the graft bed and it is apparent that lymphocytes and other cell types involved in the rejection response must migrate from the circulation into the site of inflammation and graft rejection. The process of lymphocyte migration is dependent upon an interaction between the migrating lymphocytes and the cells of the vascular endothelium. This interaction is mediated by the adhesion molecules, which are grouped into three families: selectins, integrins and those that are members of the Ig superfamily.

Selectins are transmembrane glycoproteins that are involved in the recognition of carbohydrates. Their extracellular region consists of three distinct domains; an N-terminal lectin (sugar-binding) domain, an epidermal growth factor-like domain and a variable number of short consensus repeats that are homologous to those found in complement regulatory proteins. One such molecule, which is readily induced on endothelium (Bevilacqua et al 1987) is E-selectin (ELAM-1), which is thought to be important in the initial binding of neutrophils to the vascular endothelium (Bevilacqua et al 1989).

Integrins are heterodimeric glycoproteins that are classified according to their β sub-unit into β_1, β_2 or β_3 integrins, with β_2 integrins often expressed by leukocytes. Lymphocyte function-associated antigen-1 (LFA-1) (Marlin et al 1987) and very late antigen-4 (VLA-4) (Elices et al 1990) are two β_2 integrins found on the surface of T cells. They mediate T cell-binding to the vascular endothelium via two Ig superfamily members, intercellular adhesion molecule-1 (ICAM-1) (Rothlein et al 1986) and vascular cell adhesion molecule-1 (VCAM-1) (Osborn et al 1989) respectively. Both ICAM-1 and VCAM-1, along with other Ig superfamily members, have Ig-like extracellular domains.

Upregulation of adhesion molecule expression during allograft rejection has been noted (Pober et al 1990, Gibbs et al 1993, Solez et al 1997), with several strategies using monoclonal antibodies directed against adhesion molecules demonstrating prolonged allograft survival. For example, in a fully mismatched mouse model of

cardiac transplantation, treatment of recipients with monoclonal antibodies against both ICAM-1 and LFA-1 simultaneously resulted in long-term graft survival (Isobe et al 1992). In a non-human primate model of renal transplantation, administration of an anti-ICAM-1 monoclonal antibody significantly prolonged graft survival (Cosimi et al 1990). Moreover, it was subsequently demonstrated in a phase I clinical trial, that administration of the same anti-ICAM-1 monoclonal antibody, BIRR1, to human renal allograft recipients resulted in improved graft function and delayed rejection (Haug et al 1993).

1.3.3 *Co-Stimulation*

Optimal T cell activation requires two distinct signals, the first of which is provided by ligation of the TCR and the associated CD4 or CD8 molecule with the peptide-MHC complex (Owens et al 1987, Boyce et al 1988, Jonsson et al 1989). CD4 and CD8 are transmembrane molecules that define the MHC class specificity of the T cell response to peptide. CD4 is a single glycoprotein chain with four extracellular Ig-like domains, while CD8 is a normally heterodimeric molecule with two extracellular Ig-like domains. Interaction of the TCR with an MHC-peptide complex results in the simultaneous association of either the CD4 molecule with the polymorphic β2 chain of MHC class II (Cammarota et al 1992, Konig et al 1992), or the CD8 molecule with the conserved α3 region of MHC class I (Salter et al 1990). In addition, the cytoplasmic domains of CD4 and CD8 associate with the T cell-specific intracellular protein tyrosine kinase, $p56^{lck}$, in a cysteine-dependent manner (Rudd et al 1988, Veillette et al 1988). $p56^{lck}$ activity is essential for the induction of the tyrosine kinase cascade following TCR engagement (Straus et al 1992). Interaction of the TCR and CD4 or CD8 with a peptide-MHC complex on an APC brings $p56^{lck}$ into close association with signalling motifs on CD3, thereby enhancing tyrosine phosphatase-dependent intracellular signalling events.

The two-signal hypothesis of T cell activation was originally proposed by Bretscher and Cohn (Bretscher et al 1970). It is now established that signal one alone is insufficient for generating a T cell response and may instead result in T cell anergy or unresponsiveness. The CD28/B7 pathway is generally considered the main co-

stimulatory or second signal. CD28 is a member of the Ig supergene family, and is expressed either as a monomeric or homodimeric glycoprotein on ~80% of all human T cells, and nearly all murine T cells (June et al 1990). It appears to be distributed evenly over the surface of the T cell, although following TCR ligation, its' density increases locally. In the absence of TCR ligation, CD28 ligation by itself has little effect on T cell activation. Experimentally, the use of anti-CD28 mAbs blocks T cell proliferation in response to antigen (Harding et al 1992), but studies using CD28 -/- mice have shown that in some cases CD28 is not needed for T cell activation (Shahinian et al 1993).

Two CD28 ligands, B7-1 (CD80) and B7-2 (CD86) have been characterised (Azuma et al 1993, Freeman et al 1993), which bind to CD28 with similar affinity, but differ in their cellular distribution. B7-1 is primarily found on dendritic cells (Linsley et al 1993), whilst B7-2 is expressed at moderate levels on resting T cells. Both molecules are found at low levels on resting APCs (Hathcock et al 1994).

A second member of the CD28 family, CTLA-4 (CD152), also binds B7 as its ligand, but with a much higher affinity than CD28. CTLA-4 and CD28 appear to have opposing effects on T cell activation, with CD28 promoting T cell growth and proliferation, and CTLA-4 acting as a negative regulator of T cell activation. Experimental blockade of CTLA-4 with anti-CTLA-4 mAbs may augment the progression of EAE (Karandikar et al 1996, Perrin et al 1996) and autoimmune diabetes (Luhder et al 1998), and also enhance anti-tumour responses (Leach et al 1996).

A synthetic fusion protein, CTLA-4Ig, binds B-7 (Linsley et al 1992) and inhibits co-stimulation, resulting in T cell anergy and enhancing allograft survival (Lenschow et al 1992, Turka et al 1992). Generally however, inhibition of the CD28/B7 pathway is insufficient to achieve long-term allograft survival. Other co-stimulatory pathways exist, for example the CD40-CD40L (e.g. (Grewal et al 1996) pathway, but similarly, inhibition of this pathway alone does not result in permanent graft survival (e.g. (Niimi et al 1998). These results therefore suggest that a certain degree of redundancy exists amongst the co-stimulatory pathways, and that blocking

one pathway will not necessarily inhibit T cell activation because the remaining pathways can successfully compensate. More success may be achieved by targeting more than one pathway simultaneously, and further study of the mechanisms of T cell stimulation may enable us to develop effective strategies for manipulating the T cell response.

1.3.4 *T Cell Signalling*

Following TCR ligation, the T cell is driven to proliferate and differentiate by the upregulation of IL-2 and its high affinity receptor (IL-2R). The pathways regulating IL-2 transcription are controlled by a network of kinases and phosphatases (e.g. (Weiss 1993), and are activated when the CD4 or CD8 molecule and the intracellular protein tyrosine phosphatase region of CD45, a heterogenous transmembrane molecule with a large, conserved, cytoplasmic tail, are brought into close association following ligation of the TCR with peptide-MHC complexes. This enables CD45 to activate $p56^{lck}$ and $p59^{fyn}$, both members of the src family of protein tyrosine kinases (PTK) (e.g. (Rudd et al 1994). $p56^{lck}$ associates with the CD4 and CD8 molecules (Shaw et al 1990), and $p59^{fyn}$ with the TCR ζ chain (Samelson et al 1990). CD45 activation of $p56^{lck}$ and $p59^{fyn}$ is achieved by dephosphorylation of their C-terminal negative regulatory tyrosine residues (Reviewed by (Neel 1997), and results in tyrosine phosphorylation of CD3ζ (Hall et al 1993, Chu et al 1994). In turn, this allows phosphorylation of the zeta-chain-associated protein (ZAP-70), a syk kinase, which binds to the ζ chain, by virtue of two src homology 2 (SH2) domains that interact with a doubly phosphorylated ζ chain ARAM (Iwashima et al 1994). Phosphorylation of ZAP70 initiates calcium-dependent, calcineurin-mediated phosphorylation of serine and threonine residues on signalling proteins, thereby regulating IL-2 gene transcription.

Activation of $p56^{lck}$ and $p59^{fyn}$ also results in the tyrosine phosphorylation of phospholipase C-γ (Park et al 1991, Secrist et al 1991, Weiss et al 1991), which catalyses hydrolysis of phosphatidyl inositol 4,5-biphosphate (PIP_2) into 1,2-sn-diacylglycerol (DAG), and 1,4,5 inositol triphosphate (IP_3) (Nishibe et al 1990, Kim et al 1991). DAG activates the protein serine/threonine kinase, protein kinase C

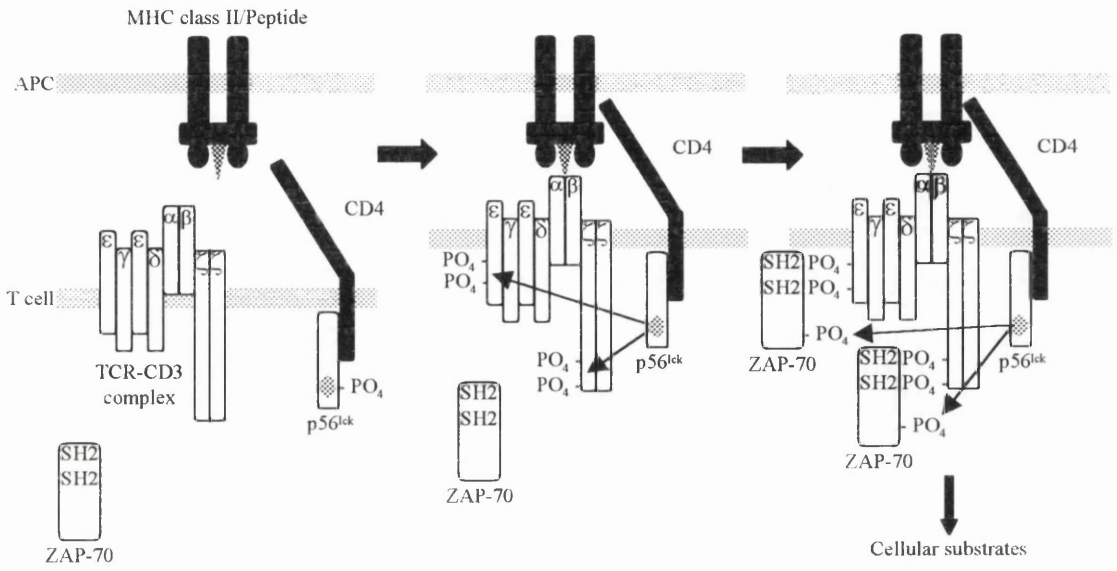

Figure 1.5: Schematic representation of the initiating events in T cell activation. Adapted from Chan et al, Annu. Rev. Immunol., 12: 555-592;1994.

(PKC) (Berridge et al 1984), and IP_3 mediates release of Ca^{2+} ions from intracellular stores (Berridge et al 1984, Imboden et al 1985), thus regulating T cell activation through cytokine gene transcription. One important consequence of elevated intracellular Ca^{2+} levels, is activation of the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase, calcineurin. Calcineurin targets the preformed subunit of NF-AT (nuclear factor of activated T cells), NF-AT1 (Loh et al 1996a, Loh et al 1996b), which, like NF-κB, is a transcription factor regulating cytokine genes. Association of NF-AT1 with calcineurin is thought to result in its dephosphorylation, allowing its translocation into the nucleus (McCaffrey et al 1993, Loh et al 1996a), where it binds to the AP-1 site in the IL-2 gene and upregulates IL-2 transcription. The immunosuppressive drugs, CsA and FK506 (Tacrolimus), when complexed to their corresponding immunophilins, cyclophilin and FKBP12, are able to bind and sequester calcineurin, thereby suppressing IL-2 gene transcription (Griffith et al 1995, Park et al 1995).

1.4 T CELL DEVELOPMENT:

The development of the mature peripheral T cell pool occurs in the thymus, with T cells arising from precursor haematopoietic stem cells (HSC) that originate from the liver in the developing foetus and the bone marrow in adults. Precursor cells enter the thymus bearing low levels of CD4 (Wu et al 1991), and from this state develop into $CD4^-CD8^-$ double negative lymphocytes. Cells then begin to express CD8 and eventually develop into $CD4^+CD8^+$ double positive (DP) cells. Maturation from the DP state involves the positive selection of T cells that possess the ability to interact with self-MHC expressing foreign antigen. It is at this stage that cells also undergo lineage commitment to either the CD4 or CD8 subclass, at which time they are classed as single positive (SP). Finally, cells that display a high affinity for self-MHC plus self-antigen are negatively selected, and undergo programmed cell death (apoptosis).

1.4.1 *Positive T Cell Selection*

As mentioned above, developing thymocytes are positively selected for their ability to interact with self-MHC bearing foreign antigen. The phenomenon of self-MHC restriction has been established for many years, and was first described as the driving force behind T cell development in the late 1970's (Fink et al 1978, Zinkernagel et al 1979). However, positive selection was not unequivocally demonstrated until the advent of transgenic mice. Kisielow and colleagues (Kisielow et al 1988a) used mice with a transgenic TCR specific for the $H\text{-}2D^b$ MHC class I allele. Mice expressing $H\text{-}2D^b$, but not other MHC alleles, developed fully mature SP CD8 cells that expressed the transgenic TCR, therefore suggesting that cells were positively selected for their ability to interact with the $H\text{-}2D^b$ allele. Similar observations were also noted by Sha et al (Sha et al 1988). Further evidence demonstrating the need for a direct TCR-MHC interaction during positive selection has been provided by experiments using transgenic mice expressing a TCR specific for $I\text{-}E^k$ and a pigeon cytochrome c peptide (Berg et al 1989). In comparison to

control H-2^b mice, transgenic H-2^k mice displayed a greater number of mature T cells, both overall and specific for the pigeon cytochrome c peptide. It was noted that in H-2^b animals T cell development was arrested at the DP stage.

It has been demonstrated that positive selection only occurs when the restricting MHC element is expressed on thymic epithelial cells (Kisielow et al 1988a, Kisielow et al 1988b). Moreover, it appears that cortical, rather than medullary, epithelial cells drive positive selection (e.g. (Hugo et al 1992). Under certain circumstances, haematopoietic cells may also be involved in positive T cell selection (Bix et al 1992), although selection invoked in this manner is relatively inefficient when compared to that mediated by thymic cells.

1.4.2 *T Cell Lineage Commitment*

The cellular and molecular mechanisms governing T cell lineage commitment in DP thymocytes remain unclear, although there does appear to be a distinct correlation between TCR MHC preference and the outcome of lineage selection. Interaction of the TCR with MHC class I results predominantly in CD8 T cell maturation (Teh et al 1988), and interaction with MHC class II results in CD4 T cell maturation (Kaye et al 1989). Moreover, it has been demonstrated that mice deficient in MHC class I have very few circulating CD8 T cells, and that class II mutant mice have very few peripheral CD4 T lymphocytes (Reviewed by (Robey et al 1994). It therefore appears that positive selection of self-MHC restricted thymocytes coincides with T cell lineage commitment.

Two hypothetical models have been forwarded to explain T cell commitment to a specific lineage. The first of these, the "instructive" model, proposes that TCR recognition of an MHC molecule is intrinsic to its lineage commitment. CD4 and CD8 molecules are the most likely candidates for MHC "class-sensing". The engagement of one or other co-receptor is thought to result in the generation of intracellular signals that downregulate the transcription of its counter-receptor. In this model, positive selection and lineage commitment are driven by the same recognition event, and occur simultaneously.

The second of the two models is referred to as the "stochastic" (or "selection") model. It suggests that contrary to the instructive model, there is no direct sensing of MHC by the TCR. It is proposed that following the binding of the TCR and the MHC molecule, cell lineage is randomly selected independently of the TCR-MHC interaction. Lineage selection triggers thymocytes to differentiate into intermediate cells ($CD4^{lo}CD8^{+}TCR^{int/hi}$ or $CD4^{+}CD8^{lo}TCR^{int/hi}$), with further differentiation only possible if they possess the appropriate co-receptor for MHC interaction. Thus, in this model, the CD4 and CD8 co-receptors play a critical role in cell survival, rather than in the choice of MHC molecule with which the TCR interacts. Evidence for the stochastic model has been provided by several groups (Chan et al 1993, Davis et al 1993). Using β_2M mutant mice (which have defective MHC class I expression) van Meerwijk and Germain (van Meerwijk et al 1993) found that despite the presence of $CD4^{int}CD8^{+}TCR^{int}$ cells in the thymus, animals did not develop mature SP CD8 T cells. This study suggests that although recognition of class I molecules is not required for lineage commitment, it is intrinsic for the development of fully mature SP CD8 T cells. Thus, in the stochastic model, lineage commitment must occur before MHC recognition and positive selection. Indeed, it appears that a large intermediate T cell population develops following lineage commitment, and that these cells are subject to death if they do not express the appropriate combination of TCR and co-receptor for MHC recognition.

More recently, a third, "asymmetric" commitment model has been proposed (Suzuki et al 1995, Suzuki et al 1997), in which non-identical rules govern CD4 and CD8 T cell commitment. The authors suggest that CD8 commitment resembles the instructive model of lineage commitment, with TCR and CD8-specific signals being required for thymocyte differentiation. In contrast, CD4 commitment is thought to occur by default, resulting from the absence of CD8-specific signals.

1.4.3 *Negative T Cell Selection*

As mentioned, negative selection is the process by which developing T cells that display too high an affinity for self-MHC plus self-antigen complexes are deleted from the emerging T cell pool. The fact that tolerance to self-antigens occurs during

the development of the immune system, and persists throughout an individual's lifetime, has been established for many years (Owen 1945, Billingham et al 1953). It was by the use of allogeneic thymic chimeras that Singer et al (Singer et al 1982) initially demonstrated that the thymus was the organ responsible for the induction of self T cell tolerance.

The mechanism responsible for self-tolerance induction in the thymus was not formally proven until the development of a mAb against the Vβ17a-expressing TCR (Kappler et al 1987). The Vβ17a TCR element is highly reactive with the mouse MHC class II molecule, I-E, and its use allowed the fate of developing T cells to be studied. It was observed that despite the immature T cell population containing $V\beta17a^+$ cells, $I\text{-}E^+$ mice did not posses mature Vβ17a-expressing T cells, thus indicating that deletion, rather than suppression, is the end-point of negative selection.

In contrast to positive T cell selection, which is driven by cortical epithelial cells, negative selection is predominantly mediated by haematopoietic cells derived from the bone marrow (Lo et al 1986, Marrack et al 1988b). It has however been demonstrated that medullary epithelial cells may also invoke negative selection (e.g. (Salaun et al 1990, Hoffmann et al 1992). Under these circumstances though, anergy rather than deletion may account for tolerance (Houssaint et al 1990). Negative selection mediated by thymic cells appears less efficient than that mediated by cells derived from the bone marrow (e.g. (Robey et al 1994).

1.4.4 *The Role of Peptides in T Cell Selection*

As self-MHC molecules are expressed on both thymic epithelial cells and haematopoietic cells, the regulation of positive versus negative selection has been open to debate. Different theories have been proposed to explain differential selection, including the suggestion that selection is dependent upon differing affinities of the TCR for the same ligand (e.g. (Sebzda et al 1994). However, it is now apparent that different thymic APCs present different populations of self-peptides. Cortical and medullary thymic epithelial cells (cTEC and mTEC) have

been shown to present peptides of different origin, with cTEC unable to present peptides from exogenous sources (Lorenz et al 1989, Kasai et al 1996, Oukka et al 1996). It has recently been shown that this is due to cTEC containing different cathepsins to mTEC and haematopoietic cells (Oukka et al 1997). Moreover, it has been shown that deletion of cathepsin L from cTEC results in the loss of positive selection (Nakagawa et al 1998). Thus, the above studies suggest that positive and negative selection are mediated by different sets of self-peptides.

1.4.5 *Programmed T Cell Death*

During development in the thymus, approximately 95% of thymocytes undergo programmed cell death (apoptosis) (Rothenberg 1992, Hueber et al 1994). The need for tightly regulated cell death is essential in the generation of a functional lymphoid system. It acts not only to remove potentially autoreactive T cells, but also those cells that express non-functional TCRs.

Bcl-2 is a mitochondrial membrane protein, involved in rescuing cells from death at several points in lymphocyte development (Reviewed by (Chao et al 1998), and is able to inhibit apoptosis in some cell lines. It is found expressed in mature SP T cells found in the medulla, but in only a few DP cells of the cortex (Hockenbery et al 1991). Thy-1, an 18kDa protein found on peripheral and thymic lymphocytes in mice however, appears to have an opposing effect upon cell survival, and is able to trigger apoptosis through a bcl-2-resistant mechanism (Hueber et al 1994). Likewise, the 35kDa Fas (CD95) antigen (Hanabuchi et al 1994), is also able to mediate apoptosis of self-reactive thymocytes (Watanabe-Fukunaga et al 1992) following ligation with its receptor, Fas ligand (FasL, CD95L).

1.5 B CELL DEVELOPMENT:

B lymphocytes derive from the same progenitor HSC as T lymphocytes, natural killer cells and dendritic cells (Galy et al 1995). Their generation is a lifelong process in both mice and humans (e.g. (Nunez et al 1996) occurring in the foetal liver and the adult bone marrow. The initial phase of B cell development, whereby

antigen specificity develops, occurs independently of antigen, which is in comparison to the highly antigen-dependent process of T cell development (see section 1.4).

The primary function of B cells is the generation of a humoral antibody response following antigenic stimulus. The mechanism by which B cells recognise antigen is fundamentally different to that of T cell recognition, as they respond to antigen in its native form, whereas T cells respond to cell-bound antigen in the form of processed peptide fragments, presented by MHC molecules.

Antibody or immunoglobulin (Ig) molecules are expressed either as cell surface molecules that comprise part of the B cell receptor (BCR) complex, or as soluble molecules that are secreted. Both forms of Ig are composed of two identical heavy (H) chains and two identical light (L) chains (see Figure 1.6) that are linked by disulphide bonds. There are five H chain isotypes: μ, δ, γ, α and ε, the use of which specifies the class of Ig produced by a B cell (IgM, IgD, IgG, IgA or IgE respectively) and two L chain isotypes, κ and λ. Each B cell makes antibodies of unique antigen specificity and this specificity is retained throughout the lifetime of the cell, even following H chain isotype class switching.

An individual's B cell repertoire is exceptionally diverse, even more so than the T cell repertoire, and there are two key mechanisms by which this diversity may be generated. Firstly, the variable region of an Ig chain, which is responsible for antigen specificity, is, at the genetic level, composed of three gene segments, known as the variable (V), diversity (D) and joining (J) gene segments, with each containing a very large number of genes. One gene from each of the three H chain gene segments is used to generate the variable region of an Ig H chain, whereas one gene from each of the V and J L chain gene segments are used to generate an Ig L chain. The process of gene rearrangement is under the control of two recombinant activator genes, RAG-1 and RAG-2 (Oettinger et al 1990). The ability to combine genes from each of these segments therefore results in a greater number of functional antigen binding sites than the total number of genes in the V, D and J

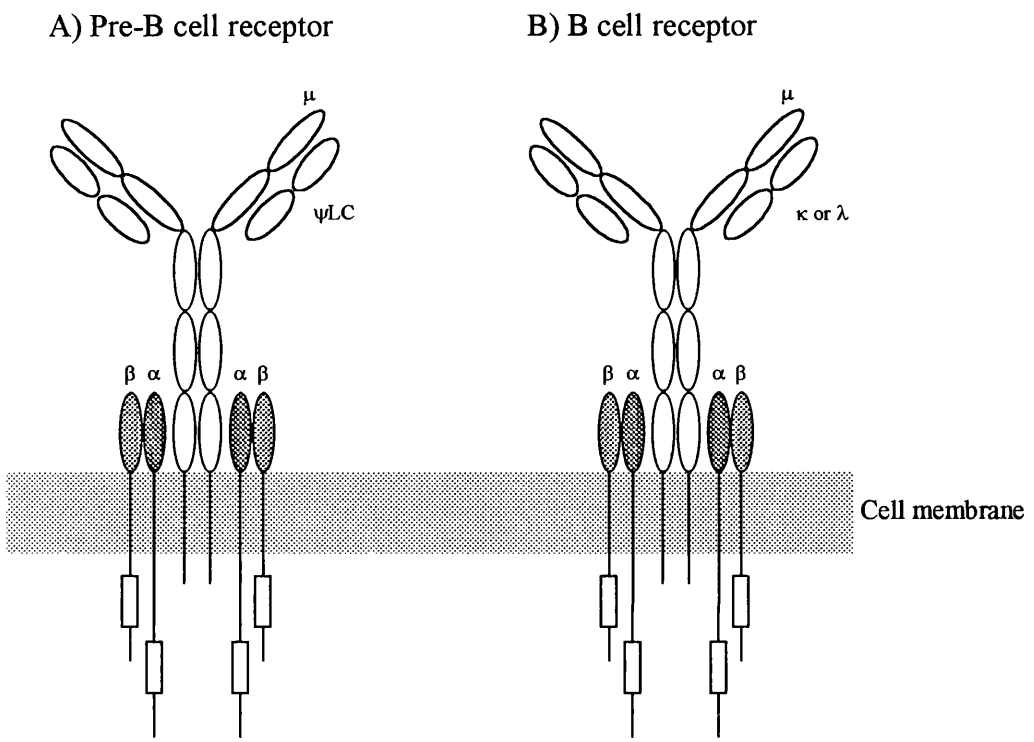

Figure 1.6: A) The pre-B cell receptor and B) The B cell receptor. Rectangles denote ARAM motifs in the cytoplasmic tails of Igα and Igβ. Adapted from Rajewsky, Nature, 381: 751-758; 1996.

pools. Gene rearrangement is also an imprecise process, with additional nucleotides potentially being incorporated or deleted at the site of gene cleavage. This

"junctional diversity" therefore further increases the potential B cell repertoire, and indeed, it is estimated that when all the possible combinations arising from gene-rearrangement are considered in combination with junctional diversity, the pre-immune Ig repertoire in humans is approximately 10^{11} (e.g. (Duchosal 1997).

During B cell development, one of the earliest markers that B cells express enabling them to be distinguished from precursor HSC and other lymphoid cells is the 95kDa Ig superfamily member, CD19, which is found on all B cells, except for antibody-secreting plasma cells, and is involved in signal transduction following ligation of the BCR (e.g. (Krop et al 1996). When they begin to express CD19, B cells are referred to as pro-B cells. Towards the end of this stage, cells start to express CD40, which is a transmembrane glycoprotein of approximately 45kDa with homology to

the nerve growth factor receptor (Stamenkovic et al 1989). The ligand for CD40, gp39, is found on activated T cells. Cross-linkage of CD40 is responsible for B cell activation, proliferation and differentiation into IgM-secreting plasma cells (Banchereau et al 1991).

Pro-B cells mature into pre-B cells, which express low levels of a pre-BCR comprising functional μ H chains in association with "surrogate" L chains (ψLC) (Brouns et al 1996) (see Figure 1.6a). The requirement for such an association is not entirely clear, but may be necessary for further B cell maturation (Brouns et al 1996). The pre-BCR complex also contains a heterodimeric Igα(CD79α)/Igβ(CD79β) complex (Reth 1992), similar in structure to the CD3 complex found in association with the TCR and likewise involved in BCR signal transduction (Sanchez et al 1993). Pre-B cells undergo further differentiation to immature B cells, at which point a functional BCR, with μ H chains associated with κ or λ L chains, is expressed on the cell surface (see Figure 1.6b). Transition to a mature B cell phenotype is accompanied by the co-expression of surface IgD.

Mature naïve B cells leave the bone marrow and migrate to the outer T cell zones in the white pulp of the spleen (Lortan et al 1987). Ligand-mediated negative selection in this area prevents cells that express autoreactive Ig molecules from entering the B cell follicles (e.g. (Cyster et al 1995). Negative selection may take the form of apoptosis (Benhamou et al 1990, Murakami et al 1992). Alternatively, cells may be induced to alter the specificity of their antigen receptor by further Ig rearrangement, termed receptor editing, and thus escape deletion, providing that the new receptor itself is not autoreactive (e.g. (Tiegs et al 1993). Cells that do not express autoreactive antibodies are able to enter the B cell follicles, and from here re-circulate through the lymphatic system and the bloodstream.

Upon antigenic stimulation, re-circulating B cells are activated in the T cell area of lymph nodes. Following ligation of the BCR by antigen, a signal is conveyed into the cytoplasm by virtue of ARAM motifs found within the intracellular domain of the Igα/Igβ complex (Reth et al 1991). B cells can respond to antigen in one of two different ways, either in a T cell-independent manner, or in a T cell-dependent

manner. If antigen is present in a multimeric form, particularly on the surface of a cell, it may be able to effectively cross-link the BCR by itself, thereby directly stimulating B cells and resulting in their proliferation and differentiation into antibody-secreting plasma cells (Mond et al 1995). i.e. cells respond to antigen in a T cell-independent fashion. If, however, an antigen is unable by itself to stimulate an antibody response, it may be internalised and processed into peptide fragments by the B cell and presented on the cell surface in the context of MHC class II molecules for recognition by CD4 T_h cells. B cells are then able to elicit "help" from these T cells, enabling their proliferation and differentiation. Such a cognate interaction is therefore T cell-dependent (Clark et al 1994).

Antigenic stimulation often results in further rearrangement of H chain DNA, with the previously rearranged $V_HD_HJ_H$ gene segments switching between constant H chain (C_H) genes. This H chain isotype class switching alters the class of antibody produced by a B cell. However, the B cell does retain its antigen specificity, which is important, as different classes of antibody stimulate different effector mechanisms, thereby rendering the same antigen susceptible to different forms of attack.

1.6 TRANSPLANTATION IMMUNOLOGY:

The ability of the immune system to distinguish self from non-self is critical for protecting the body against invading pathogens, but works against the therapeutic aim of transplantation where graft rejection is the inevitable outcome. The immune response to an allograft is initiated by T cell recognition of allogeneic-MHC molecules. Indeed, it was such recognition that led to the discovery of the MHC antigens, which were initially described as "transplantation antigens" (Gorer 1936, Gorer 1937). The immune response to a mismatched allograft is characterised by its uniquely powerful primary response *in vitro*, as demonstrated by the mixed leukocyte reaction (MLR). An explanation for the strength of this response comes from the unusually high precursor frequency of CTLs displaying specificity for

alloantigen; this is at least 100-fold higher than the number of T cells specific for nominal protein antigen in the periphery (Lindahl et al 1977, Liu et al 1993).

Two hypotheses have been forwarded to account for the strength of the primary alloresponse. Matzinger and Bevan (Matzinger et al 1977) initially proposed that a single MHC antigen may represent multiple conformational T cell epitopes, due to the large number of exogenous proteins with which it can associate. This "multiple binary complex" theory acquired further credence upon elucidation of the finer details of peptide processing and presentation. However, even discounting the variance provided by bound peptides, an allogeneic MHC molecule is present upon the surface of a donor APC at a much higher frequency than would normally occur in the presentation of a nominal antigen in the context of self-MHC class II upon processing by self-APC. Therefore, such "high determinant density" (Bevan 1984) may by itself explain the greater precursor frequency of alloreactive T cells.

The two above theories of allorecognition differ fundamentally with regards to their requirement for bound peptide and consequently, numerous studies have been performed to establish its relative importance. One indication that peptide may be important in allorecognition stems from the amino acid residues found within the groove, or cleft of MHC molecules (Lechler et al 1990, Reinsmoen et al 1990). This area is inaccessible to the TCR, but MHC alleles differing only in this region have been shown to stimulate separate T cell clone populations. This indicates that different peptides bound into the groove are likely to play a key role in T cell stimulation.

Furthermore, species and cell type-specific allorecognition have been reported. Heath et al (Heath et al 1989), described experiments whereby CTLs specific for K^b expressed on the surface of the EL4 murine cell line, were unable to lyse human cells transfected with the K^b gene, unless cells were additionally pulsed with a source of murine protein. Marrack and Kappler (Marrack et al 1988a) found that such peptide specificity might even be limited to different types of antigen presenting cell. It was reported that murine Vβ17a-expressing T cell receptors displaying specificity for I-E were only able to detect the ligand when expressed on

B cells, and not when expressed on either I-E^+ macrophages or I-E transfected fibroblasts.

Nonetheless, lysis of cells bearing MHC independent of bound-peptide has been documented (Aosai et al 1991), but the physiological significance of such an interaction remains unclear.

Lechler and colleagues (Barber et al 1991, Lombardi et al 1991) have attempted to combine the multiple binary complex and high determinant density hypotheses, by suggesting that allo-MHC molecules may be viewed as having two functional regions. The first region, the outward-facing surfaces of the α-helices, forms the TCR-contact site, and the second area, the MHC cleft or groove, forms the antigen-binding region. They proposed that T cell alloresponses are dependent upon the nature of the MHC disparity between the donor and recipient. If the TCR-binding region between the two individuals display sufficient similarity, then allorecognition will be akin to self-restricted T cell recognition of nominal antigen in the periphery, with T cells responding to novel MHC-bound peptides. However, if the TCR-contact regions show great dissimilarity, bound peptide may become unimportant, and the allo-MHC molecule itself will act as the T cell ligand. In this case, allorecognition may take the form of "molecular mimicry", with the T cell recognising allo-MHC at the level of its three-dimensional structure.

Taken together, the above studies suggest that whilst peptide-independent T cell recognition of MHC molecules may occur, the physiological significance of this is uncertain, and the majority of data suggest that MHC-bound peptide is instrumental in initiating the alloimmune response.

1.7 ALLORECOGNITION:

1.7.1 *Direct and Indirect Allorecognition in Graft Rejection*

It is widely appreciated that two pathways of allorecognition may contribute to graft rejection. The first of these, direct recognition (Figure 1.7a), is unique to

transplantation, and involves T cell recognition of intact donor MHC molecules upon the surface of donor cells, with CD8 T cells apparently recognising MHC class I, and CD4 T cells recognising MHC class II.

Evidence for this pathway of allorecognition was first provided in 1982 by Lechler and Batchelor (Lechler et al 1982). They found that depleting donor passenger leukocytes from (AS x AUG)F_1 kidney allografts, by temporarily "parking" them in immunosuppressed AS hosts, resulted in prolonged allograft survival upon re-transplantation into secondary AS hosts. Injecting small numbers of donor-strain dendritic cells at the time of re-transplant was seen to restore graft immunogenicity. It was therefore postulated that effective allograft rejection required the direct stimulation of recipient T cells by the intact MHC molecules expressed on donor dendritic cells.

It was observed however, that passenger cell-depleted grafts were still rejected by their secondary hosts, albeit in a delayed fashion. This finding led to the proposal of a second route by which alloantigen may be recognised. It was suggested that via this alternative route, alloantigen may be dealt with in a manner analogous to that of nominal protein antigen in the periphery. Thus, antigen would be internalised by the recipient's own APCs, and presented upon the cell surface in the context of self MHC class II as peptide to recipient CD4 T cells, thereby complying with the dogma of MHC restriction (Zinkernagel et al 1979). This second route of allorecognition is now termed the indirect pathway (Figure 1.7b).

1.7.2 *Indirect Allorecognition Plays a Major Role in Graft Rejection*

The first *in vivo* demonstration of indirect recognition was presented in 1986 (Sherwood et al 1986). Adoptive transfer of T cell-depleted splenocytes or peritoneal cells from alloimmunised mice to naive syngeneic recipients was seen to provoke the accelerated rejection of subsequent donor-strain skin grafts. Although the presence of some donor class II MHC was noted in the transferred cell population, the authors argued that this was not in sufficient quantity to stimulate a direct alloresponse. They therefore concluded that the observed rejection kinetics

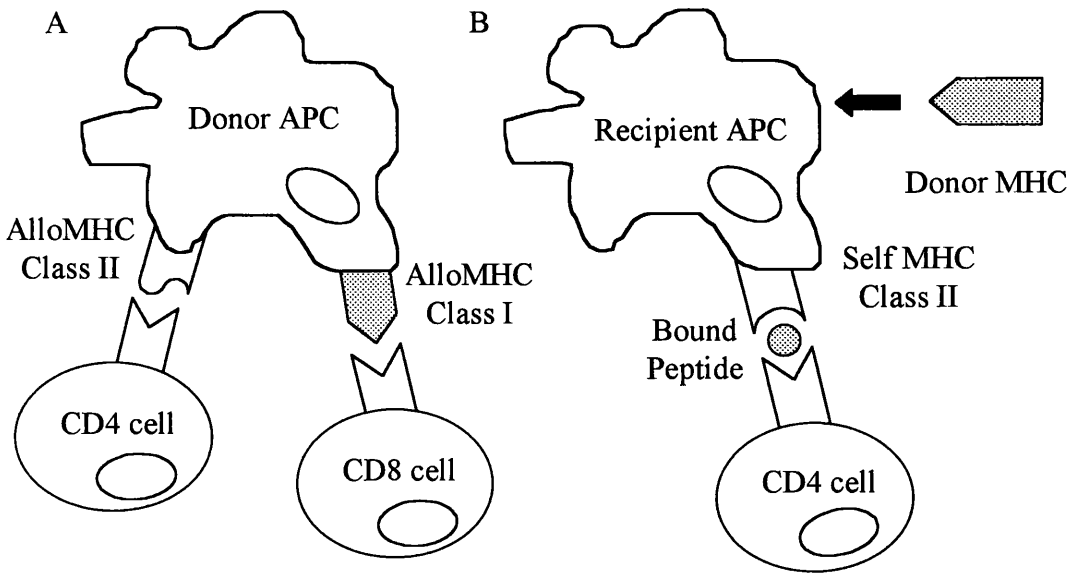

Figure 1.7: Schematic Representations of A) Direct Allorecognition, and B) Indirect Allorecognition.

were due to the indirect activation of naive recipient CD4 T cells by the transferred APCs.

Confirmation that indirect recognition occurs following transplantation has been provided by two different experimental approaches. The first of these examines the incidence of indirect T cell priming *in vivo* following immunisation with allogeneic material. Using this approach, Benichou et al (Benichou et al 1992) demonstrated that T cells from Balb/c and SJL mice immunised with allogeneic (A^k) splenocytes or a skin graft, were able to proliferate *in vitro* to synthetic A^k allopeptides, thereby indicating processing of donor MHC and presentation of the resulting allopeptides by recipient MHC class II. Likewise, it was observed that CD4 T cells obtained from Lewis ($RT1^l$) rats doubly immunised with DA ($RT1^a$) skin and kidney grafts, proliferated *in vitro* to a synthetic MHC class I A^a-derived peptide (Fangmann et al 1992b). Additionally, in a fully vascularised model of graft rejection (Watschinger et al 1994), the transplantation of WF ($RT1^u$) cardiac allografts into Lewis recipients was also shown to stimulate T cell alloreactivity, this time towards donor MHC class II peptides. Proliferation could be blocked by the addition of an mAb against the recipient MHC class II, and was therefore attributed to CD4 T cells.

The above studies, whilst demonstrating that indirect allorecognition may be stimulated by transplantation, do not necessarily confirm a role for the indirect pathway in effecting allograft rejection. Therefore, the second approach adopted to examine the incidence of indirect recognition in graft rejection has been to prime recipients to donor alloantigen via the indirect pathway prior to challenge with an allograft.

Two key studies to address this issue have examined A^a skin graft survival in Lewis strain rats primed with either A^a allopeptide (Fangmann et al 1992a), or isolated, denatured RT1.A^a and RT1.B^a protein chains (Dalchau et al 1992). Such forms of antigen, which lack the conformational structure of the intact MHC molecule, negate the possibility of directly priming recipient T cells. Both studies observed accelerated rejection of subsequent DA ($RT1^a$) skin grafts, associated with a concomitant acceleration in alloantibody response. The work of Fangmann et al (Fangmann et al 1992a), observed that pre-treatment with two allopeptides derived from the hypervariable α-helical regions of the RT1.A^a class I molecule, resulted in accelerated rejection of subsequent DA skin grafts. Interestingly, a third peptide arising from the β-pleat of the $\alpha 1$ domain (which comprises part of the floor of the peptide-binding cleft), did not appear immunogenic. This finding was in contrast to a later study performed by Shirwan et al (Shirwan et al 1995), who demonstrated the immunogenicity of a shorter variant of this peptide in the PVG.1U (RT1.A^u) rat strain. This discrepancy is likely to be due to strain-dependant differences in antigen processing (Benham et al 1994).

Studies from our own laboratory (Pettigrew et al 1998) have recently demonstrated that indirect T cell activation may also be important in priming for accelerated organ allograft rejection. A DNA-based strategy was adopted to prime $RT1^u$ animals with a soluble version of the RT1.A^a molecule. Despite incorporating appropriate conformational epitopes, the absence of cell surface presentation, and therefore co-stimulation, prevented direct T cell activation. Nevertheless, such pre-treatment markedly accelerated the rejection of subsequent R8 (RT1.A^a) cardiac allografts.

Furthermore, this response could be abrogated by treatment with an anti-CD4 monoclonal antibody.

1.7.3 *Indirect Allorecognition in Human Transplantation*

Relatively little data has been published with reference to indirect recognition in human transplantation. Two studies of note however (Muluk et al 1991, Liu et al 1996b) have shown the presence of IL-2 responsive self-restricted T cells specific for mismatched HLA antigens in the circulation of human renal (Muluk et al 1991), and cardiac (Liu et al 1996b) transplant patients. In the latter study, the presence of T cells specific for DRβ1*0101 peptide in the circulation of DRβ1*1101 patients bearing a DRβ1*0101 cardiac allograft, was observed both before and during acute and chronic rejection episodes, but no such cells were observed during quiescence.

Taken together, all the above data undoubtedly confirm the incidence of indirect allorecognition in graft rejection, although its relative role in effecting rejection will be discussed later.

1.7.4 *Immunodominance and Epitope Spreading*

Elution studies have demonstrated that from a theoretically large pool of potential peptide epitopes, a relatively small number are generated and presented. A concept of immunodominance has been established concerning both immunity to complex antigens, and autoimmune responses (Sercarz et al 1993), in which the immune response appears to be initially restricted to a single, or few, dominant epitope(s) within the immunogenic protein. There is evidence that the intial indirect alloresponse is similarly directed at a limited number of immunodominant epitopes (Dalchau et al 1992, Fangmann et al 1992a, Fangmann et al 1992b). Benichou et al (Benichou et al 1994a) explored this concept more fully. In the mouse he demonstrated that, following either the injection of donor strain cells or a donor strain skin graft, the alloimmune response in three donor-recipient combinations was limited to a single dominant epitope. If all alloresponses were restricted to isolated epitopes, the possibility of targeting such epitopes to modify the immune response to an allograft would appear to be eminently feasible. However, in clinical practice the

indirect response is unlikely to be so defined, and is likely to depend on both the discrepancy between the recipient and donor MHC, and upon the recipient's antigen processing mechanisms (Parker et al 1992, Gallon et al 1995). This is supported by a recent study examining the fine specificity of the T cell response to a 24-mer peptide by three different rat strains. Using a nested set of 15-mer peptides, it was observed that the T cell response was localised to a different area of the parent peptide for each of the three rat strains (Benham et al 1994).

Although the initial immune response to an allograft appears to be of relatively limited repertoire, it is evident that the response to nominal protein antigen is a dynamic process (Mamula 1998). As the response progresses, T cell reactivity spreads from the initial immunodominant determinant to additional sub-dominant and cryptic epitopes (Sercarz et al 1993). This so-called epitope spreading is a fundamental mechanism of the immune system that has evolved for the elimination of invading pathogens; clearly, targeting multiple epitopes on a foreign antigen will increase the chances of effective clearance. Whilst important in the fight against infection and malignancy, this phenomenon has proved highly detrimental in the progression of autoimmune disease (Lehmann et al 1992).

Liu et al (Liu et al 1996a) have demonstrated that diversification of the immune response may also take place following transplantation. In patients bearing a cardiac allograft differing at two HLA-DR alleles, alloreactivity during early rejection episodes was restricted to a single determinant on one of the DR alleles. However, following multiple rejection episodes, intermolecular spreading of the immune response to encompass the second allele was observed.

Further studies from the same group have explored the relationship between T cell alloreactivity, epitope spreading and chronic allograft rejection (Ciubotariu et al 1998, Suciu-Foca et al 1999). Patients who displayed late, persistent alloreactivity were at a greater risk of developing coronary artery vasculopathy (CAV) (a hallmark of chronic cardiac rejection), than those subjects who showed no reactivity after the first 6 months. In addition, a greater proportion of patients who developed CAV showed inter- and intramolecular spreading of the immune response than those

patients who did not develop CAV. It therefore appears that a correlation between the diversification of T cell alloreactivity, to include cryptic epitopes and chronic rejection exists.

In summary, although the indirect pathway contributes to allorecognition, the exact mechanics of antigen processing remain unclear. In particular, there appears to be a balance between focusing upon a single dominant epitope and spreading to multiple epitopes. The regulation of the balance requires further clarification as this has important implications upon the design of therapeutic strategies that target the indirect pathway. The data available to date suggests that the design of such strategies may not be as straightforward as once thought (Sayegh et al 1992, Shirwan et al 1997b).

1.8 THE ROLE OF INDIRECTLY ACTIVATED T CELLS IN EFFECTING ALLOGRAFT REJECTION:

Several mechanisms are traditionally thought to play a role in allograft rejection. Firstly, a delayed type hypersensitivity (DTH) response could occur upon recipient T cell infiltration into the graft, with recognition of donor antigenic determinants resulting in T cell activation. The subsequent release of chemokines may damage the graft directly, or lead to the recruitment of non-specific effector cells, such as macrophages, to the graft bed. Secondly, as first demonstrated by the MLR, alloreactive T cells may directly damage the graft, by developing cytotoxic properties, which enables them to lyse donor cells. Cytotoxic T cells are generally of the CD8 T cell subset and therefore recognise allogeneic MHC class I. Finally, the development of alloantibody and its subsequent binding to allogeneic MHC on the surface of donor cells can also result in graft damage through the activation of complement. Although it is well recognised that pre-formed antibody plays a critical role in hyperacute rejection (Kissmeyer-Nielsen et al 1966), as a mechanism for acute and chronic rejection, alloantibody has been largely neglected. It has, however been demonstrated that alloantibody may play a significant role in the rejection of

human renal allografts (Halloran et al 1992). In addition, alloantibody has been implicated in the rejection of skin (Fangmann et al 1992a, Steele et al 1996), kidney (Benham et al 1995) and cardiac (Gracie et al 1990) allografts in several rodent models. It is likely however, that specific rejection responses are dependent upon several factors, including the nature of MHC disparity between donor and recipient, the type of graft transplanted, and the state of sensitisation of the host.

Although the relative importance of the indirect pathway of allorecognition in initiating graft rejection remains unclear, it is of note that indirectly primed T cells can theoretically contribute to each of the above mentioned pathways. The stimulation of recipient CD4 T cells by processed alloantigen in the context of self-MHC class II may result in nonspecific DTH-like responses, as discussed by Parker et al (Parker et al 1992). Indirectly primed T cells may also provide help to CD8 T cells for the development of an antigen-specific CTL response (Mitchison et al 1987, Lee et al 1994). Finally, cognate (antigen-specific) help may be provided to B cells for the generation of an antibody response (Noelle et al 1990) (Figure 1.8b), and it is with this particular aspect of the indirect alloresponse that this thesis is principally concerned.

T cell help for an antibody response directed at a nominal antigen generally requires a cognate interaction between the TCR and the class II-peptide complex of the B cell (Noelle et al 1990). In other words, only T cells specific for the peptide fragment presented by the B cell upon processing of the complete antigen are able to provide the necessary co-stimulatory signals required for an antibody response. Regarding the alloantibody response to MHC mismatched allografts, such cognate help could only be provided by indirectly activated T cells. Directly activated T cells would not be able to provide help in this manner, as their target MHC class II molecule will differ to the class II molecule expressed by the recipient B cell.

Evidence supporting the theory that indirect T cell priming is intrinsic in the development of the alloantibody response was provided by Bradley and colleagues, using a vascularised class I disparate allograft model. Using a series of T cell-depletion and passive transfer studies, it was demonstrated that the ability of

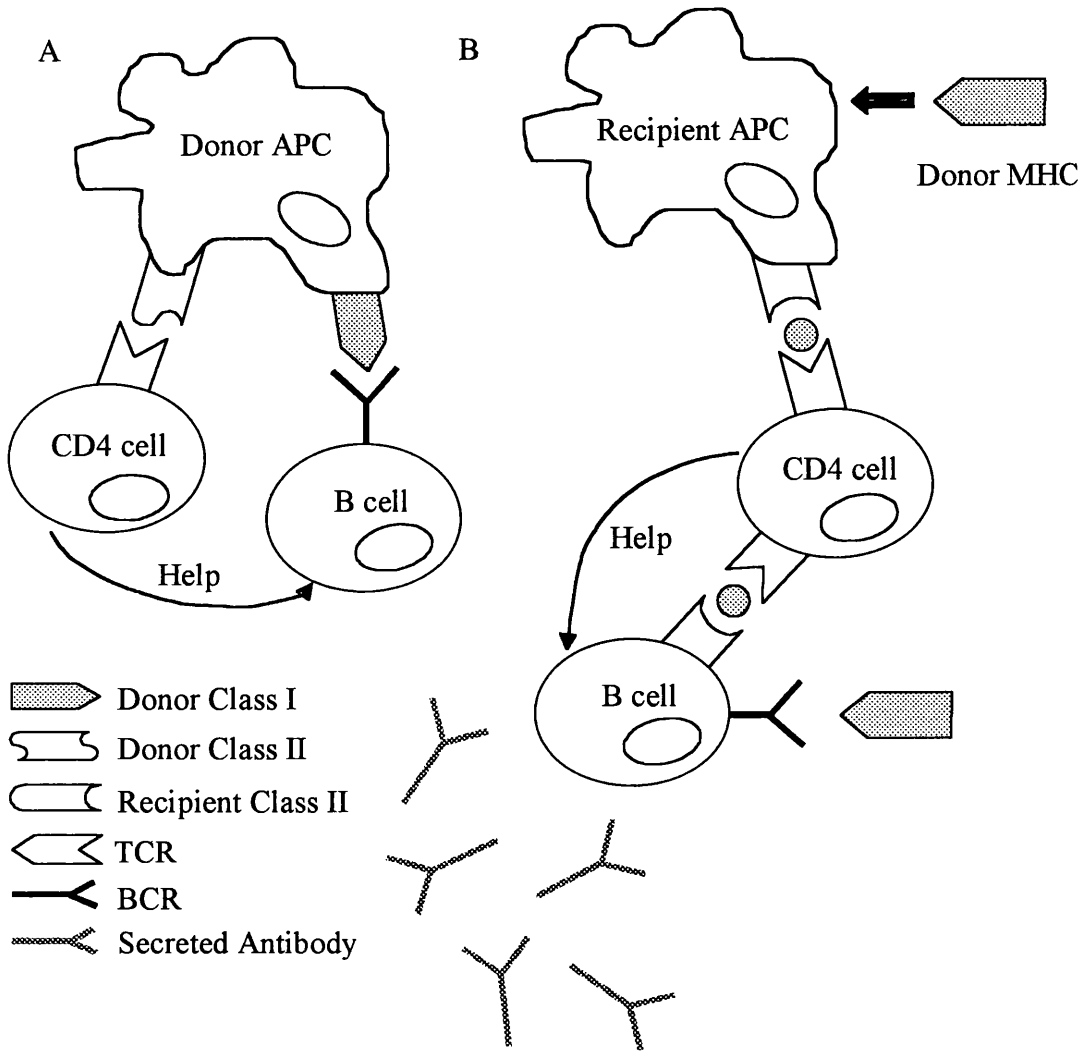

Figure 1.8: Mechanisms of T-B Collaboration for Alloantibody Production. A) Non-cognate T Cell Help through the Direct Pathway of Allorecognition, B) Cognate T Cell Help via the Indirect Pathway of Allorecognition.

PVG.RT1^{u} (RT1.A^{u}) to reject PVG-R8 (RT1.A^{a}) allografts was CD4, not CD8 T cell-dependent (Gracie et al 1990, Morton et al 1993). Thus, it is likely that CD4 cells recognised the disparate class I molecule, not as an intact antigenic determinant on the surface of graft cells, but as processed peptide fragments presented by recipient APCs. Furthermore, rejection in this strain combination appears to be mediated by alloantibody, as passive transfer of immune serum to naive RT1^{u}

recipients resulted in accelerated rejection of subsequent donor-strain, but not third-party, skin and cardiac allografts (Morton et al 1993). More recent studies using this model (MacDonald et al 1997) have suggested that indirect T cell activation, by providing cognate B cell help, is central to the development of an alloantibody response. By specifically targeting synthetic A^a-derived allopeptides to small resting B cells presumed devoid of co-stimulatory signals, a marked reduction in the anti-A^a alloantibody response to a subsequent R8 cardiac allograft was achieved.

However, it has also been proposed that directly activated T cells may provide help to B cells for alloantibody production (Kelly et al 1996) (See Figure 1.8a), particularly during the early stages of graft rejection. This hypothesis was based on the observation that donor interstitial dendritic cells expressing intact donor MHC class II were integral to the development of the alloantibody response. Moreover, the early alloantibody response to an accompanying allogeneic MHC class I molecule was also dependent on the donor class II molecule, as its development was impeded by the presence of pre-formed anti-class II antibodies. It was therefore suggested that recipient CD4 T cells recognising donor class II alloantigen directly could provide help for the alloantibody response against other antigenic determinants on the graft. However, it is not possible for such help to involve a standard T-B cell cognate interaction, as the T and B cell determinants are obviously different. Analogous to the three cell cluster model proposed for the generation of a cytotoxic response (Mitchison et al 1987), Kelly and colleagues suggested that linkage of T and B cells in this model could occur via the donor APC, through its presentation of both determinants simultaneously (see Figure 1.8a).

Recent studies by Steele and co-workers (Steele et al 1996) may help to reconcile the discrepancy between our results and those of Fabre and colleagues. Employing transgenic class II-deficient mice as either donors or recipients of skin grafts, it was demonstrated that class II-deficient grafts were rejected with normal kinetics, and that the IgM and IgG responses to the residual graft antigens were similar to that of control animals receiving normal grafts. For similar reasons to those described for the class I disparate PVG-R8 to PVG-$RT1^u$ model, this experiment confirms that the

restriction of T cell help to the indirect pathway is sufficient for a normal alloantibody response. Class II-deficient recipients (but with a normal CD4 repertoire) however, produced only moderate levels of alloantibody, which was exclusively of the IgM subclass. In this situation, indirect T cell recognition cannot occur. Thus, it appears that directly activated T cells are capable of initiating the primary IgM response, but are incapable of providing the help required to induce heavy chain switching to the IgG subclasses, which is a characteristic of the secondary antibody response.

Agarwal et al have also suggested that the various stages of the antibody response require varying levels of T cell help (Agarwal et al 1997). As the alloantibody response switches to IgG, they noted that the specificity of T cell help focuses upon a limited number of epitopes. Such restriction appears to be a result of competition amongst the putative T cell epitopes that B cells present, for the limited available T cell help. There is, of course, no reason why a limited allo-IgM response may not be equally as damaging to the graft as a more differentiated IgG response. Certainly, antibody of an IgM isotype generally exhibits powerful cytotoxic properties.

In conclusion, the indirect alloresponse can undoubtedly contribute to alloantibody production. However, it still remains unclear whether the initial rapid antibody response can be accounted for by indirect recognition alone, or whether a unique form of non-cognate help, as provided by directly activated T cells is largely responsible.

1.9 TOLERANCE INDUCTION:

Tolerance generally exists to self-antigens, and the realisation by Billingham et al that tolerance could be extended to alloantigens by the injection of foetal mice with donor strain cells (Billingham et al 1953), suggests that antigen-specific tolerance in the absence of long-term immunosuppression is possible. There are two possible mechanisms by which tolerance may occur, either through intrathymic deletion of developing T cells, or through peripheral tolerogenic mechanisms. Recent animal

studies have suggested that peripheral tolerance can occur by several different means, namely T cell ignorance, T cell anergy and T cell suppression (Reviewed by (Charlton et al 1994).

T cell ignorance, as its name suggests, implies that the antigen in question escapes immune surveillance either through sequestration, or through presentation by non-professional APC, such that recipient T cells that recognise the antigen are neither activated nor anergised, and remain capable of responding to the antigen if it is later presented by a professional APC (Heath et al 1992). T cell anergy however occurs when the TCR complex receives a sub-optimal activation signal, usually due to the absence of a suitable secondary co-stimulatory signal (Lafferty et al 1977, Schwartz 1992). Suppressor T cell mechanisms are more difficult to define, largely because a distinct effector T cell population has not been identified, but broadly imply that potentially auto- or alloreactive T cells are suppressed from activation by a further T cell population. Suppressor T cells are also thought to be involved in the prolonged survival of both rat cardiac allografts following CsA treatment (Hall et al 1990), and mouse skin grafts following monoclonal antibody therapy (Qin et al 1993). In several animal models of transplantation tolerance, more than one mechanism appears to be responsible for allograft non-responsiveness (Oluwole et al 1995, Zhang et al 1996, Chen et al 1998). This will also likely prove to be the case in the more complicated setting of graft acceptance in humans.

As this thesis explores the use of donor-derived allopeptides to tolerise the indirect pathway of allorecognition, it is simpler to consider tolerance using a functional, rather than a mechanistic, approach and to examine specifically those protocols that result in donor specific tolerance (DST). DST is achieved by using tolerogenic protocols that incorporate donor antigen, initially in the form of intact donor cells, but more recently as allopeptide fragments.

Pre-treatment of potential organ recipients with a blood transfusion, a once common clinical practice, provides an early example of antigen-specific tolerance. However, because grouping of the pre-administered blood was chosen randomly, only indirect evidence exists to suggest that the tolerance achieved was an antigen-specific effect

and due to the subsequent donor organ sharing one or more HLA antigen with the pre-immunising blood (Lagaaij et al 1989, van Twuyver et al 1991).

The beneficial effect of blood transfusions on allograft survival can be reproduced with preparations of purified erythrocytes, which express only minimal levels of MHC class II (Wood et al 1985). In addition, pre-treatment of mice with syngeneic fibroblasts transfected with donor MHC molecules was shown to prolong subsequent cardiac allograft survival, sometimes indefinitely, in a fully mismatched mouse strain combination (Madsen et al 1988).

In the latter experiment, DST was achieved without incorporating all of the mismatched donor MHC antigens into the tolerogenic protocol, although the mechanism by which such "linked epitope suppression" occurs has yet to be established. It appears however, that this phenomenon is restricted to only those additional antigenic epitopes that are physically linked with the donor antigen (i.e. on the same allograft) to which tolerance has already been established, since the effect cannot be reproduced if the additional antigens are instead expressed on a second, concurrently administered graft (Davies et al 1996, Wong et al 1997). Presumably therefore, both the tolerising, or "suppressor", epitope and the additional antigenic donor epitope are presented on the surface of a single APC (either recipient or donor), resulting in down-regulation of the T cell response to the additional determinant. It is likely that the interaction of recipient T cells with the suppressor epitope either downregulates the co-stimulatory activity of the APC or results in the release of locally-acting, inhibitory cytokines (Lombardi et al 1994).

Linked epitope suppression is also apparent in models of intrathymic (IT) injection of donor antigen and intravenous administration of donor bone marrow cells (BMC). For example, it has been demonstrated that intravenous administration of BMC from transgenic CBK ($H\text{-}2^k + K^b$) mice into CBA.Ca ($H2^k$) recipients not only results in the survival of subsequent CBK cardiac allografts, but also of all heart grafts expressing the K^b antigen, irrespective of the presence of additional mismatched MHC antigens (Wong et al 1997).

Similarly, it has been shown that IT injection of purified soluble donor class I antigen is able to prolong cardiac allograft survival indefinitely in the completely mismatched Wistar Furth (WF, $RT1^u$) to Lewis ($RT1^l$) rat strain combination, but only in the presence of transient immunosuppression (Oluwole et al 1993). The same protocol, instead using Lewis rats as pancreatic islet donors, and low responder WF rats as recipients, resulted in permanent graft survival, but this time without immunosuppression (Oluwole et al 1994). A slightly different approach also demonstrating linked epitope suppression was used by Knechtle et al, who administered donor $RT1.A^a$ antigen IT either as a 'naked' DNA injection (Knechtle et al 1997), or expressed on the surface of genetically modified, syngeneic Lewis hepatocytes (Knechtle et al 1994). Both models resulted in long term survival of ACI ($RT1^a$) livers in mismatched Lewis recipients, although transient immunosuppression was again required.

Attempting to interpret the mechanisms that are responsible for IT tolerance in the above experiments is complicated by the use of soluble class I alloMHC as the thymic inoculum in certain experiments (Oluwole et al 1993, Oluwole et al 1994), since soluble MHC may itself exhibit intrinsic tolerogenic properties (Calne et al 1967). For example, Sumimoto et al observed prolonged DA ($RT1.A^a$) cardiac allograft survival in PVG ($RT1^c$) recipients following the continuous infusion of purified donor class I antigen (Sumimoto et al 1990). However, neither Spencer and Fabre (Spencer et al 1987) nor Priestley et al (Priestley et al 1989) were able to reproduce this effect in the DA to PVG, or DA to Lewis rat strain combinations. An explanation for this discrepancy has however been offered by Foster et al. They demonstrated that, although the administration of soluble, donor $RT1.A^a$ antigen as purified membrane-bound molecules resulted in the indefinite survival of DA kidney allografts in Lewis recipients, its' administration as a water-soluble preparation required simultaneous immunosuppression for prolonged graft survival (Foster et al 1992). This suggests that different physical conformations of soluble class I molecules have different tolerogenic effects. For example, Spencer and Priestley both used water-soluble MHC class I preparations (Spencer et al 1987, Priestley et al 1989), and in this state soluble class I molecules cannot form complexes with each

other. In comparison, it is likely that in Sumimoto's study, the administered serum contained soluble donor class I protein that was able to form multivalent complexes (Sumimoto et al 1990). That aggregation of soluble donor class I antigen into multivalent complexes is required to down-regulate the CD8 T cell alloresponse has been verified in several subsequent *in vitro* studies (Dal Porto et al 1993, Abastado et al 1995, Zavazava et al 1996). The mechanism through which multivalent soluble class I complexes are able to downregulate T cell activation appears to be through the process of apotosis (Zavazava et al 1996), presumably resulting from the absence of an additional second signal from the co-stimulatory molecules that are normally co-presented on encounter with cell-bound antigen (Schwartz 1990).

It is possible that the KCl-extracted soluble antigens used by Oluwole et al in their IT injection experiments as discussed above (Oluwole et al 1993, Oluwole et al 1994) retained their hydrophobic transmembrane and cytoplasmic tails, and thus, their ability to aggregate into multivalent complexes, which may explain their tolerogenic effect. A similar mechanism has been proposed to be responsible for the beneficial effects of pre-transplant blood transfusion (Buelow et al 1995). However, it has subsequently been demonstrated by Oluwole and colleagues that recipient APC are intrinsically involved in their model of IT injection (Chowdhury et al 1995, Oluwole et al 1995), therefore suggesting that indirect T cell allorecognition of donor antigen may be involved in the induction of tolerance. Since fragmented donor antigen (for example in the form of peptides) will be recognised exclusively through the indirect pathway, examining whether the same tolerogenic effect can be achieved following the administration of allopeptide fragments, rather than intact donor antigen, could test this hypothesis.

Several groups have now explored this possibility, and have confirmed that the indirect recognition of donor antigen is involved in some tolerisation protocols. For example, in the completely MHC mismatched rat strain combination, WF ($RT1^u$) to Lewis ($RT1^l$), indefinite renal allograft survival was achieved by the IT injection of a mixture of four 25-mer donor-strain MHC class II allopeptides (Sayegh et al 1993, Sayegh et al 1994). Oral administration of the same peptides also downregulated the

response to allogeneic cells as judged by *in vivo* DTH responses, however, the effectiveness of this route of peptide administration on graft survival was not examined (Sayegh et al 1992, Hancock et al 1994). Using a similar protocol, but with a single class I peptide derived from the α helix of the α1 domain of the donor RT1.A^a molecule, Shirwan et al were able to achieve permanent cardiac allograft survival in the PVG.R8 (RT1.A^a) to PVG.1U (RT1.A^u) rat strain combination (Shirwan et al 1997b). Notably, the dose of allopeptide required could be reduced if a second donor peptide, derived from the α2 domain, was co-administered (Shirwan et al 1997a).

These latter experiments illustrate two important concepts. Firstly, they provide the strongest evidence to date that the indirect pathway of allorecognition may be at least as important as the direct pathway in mediating acute allograft rejection, and secondly, they suggest that linked epitope suppression can exert a powerful effect in preventing additional graft epitopes from stimulating the alloimmune response. For example, Chowdhury et al were able to achieve permanent WF ($RT1^u$) cardiac allograft survival in completely mismatched ACI ($RT1^a$) rats by IT injection of a single 17 amino acid immunodominant peptide derived from the α2 domain of the A^u class I MHC antigen (Chowdhury et al 1998). Wang et al have provided an even more profound example of this effect (Wang et al 1997). A genetically engineered chimeric MHC class I molecule was constructed using the ACI RT1.A^a antigen as a backbone, but which was altered at the last four, of nine, disparate residues in the α helical region of the α1 domain to resemble the donor WF RT1.A^u antigen. The other five residues in the α1 domain that differed between the two rat strains were left unaltered. Despite these residual differences, as well as those within the α2 and α3 domains, a single injection of the chimeric protein into the portal vein of ACI recipients resulted in the indefinite survival of WF cardiac allografts without the need for additional immunosuppression. Notably, this result could not be reproduced with a second chimeric protein, which had been changed to incorporate all nine amino-acid differences in the hypervariable region of the α1 domain between the two strains.

The ability to minimise the amount of donor antigen that is required to achieve tolerance would be undoubtedly beneficial in the clinical setting, as a single protocol could be used to tolerise to several mismatched donor organs that share the specific tolerising antigen. This principal may also be applied to the prevention of the development of chronic rejection, as this is associated with alloreactivity spreading to additional donor epitopes (e.g. (Ciubotariu et al 1998, Suciu-Foca et al 1998), and moreover, is currently the major cause of graft rejection.

1.10 AIMS:

The primary aim of this thesis was to study in greater depth the nature of the indirect T cell response to alloantigen. As discussed, it has been suggested that the indirect response to alloantigen is, at least initially, directed towards a single dominant epitope (Benichou et al 1994a). However, it is likely that as the alloresponse progresses, or upon manipulation of the host's immune system, T cell reactivity may diversify to include further sub-dominant epitopes (Benichou et al 1998, Mamula 1998). This may have important implications in the design of peptide-based tolerogenic strategies, and experiments were therefore designed to identify all the dominant and sub-dominant T cell epitopes that may potentially contribute to allograft rejection in the MHC class I disparate rat strain combination, PVG-R8 ($RT1.A^aB/C^uD^u$) to PVG-$RT1^u$ ($RT1.A^uB/C^uD^u$). Although these two strains differ only at the classical MHC class I locus A, it has been established that CD4 T cells mediate graft rejection (Gracie et al 1990, Morton et al 1993), and therefore proposed that their activation occurs through indirect recognition of $RT1.A^a$ peptide fragments. Since graft rejection in this strain combination is mediated by alloantibody (Gracie et al 1990, Morton et al 1993), the principal mode by which CD4 T cells effect graft rejection is likely to be through the provision of cognate help for B cells which present similar peptide fragments upon processing and presentation of the intact donor A^a antigen.

I chose to map the immunogenic epitopes of the A^a molecule by using a series of A^a-derived allopeptides. Previous work has suggested that A^a-derived allopeptides do not contain the appropriate 3-dimensional conformational epitopes necessary for direct T cell recognition and activation (MacDonald et al 1997, Pettigrew et al 1998). Therefore, the influence of peptide priming on the response to the intact A^a antigen is a result of exclusive priming of the indirect pathway of allorecognition. Previous studies adopting a similar peptide-based approach in this strain combination have focused upon the immunogenic and tolerogenic properties of peptides derived from only the hypervariable regions of the A^a molecule (Shirwan et al 1995, MacDonald et al 1997, Mhoyan et al 1997, Shirwan et al 1997b). However, since other regions of the molecule may contain additional $RT1^u$ T cell determinants, I decided to extend the above work by using a series of 18 overlapping 15-mer allopeptides spanning the length of the $\alpha 1$ and $\alpha 2$ domains of the A^a molecule. The 15 amino acid length of these peptides was chosen somewhat empirically, based on the published data for the optimal length of peptides binding to MHC class II molecules (Rudensky et al 1991, Chicz et al 1992). This approach can however be validated by comparing the results obtained with the 15-mer peptides, to those obtained with a longer 24 amino acid peptide derived from the hypervariable region of the $\alpha 1$ domain. This peptide in effect acts as a positive control as it has been shown not only to evoke an accelerated anti-A^a immune response in $RT1^u$ animals, but has also been used successfully to downregulate their immune response to the intact A^a molecule when administered in a tolerogenic fashion (MacDonald et al 1997).

Three different methods were chosen to examine whether individual allopeptides had the potential to be involved in the generation of the anti-A^a immune response. Firstly, I wished to examine the immunogenicity of the peptides in the $RT1^u$ rat strain, by immunising animals with individual allopeptides in CFA, then observing both the *in vitro* T cell proliferative responses and the anti-peptide antibody responses that they evoked. I predicted that within the A^a molecule there would be several immunogenic epitopes, but that the primary indirect response to the donor A^a antigen would focus upon a limited number of dominant epitopes from this set. To

determine the location of these dominant epitope(s) in the A^a molecule, I decided to prime $RT1^u$ animals with the intact A^a molecule, in the form of an R8 allograft, and examine the *in vitro* proliferative recall responses stimulated by individual allopeptides. Finally, I sought to examine the *in vivo* T cell response to those A^a-derived allopeptides that are specifically involved in the indirect response against the intact A^a antigen by monitoring their ability to influence the antibody response to, and the rejection kinetics of, a subsequent A^a-bearing allograft. This may provide a more accurate assessment of those T cell epitopes that can potentially contribute to the CD4 T cell response to the A^a molecule than by using T cell proliferation studies alone, since standard proliferation assays are only a relatively crude reflection of the *in vivo* T cell response (Nevala et al 1997).

This latter approach may be particularly relevant for those A^a peptides that share the same amino acid sequence as the corresponding region of the A^u molecule, although logically, one would not expect such consensual sequences to stimulate an indirect T cell response. However, Fedoseyeva et al (Fedoseyeva et al 1996) have recently demonstrated that whilst priming Balb/c ($H\text{-}2^d$:$K^dA^dE^dD^dL^d$) mice with the self-MHC peptide $H\text{-}2D^d$ 61-80 did not stimulate a proliferative response, a response could be invoked upon challenge with a B10.A ($H2^a$:$K^kA^kE^kL^dD^d$) allograft bearing the D^d antigen. This study confirms that during thymic selection of the T cell repertoire, not all self-reactive T cells are deleted, but that some persist in a state of anergy. Furthermore, this highlights that one cannot necessarily predict those epitopes that will be involved in the indirect response to an alloantigen.

The aim of these experiments therefore was to provide a functional *in vivo* map of the dominant and sub-dominant epitopes of the $RT1.A^a$ MHC class I molecule that could be used to favourably modulate the alloimmune response to the A^a molecule. It was hoped that a tolerogenic protocol incorporating both dominant and sub-dominant epitopes, as revealed by this more accurate mapping, would result in a more effective downregulation of the alloimmune response than has to date been accomplished by other peptide-based strategies (MacDonald et al 1997). In order to

achieve this, I chose a protocol of intravenous injection of relatively high doses of allopeptide.

CHAPTER 2:

MATERIALS AND METHODS

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CHAPTER 2:

MATERIALS AND METHODS

2.1 ANIMALS

Congenic PVG-RT1^u (A^uB/D^uC^u) and recombinant PVG-R8 ($A^{av1}B/D^uC^u$) rats were purchased from Harlan UK Ltd. (Bicester, Oxon, UK). The derivation of PVG-RT1^u and PVG-R8 rat strains (subsequently referred to as RT1^u and R8) is referenced elsewhere (Gracie et al 1990). All animals were housed and maintained under standard conditions, initially at the University of Glasgow Central Research Facility, and subsequently at the University of Cambridge Central Biomedical Services at Laundry Farm. Animals were used when 8-12 weeks old.

2.2 TISSUE CULTURE MEDIA

2.2.1 *Washing Medium*

Hanks Buffered Salt Solution (HBSS) (Gibco BRL Life Technologies, Paisley, UK) supplemented with 2% heat-inactivated (HI) Foetal Calf Serum (FCS) (Sigma Biosciences, Poole, Dorset, UK), 100U/ml Penicillin, 100μg/ml Streptomycin and 10mM Hepes solution (all Gibco BRL), was used in all preparative procedures for cells, unless otherwise stated.

2.2.2 *Culture Medium*

RPMI 1640 (Gibco BRL) supplemented with 2mM L-glutamine (Gibco BRL), 100U/ml Penicillin, 100μg/ml Streptomycin, and 5μM 2-Mercaptoethanol (Fischer Scientific, Loughborough, Leicestershire, UK) was used as culture medium for cells, supplemented with HI normal syngeneic rat serum or FCS as stated.

2.3 CELL PREPARATIONS

2.3.1 *Lymph Node Cells*

Cervical, mesenteric and popliteal lymph nodes were removed from sacrificed rats under sterile conditions. Pooled lymph nodes were finely chopped with a scalpel blade, and gently pushed through a fine stainless steel mesh, using a sterile 5ml syringe plunger, to yield a single-cell suspension in 10mls of washing medium.

The cell suspension was transferred into a conical based test-tube (Sterilin, Stone, Staffs, UK), and left to stand on ice for a few minutes to allow the debris to settle. Cells were transferred to a clean test-tube, centrifuged at 1200 rpm for 7 minutes, re-suspended, and washed a further 3 times. Cells were counted using a haemocytometer, and viability was determined by trypan blue exclusion.

2.3.2 *Splenocytes*

Spleens were removed under sterile conditions, and gently teased apart in washing medium using plastic forceps. The resulting cell suspension was transferred to a 10ml conical based test-tube, and left on ice for a few minutes, to allow cell debris to settle out. Cells were transferred to a fresh test-tube and centrifuged at 1200 rpm for 7 minutes. The cell pellet was re-suspended and erythrocytes removed by hypotonic lysis, by the addition of 5mls distilled water followed immediately by the addition of 5mls 1.8% NaCl. Splenocytes were washed twice more, and counted by trypan blue exclusion.

2.3.3 *Concanavalin A-Transformed Lymphoblasts*

^{51}Chromium (^{51}Cr)-labelled Concanavalin A (ConA)-stimulated lymphoblasts were used as target cells in antibody-mediated cytotoxicity assays.
Splenocytes from donor-strain rats were prepared as described (section 2.3.2), but were not depleted of erythrocytes. Cells were adjusted to a concentration of 2-2.5 x 10^6 cells/ml in 50mls of culture medium enriched with 10% HI FCS, and incubated

with 5μg/ml of ConA (Sigma) in a $75cm^3$ tissue culture flask (Nunclon, Denmark) for 48 hours at 37^0C in 5% CO_2.

2.4 SYNTHETIC ALLOPEPTIDES

A series of 18 synthetic 15-mer peptides, overlapping by 5 amino acids, and spanning the α1 and α2 domains of the rat classical class I MHC molecule $RT1.A^{av1}$ (residue 25 (glycine) to residue 209 (phenylalanine) inclusive), were purchased from Immune Systems Ltd. (Paignton, UK). All peptides were synthesised by Fmoc and HBTU chemistry, purified by HPLC, and assessed by Mass Spectrometry for purity. All peptide preparations were shown to be greater than 80% pure.

In addition, one peptide of 24 amino acids (designated α1), and a control peptide (designated irrelevant) were used. The α1 peptide corresponds to the hypervariable region of the α1 domain (amino acid residues 81-104). The irrelevant peptide contains the same amino acids as peptide number 7, but in a random order.

All 15-mer peptide amino acid sequences are shown in Table 2.1.

2.5 INJECTIONS

2.5.1 *Subcutaneous Injection with Peptide*

$RT1^u$ rats were injected subcutaneously with a 19G needle (Microblane, Becton Dickinson, Oxford, UK) into each hind footpad with 50μg of peptide dissolved in 50μl of distilled water, and emulsified with a comparable volume of Freund's Complete Adjuvant (CFA) (Sigma). Control animals received injections of distilled water emulsified with CFA alone. Animals were anaesthetised by halothane (Zeneca Ltd. Macclesfield, Cheshire, UK) inhalation.

Table 2.1: The amino acid sequences of synthetic peptides.

PEPTIDE	SEQUENCE	POSITION
P1	G S H S L R Y F Y T A V S R P	25-39
P2	A V S R P G L G E P R F I A V	35-49
P3	R F I A V G Y V D D T E F V R	45-59
P4	T E F V R F D S D A E N P R M	55-69
P5	E N P R M E P R A R W M E R E	65-79
P6	W M E R E G P E Y W E Q Q T R	75-89
P7	E Q Q T R I A K E W E Q I Y R	85-99
P8	E Q I Y R V D L R T L R G Y Y	95-109
P9	L R G Y Y N Q S E G G S H T I	105-119
P10	G S H T I Q E M Y G C D V G S	115-129
P11	C D V G S D G S L L R G Y R Q	125-139
P12	R G Y R Q D A Y D G R D Y I A	135-149
P13	R D Y I A L N E D L K T W T A	145-159
P14	K T W T A A D F A A Q I T R N	155-169
P15	Q I T R N K W E R A R Y A E R	165-179
P16	R Y A E R L R A Y L E G T C V	175-189
P17	E G T C V E W L S R Y L E L G	185-199
P18	Y L E L G K E T L L R S D P P	195-209
Irrelevant	Y A Q W E I Q K E R E R Q T I	N/A

The α1 peptide spans amino acid residues 81-104, and its sequence is as follows:

P E Y W E Q Q T R I A K E W E Q I T R V D L R T

N.B. Residues 1-24 represent the signal sequence, which is cleaved during final transit of the nascent MHC molecule through the endoplasmic reticulum.

2.5.2 *Intravenous Injection with Peptide*

Male RT1^{u} rats were injected intravenously into the penile dorsal vein using a 23G needle, whilst anaesthetised. Animals received one dose of 300μg of peptide dissolved in 300μl of normal saline (Fresenius Ltd., Basingstoke, UK). Control animals received injections of saline.

2.5.3 *Intravenous Injection with Blood*

Male RT1^{u} rats were injected intravenously with 1.5mls of heparinised blood, into the penile dorsal vein using a 23G needle, whilst anaesthetised.

2.6 SKIN TRANSPLANTATION

Anaethestised recipient $RT1^u$ animals were grafted on the flank with $1cm^2$ full thickness R8 abdominal skin, secured by four 4/0 sutures (Ethicon Ltd. Edinburgh, UK) applied at the corners of the graft. Wounds were dressed with a saline-soaked swab held in place with sleek tape. Dressings were removed after 4 days, and the grafts assessed daily. Rejection was defined as 50% necrosis of the graft.

2.7 CARDIAC TRANSPLANTATION

2.7.1 *Donor Heart Retrieval*

The donor rat was anaesthetised by continuous halothane inhalation. A midline incision was used to expose the inferior vena cava (IVC), into which 400 units of heparin (CP Pharmaceuticals Ltd. Wrexham, UK) was injected using an insulin syringe (Becton Dickinson). The aorta was transected in order to exsanguinate the animal. A thoracotomy was immediately performed, and cardioplegia achieved by packing the thoracic cavity with ice. The operating field was visualised using an operating microscope (Wild Heerbrug, Switzerland) at x10 magnification, and the heart and great vessels were dissected free from the surrounding connective tissue, so that the aorta, pulmonary artery and great veins were identified. The right superior vena cava (SVC) and IVC were ligated with 5/0 silk sutures (Ethicon Ltd., UK) and divided flush at their junction with the right atrium. Similarly, the left SVC and the azygous vein were ligated and divided at their common insertion into the left atrium. The ascending aorta was divided proximal to its bifurcation. Finally, the pulmonary veins draining into the back of the heart were ligated as a pedicle, and the heart removed by dividing between this tie and the posterior mediastinal structures.

2.7.2 *Recipient Transplantation*

Heterotopic cardiac transplantation was performed using the modified technique of Ono and Lindsey (Ono et al 1969). Recipient rats were anaesthetised by continuous halothane inhalation. A midline abdominal incision was made, and the intestines

displaced and wrapped in a saline-soaked swab. The aorta and IVC were exposed, cleared of surrounding connective tissue, and a length of approximately 5mm of each vessel cleared of any tributaries or branches, to allow haemostatic control by the application of a proximal and distal occlusion clamp. The operating field was visualised through an operating microscope at x16 magnification, and a 3mm arteriotomy and venotomy were fashioned in the recipient's aorta and IVC respectively. Thereafter, continuous end to side anastomosis of the donor aorta to the recipient aorta was performed using a 9/0 nylon suture (Ethicon Ltd.). A similar technique was used to anastomose the donor pulmonary aorta to the recipient IVC. During anastomosis, the donor heart was kept cool by the application of a gauze swab to which cold saline was intermittently applied. Upon completion, the clamps were slowly removed and any haemorrhage from the suture line controlled by a combination of pressure and the application of haemostatic alginate (Surgicel, ?). Cold ischaemic times for the heart ranged from 30 to 40 minutes. On confirmation that the heart had begun to beat, the intestinal contents were replaced, and the wound closed in two layers with continuous 3/0 vicryl sutures (Ethicon Ltd.). Grafts were assessed daily by palpation, and rejection was defined as the complete cessation of myocardial contraction.

2.8 T CELL PROLIFERATION ASSAYS

Purified splenocyte and lymph node cell populations were prepared as described in section 2.3. Cells were re-suspended in culture medium enriched with 2% HI normal syngeneic rat serum, at a concentration of 4 x 10^6 cells/ml. 100µl of cells were placed into each experimental well of 96-well U-bottomed plates (Nunclon) (4 x 10^5 cells/well). Stimulator RT1.A^{avl} peptides were added to a final concentration of 40µg/ml in each well, and the volume in each well was adjusted to 200µl. In addition, 10µl of ConA, at a final concentration of 5µg/ml was added to wells as a positive control. Cells in culture medium alone were used to indicate base-line proliferation. Plates were incubated at 37^0C in 5% CO_2

After 48 hours in culture, cells were pulsed with 1μCi/well of ^{3}H-Thymidine (TRK418, Amersham International plc, Little Chalfont, Bucks, UK), and incubated for a further 24 hours. Plates were harvested using either a semi-automatic 12-well cell harvester (Skatron Instruments, Sweden), or a semi-automatic 196 Filtermate plate harvester (Canberra-Packard, Pangbourne, Berks, UK). Cellular ^{3}H-Thymidine incorporation was assessed using a liquid scintillation beta counter (Wallac, Milton Keynes, UK), or a Topcount.NXT microplate scintillation and luminescence counter (Canberra-Packard). Results were expressed as counts per minute (cpm), or as a stimulation index, calculated by the formula:

$$\frac{\text{Experimental counts per minute}}{\text{Control counts per minute}}$$

2.9 DETECTION OF CYTOTOXIC ALLOANTIBODY

Donor alloantigen-specific cytotoxic alloantibody levels were determined in serum samples from modified RT1^{u} rats using a ^{51}Cr-release assay.

Specific cytotoxicity was assessed using ^{51}Cr-labelled donor strain R8 ConA-transformed splenoblasts (as described in section 2.3.3) as target cells. Target cells in 1ml of serum-free culture medium were incubated with 5.0MBq ^{51}Na-Chromate (CJS11, Amersham International plc.) for 90 minutes in a water-bath at 37^{0}C with regular agitation. Cells were washed once with washing medium, re-suspended, and erythrocytes removed by hypotonic lysis. Cells were washed a further 3 times to remove excess sodium chromate, counted by trypan blue exclusion, and re-suspended at a concentration of 1 x 10^{6} cells/ml in RPMI 1640 + 5% HI FCS.

Serum samples were triple diluted in RPMI 1640 + 5% HI FCS to produce dilutions of 1:3, 1:9, 1:27, 1:81, 1:243, 1:729, 1:2187 and 1:6561. Triplicate 50μl aliquots of diluted serum samples were incubated in 96-well V-bottomed plates (Serowell, Bibby Sterilin Ltd., Stone, Staffs, UK) with 50μl of target cells at 37^{0}C in 5% CO_2 for 45 minutes. 100μl of guinea pig complement (Harlan Sera Labs, Bicester, Oxon, UK) was added to each well, and the plates incubated for a further 60 minutes.

Spontaneous cellular release of chromium was determined using target cells incubated without complement or serum, and maximum release was determined using cells incubated with Triton X 100 (Sigma).

Plates were centrifuged for 7 minutes at 1200 rpm, and 100µl of supernatant was harvested from each well. Released ^{51}Cr was determined using either a Compugamma Counter (LKB Pharmacia, Milton Keynes, UK), or a Cobra autogamma counter (Packard).

Percent specific ^{51}Cr-release was calculated by the formula:

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

2.10 DETECTION OF ANTI-PEPTIDE ANTIBODY

Serum samples were tested for the presence of antibodies to peptide using an indirect radioactive binding assay, modified from the method previously described by Fangmann et al (Fangmann et al 1992a). All incubations, except for the blocking of non-specific binding sites were carried out at 4^0C.

96-well optiplates (Canberra-Packard) were coated with 50µl/well of peptide at 100µg/ml in 0.15M NaCl. Plates were incubated for 18 hours, then washed x 3 with PBS/0.1% Bovine Serum Albumin (BSA) (Sigma). Non-specific binding sites were blocked with 200µl of Meggablock III (Bionostics, Wyboston, Beds, UK) for 2 hours at 37^0C. Plates were washed as before. Tripling dilutions of test serum were added in triplicate to the plates, diluted in PBS/0.5% BSA (50µl/well). Plates were incubated for a further 2 hours, and then washed again. Anti-peptide antibody was determined by the addition of 50µl/well of ^{125}I-labelled sheep F(ab`)2 anti-rat Ig (Amersham International plc.) diluted 100-fold in PBS/0.5% BSA. Following a 60 minute incubation, plates were washed x 3 with PBS/0.1% BSA, and 100µl/well of Microscint 40 (Canberra-Packard) added. Bound radioactivity was measured using a Topcount.NXT. Results are expressed as cpm.

2.11 DETERMINATION OF CLASS AND SUBCLASS OF ANTI-A^a ALLOANTIBODY

IgM and IgG2b anti-A^a alloantibodies were detected by flow cytometry in serum samples from experimental $RT1^u$ rats using R8 lymph node target cells at a concentration of 1×10^7 cells/ml in PBS/0.2% BSA/0.1% sodium azide (Sigma).

Serum samples were triple diluted in PBS/0.2% BSA/0.1% sodium azide (PBA) to produce dilutions of 1:3, 1:9, 1:27, 1:81, 1:243, 1:729, 1:2187 and 1:6561. 50µl aliquots of diluted serum samples were incubated in 96-well U-bottomed plates with 50µl of target cells for 30 minutes at 4^0C. Cells were then washed by adding 100µl of PBA, and plates centrifuged for 7 minutes at 1800 rpm. The supernatant was discarded, and the plates washed again with 200µl of PBA.

FITC-conjugated mouse anti-rat IgM (Harlan Sera Labs) and IgG2b (Sigma) monoclonal antibodies were used to detect IgM and IgG2b alloantibodies respectively. Each antibody was diluted 400-fold in PBA, and 100µl added to experimental wells. Cells were re-suspended using a pipette, and plates incubated for 30 minutes at 4^0C in the dark. Plates were washed x 2 as before, and cells re-suspended in 100µl of PBA.

IgM and IgG2b alloantibodies were detected using a FACSCaliber (Becton Dickinson) flow cytometer with CellQuest software.

CHAPTER 3:

THE IMMUNE RESPONSE TO RT1.A^a-DERIVED ALLOPEPTIDES IN THE RT1^u RAT STRAIN

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CHAPTER 3:

THE IMMUNE RESPONSE TO RT1.A^a-DERIVED ALLOPEPTIDES IN THE RT1^u RAT STRAIN

3.1 *Introduction*

The work described in this thesis examines the role of the indirect pathway of recognition in allograft rejection using a class I disparate rat strain combination, PVG-R8 (RT1.A^{a}B/D^{u}C^u) to PVG-RT1^u (RT1.A^{u}B/D^{u}C^u). Previous work in our laboratory has demonstrated that graft rejection in this strain combination is due to CD4 T cell-dependent alloantibody-mediated effector mechanisms (Gracie et al 1990, Morton et al 1993). MacDonald et al have also shown that priming RT1^u recipients to the indirect pathway, with allopeptides derived from the hypervariable regions of the RT1.A^a molecule, results in the accelerated rejection of R8 cardiac allografts (MacDonald et al 1997). The R8 and RT1^u rat strains are genotypically identical except at the MHC region, where they differ at a single class I locus, RT1.A (Figure 3.1a). Since these two strains share the same MHC class II molecules, it is likely that recipient CD4 T cells recognise the allogeneic donor class I molecule as processed antigen presented by MHC class II on either donor or recipient APC.

The indirect presentation of an antigenic molecule, such as the RT1.A^a MHC class I molecule, is a controlled process, such that only certain peptide sequences from the antigen are presented as T cell epitopes. Furthermore, a pattern of hierarchical dominance exists amongst presented epitopes, wherein a limited number of dominant epitopes are involved in the initial response, and the remaining sub-dominant, or cryptic epitopes may, under certain circumstances, become more relevant (Sercarz et al 1993). To analyse more fully the functional T cell

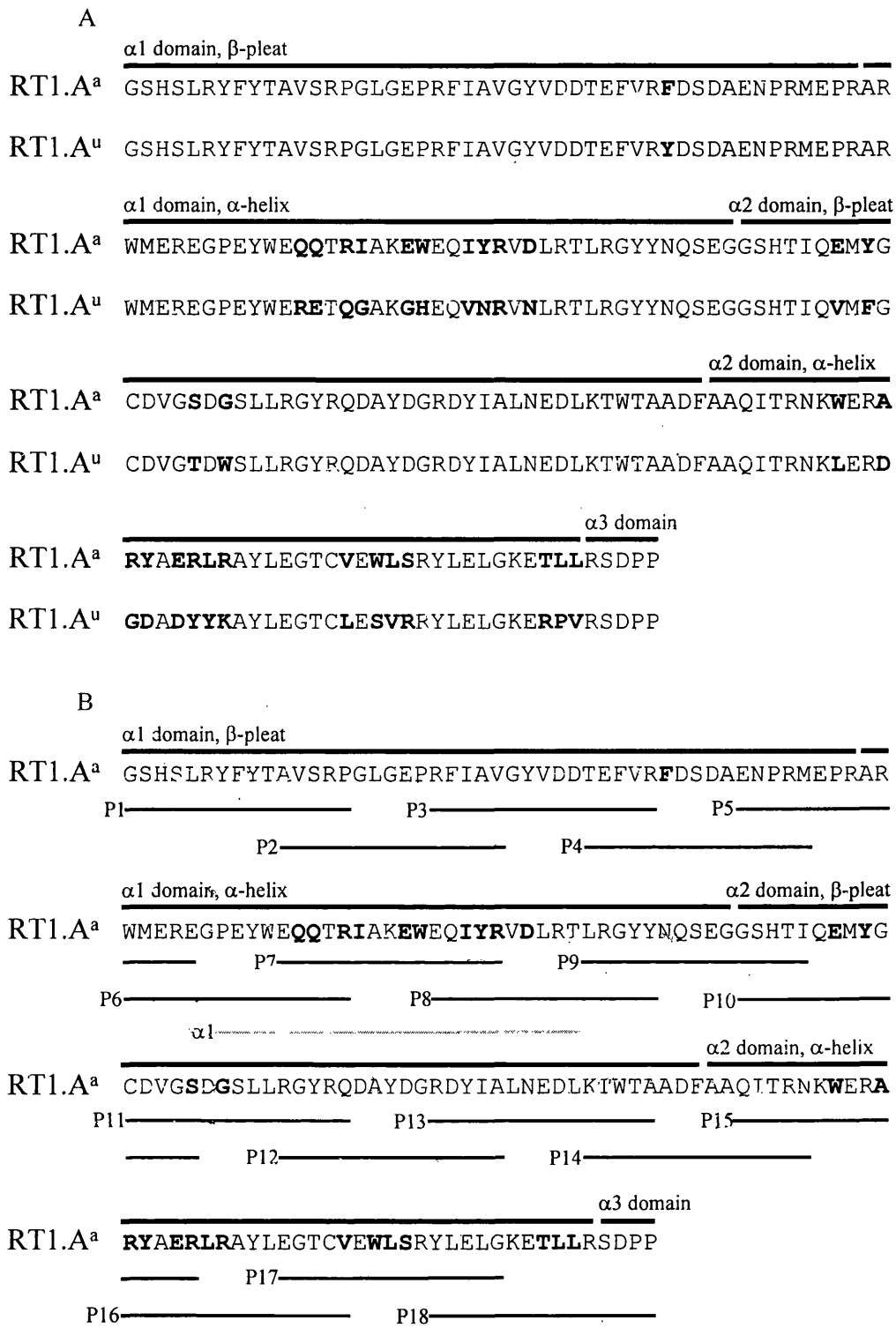


Figure 3.1: A) The nature of the amino acid disparity between the RT1.A^a and RT1.A^u MHC class I molecules. B) The positions of the α1 and 15-mer peptides in the RT1.A^a molecule. Disparate amino acids are in bold type.

determinants that are contained within the A^a molecule, and their relative importance in the indirect response to alloantigen, I chose to examine the *in vitro* and *in vivo* responses of $RT1^u$ rats to a series of 18 synthetic peptides spanning the $\alpha 1$ and $\alpha 2$ domains of A^a.

The 18 peptides (P1-P18) were synthesised by Immune Systems (UK). Each peptide is 15 amino acids long, and overlaps with each of its adjacent peptides by five amino acids. 15-mer peptides were chosen for this study, as it has been shown that this is the optimal length for peptide binding to MHC class II (Rudensky et al 1991, Chicz et al 1992). The positions of the peptides in the A^a molecule are illustrated in Figure 3.1b. Two additional peptides were used, one of 24 amino acids, derived from the hypervariable region of the $\alpha 1$ domain (designated $\alpha 1$), and a control peptide (designated irrelevant). The irrelevant peptide contains the same amino acids as peptide 7 (P7), but in a random order.

I initially examined which of these peptides were able to elicit an immune response in $RT1^u$ rats, by priming animals subcutaneously with individual peptides emulsified in CFA. These results were compared with the recall responses to peptides following immunisation with the intact A^a molecule in the form of R8 skin and cardiac allografts. It was anticipated that only a limited number of peptides would be involved in the indirect response to the intact molecule, and that these would be contained within a wider subset of immunogenic peptides, as revealed following peptide priming experiments. A comparison of the spectrum of responses resulting from different immunisation protocols not only enables mapping of dominant epitopes, but can also identify potentially sub-dominant or cryptic epitopes.

3.2 *The Immune Response Following Immunisation with Allopeptide*

To examine the immunogenicity of allopeptides, naïve $RT1^u$ animals were subcutaneously immunised with 100μg of individual peptides emulsified in CFA. After 12 days, LNCs and sera from the immunised animals were used in *in vitro* T cell proliferation assays and analysed for alloantibody content respectively. Optimum conditions for these assays, with regards to peptide concentration, numbers of responder cells and the constituents of the media, had been previously established in our laboratory (Catherine MacDonald – personal communication), and were consequently re-employed in the present study. However, preliminary experiments were performed to establish the optimum time-point at which to harvest the *in vitro* T cell proliferation assays. Figure 3.2 is a representative graph of these experiments. No significant difference in the level of T cell proliferation was noted in samples that were harvested 96 or 120 hours after culture initiation. However, those cells harvested at 96 hours did appear to exhibit slightly higher levels of ^{3}H-Thymidine incorporation, and I consequently employed this time-point for the remainder of my study.

3.2.1 *The T Cell Proliferative Response*

To examine the *in vitro* T cell proliferative response to the allopeptides, LNCs were prepared from $RT1^u$ rats 12 days after peptide priming, cultured *in vitro* with the immunising peptide for 72hrs, and pulsed with ^{3}H-Thymidine for an additional 24hrs before harvesting. Assays were performed 12 days after peptide immunisation, as work from other laboratories would suggest that 10-12 days is the minimum time at which *in vitro* proliferation can be detected (Benichou et al 1992, Benichou et al 1994a). Specific *in vitro* T cell proliferative responses to immunising peptides are illustrated in Figure 3.3. Only P1, P7 and the α1 peptide stimulated recall responses greater than the proliferative response to the same peptides by LNCs from naive $RT1^u$ rats. Of these three peptides, α1 stimulated the greatest response, with proliferation five times greater than that of naive cells. P7 stimulated a response

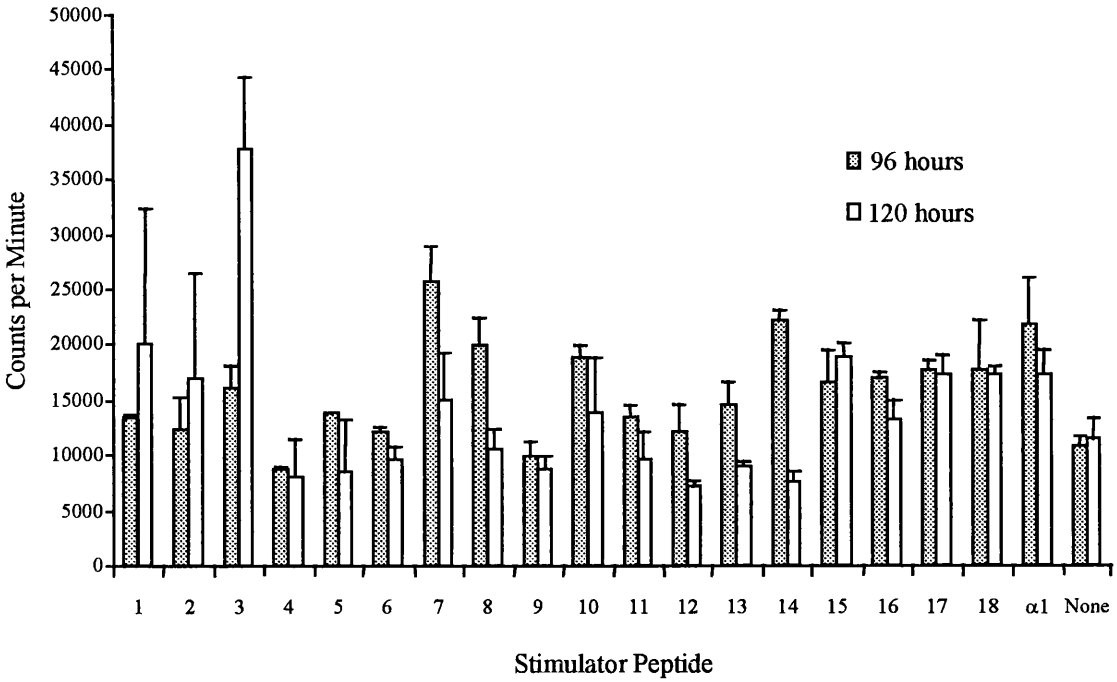


Figure 3.2: Comparison of *In Vitro* T Cell Proliferation of LNCs Harvested 96 and 120 Hours after Initiation of Culture.
An $RT1^u$ animal was immunised with a heterotopic R8 cardiac allograft, and LNCs used in T cell proliferation studies 11 days later. Cells were cultured with individual allopeptides at 40μg/ml for 96 or 120hrs. Cells were pulsed with ^{3}H-Thymidine for the last 24hrs of culture. Results are expressed as cpm. Standard deviations derive from original triplicate values.

approximately three times that of background and P1 approximately twice that of background. *In vitro* culture with any of the other 15-mers, including the irrelevant peptide, failed to generate a proliferative response greater than that of naive cells. It is unsurprising that the α1 peptide stimulated a strong proliferative response, as its sequence encompasses the ten disparate A^a/A^u amino acid residues found in the α-helical region of the α1 domain. In comparison, P7 encompasses nine of these disparate amino acid residues (see Figure 3.1b). Interestingly however, neither P15 nor P16 generated a proliferative response, despite their sequences deriving from the hypervariable α-helical region of the α2 domain. There are 15 amino acid residue differences in this second hypervariable region between the two rat strains, with P15 and P16 encompassing nine of the disparate residues in total (see Figure 3.1b).

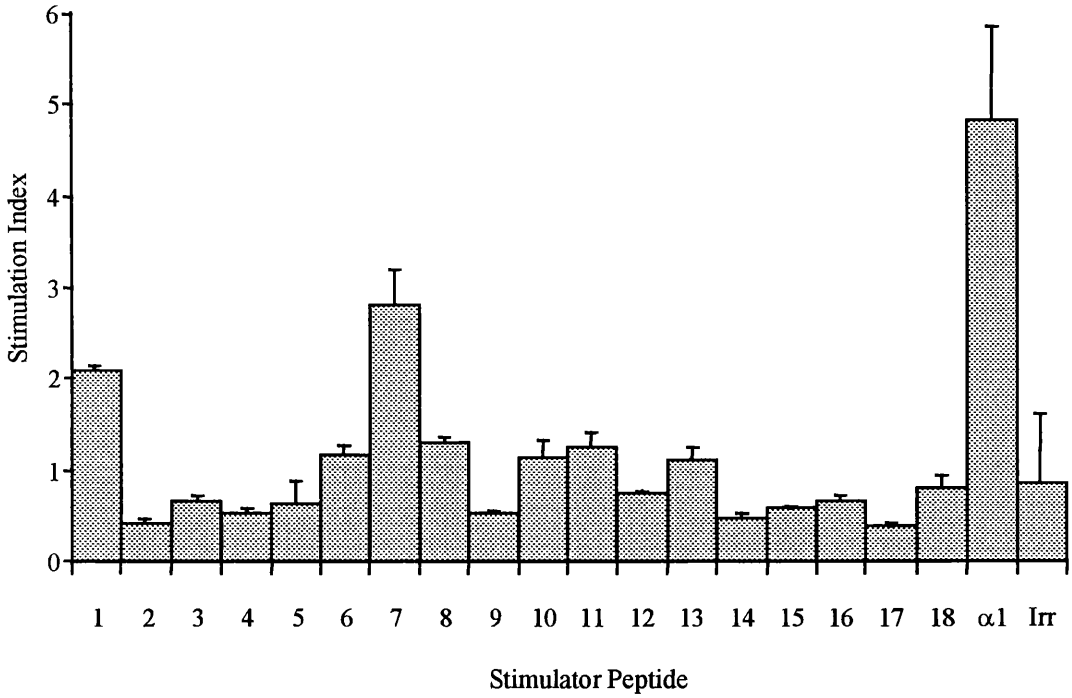


Figure 3.3: Specific *In Vitro* T Cell Proliferative Responses of RT1^u Animals Immunised with A^a Allopeptides.

RT1^u animals were immunised subcutaneously with 100μg of peptide emulsified in CFA and LNCs used in T cell proliferation studies 12 days later. Cells were cultured for 72hrs with individual allopeptides at 40μg/ml, and pulsed with ^{3}H-Thymidine for an additional 24hrs before harvesting. LNCs from naïve RT1^u rats were cultured in the same manner to obtain background levels of proliferation. Standard deviations derive from original triplicate values.

Notably, immunisation with P7 stimulated a recall response to the α1 peptide that was slightly stronger, although not significantly so, than the response observed to P7 itself (Figure 3.4a). In addition, immunisation with P8 also stimulated a response to the α1 peptide, but interestingly, the recall response to P8 itself was not greater than that observed in naïve LNCs (Figure 3.4b). Given that P7 and P8 both share common sequences with the α1 peptide, and that no proliferation to α1 was observed following immunisation with any of the other peptides (for example, see Figure 3.4c), the proliferative response to α1 upon immunisation with P8 is probably not an artifact. This would suggest that priming with P8 does in fact stimulate an *in vivo* T cell response, but that the *in vitro* proliferation assay does not reveal this.

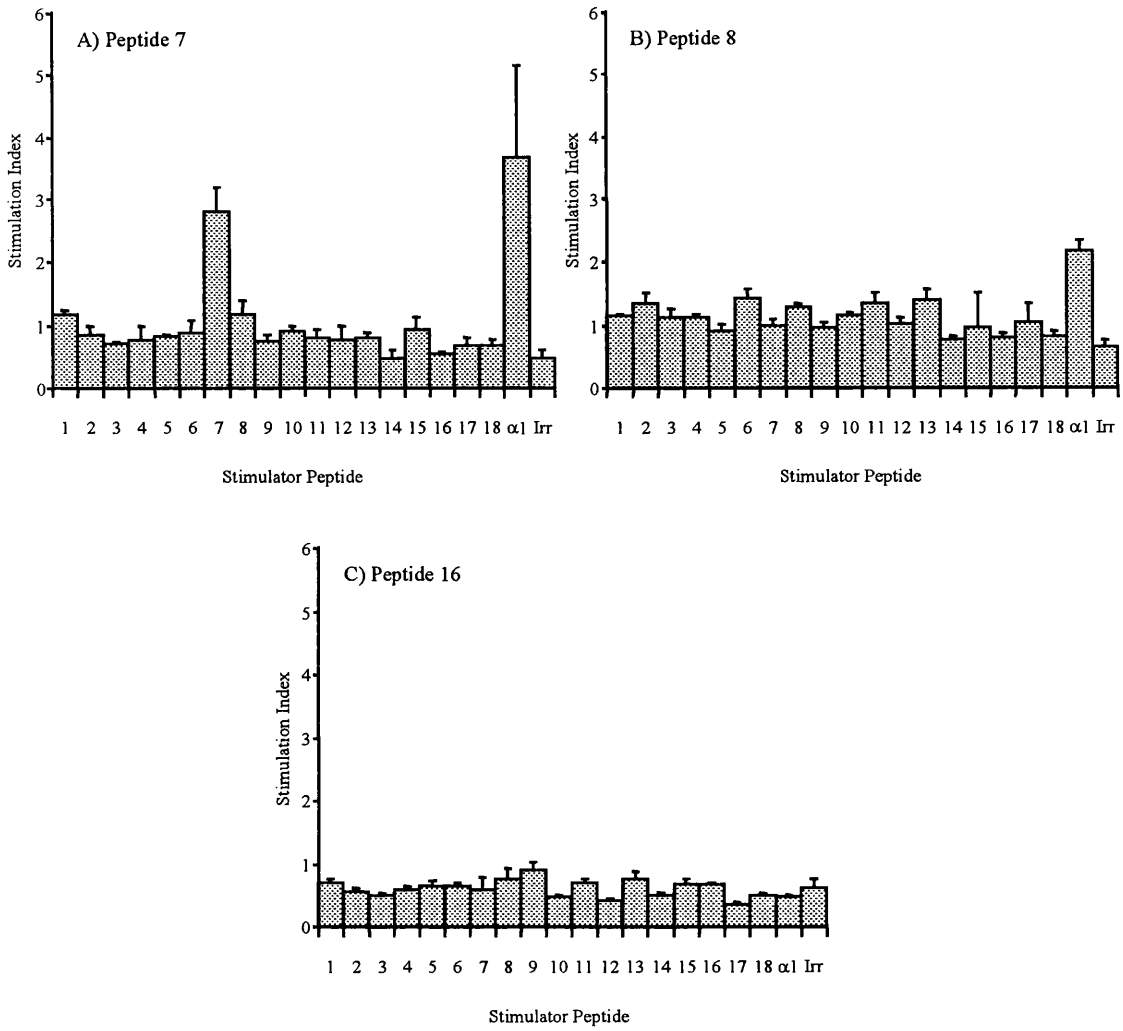


Figure 3.4: *In Vitro* T Cell Proliferation of LNCs from RT1^u Animals Immunised with Peptides P7, P8 or P16.

RT1^u animals were immunised subcutaneously with 100μg of A) P7, B) P8 or C) P16 emulsified in CFA and LNCs used in T cell proliferation studies 12 days later. Cells were cultured for 72hrs with individual allopeptides at 40μg/ml, and pulsed with ^{3}H-Thymidine for an additional 24hrs before harvesting. LNCs from naïve RT1^u rats were cultured in the same manner to obtain background levels of proliferation. Standard deviations derive from original triplicate values.

This discrepancy between the *in vivo* T cell response and the *in vitro* proliferative response will be discussed later.

3.2.2 *Anti-Peptide Alloantibody Analyses*

Because of the possibility that T cell proliferation assays do not reveal all potentially immunogenic epitopes, peptide immunogenicity was alternatively examined by

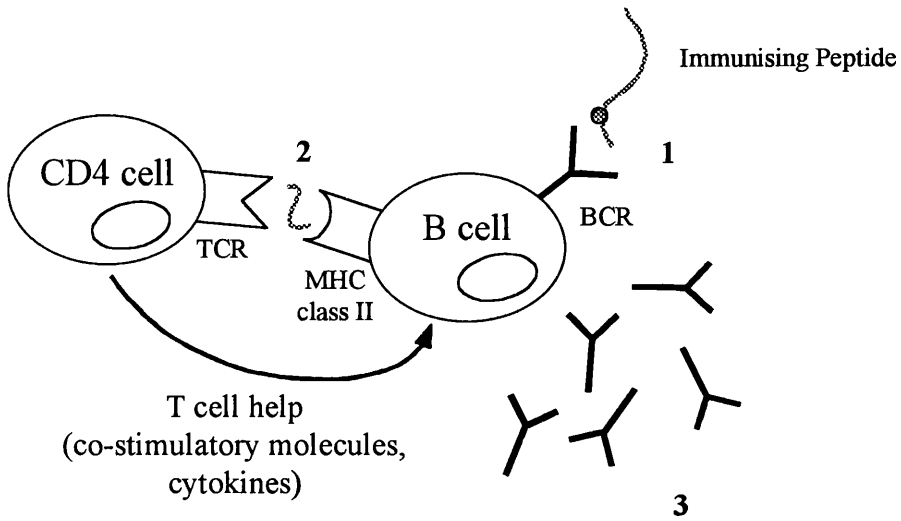
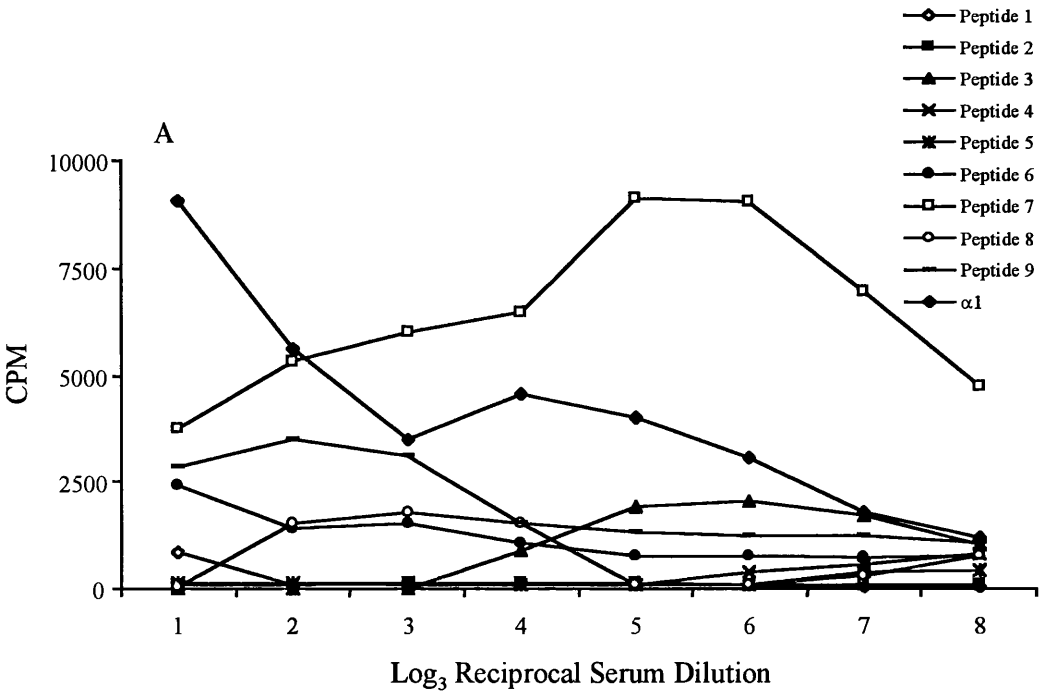


Figure 3.5: Cognate T and B Cell Collaboration for the Production of Anti-peptide Antibodies.
1) Peptide is internalised by the B cell through recognition of its B cell determinant.
2) T cells specific for the peptide epitope presented by the B cell provide help for the production of anti-peptide antibodies (3).

measuring the antibody responses that peptides engendered following their subcutaneous immunisation. Analysis of the anti-peptide antibody response in serum samples obtained at different time points following immunisation permits the kinetics of the T cell response to be observed indirectly through its influence on the B cell response. As can be seen from Figure 3.5, the development of the anti-peptide antibody response is dependent not only upon T cell help, but also on the presence of B cells that have receptors for the appropriate peptide epitopes. Consequently, although this approach may detect additional immunogenic epitopes compared with the proliferation assay, it may not be all encompassing, as other peptides that contain potential T cell epitopes may not be revealed due to the absence of a B cell epitope.

Anti-peptide antibody in serum samples taken from $RT1^u$ rats 12 days after subcutaneous immunisation with peptide was assessed using an indirect radioactive binding assay in which sera were incubated in microtitre plates coated with the immunising peptide, and antibody detected using an ^{125}I-labelled sheep F(ab`)2 anti-rat Ig. Figure 3.6a demonstrates the levels of peptide-specific antibody detected in



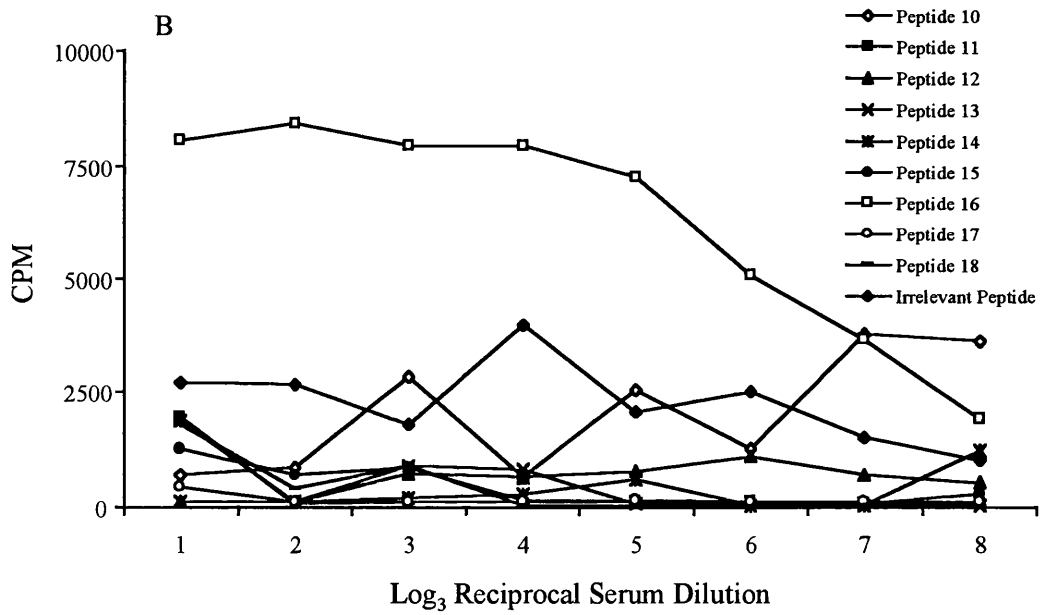


Figure 3.6: Anti-peptide Antibody Production Following Immunisation of RT1^u Rats with Allopeptide.

Specific anti-peptide antibody levels in serum samples from RT1^u rats immunised with peptides derived from A) the α1 domain, and B) the α2 domain of the RT1.A^a molecule. Samples were taken 12 days after subcutaneous administration of 100μg of peptide emulsified in CFA. Alloantibody levels were assessed using an indirect radioactive binding assay in microtitre plates coated with immunising peptides. Results are the mean of two animals per group.

serum samples from animals immunised with peptides derived from the α1 domain (i.e. peptides P1-P9 and the α1 peptide). Similarly, Figure 3.6b illustrates levels of peptide-specific antibody found in serum samples from $RT1^u$ rats immunised with allopeptides derived from the α2 domain (i.e. peptides P10-P18) and also the irrelevant peptide. The two graphs demonstrate that only the α1 peptide and P7 from the α1 domain, and P16 from the α2 domain provoked an anti-peptide antibody response. All other samples tested displayed negligible levels of anti-peptide antibody.

Although this assay demonstrates that $RT1^u$ rats mount an antibody response to certain peptides (P7, P16 and α1), the naturally high background levels associated with the indirect radioactive binding assay prevents accurate titration and consequently prohibits precise quantitative comparisons between the peptides that stimulated an anti- antibody response. I therefore attempted to measure the antibody response quantitatively using an ELISA (as described by Shirwan et al 1995). However, repeatedly high background levels in these assays prevented a distinction between control and sample wells.

3.2.3 *Cytotoxic Alloantibody Analyses*

Sera were also tested for their ability to lyse R8 lymphoblast target cells in a standard ^{51}Cr-release assay, in which target cells were labelled with ^{51}Cr before incubation with experimental serum samples, and donor-specific alloantibody activated by the addition of guinea pig complement. No cell lysis was observed in response to any of the samples, thus indicating that anti-peptide antibodies were unable to cross-react with the intact A^a molecule (data not shown, but the average maximum and spontaneous ^{51}Cr-release values were 1040 and 104 cpm respectively).

3.3 *T Cell Proliferation Studies Following Immunisation with the Intact RT1.A^a Class I Molecule.*

The indirect response to the intact A^a molecule, as would occur upon challenge with an R8 graft, presumably centres upon the subset of peptides established as

immunogenic in the preceding experiments. It is possible however, that the indirect response is further focused upon a single dominant epitope (Benichou et al 1994a). To explore this possibility, the *in vitro* T cell proliferative responses to individual peptides were assessed following *in vivo* challenge with the intact A^a molecule in the form of an R8 skin graft followed by an R8 cardiac allograft. Naïve $RT1^u$ animals were immunised with a full thickness R8 skin graft 7 days before receiving a fully vascularised heterotopic R8 cardiac allograft. 7 days after heart grafting, LNCs were cultured *in vitro* with individual allopeptides for 72hrs, and pulsed with ^3H-Thymidine for a further 24hrs before harvesting.

A typical pattern of *in vitro* T cell proliferation to individual allopeptides following such immunisation is shown in Figure 3.7. The α1 peptide, P7 and P8 stimulated a greater T cell proliferative response in LNCs that had been previously exposed to the A^a molecule than in naive cells, with the response to the α1 peptide stronger than that to P7 or P8. Proliferative responses to the other 15-mer peptides were comparable in primed and naïve LNCs.

Proliferation to the α1 peptide, but not to any of the 15-mer peptides, was generally observed following immunisation of $RT1^u$ animals with a single heart graft, although results were more variable as can be seen in Figure 3.8. Skin grafting alone did not evoke any constant pattern of *in vitro* T cell proliferation as is demonstrated in Figure 3.9.

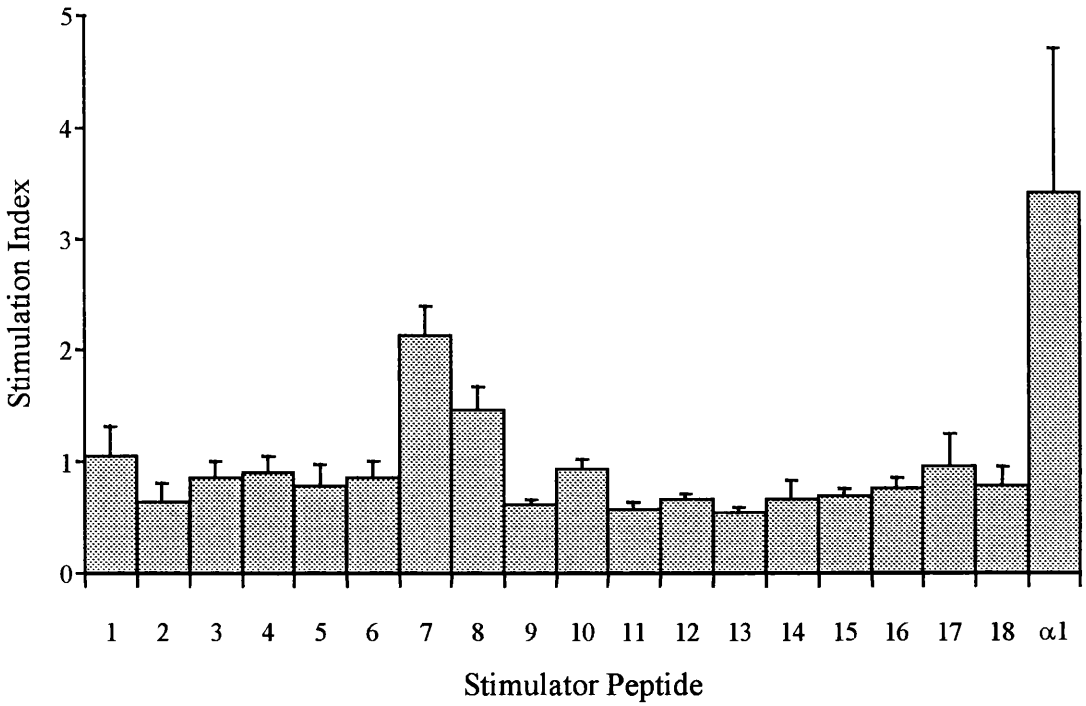


Figure 3.7: *In Vitro* T Cell Proliferation to Allopeptide Following Immunisation with an R8 Skin Graft and an R8 Cardiac Allograft.

$RT1^u$ animals were immunised with a full thickness R8 skin graft 10 days before challenge with a fully vascularised R8 cardiac allograft. LNCs from experimental rats were used in T cell proliferation studies 7 days after cardiac transplantation. Cells were cultured for 72hrs with individual allopeptides at 40μg/ml, and pulsed with ^3H-Thymidine for an additional 24hrs before harvesting. LNCs from naïve $RT1^u$ rats were cultured in the same manner to obtain background levels of proliferation. Results shown are the mean and standard deviation of three animals.

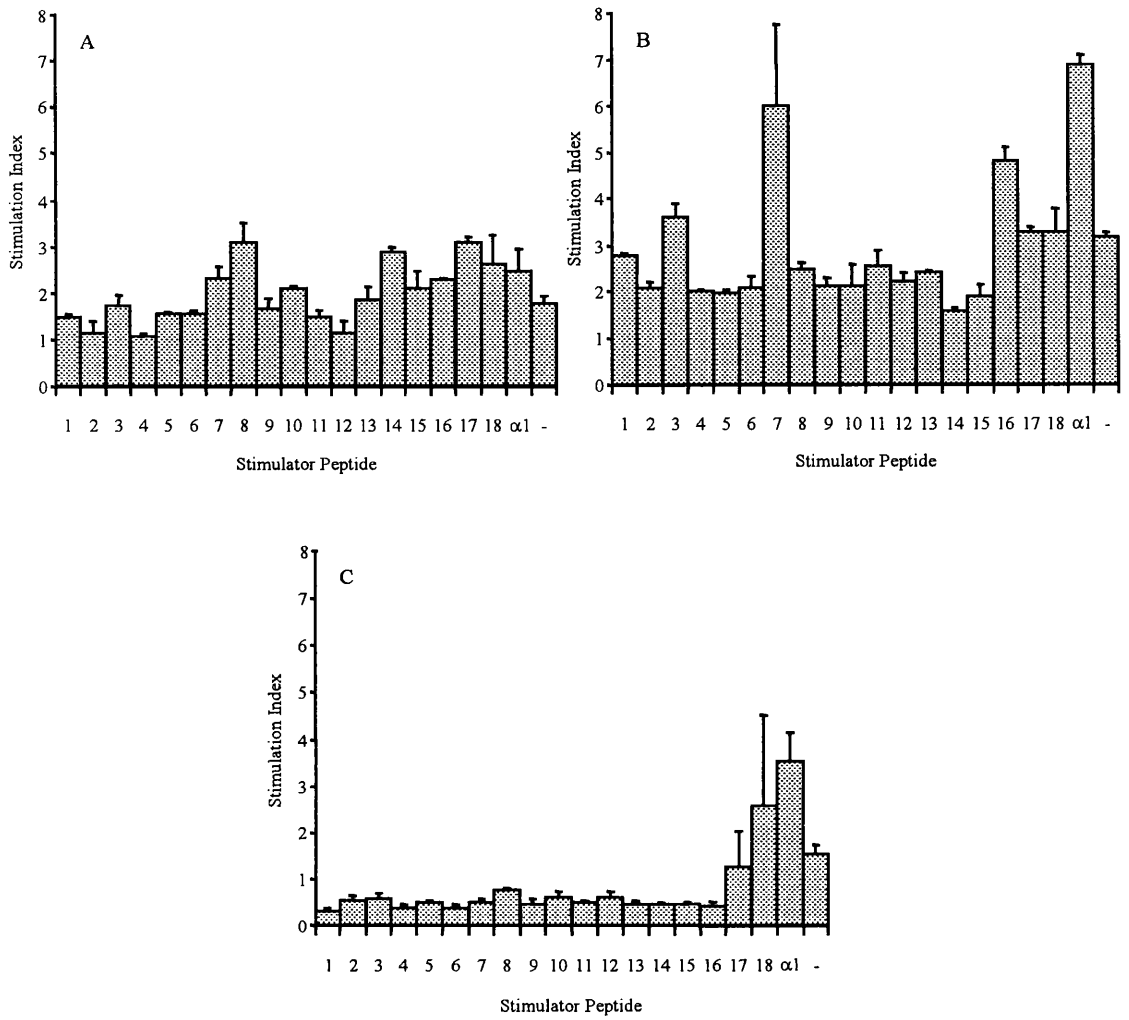


Figure 3.8: *In Vitro* T Cell Proliferation of LNCs from RT1^u Rats Immunised with an R8 Cardiac Allograft.

RT1^u animals were immunised with a heterotopic R8 cardiac allograft, and LNCs used in T cell proliferation studies 7 days later. Cells were cultured for 72hrs with individual allopeptides at 40μg/ml, and pulsed with ^{3}H-Thymidine for an additional 24hrs before harvesting. LNCs from naïve animals were treated in the same manner to obtain background levels of proliferation. Each graph represents the proliferative response of a single animal. Standard deviations derive from original triplicate values.

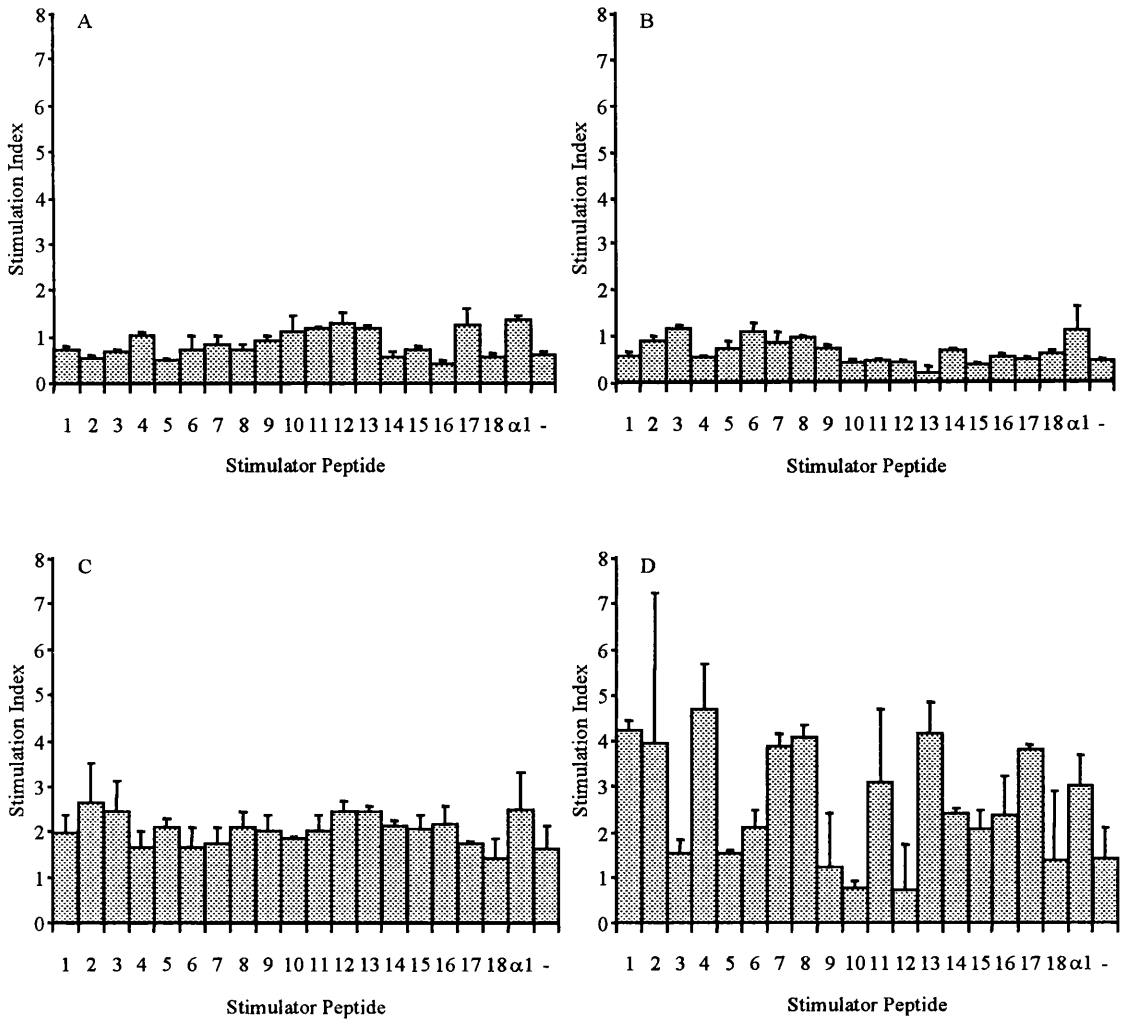


Figure 3.9: *In Vitro* T Cell Proliferation of LNCs from RT1^u Rats Immunised with an R8 Skin Graft.

RT1^u animals were immunised with a full thickness R8 skin graft, and LNCs used in T cell proliferation studies 7 days later. Cells were cultured for 72hrs with individual allopeptides at 40μg/ml, and pulsed with ^{3}H-Thymidine for an additional 24hrs before harvesting. LNCs from naïve animals were treated in the same manner to obtain background levels of proliferation. Each graph represents the proliferative response of a single animal. Standard deviations derive from original triplicate values.

3.4 *Discussion*

Following immunisation with individual peptides, proliferative recall responses to only P7, the α1 peptide and to a lesser extent P1, were observed (see Figure 3.3). Of these, the response to the α1 peptide, which encompasses all of the ten amino acid differences found between the A^a and A^u sequences in the α-helical region of the α1 domain, appeared stronger than that to P7, which encompasses nine of the same amino acid differences. Since the 24 amino acid α1 peptide encompasses all of P7 and part of P8 (see Figure 3.1b), it is possible that the epitope most frequently generated upon processing of the α1 peptide, or indeed the intact A^a molecule, bridges both P7 and P8 (see Figures 3.4a, 3.4b and 3.7).

It was notable however, that following immunisation with P7, the proliferative recall response to the α1 peptide was stronger than that to P7 itself (Figure 3.4a). The explanation for this is not readily obvious, but possibly reflects deficiencies in the *in vitro* presentation of P7. In other words, although P7 can efficiently stimulate T cells *in vivo*, it may be that under the conditions of an *in vitro* T cell proliferation assay, processing of the α1 peptide generates the same epitope contained within P7, only more efficiently. The same reasoning could equally be used to explain why, upon *in vivo* priming with P8, an *in vitro* proliferative response can be demonstrated to the α1 peptide but not to P8 itself (Figure 3.4b).

The generation of an anti-peptide antibody response requires a peptide to contain both T and B cell determinants, and it is likely that certain peptides contain a T cell epitope but lack a suitable determinant for triggering the B cell receptor. It was interesting therefore, that whereas the T cell proliferation assays suggested *in vivo* responses only to P1, P7 and α1 (Figure 3.3), the anti-peptide antibody assay additionally revealed a humoral response to P16 (Figure 3.6). There are two possible explanations for this unexpected finding. Firstly, the indirect radioactive binding assay used for detecting anti-peptide antibodies may be more sensitive than the proliferation assay for examining T cell responses to peptide. Secondly, the ability of peptides to provoke a response *in vivo* might not necessarily correspond to *in*

vitro T cell proliferation. Evidence for this has been provided by Nevala et al (Nevala et al 1997), who showed that certain peptides that were able to influence allograft rejection, did not stimulate an *in vitro* CTL response. This emphasises the complexities of the *in vivo* alloimmune response, and demonstrates that *in vitro* assays may not always represent accurately *in vivo* T cell specificities.

Epitopes involved in the indirect response to the A^a molecule appear to exhibit a hierarchical pattern of dominance. Following immunisation of $RT1^u$ animals with the intact A^a molecule, in the form of a skin graft followed by a heart graft, it was observed that proliferative T cell responses were directed towards only P7 and the α1 peptide, which are derived from the hypervariable region of the α1 domain (see Figure 3.7). In a similar fashion, Fangmann et al demonstrated that proliferation could be detected to a peptide derived from the α1 α-helical region of the donor RT1.A^a molecule following immunisation of Lewis ($RT1^l$) recipients with a DA ($RT1^a$) skin graft followed by a DA kidney graft 4-6 weeks later (Fangmann et al 1992b). No proliferative response was observed to peptides derived from either the α-helical or β-pleated sheet regions of the α2 domain. Benichou et al subsequently reported that following skin grafting in mice, the T cell response to the β1 domain of the donor class II H2-A^k β chain similarly focused upon a single dominant epitope. In this study, a series of 15-mer peptides progressing along the length of the donor MHC molecule in single amino acid residue steps were used. This approach results in a more thorough examination of the epitopes involved in the indirect pathway since the T cell response to all possible epitopes from the donor MHC molecule is examined. Furthermore, in this study, second-set grafting was not required, with proliferation to the dominant epitope detected 10 days after skin grafting alone (Benichou et al 1994a). In my experiments, focusing of the T cell proliferative response onto a dominant epitope was more clearly demonstrated following second-set grafting, concurring with the results of Fangmann (Fangmann et al 1992b). This possibly represents a species-specific variation in T cell proliferative responses.

Proliferation to the α1 peptide following challenge with the intact A^a molecule was again greater than that observed to P7 (Figure 3.7), and once again, it is possible that

the α1 peptide contains, in terms of antigen processing, a more optimal T cell epitope than its shorter variant P7. However, as mentioned above, we cannot exclude the possibility that an artifact exists surrounding the *in vitro* presentation of P7. For example, it has been suggested by Viner et al that APCs may internalise and process peptides through different compartments in *in vitro* T cell proliferation assays than in an *in vivo* situation. Therefore, the same peptide epitope may be presented in sufficiently different configurations as to alter its T cell epitope depending on the nature of its surrounding environment, thus resulting in the activation of different subsets of peptide-reactive T cell clones *in vitro* and *in vivo* (Viner et al 1996).

In conclusion, these experiments reveal that several immunogenic epitopes exist within the RT1.A^a molecule, and that during the indirect response to the intact A^a molecule, as provided by R8 allografts, the alloimmune response is further focused onto a dominant epitope. This dominant epitope is located within the hypervariable region of the α1 domain, which concurs with previous results both in this strain combination (Shirwan et al 1995), and in the DA (RT1.A^a) to Lewis (RT1.A^l) rat strain combination (Fangmann et al 1992b). The α1 peptide generates a stronger *in vitro* T cell proliferative response than P7, and this would suggest that additional amino acid sequences of the hypervariable region are involved in the generation of the optimal dominant epitope. These amino acids would be contained within P8, and it is perhaps surprising that a stronger response to this peptide was not observed.

There are several possible reasons to explain why the hypervariable regions of an allo-MHC molecule contain the epitopes principally involved in the indirect alloresponse. Most obviously, it is this area that contains the greatest number of amino acid differences as compared to the recipient's own MHC molecule. One would therefore expect that processing of an alloMHC molecule would generate a greater number of non-self epitopes derived from this region than from other less variable areas of the molecule. However, it is also possible that the recipient T cell response is intrinsically biased towards epitopes from this region, either because similar areas of self-MHC molecules are involved in positive selection of the T cell repertoire, or perhaps because processing of the donor MHC molecule by recipient

APCs favours the presentation of epitopes from the hypervariable region. Given this, it is surprising that the response to peptides derived from the second hypervariable region in the α2 domain were weaker and inconsistent. In fact, P1, in effect a self-$RT1^u$ peptide (see Figure 3.1b) appeared to stimulate a stronger T cell proliferative recall response than peptides derived from this second hypervariable region (Figure 3.3). As mentioned above, Fangmann et al also reported a lack of response to the second hypervariable region of the $RT1.A^a$ molecule upon challenge of Lewis, rather than $RT1^u$ animals, with an A^a-bearing graft (Fangmann et al 1992b).

These results suggest that epitopes derived from areas of the A^a molecule other than the hypervariable regions may potentially be immunogenic, and further experiments were therefore designed to examine the influence of peptide priming on the immune response to the intact A^a molecule as confirmation of this.

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THE INFLUENCE OF A^a ALLOPEPTIDE IMMUNISATION ON THE IMMUNE RESPONSE TO THE INTACT A^a CLASS I MOLECULE

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THE INFLUENCE OF A^a ALLOPEPTIDE IMMUNISATION ON THE IMMUNE RESPONSE TO THE INTACT A^a CLASS I MOLECULE

4.1 *Introduction*

The findings from Chapter 3 suggest that the indirect $RT1^u$ alloimmune response to the $RT1.A^a$ MHC class I molecule is restricted to a limited number of dominant T cell determinants, contained within the hypervariable region of the $\alpha 1$ domain. That the initial immune response to a protein antigen focuses on a limited number of dominant epitopes is well established (reviewed in Sercarz et al 1993). For example, the response to hen egg lysozyme (HEL) is limited to determinants from a small area of the molecule, despite the ability of epitopes from other regions to induce T cell responses when used as immunogens (Maizels et al 1980). Equally, this phenomenon has been shown to apply during the indirect response to an allo-MHC molecule following transplantation (Fangmann et al 1992b, Benichou et al 1994a, Liu et al 1996a). There is no direct evidence to explain the exact mechanisms responsible for immunodominance, but factors such as antigen processing, MHC affinity for processed antigenic peptide and the existing T cell repertoire are all likely to be involved. Moreover, the response to other potentially immunogenic epitopes must be downregulated, either through competitive inhibition, for example during antigen processing, or through active suppression perhaps as a result of inhibitory factors released by T cells specific for the dominant epitope.

Despite initial immune focusing, there is strong evidence to suggest that with time, the immune response to nominal antigen diversifies to include further T cell epitopes. This has been clearly demonstrated by Lehmann et al, who noted that during the inductive phase of EAE in mice, the immune response focused onto a single dominant determinant of the pathogenic myelin basic protein. However, in

mice with chronic EAE, proliferative recall responses could be detected towards several additional determinants of the same protein (Lehmann et al 1992). This phenomenon of "epitope spreading" has also recently been demonstrated as occuring following transplantation in both rodents (Benichou et al 1998), and humans (Ciubotariu et al 1998, Suciu-Foca et al 1998).

Epitope spreading appears to happen in a coordinated fashion, but the reasons as to why certain epitopes are preferentially involved in the secondary response are unclear. Those epitopes that are not involved in the initial dominant response to an antigen are generally termed as being cryptic, but Sercarz et al have classified an additional subset of epitopes referred to as sub-dominant. Sub-dominant epitopes can be differentiated from cryptic epitopes by their ability, when injected as peptides in an immunogenic fashion, to induce strong recall responses not only to themselves, but more importantly, to the parent protein from which they are derived (Sercarz et al 1993). It therefore seems likely that the immune response to a complex protein, such as an allo-MHC molecule, will spread to sub-dominant, rather than cryptic, epitopes. Such sub-dominant epitopes may therefore be of critical importance in the development of peptide-based tolerogenic strategies to counter the indirect alloimmune response.

Using Sercarz's definition of sub-dominance, it is likely that sub-dominant epitopes involved in the indirect alloimmune response may be identified by monitoring the effect that allopeptide immunisation has upon the immune response to an allograft bearing the donor MHC antigen from which the peptide was derived. This has been demonstrated by Fangmann et al (Fangmann et al 1992a), who showed that priming Lewis ($RT1^l$) rats with peptides derived from the two hypervariable regions of the $RT1.A^a$ class I MHC molecule moderately accelerated the rate of rejection of subsequent A^a-bearing DA skin grafts. In comparison, a third peptide, derived from the β-sheet of the α2 domain was unable to influence the rejection kinetics of DA skin grafts. All three peptides were however, independently capable of enhancing alloantibody production to the intact A^a molecule, even though this heightened response did not appear to correlate with accelerated rejection. These experiments

nevertheless suggest that all three peptides can be classed as either dominant or sub-dominant in that they are able to influence the immune response to the intact A^a molecule. As the indirect T cell response was assayed against only a limited sequence of the donor MHC molecule, specifically the highly variable regions, it is unsurprising that no cryptic epitopes were identified.

Vella et al have performed similar experiments in the WF ($RT1^u$) to Lewis ($RT1^l$) rat strain combination (Vella et al 1999). These studies were more extensive however, as animals were pre-immunised with one of eight peptides that together spanned the full-length of the $RT1.B^u\beta$ and $RT1.D^u\beta$ chains. In comparison to the results obtained by Fangmann and colleagues (Fangmann et al 1992a), although four of the peptides were immunogenic, only one was able to influence the rate of rejection of a subsequent WF cardiac allograft. The inability of the remaining seven peptides to influence the recall response to the intact protein suggests that they contain cryptic epitopes.

In the PVG-R8 ($RT1.A^a$) to PVG-1U ($RT1.A^u$) rat strain combination, a similar model upon which the experimental work of this thesis is based, Shirwan et al have demonstrated that immunisation with each of three donor A^a-derived allopeptides was able to enhance the immune response to a subsequent A^a-bearing R8 cardiac allograft. However, as with the work of Fangmann (Fangmann et al 1992a), all three peptide sequences corresponded to the hypervariable regions of the A^a molecule, with the less variable regions of the molecule, which are more likely to contain cryptic epitopes, not being tested. Experiments from our own laboratory have previously demonstrated that priming $RT1^u$ rats with peptides derived from the hypervariable regions of the A^a molecule marginally accelerates the rate of rejection of subsequent A^a-bearing R8 cardiac allografts (MacDonald et al 1997). In these studies, allograft rejection was associated with an enhanced anti-A^a alloantibody response, which passive transfer experiments, also performed in this laboratory, have shown to be the main effector mechanism associated with graft rejection in this strain combination (Gracie et al 1990, Morton et al 1993). The means by which augmentation of the indirect pathway through peptide priming are able to influence

the antibody response to the intact A^a molecule do, however, require further consideration.

Peptide-specific alloantibodies generated following priming with peptide alone are unable to cross-react with the intact A^a molecule (see Section 3.2.3). Therefore, the influence of peptide priming on the alloantibody response to the intact A^a molecule must instead relate to the ability of peptide-specific T cells to provide help to B cells that are themselves specific for the intact A^a antigen (see Figure 4.1). Thus, for a peptide to enhance the rate at which an allograft is rejected by accelerating the anti-donor alloantibody response, it must fulfil two criteria. Firstly, it must stimulate a peptide-specific T cell response and secondly, upon processing of the intact A^a molecule, B cells must present an epitope corresponding to the immunising peptide.

I therefore hypothesised that monitoring the effect of peptide priming on the kinetics of rejection of a subsequent A^a-bearing graft and on the development of the anti-A^a alloantibody response, would permit clarification of other sub-dominant epitopes that may contribute to allograft rejection.

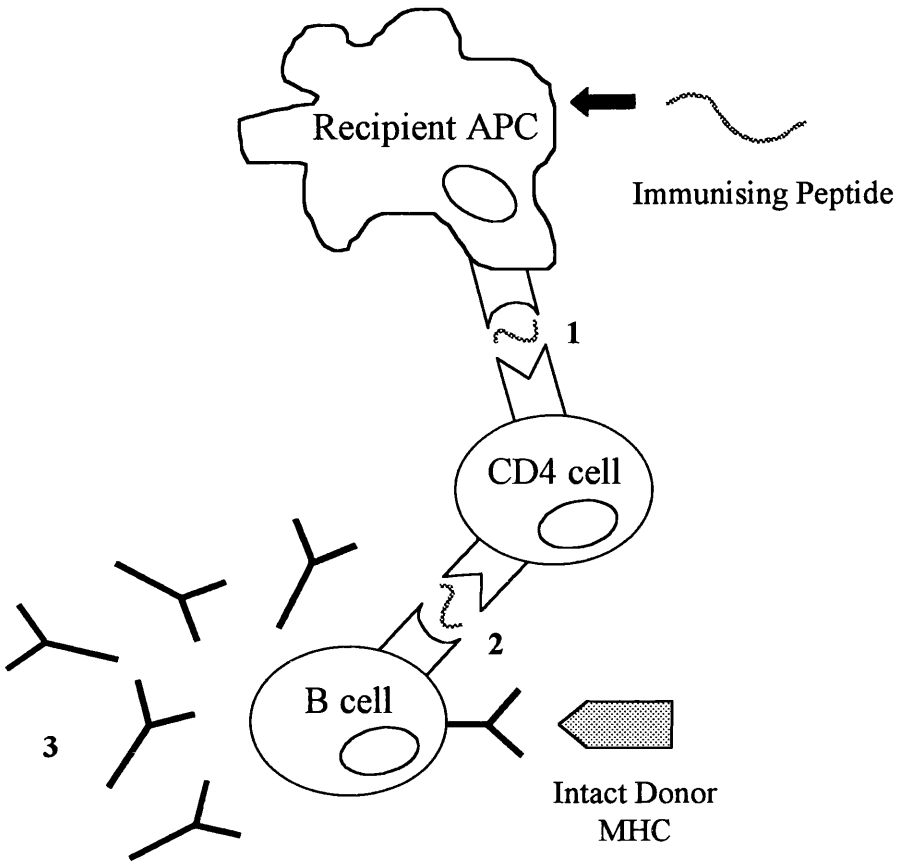

Figure 4.1: The Mechanism by which A^a Allopeptide Priming Accelerates the Alloantibody Response to the Intact A^a Molecule.

1) CD4 T cell recognises a peptide-derived epitope processed and presented by recipient APC.

2) B cells present the same epitope upon processing of the intact donor A^a molecule, thereby eliciting cognate T cell help for accelerated alloantibody production (3).

Adapted from Fabre, 1996.

4.2 *The Immune Response to an R8 Blood Transfusion*

In the following experiments, $RT1^u$ animals were immunised with individual allopeptides to assess their ability to activate T cells capable of providing more rapid help to B cells for an accelerated anti-A^a alloantibody response upon challenge with an R8 blood transfusion. As well as examining the development of the cytotoxic alloantibody response, the IgM and IgG2b responses were also examined. IgM is the first Ig isotype to be produced during an immune response, but as the response progresses, heavy chain class switching to IgG occurs. It has been demonstrated that IgG2b is the most effective IgG subclass for mediating complement-dependent lysis in the rat (Hughes-Jones et al 1983) and has been demonstrated to effect allograft rejection in the R8 to $RT1^u$ strain combination (Gracie et al 1996, Pettigrew et al 1998). Consequently, I decided to examine the development of both the IgM and IgG2b responses, to examine whether allopeptide priming was associated with an accelerated IgM and/or an accelerated IgG2b response.

4.2.1 *Alloantibody Analyses*

$RT1^u$ animals were immunised subcutaneously with 100μg of peptide emulsified in CFA 7 days before challenge with an R8 blood transfusion. Serum samples taken on days 4, 7, 10 and 12 after blood transfusion were assessed for donor specific cytotoxic, IgM and IgG2b alloantibody content.

Figure 4.2 illustrates that priming with the dominant A^a peptide, P7, significantly heightens the cytotoxic alloantibody response to a subsequent A^a-bearing R8 blood transfusion in comparison to the response mounted by control animals immunised with an R8 blood transfusion alone. Figure 4.3 illustrates the development of the A^a-specific cytotoxic alloantibody response in animals immunised with P7, P1 or the irrelevant peptide. The two latter peptides were chosen to examine whether immunisation with the dominant peptide could enhance the $RT1^u$ cytotoxic alloantibody response to an R8 blood transfusion in comparison to the response generated by animals immunised with the possible sub-dominant peptide, P1 (see Figure 3.3), or the control irrelevant peptide. Interestingly, the kinetics of the

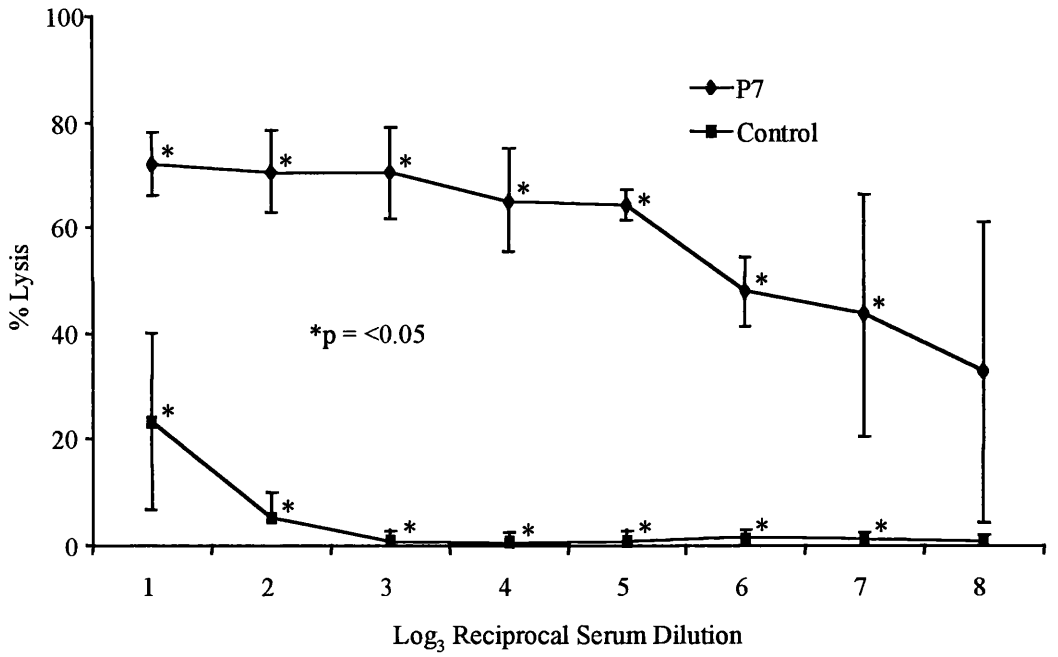

Figure 4.2: Cytotoxic Alloantibody Production to an R8 Blood Transfusion.

RT1^u rats were immunised subcutaneously with 100μg of P7 emulsified in CFA and challenged 7 days later with an R8 blood transfusion. Control animals received an R8 blood transfusion alone. Serum samples taken on day 4 after blood transfusion were assessed for cytotoxic alloantibody content using a standard ^{51}Cr-release assay against R8 lymphoblast target cells. Results are the mean and standard deviation of 3 animals per group. Statistical analysis was performed using the Mann Whitney U test. Only significant results are indicated.

response were markedly accelerated in those animals immunised with both P1 and P7. Four days after blood transfusion it can be seen that priming with P7 induced a significantly greater cytotoxic alloantibody response than pre-immunisation with either P1 or the irrelevant peptide (*p = <0.05). Similarly, P1-immunised animals mounted a significantly greater antibody response than animals primed with the irrelevant peptide (**p = 0.048). Experiments were repeated for each of the individual 15-mer peptides, and results expressed as the final dilution of serum at which donor cell lysis was greater than or equal to 20%. The results for all peptides were charted on composite graphs (Figures 4.4a and 4.4b). Figure 4.4a confirms that pre-immunisation with P7 results in significantly higher levels of circulating

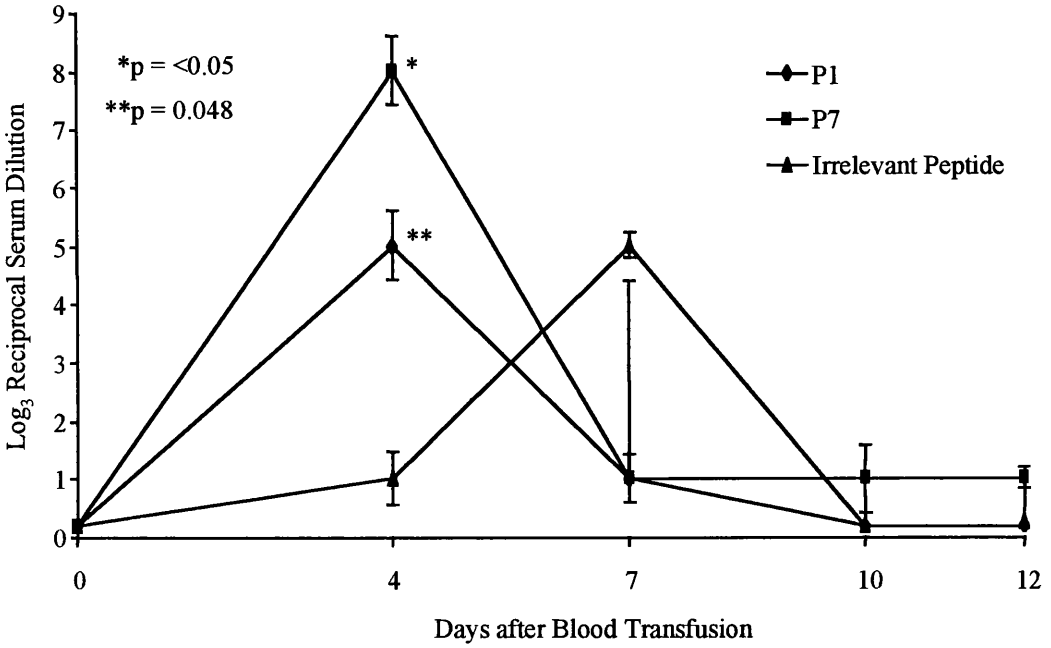

Figure 4.3: Development of the Cytotoxic Alloantibody Response to an R8 Blood Transfusion Following Subcutaneous Immunisation with Peptide.

$RT1^u$ rats were immunised subcutaneously with 100mg of peptide emulsified in CFA and challenged 7 days later with an R8 blood transfusion. Serum samples taken at days 4, 7, 10 and 12 after blood transfusion were assessed for cytotoxic alloantibody content using a standard ^{51}Cr-release assay against R8 lymphoblast target cells. Results are expressed as the final dilution of serum at which target cell lysis was greater than or equal to 20%. Results are the mean and standard deviation of 3 animals per group. Statistical analysis was performed using the Mann Whitney U test. Only significant results are indicated.

* Indicates that the alloantibody response generated following immunisation with P7 is significantly greater than that induced by priming with either P1 or the irrelevant peptide.

** Indicates that the alloantibody response mounted following P1-immunisation is significantly greater than that mounted following immunisation with the irrelevant peptide.

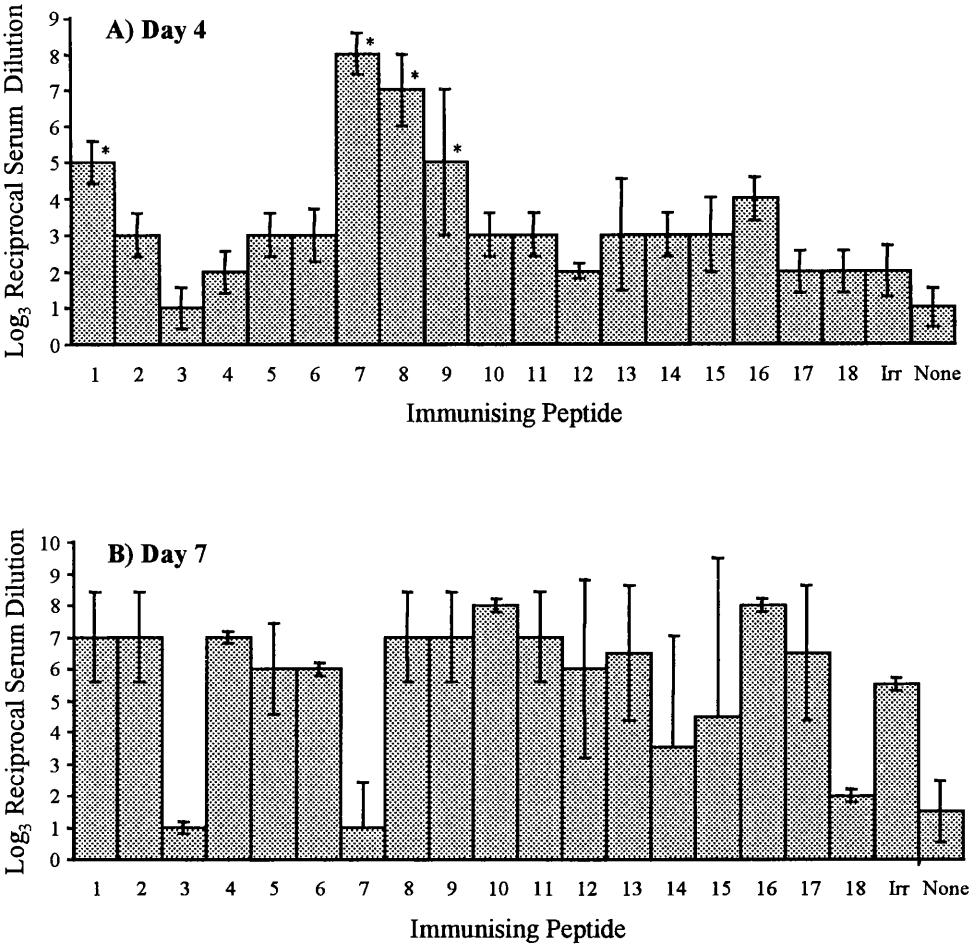

Figure 4.4: Cytotoxic Anti-A^a Alloantibody Production Following Immunisation with Allopeptide and Challenge with an R8 Blood Transfusion.

RT1^u rats were immunised with 100μg of peptide emulsified in CFA and challenged 7 days later with an R8 blood transfusion. Control animals received an R8 blood transfusion alone. Serum samples taken on A) day 4 and B) day 7 after blood transfusion were assessed for cytotoxic alloantibody content using a standard ^{51}Cr-release assay against R8 lymphoblast target cells. Results are expressed as the final dilution of serum at which target cell lysis was greater than or equal to 20%, and are the mean and standard deviation of 2-3 animals per group. Statistical analysis was performed using the Mann Whitney U test. Only significant results are indicated.

* Indicates that alloantibody levels are significantly higher than those produced in response to an R8 blood transfusion alone ($p = <0.05$).

lymphocytotoxic alloantibody 4 days after a blood transfusion in comparison to animals that received an R8 blood transfusion alone ($p = 0.026$). However, pre-immunisation with other peptides, in particular P8 ($p = 0.028$), and to a lesser extent P1 ($p = 0.049$), P9 ($p = 0.028$) and P16 (not significant), was also associated with the development of a more rapid anti-A^a alloantibody response than was seen in control animals. It is noteworthy that the sequences of P1 and P9 correspond to regions of the A^a molecule that are identical in sequence to the corresponding region of the A^u molecule, and are therefore self peptides in the $RT1^u$ rat strain (see Figure 3.1b).

Figure 4.4b demonstrates that by day 7 after blood transfusion, the increased day 4 cytotoxic alloantibody responses to the A^a molecule that were seen following immunisation with P1, P7, P8, P9 and P16 were no longer apparent. This suggests that following priming with immunogenic peptides, the overall strength of the anti-A^a response does not necessarily change, but rather that the kinetics of its development are accelerated. i.e. the cytotoxic antibody response at day 7 following pre-immunisation with P7 is lower than that in animals primed with the other peptides (Figure 4.4b), but this is due to the earlier surge in alloantibody not being sustained. That it is mainly the kinetics of the antibody response that are accelerated, rather than the magnitude is also suggested by the results shown in Figure 4.3.

To determine the chronological aspects of the anti-A^a alloantibody response more fully, the isotype subclasses of antibody were also examined at days 10 and 12 following blood transfusion, as previous work in this laboratory examining the alloantibody response to a soluble A^a class I MHC product has suggested that heavy chain class switching from IgM to IgG occurs by day 12 (G. Pettigrew – personal communication). Thus, by examining individual antibody isotypes, I hoped to ascertain whether heavy chain class switching, in addition to the cytotoxic alloantibody response, also occurred more rapidly following peptide priming.

Figure 4.5a depicts the IgM response to the intact A^a antigen upon priming with the dominant peptide (P7), the presumed sub-dominant peptide (P1) or the control irrelevant peptide. This graph confirms that priming with P7 significantly accelerated the kinetics of the IgM response in comparison to that seen in control

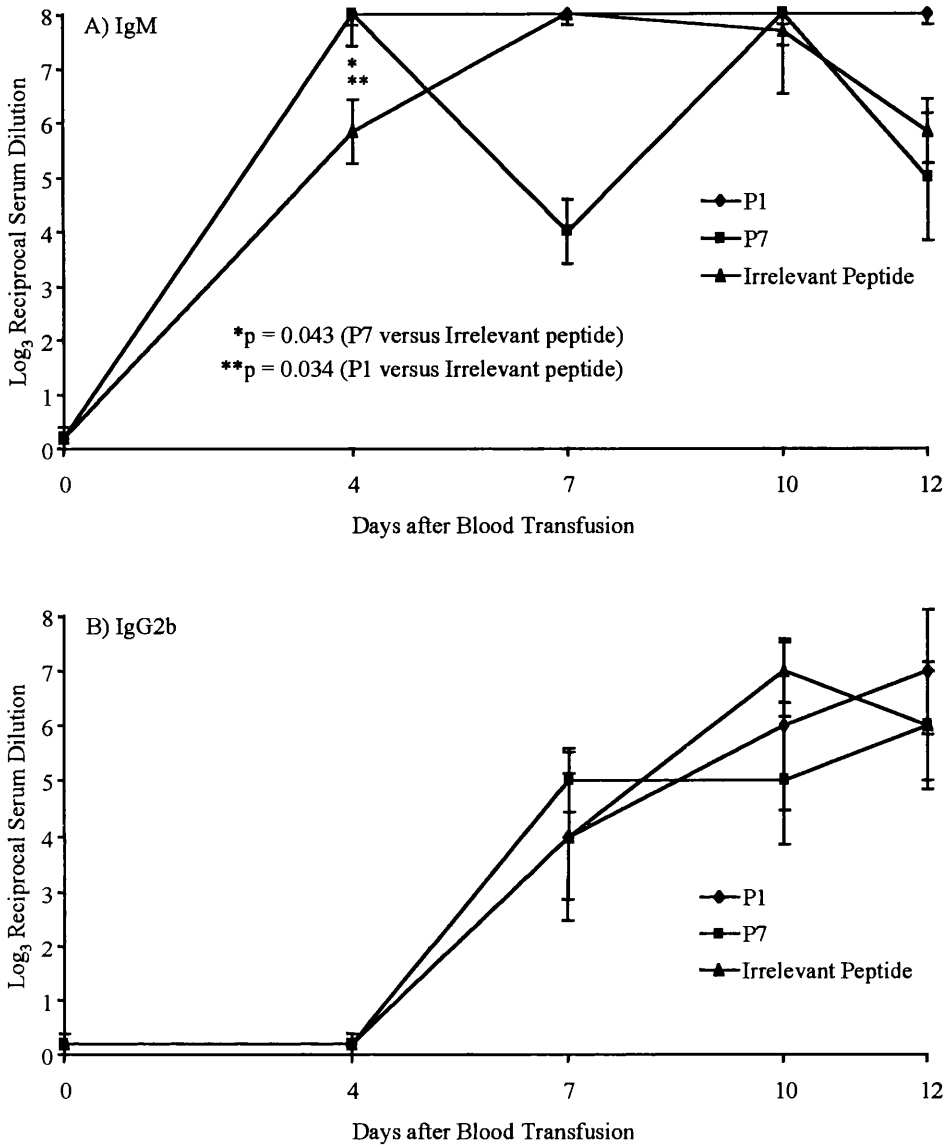

Figure 4.5: The A^a-Specific IgM and IgG2b Alloantibody Responses Following Subcutaneous Immunisation with Allopeptide and an R8 Blood Transfusion.

RT1^u rats were immunised subcutaneously with 100μg of P1, P7 or the irrelevant peptide emulsified in CFA and challenged 7 days later with an R8 blood transfusion. Serum samples taken on days 4, 7, 10 and 12 after blood transfusion were assessed for A) IgM and B) IgG2b alloantibody content by Flow Cytometry, using R8 LNCs as target cells and FITC-conjugated anti-Ig mAbs. Results are expressed as the final dilution of serum at which antibody levels were greater than or equal to twice background values, with background representing the level of fluorescence observed with cells and antibody alone. Results are the mean and standard deviation of three animals per group. Statistical analysis was performed using the Mann Whitney U test. Only significant results at day 4 are indicated.

animals (*p = 0.043). Priming with P1 resulted in an equally rapid antibody response at day four (**p = 0.034) to that observed upon priming with P7. Figure 4.5b demonstrates the kinetics of the IgG2b response: IgG2b was first detected at day 7, but there was little difference in the levels of alloantibody observed in the three groups of animals. These experiments illustrate that the characteristics, and in particular the strength of the overall IgM and IgG2b alloantibody responses in these different experimental groups is broadly similar, and that the principal difference upon priming with immunogenic peptides is in the day 4 IgM levels.

4.3 *The Immune Response to an R8 Cardiac Allograft*

I next wished to examine whether the pattern of sub-dominant epitopes, as revealed by the above blood transfusion experiments, was equally relevant in the immune response to a fully vascularised cardiac allograft, and to confirm that peptides were able to sensitise for accelerated graft rejection. The above experiments were therefore repeated, but after priming with individual peptides, $RT1^u$ animals were challenged with an R8 heart graft rather than a blood transfusion. In this series of experiments, P7 and P8 were chosen as representing the dominant epitopes from the A^a molecule, P4 and P12 as negative controls and P1 as a potential sub-dominant epitope (see Figure 4.4a).

4.3.1 *Cytotoxic Alloantibody Production*

The levels of circulating anti-A^a alloantibody in day 4 serum samples from $RT1^u$ rats immunised with peptide and challenged with an R8 cardiac allograft, are shown in Figure 4.6. At day 4 after grafting, animals that were pre-immunised with either P4 or P12 produced lower levels of lymphocytotoxic antibody than control animals. Priming with P1 before heart grafting however resulted in slightly increased levels of alloantibody. Pre-treatment with P8 and, in particular, P7, stimulated a significantly elevated alloantibody response in comparison to control animals ($p = <0.05$). These results reflect a similar pattern to that observed towards a blood transfusion following peptide immunisation (see Figure 4.4a).

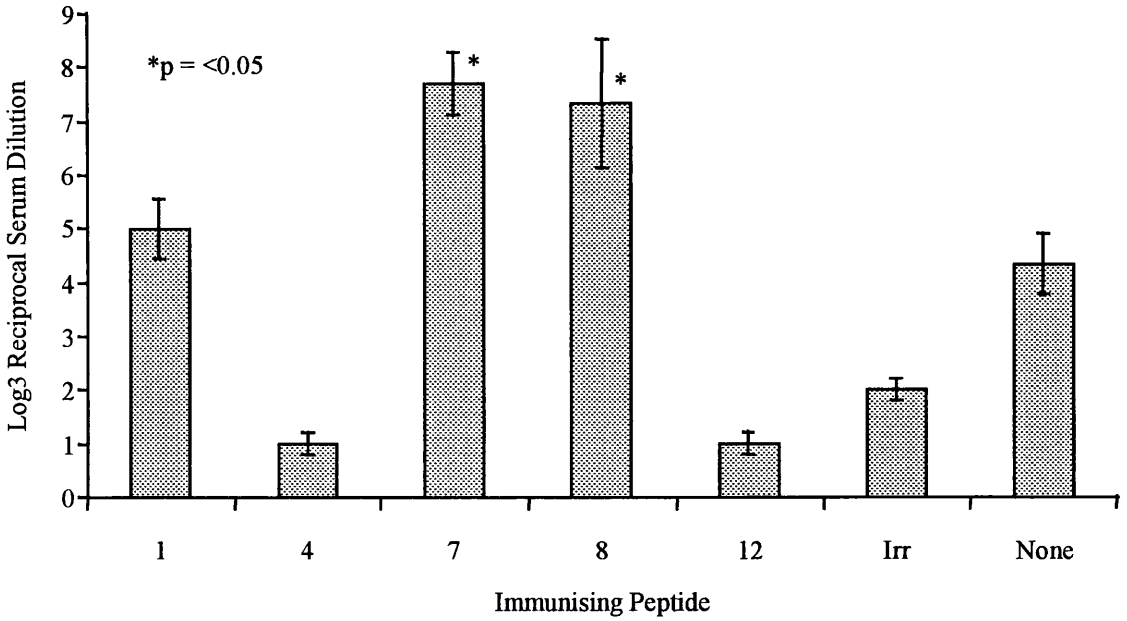

Figure 4.6: Cytotoxic Anti-A^a Alloantibody Production Following Immunisation with Allopeptide and Challenge with an R8 Cardiac Allograft.
RT1^u rats were immunised subcutaneously with 100μg of peptide emulsified in CFA and challenged 7 days later with an R8 cardiac allograft. Control animals received an R8 cardiac allograft alone. Serum samples taken on day 4 after heart grafting were assessed for cytotoxic alloantibody content using a standard ^{51}Cr-release assay against R8 lymphoblast target cells. Results are expressed as the final dilution of serum at which target cell lysis was greater than or equal to 20%. Results are the mean and standard deviation of 2-3 animals per group. Statistical analysis was performed using the Mann Whitney U test. Significantly enhanced antibody responses, in comparison with control responses, are indicated.

4.3.2 *Allograft Rejection*

Table 4.1 demonstrates the rejection times of R8 cardiac allografts by modified and unmodified RT1^u recipients. Transplanted hearts were rejected by unmodified recipients with an MST of 7 days (Group 1). Groups 2-7 illustrate the fate of grafts transplanted into recipients pre-immunised with different allopeptides 7 days before grafting. Pre-treatment with P4, P12 or the irrelevant peptide (Groups 3, 6 and 7 respectively) had little or no effect upon the outcome of graft survival. Priming with P1 (Group 2) or P8 (Group 5) resulted in significantly accelerated rates of rejection in comparison to that seen in control animals ($p = 0.006$, $p = 0.014$ respectively),

Table 4.1: The Rejection of MHC Class I-Disparate PVG-R8 Cardiac Allografts by PVG-RT1^u Recipients.

Group	Pre-treatment[a]	n	Graft Survival[e] (days)	MST
1	None	6	6,6,7,7,7,7	7
2	P1	4	5,5,5,5	5
3	P4	3	6,7,7	7
4	P7	5	4,4,4,4,4	4
5	P8	3	4,5,5	5
6	P12	3	7,7,7	7
7	Irrelevant Peptide	3	6,7,7	7
8	All 18 Peptides[b]	4	4,4,4,4	4
9	CFA Alone	3	5,6,6	6
10	R8 Blood Transfusion[c]	3	1,1,1	1
11	R8 Skin Graft[d]	4	1,1,1,1	1

[a] Recipient RT1^u animals were immunised subcutaneously with 100μg of peptide emulsified in CFA 7 days before receiving an A^a-disparate cardiac allograft.

[b] Recipient animals were immunised with a cocktail of all 18 peptides (100μg of each peptide) emulsified in CFA 7 days before allografting.

[c] Recipient animals were immunised with 1.5mls of R8 blood 7 days before heart grafting.

[d] Recipient rats were immunised by the application of a full thickness R8 skin allograft 7 days before heart grafting.

[e] Animals were assessed daily, and allograft rejection was defined as the complete cessation of myocardial contraction.

with grafts rejected 5 days after transplantation. Pre-treatment with P7 (Group 4) however, resulted in the most rapid rate of rejection, with grafts surviving for just 4 days ($p = 0.003$).

Another group of animals (Group 8) were primed with a cocktail of all 18 allopeptides, to examine the effect that administration of all potential T cell epitopes afforded by the peptides had upon graft survival. As a further comparison, groups of RT1^u animals were pre-immunised with the intact A^a molecule, either in the form of an R8 blood transfusion or skin graft (Groups 10 and 11 respectively). It was expected that the administration of all 18 peptides would result in maximal stimulation of the indirect pathway. Rejection kinetics in this group however, were the same as seen following priming with P7 alone, which suggests that sole administration of the dominant peptide achieves the same level of enhancement of the indirect pathway. In comparison, those animals primed with an R8 blood

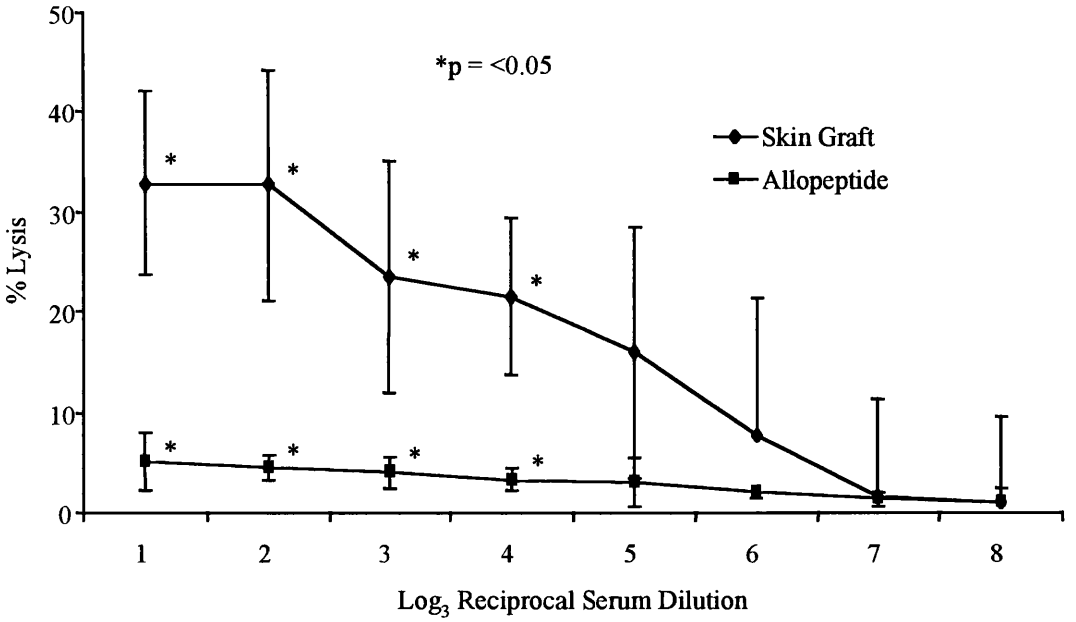

Figure 4.7: Cytotoxic Anti-A^a Alloantibody Production Following Immunisation with an R8 Skin Graft or R8 Allopeptides. RT1^u rats were immunised with a full thickness R8 skin graft or a mixture of all 18 allopeptides emulsified in CFA subcutaneously (100μg of each peptide). Serum samples taken on day 7 after treatment were assessed for cytotoxic alloantibody content using a standard ^{51}Cr-release assay against R8 lymphoblast target cells. Results are the mean and standard deviation of 4 animals per group. Statistical analysis was performed using the Mann Whitney U test. Only significant results are indicated.

transfusion or skin graft rejected their cardiac allografts significantly more rapidly, within 24hrs of grafting ($p = 0.014$, $p = 0.008$ respectively). This finding may suggest that mechanisms other than the indirect T cell alloresponse are primed. Analysis of the cytotoxic alloantibody 7 days after peptide priming or skin grafting, i.e. the day of cardiac transplantation, revealed that only skin grafting resulted in the generation of an anti-A^a cytotoxic alloantibody response that was able to cross-react with the antigens of the heart graft (Figure 4.7). Therefore, it is likely that the presence of pre-formed antibody, through its ability to provoke hyperacute rejection, rather than any intrinsic difference in the mode of T cell activation, is accountable for the differences observed in graft rejection times by recipients primed with allopeptide and an R8 skin graft or blood transfusion.

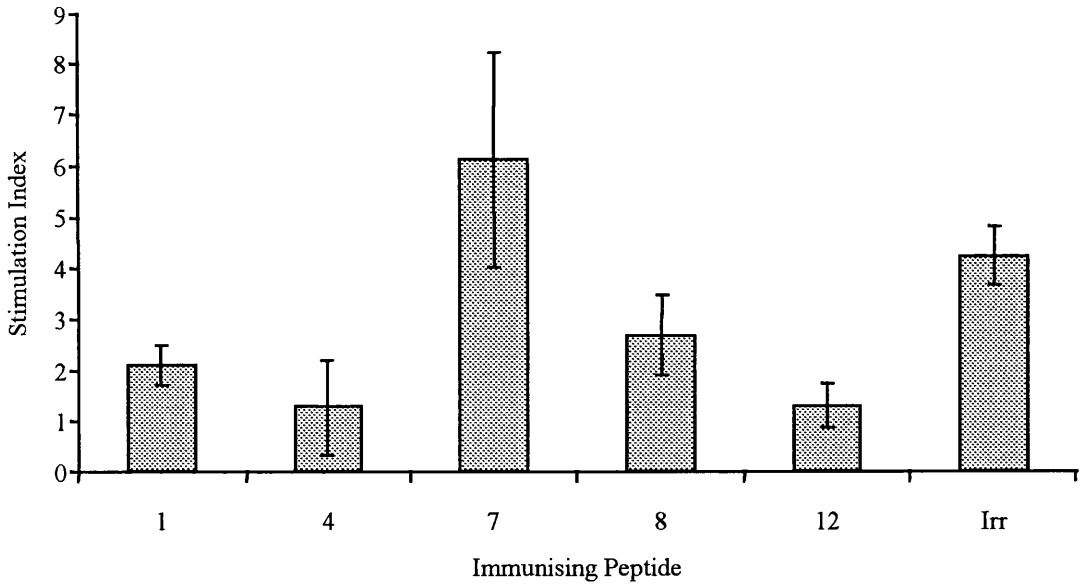

Figure 4.8: Specific *In Vitro* T Cell Proliferative Responses of RT1^u Animals Immunised with Allopeptide and Challenged with an R8 Cardiac Allograft.

RT1^u rats were immunised subcutaneously with 100μg of peptide emulsified in CFA and challenged 7 days later with an R8 cardiac allograft. LNCs were used in T cell proliferation studies 7 days after transplantation. Cells were cultured for 72hrs with individual allopeptides at 40μg/ml, and pulsed with ^{3}H-Thymidine for an additional 24hrs before harvesting. LNCs from naïve RT1^u rats were treated in the same manner to obtain background levels of proliferation. Results are expressed as the mean and standard deviation of 3-6 animals per group.

4.3.3 *T Cell Proliferation*

The initial T cell response to the intact A^a molecule focuses to a single dominant determinant located within the hypervariable region of the α1 domain (see Figure 3.7). However, pre-immunisation with sub-dominant peptides results in a T cell response that can influence both the antibody response to and the rejection kinetics of R8 cardiac allografts. One can postulate that the response to the dominant epitope following priming with sub-dominant epitopes will either be unaffected, or may alternatively be downregulated. The latter would be expected to occur if the same mechanisms that are responsible for limiting the initial T cell alloresponse to the dominant determinant in naïve animals are instead focused onto the immunising sub-dominant epitope. Such alteration in the hierarchy of dominance by peptide priming

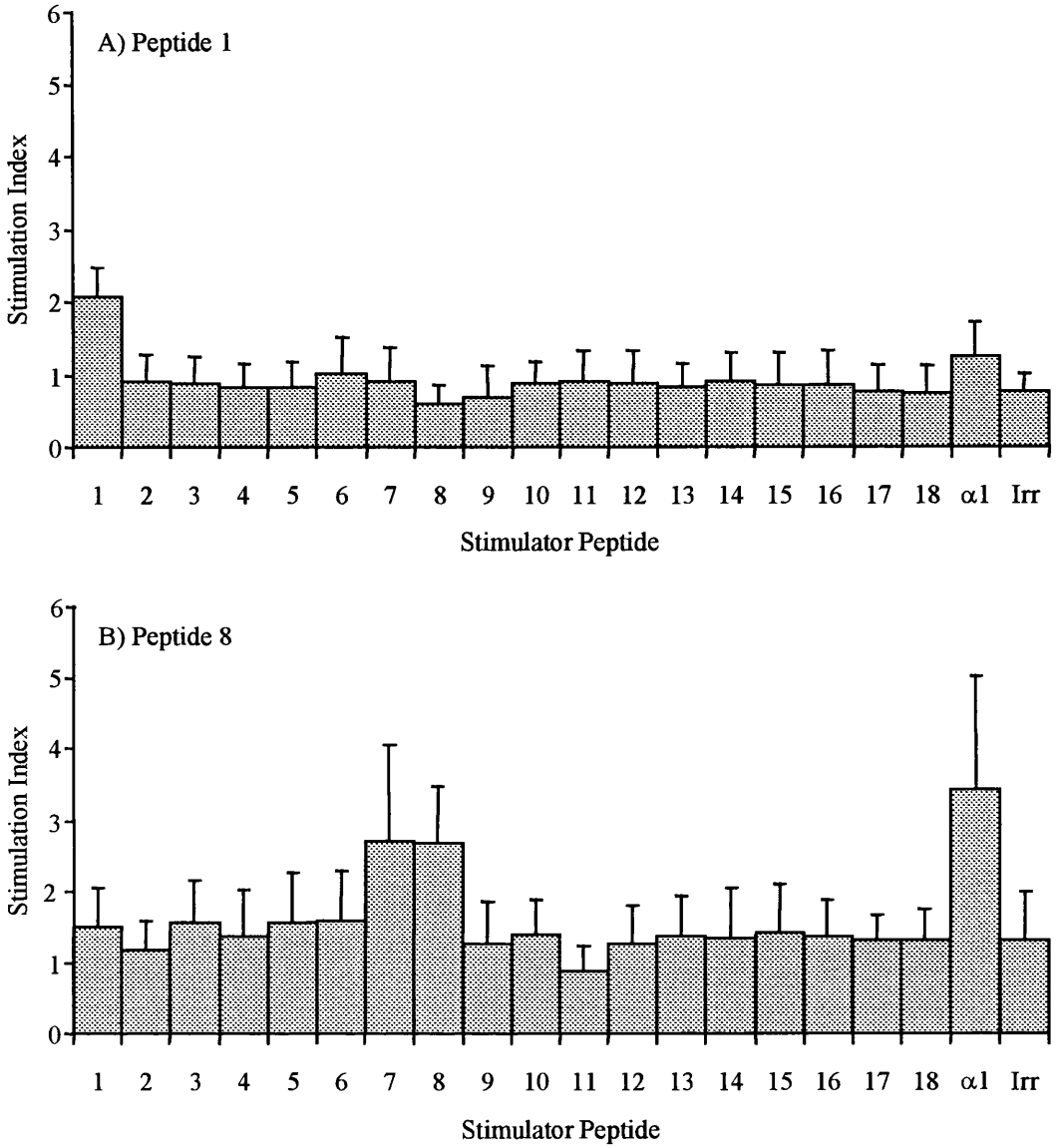

Figure 4.9: *In Vitro* T Cell Proliferation of LNCs from RT1^u Animals Primed with P1 or P8 and Challenged with an R8 Cardiac Allograft.

RT1^u rats were immunised with 100μg of A) Peptide 1 or B) Peptide 8 emulsified in CFA 7 days before challenge with an R8 cardiac allograft. LNCs were used in T cell proliferation studies 7 days after transplantation. Cells were cultured for 72 hrs with individual allopeptides at 40μg/ml, and pulsed with ^{3}H-Thymidine for an additional 24hrs before harvesting. LNCs from naïve RT1^u rats were cultured in a similar manner to obtain background levels of proliferation. Results are the mean and standard deviation of 3-6 animals per group.

has been reported for autoimmune responses (Benichou et al 1994b). The proliferative responses to both the immunising peptide and to P7 were therefore examined to see which of these hypotheses were correct. Figure 4.8 demonstrates that generally, T cell proliferation can be detected to those peptides that stimulate an accelerated alloantibody response and accelerated graft rejection (see also Figure 4.6 and Table 4.1). However, the proliferative response does not completely switch from P7. Often, as illustrated in Figure 4.9, responses to both the immunising peptide and P7 are detected. These proliferative responses therefore reflect more accurately the *in vivo* effects of the peptides than earlier T cell proliferation experiments (see Figures 3.3 and 3.7). This could be a result of double priming (i.e. allopeptide followed by a heart), or is perhaps due to the slightly longer time interval between initial priming and cell harvest for proliferation.

4.4 *Discussion*

Priming with four of the 15-mer allopeptides other than the dominant peptide, P7 accelerated the antibody response upon challenge with an R8 blood transfusion (Figure 4.4a). The first of these peptides, P8 stimulated a very strong response. It is unclear however, as to whether the epitope contained within P8 is totally different to that within P7, or whether the optimal dominant epitope of the A^a molecule spans these two peptides. Three other peptides stimulated an anti-A^a response, P1, P9 and P16 and these can therefore be classed as sub-dominant (Sercarz et al 1993) and it is likely that the epitopes that they contain are presented by B cells upon processing of the intact A^a antigen. It is not surprising that P16, which is derived from the hypervariable region of the $\alpha 2$ domain, can influence the response to the intact A^a molecule. However, that P1 and P9, which are in effect self $RT1^u$ peptides, can also contribute to the immune response was unexpected. This suggests that not all potentially autoreactive T cells are deleted during the development of the $RT1^u$ T cell repertoire, with some escaping from the thymus and maintained in an anergic state by peripheral suppressor mechanisms. It is likely that the stimulus provided by priming with peptide in CFA results in presentation of epitopes by professional APCs that provide co-stimulatory signals of a level able to reverse the anergic state of the autoreactive T cells. This phenomenon is not unprecedented, with Fedoseyeva et al (Fedoseyeva et al 1996) recently documenting the activation of autoreactive T cells following allografting.

These results offer an insight into the nature of antigen processing and presentation by B cells, and in particular, how this affects the indirect response to the A^a antigen. That priming with sub-dominant epitopes enhances the antibody response to the intact A^a molecule suggests firstly, that B cell processing of the intact A^a antigen results in the presentation of peptide fragments that correspond to the sub-dominant epitopes, and secondly, that T cells specific for the sub-dominant epitopes exist. In the current study however, it appears that only a single dominant epitope is normally involved in the T cell response to the A^a molecule in naïve animals (see Figure 3.7). Therefore, the T cell response to the sub-dominant epitopes must, at least initially,

be inhibited, with only those T cells specific for the dominant epitope undergoing activation and proliferation. Figure 4.9 would suggest however, that the hierarchical pattern of dominance can be altered by pre-immunisation with sub-dominant peptides, and that in addition to those T cells specific for the dominant region of the A^a molecule, T cells specific for the immunising peptide are also able to undergo activation and proliferation.

Antibody isotype analysis (Figures 4.5a and 4.5b) suggested that the accelerated alloantibody response to an R8 blood transfusion following peptide immunisation was, within the constraints of the assay, solely of the IgM subclass. It was additionally demonstrated that peptide priming could potentially result in the accelerated rejection of R8 cardiac allografts to just 4 or 5 days. Therefore, by extrapolation of the results obtained from the blood transfusion experiments, it is unlikely that an IgG2b response would be detected at this point. Moreover, the strength and kinetics of the IgG2b response, which has previously been demonstrated as the most effective IgG subclass for mediating complement-dependent red cell lysis (Hughes-Jones et al 1983), are unaltered by peptide immunisation. This is somewhat surprising, as one would naturally expect that the more rapid IgM response associated with peptide priming would also result in earlier heavy chain class switching. The reason for its failure to do so is not readily obvious, but may reflect the fact that different levels of T cell help are required during different phases of the antibody response. Agarwal et al (Agarwal et al 1996, Agarwal et al 1997) have suggested that the process of isotype switching requires a greater level of T cell help than the initial IgM response. Consequently, at this point the helper T cell response is more stringently focused towards those T cell clones with sufficiently high affinity for the epitopes that B cells present. Similar results have been observed in a mouse skin graft model (Steele et al 1996). MHC class II-deficient recipients, which are only able to provide limited CD4 T cell help for antibody production against an allogeneic skin graft, mounted an efficient IgM response, but isotype switching did not occur. These observations may offer an explanation for my findings if one assumes that peptide priming augments the

amount of T cell help available for the IgM response, but not to an extent sufficient to induce isotype switching.

It is of interest that priming with peptide, regardless of whether it is dominant or sub-dominant, appears to have a similar effect on the IgM response to the A^a molecule (see Figure 4.5a), and more importantly, on the kinetics of allograft rejection (see Table 4.1). Furthermore, immunisation with a mixture of all 18 peptides does not result in a rate of rejection that is any more rapid than that seen upon immunisation with P7 alone, suggesting that immunisation with a single peptide can achieve maximal stimulation of the indirect pathway. In the same strain combination as that used in this study, Wang et al have used an alternative approach to achieve maximal stimulation of the indirect pathway (Wang et al 1997). $RT1^u$ animals were immunised with the complete α heavy chain of the $RT1.A^a$ antigen, a protocol that resulted in an acceleration in graft rejection times similar to that observed upon administration of all 18 peptides in my own experiments (i.e. grafts survived for just 4 days). It was reasoned that the full length heavy chain would be processed such that the optimal epitopes involved in the indirect response would be generated, but that a direct T cell response would not occur since the α heavy chain was not associated with β_2-microglobulin (which is required for correct conformational folding of MHC class I). In comparison, priming with either an R8 skin graft or blood transfusion resulted in much more rapid rejection of a subsequent R8 cardiac allograft. Work from our own laboratory has suggested that this is not necessarily due to more efficient activation of the indirect pathway, but rather to the presence of conformational B cell epitopes that result in the generation of an anti-A^a alloantibody response that effects hyperacute, humoral rejection of the subsequent cardiac allograft (Pettigrew et al 1998).

CHAPTER 5:

THE IMMUNE RESPONSE TO THE INTACT A^a MOLECULE FOLLOWING INTRAVENOUS IMMUNISATION WITH ALLOPEPTIDE

CONTENTS PAGE

CHAPTER 5:

THE IMMUNE RESPONSE TO THE INTACT A^a MOLECULE FOLLOWING INTRAVENOUS IMMUNISATION WITH ALLOPEPTIDE

5.1 *Introduction*

The general relevance of dominant and sub-dominant epitopes in the design of tolerogenic strategies remains unclear. There have, however, been several examples of tolerance induction where not every conceivable donor T cell determinant was incorporated into the tolerisation protocol. This has been clearly demonstrated in rodent models of allograft rejection where the donor and recipient are mismatched at more than one MHC locus. For example, Wong et al have demonstrated that the intravenous administration of bone marrow cells from transgenic CBK ($H2^k$ + $H2^b$) mice into CBA.Ca ($H2^k$) recipients not only results in the long term survival of subsequent CBK heart grafts, but also of all cardiac allografts bearing the K^b antigen, irrespective of the presence of additional mismatched MHC antigens (Wong et al 1997). Similar results have also been seen with minor histocompatibility antigens in a mouse model of skin graft rejection (Davies et al 1996). The above studies introduce the concept of "linked epitope suppression", whereby tolerance induced to a single foreign epitope before allografting can spread to include additional mismatched antigens present on a subsequently transplanted organ. The mechanisms responsible for this effect have not been established, but it is notable that the additional antigens to which tolerance spreads must be present on the same graft as the initial suppressor epitope, rather than upon a second concurrently applied graft (Davies et al 1996, Wong et al 1997). It is therefore likely that linked epitope suppression involves the presentation of both the suppressor and the additional mismatched antigens by the same APC (either donor or recipient).

Whilst Wong's studies were principally concerned with the spreading of tolerance to additional allo-MHC antigens, i.e. intermolecular linked epitope suppression,

intramolecular suppression can also occur. For example, it has been shown that long term survival of fully mismatched allografts can be achieved using tolerogenic protocols that employ peptides from limited stretches of the donor MHC molecule. Sayegh et al demonstrated that the intrathymic injection of a mixture of 8 synthetic 25-mer allopeptides, representing the full length Wistar Furth (WF, $RT1^u$) $RT1.B^u\beta$ and $RT1.D^u\beta$ sequences, into Lewis ($RT1^l$) recipients, resulted in the permanent survival of subsequent WF renal allografts (Sayegh et al 1993). Moreover, although administration of either the four $RT1.B^u\beta$ or four $RT1.D^u\beta$ peptide mixtures alone had no effect upon graft survival (Sayegh et al 1993), injection of the four immunogenic peptides (2 $RT1.B^u\beta$ and 2 $RT1.D^u\beta$ peptides) was sufficient to achieve long-term graft survival (Sayegh et al 1994). In addition, Chowdhury et al have shown that long term survival of WF cardiac allografts in ACI ($RT1^a$) recipients can be achieved by intrathymic injection of a single immunogenic peptide (of 17 amino acids) derived from the $\alpha 2$ domain of the donor $RT1.A^u$ molecule (Chowdhury et al 1998). However, in this model, tolerance was only achieved when the peptide was administered in combination with a sub-therapeutic dose of ALS. These results have important implications as they demonstrate that targeting the indirect pathway of allorecognition with only small amounts of donor antigen can achieve long-term survival of completely mismatched allografts.

Shirwan et al (Mhoyan et al 1997, Shirwan et al 1997b) have shown similar results in the PVG-R8 to PVG-1U rat strain combination, but have additionally demonstrated that peptides encompassing dominant epitopes are more effective than other immunogenic peptides at inducing allograft tolerance. The rat strains used in these latter studies are similar to those employed in this thesis. It was hoped that the more complete mapping of the dominant and sub-dominant epitopes of the $RT1.A^a$ molecule, as achieved in the previous two chapters, would permit a more thorough exploration of Shirwan's observations, thereby allowing further, more effective peptide-based tolerogenic protocols to be devised.

To test the capability of the dominant and sub-dominant A^a allopeptides to induce non-responsiveness to the intact A^a molecule, I chose to administer relatively high

doses of the peptides intravenously to $RT1^u$ recipients and monitor the effect on the alloimmune response following subsequent challenge with an R8 blood transfusion or cardiac allograft. Intravenous administration of antigen is a recognised route for the induction of non-responsiveness, and has been used both in models of autoimmunity (e.g. Leadbetter et al 1998) and transplantation (e.g. Benichou et al 1994b). Antigen is generally administered at a high dose, and the mechanism behind this "high zone" tolerance possibly results from swamping of the available professional APC repertoire, such that non-professional APCs present antigen in a tolerogenic fashion (Bishop et al 1997). Benichou et al have demonstrated that intravenous administration of dominant allopeptide sequences can result in T cell non-responsiveness in autoimmunity (Benichou et al 1994b, Tam et al 1996), and the work in this thesis was broadly based upon this experimental protocol.

5.2 *The Immune Response to an R8 Blood Transfusion*

To examine the effect of intravenous administration of a "high dose" of allopeptide on the immune response to the intact A^a molecule, $RT1^u$ animals were immunised with 300μg of the dominant (P7) or sub-dominant (P1) allopeptides in 300μl of saline 12 days before challenge with an R8 blood transfusion. Control animals were immunised in a similar manner, replacing allopeptide with the irrelevant peptide. Animals were bled for serum 4, 7, 10 and 12 days after challenge with blood to investigate the development of the cytotoxic, IgM and IgG2b alloantibody responses. In addition, LNCs were used in *in vitro* T cell proliferation studies 12 days after blood transfusion to examine the influence of peptide priming on the *in vivo* activation of T cells.

5.2.1 *Alloantibody Analyses*

Figure 5.1 illustrates the allospecific cytotoxic antibody response to an R8 blood transfusion following pre-immunisation with peptide intravenously. The alloantibody response to the intact A^a molecule, as detected by using a standard ^{51}Cr-release assay against R8 lymphoblast target cells, was markedly diminished in animals pre-treated with P1 or P7 in comparison to control rats pre-treated with the irrelevant peptide. The kinetics of the responses of control and P7-treated animals appeared to be similar however, with antibody levels peaking on day 7, and declining thereafter. It is notable that intravenous immunisation with either P1 or P7 did not result in a detectable cytotoxic alloantibody response at day 4 following blood transfusion. This is in comparison to the results observed in Chapter 4 (Figure 4.3), wherein subcutaneous immunisation with the same peptides resulted in an accelerated cytotoxic anti-A^a alloantibody response detectable 4 days after blood transfusion, which appeared to correlate with accelerated allograft rejection.

Figure 5.2 illustrates the development of the IgM response in the same three groups of animals. Again, pre-treatment with both P1 and P7 diminished levels of alloantibody production in comparison to controls, with P1 marginally more effective, but this was statistically non-significant (Figure 5.2b). The IgM response

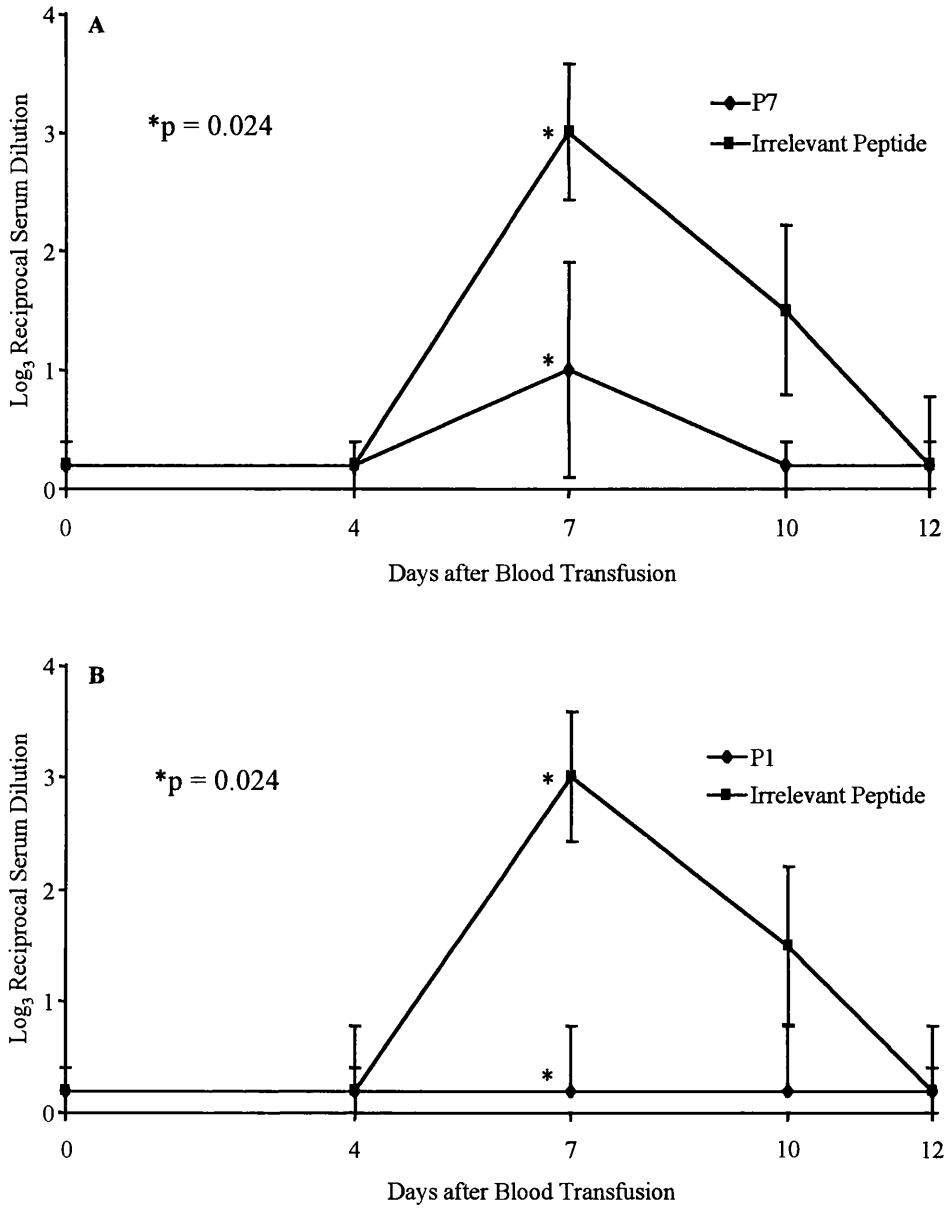

Figure 5.1: Cytotoxic Alloantibody Production to an R8 Blood Transfusion Following Immunisation with a High Dose of Allopeptide.
RT1^u rats were immunised intravenously with 300μg of A) P7, or B) P1 in 300μg of saline and challenged 12 days later with an R8 blood transfusion. Control animals received 300μg of the irrelevant peptide in saline intravenously before challenge with blood. Serum samples taken on days 4, 7, 10 and 12 after blood transfusion were assessed for cytotoxic alloantibody content using a standard ^{51}Cr-release assay against R8 lymphoblast target cells. Results are expressed as the final dilution of serum at which target cell lysis was greater than or equal to 20%, and are the mean and standard deviation of 2-6 animals per group. Statistical analysis was performed using the Mann Whitney U test. Only significant results are indicated.

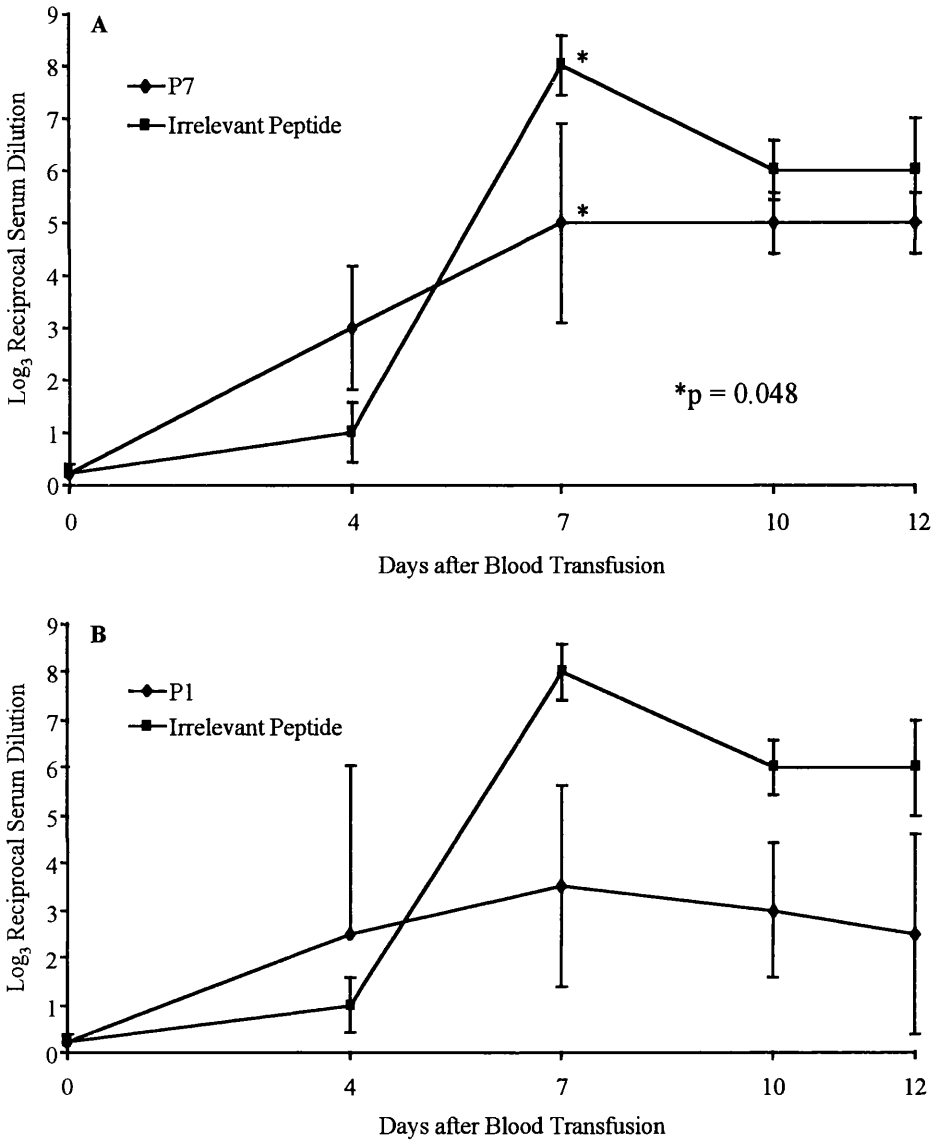

Figure 5.2: The Allospecific IgM Response to an R8 Blood Transfusion Following Immunisation with a High Dose of Allopeptide.
RT1^u rats were immunised intravenously with 300μg of A) P7, or B) P1 in 300μl of saline and challenged 12 days later with an R8 blood transfusion. Control animals were immunised with 300μg of the irrelevant peptide in saline before challenge with blood. Serum samples taken on days 4, 7, 10 and 12 after blood transfusion were assessed for IgM content by Flow Cytometry, using R8 LNCs as target cells and a FITC-conjugated anti-IgM mAb. Results are expressed as the final dilution of serum at which antibody levels were greater than or equal to twice background values, with background representing the level of fluorescence observed with cells and antibody alone. Results are the mean and standard deviation of 2-6 animals per group. Statistical analysis was performed using the Mann Whitney U test. Only significant results are indicated.

kinetics were similar in all three groups, with antibody levels peaking on day 7. Levels declined slightly from this point onwards in animals immunised with P1 or the irrelevant peptide, but remained constant in those animals immunised with P7 (Figure 5.2a). As might be expected, an IgM response was detectable as early as day 4, but notably, with little difference between groups. If anything, P1 and P7-treated animals showed a slightly greater response, but this was non-significant. This is of interest, since it was also shown in the previous chapter that the early IgM response may account for the accelerated rate of graft rejection observed in $RT1^u$ animals subcutaneously immunised with P1 or P7 in CFA. It is notable that the kinetics of the IgM response are broadly similar to those of the cytotoxic alloantibody response.

The development of the IgG2b response in the above groups of animals is shown in Figure 5.3. Pre-immunisation with a high dose of allopeptide was less effective at modifying the IgG2b response than either the cytotoxic or IgM responses. P7 was able to downregulate IgG2b levels to a certain extent (Figure 5.3a), but P1 did not appear able to alter the response (Figure 5.3b). The greatest difference in the IgG2b levels observed in the serum samples of $RT1^u$ rats treated with P7 or the irrelevant peptide was seen at day 7. From this point onwards, alloantibody levels in samples from P7-treated animals increased, until at day 12, levels were comparable to those seen in control animals.

5.2.2 *T Cell Proliferation Studies*

Having established that intravenous administration of peptide was able to downregulate the antibody response to an R8 blood transfusion, I next examined whether the T cell response, as measured by a standard *in vitro* T cell proliferation assay, was similarly downregulated. Such assays would also enable me to examine how the responses to other epitopes within the A^a molecule, particularly those established as sub-dominant, were simultaneously affected. One could hypothesise that the response to the sub-dominant epitopes may be augmented if the hierarchical pattern of dominance was switched to these epitopes as a compensatory mechanism. Alternatively, these responses may be downregulated if the principals of linked epitope suppression as discussed above are applicable. To examine whether pre-

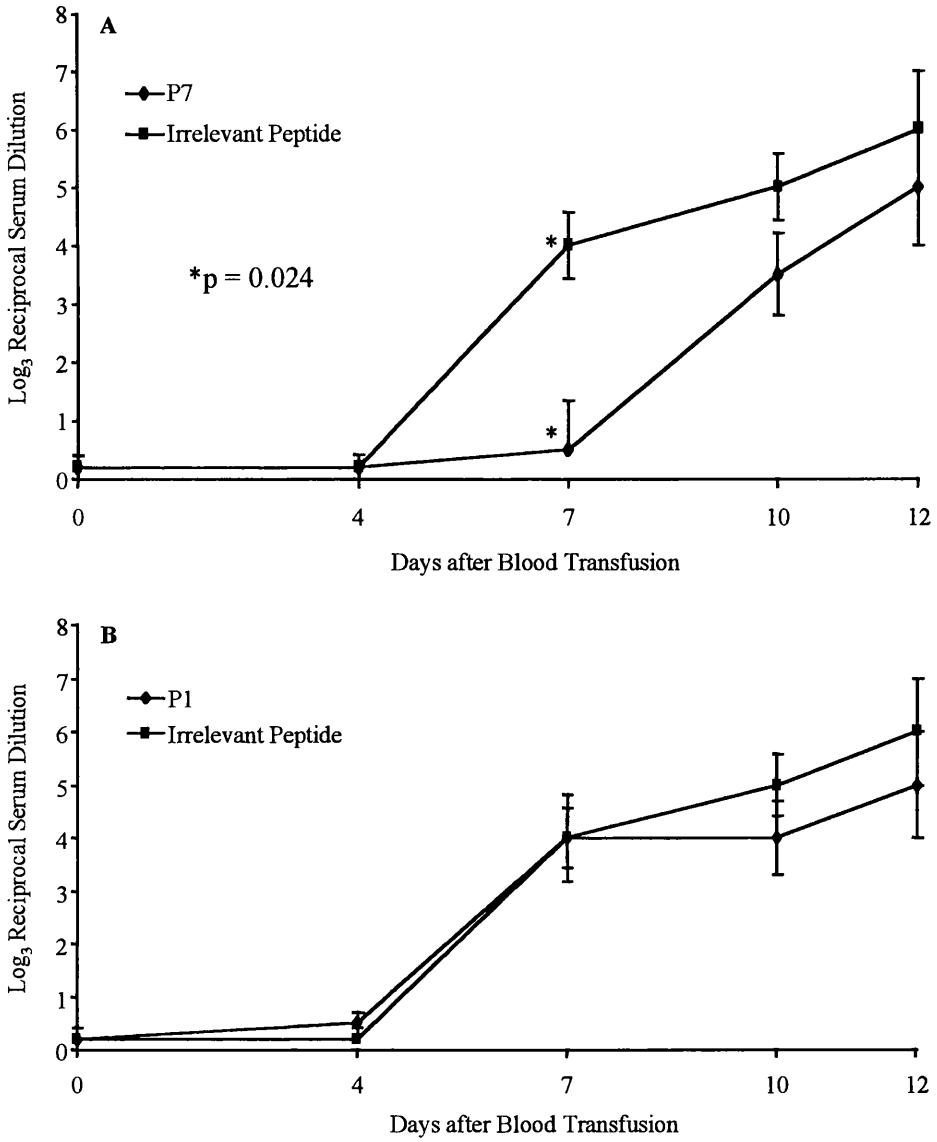

Figure 5.3: The Allospecific IgG2b Response to an R8 Blood Transfusion Following Immunisation with a High Dose of Allopeptide.
RT1^u rats were immunised intravenously with 300μg of A) P7, or B) P1 in 300μl of saline and challenged 12 days later with an R8 blood transfusion. Control animals were immunised with 300μg of the irrelevant peptide in saline intravenously before challenge with blood. Serum samples taken on days 4, 7, 10 and 12 after blood transfusion were assessed for IgG2b content by Flow Cytometry using R8 LNCs as target cells and a FITC-conjugated anti-IgG2b mAb. Results are expressed as the final dilution of serum at which antibody levels were greater than or equal to twice background values, with background representing the level of fluorescence observed with cells and antibody alone. Results are the mean and standard deviation of 2-6 animals per group. Statistical analysis was performed using the Mann Whitney U test. Only significant results are indicated.

immunisation of $RT1^u$ rats with a high dose of an allopeptide intravenously was able to influence the *in vivo* T cell response, LNCs from the above animals were used in *in vitro* T cell proliferation studies 12 days after challenge with blood. Cells were incubated with individual allopeptides at a concentration of 40μg/ml for 72hrs, and pulsed for an additional 24hrs with ^{3}H-Thymidine before harvesting. T cell proliferative responses are illustrated in Figure 5.4.

Following immunisation with the irrelevant peptide (Figure 5.4a), proliferation to the α1 peptide was observed. Likewise, all three animals in this group displayed moderately high levels of proliferation to P14. That these cells responded to P14 is difficult to explain, particularly as analysis of the amino acid sequences demonstrates that there is no obvious similarity between this peptide and the irrelevant peptide. No T cell proliferation was seen upon priming with P1 (Figure 5.4b). However, despite the apparent downregulation of alloantibody responses following pre-treatment with P7 (Figures 5.1a, 5.2a and 5.3a), the *in vitro* T cell proliferative response to P7 was slightly augmented (Figure 5.4c), as no proliferation was seen to P7 upon pre-treatment with saline alone (data not shown) or the irrelevant peptide (Figure 5.4a).

5.3 *The Immune Response to an R8 Cardiac Allograft*

I next examined whether the ability of intravenous peptide treatment to downregulate the alloantibody response to an R8 blood transfusion would equally influence the antibody responses to, and the rejection kinetics of, R8 cardiac allografts. I chose to study primarily the influence of P7 on the immune response to a subsequent heart graft, as it is the dominant A^a epitope, and therefore the most obvious candidate for peptide therapy. Groups of animals were intravenously pre-treated with either 300μg of P7, a mixture of the identified dominant and sub-dominant peptides, P1, P7, P8 and P9 (300μg of each peptide) or a cocktail of all 18 peptides (again 300μg of each peptide). Controls for animals immunised with P7 or the mixture of P1, P7, P8 and P9 were treated with saline alone, whereas controls for those rats primed with all 18 peptides were immunised with an equivalent amount of the irrelevant peptide (5.4mgs) in saline. Rats received an R8 cardiac allograft 12

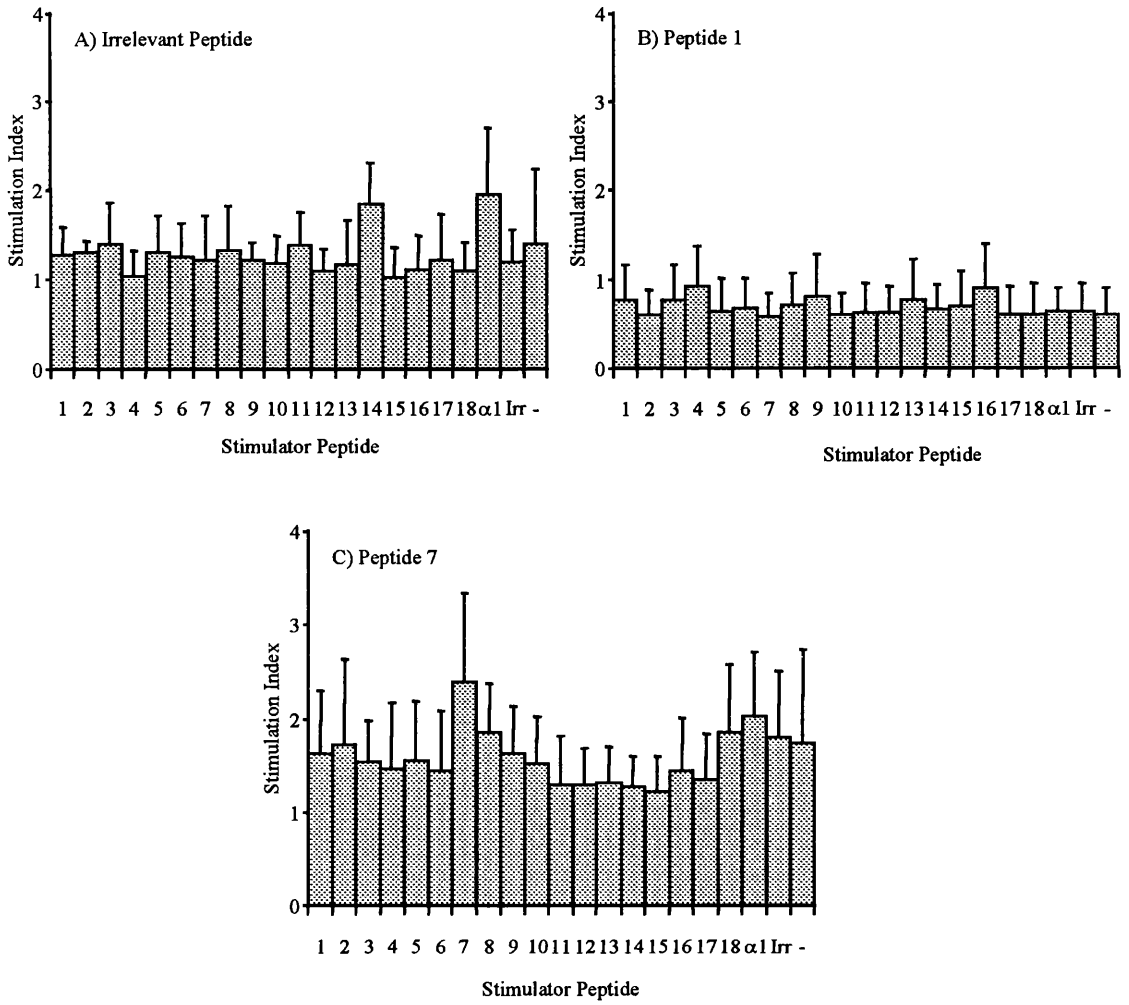

Figure 5.4: *In Vitro* Proliferation of LNCs from RT1^u Rats Immunised with a High Dose of Allopeptide Intravenously and Challenged 12 Days Later with an R8 Blood Transfusion.

RT1^u rats were immunised intravenously with 300μg of A) the irrelevant peptide, B) P1 or C) P7 in 300μl of saline and challenged 12 days later with an R8 blood transfusion. LNCs were used in T cell proliferation studies 12 days after challenge with blood. Cells were cultured for 72hrs with individual allopeptides at 40μg/ml, and pulsed with ^{3}H-Thymidine for an additional 24hrs before harvesting. LNCs from naïve RT1^u rats were cultured in the same manner to obtain background levels of proliferation. Results are the mean and standard deviation of 2-6 animals per group.

days after peptide treatment, and were bled for serum on days 4, 7, 10 and 12 with respect to grafting. On day 12 after grafting, animals were sacrificed and their LNCs used in *in vitro* T cell proliferation studies.

5.3.1 *Alloantibody Analyses*

Figure 5.5 demonstrates the development of the A^a-specific cytotoxic alloantibody response to an R8 cardiac allograft by the above groups of animals. Pre-immunisation with a high dose of P7 (Figure 5.5a) diminished the antibody response in comparison with saline-treated control animals, but did not influence the kinetics of the response. The difference in the two treatments appeared to be most marked at days 7 and 10 following transplantation. Figure 5.5b also shows that pre-immunisation with allopeptide, in this case a combination of dominant and sub-dominant peptides, reduced the cytotoxic alloantibody response. Immunisation with a cocktail of all 18 allopeptides failed to decrease the cytotoxic antibody response to an R8 cardiac allograft in comparison to control animals immunised with an equivalent amount of the irrelevant peptide (Figure 5.5c). However, the overall response in both of these groups appeared to be smaller than that observed upon pre-treatment with either P7 (Figure 5.5a) or the mixture of dominant and sub-dominant peptides (Figure 5.5b), although after day 10, levels of antibody in allopeptide-treated animals begins to increase.

IgM and IgG2b donor-specific alloantibody levels were measured in day 4, 7, 10 and 12 serum samples from the above groups of $RT1^u$ animals. Pre-treatment with P7 (Figure 5.6a) did not influence the IgM response compared with saline-treated control animals. Pre-treatment with a mixture of P1, P7, P8 and P9 however, actually resulted in a statistically significant ($p = 0.046$) increase in IgM levels in day 4 serum samples in comparison to the response mounted by control animals (Figure 5.6b). Figure 5.6c shows a similar IgM response in animals immunised with either all 18 peptides or with the same quantity of control irrelevant peptide. In each experiment, the IgM response peaked at day 7 in allopeptide-treated animals and declined thereafter.

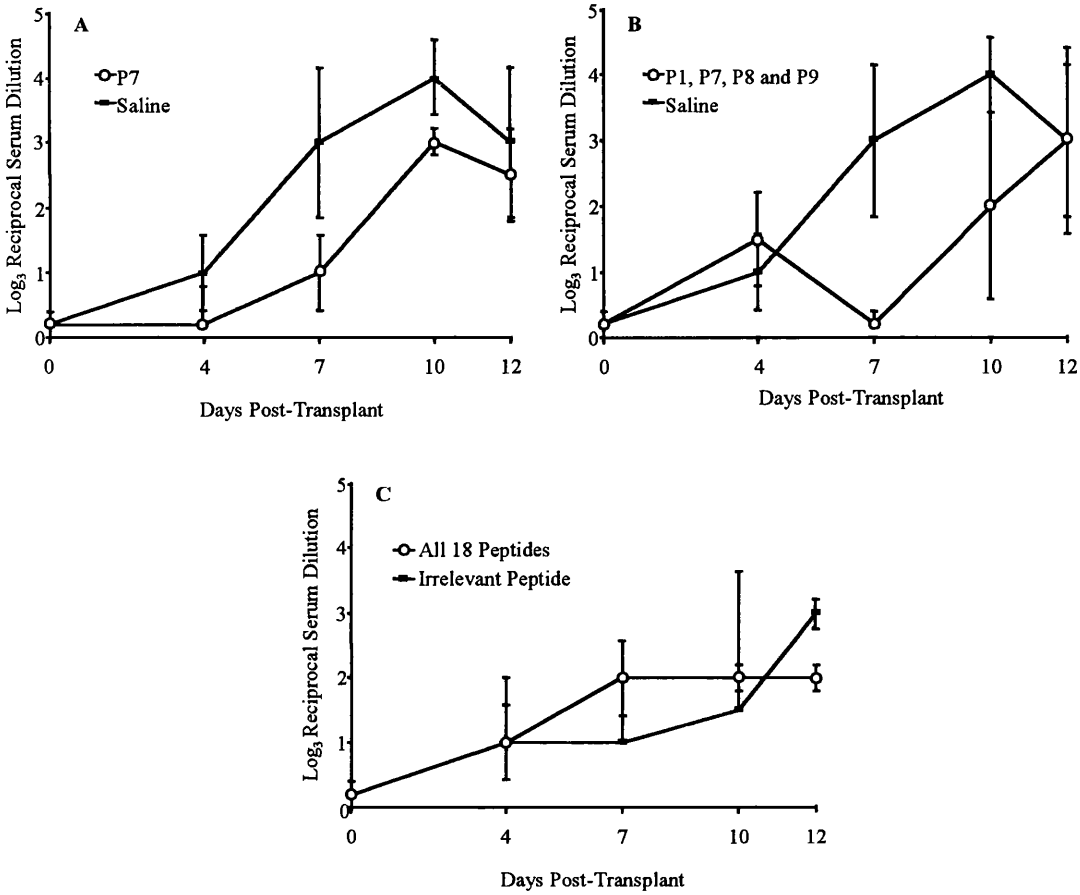

Figure 5.5: Cytotoxic Alloantibody Production to an R8 Cardiac Graft Following Immunisation with a High Dose of Allopeptide.

RT1^u rats were immunised intravenously with A) 300μg of P7, B) A mixture of P1, P7, P8 and P9 (300μg of each peptide) or C) A cocktail of all 18 peptides (300μg of each peptide) in 300μl of saline and challenged 12 days later with an R8 cardiac allograft. Control animals were immunised with saline alone (Graphs A and B), or 5.4mg of the irrelevant peptide in saline (Graph C) 12 days before transplantation. Serum samples taken on days 4, 7, 10 and 12 after transplantation were assessed for cytotoxic alloantibody content using a standard ^{51}Cr-release assay against R8 lymphoblast target cells. Results are expressed as the final dilution of serum at which target cell lysis was greater than or equal to 20%, and are the mean and standard deviation of 2-3 animals per group.

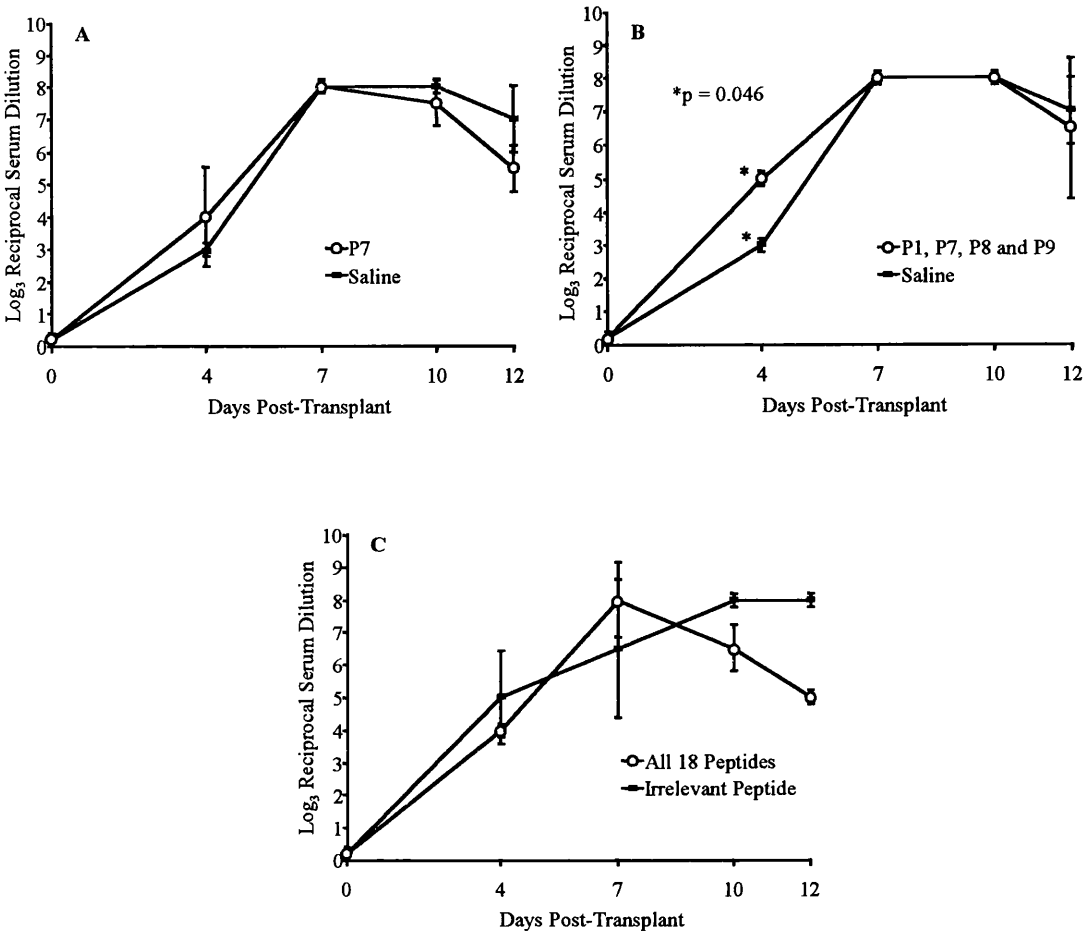

Figure 5.6: The Allospecific IgM Response to an R8 Cardiac Graft Following Immunisation with a High Dose of Allopeptide.

RT1^u rats were immunised with A) 300mg of P7, B) A mixture of P1, P7, P8 and P9 (300mg of each peptide) or C) A cocktail of all 18 peptides (300mg of each peptide) in 300ml of saline and challenged 12 days later with an R8 cardiac allograft. Control animals were immunised with saline alone (Graphs A and B), or 5.4mg of the irrelevant peptide in saline (Graph C) 12 days before transplantation. Serum samples taken on days 4, 7, 10 and 12 after transplantation were assessed for IgM alloantibody content by Flow Cytometry, using R8 LNCs as target cells and a FITC-conjugated anti-IgM mAb. Results are expressed as the final dilution of serum at which antibody levels were greater than or equal to twice background values, with background representing the level of fluorescence observed with cells and antibody alone. Results are the mean and standard deviation of 2-3 animals per group. Statistical analysis was performed using the Mann Whitney U test. Only significant results are indicated.

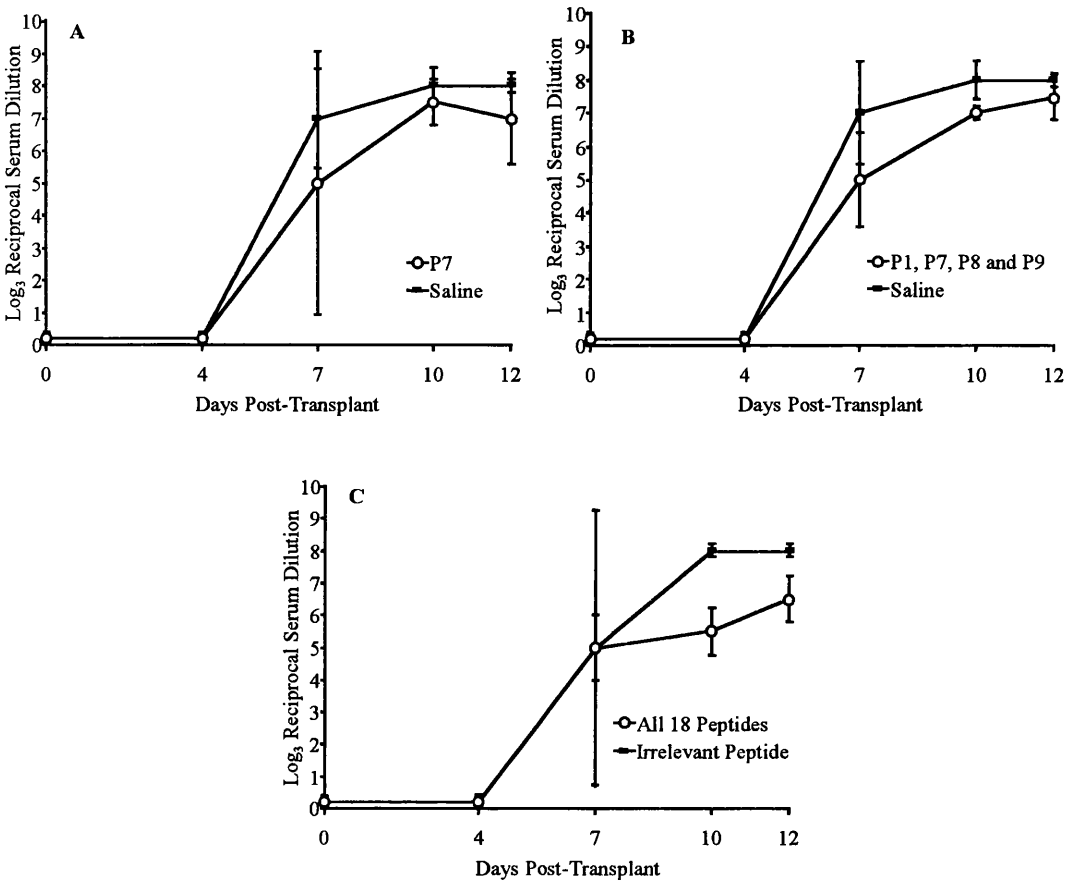

Figure 5.7: The Allospecific IgG2b Response to an R8 Cardiac Graft Following Immunisation with a High Dose of Allopeptide.

$RT1^u$ rats were immunised with A) 300μg of P7, B) A mixture of P1, P7, P8 and P9 (300μg of each peptide) or C) A cocktail of all 18 peptides (300μg of each peptide) in 300μl of saline and challenged 12 days later with an R8 cardiac allograft. Control animals were immunised with saline alone (Graphs A and B), or 5.4mg of the irrelevant peptide in saline (Graph C) 12 days before transplantation. Serum samples taken on days 4, 7, 10 and 12 after transplantation were assessed for IgG2b content by Flow Cytometry, using R8 LNCs as target cells and a FITC-conjugated anti-IgG2b mAb. Results are expressed as the final dilution of serum at which antibody levels were greater than or equal to twice background values, with background representing the level of fluorescence observed with cells and antibody alone. Results are the mean and standard deviation of 2-3 animals per group.

The IgG2b response to an R8 cardiac allograft in the same groups of rats is illustrated in Figure 5.7. As observed with the IgM response, pre-treatment with P7 (Figure 5.7a) or a mixture of P1, P7, P8 and P9 (Figure 5.7b) had little effect on the allospecific IgG2b response in comparison to controls. Levels of antibody did appear slightly diminished in allopeptide-treated rats, but this was statistically non-significant. The response at day 10 in animals pre-treated with all 18 peptides was marginally less than in control animals, but again, this difference was non-significant.

5.3.2 *Allograft Rejection*

The effect of A^a allopeptide-immunisation on the survival of subsequent R8 cardiac allografts is shown in Table 5.1. Unmodified $RT1^u$ recipients normally rejected R8 hearts in 7 days (Group 1). Pre-treatment with a mixture of all 18 allopeptides or P7 alone in CFA subcutaneously reduced graft survival to just 4 days (Groups 4 and 2 respectively), whereas pre-treatment with the irrelevant peptide in CFA had no effect upon the rate of graft survival (Group 6).

Intravenous immunisation with either P7 alone (Group 3) or a mixture of P1, P7, P8 and P9 (Group 8) in saline did not prolong graft survival, despite decreasing the rate of cytotoxic anti-A^a alloantibody production. In fact, such pre-treatments actually decreased graft survival times (to 5 and 5.5 days respectively) with respect to naive controls. Pre-immunisation with all 18 peptides in saline intravenously appeared to have no effect on graft survival, with hearts surviving for 7 days (Group 5). Interestingly however, treatment of rats with a very high dose of the irrelevant peptide resulted in an MST of just 5.5 days (Group 7). This was unexpected, as being a control, this peptide should be unable to contribute to allograft rejection. It may, however, cause non-specific enhancement of the alloimmune response, especially at the very high dose used in this particular experiment, and certainly, its intravenous administration appeared to slightly augment the proliferative response to allopeptide upon heart grafting (Figure 5.8a). Results in Groups 7 and 8 are from only two animals, as one animal in each group died from post-operative complications.

Table 5.1: The Rejection of MHC Class I-Disparate Cardiac Allografts by $RT1^u$ Recipients.

Group	Pre-treatment	n	Graft Survival[f] (days)	MST
1	None	6	6,6,7,7,7,7	7
2	P7 +CFA[a]	5	4,4,4,4,4	4
3	P7 + Saline[b]	3	5,5,6	5
4	All 18 + CFA[c]	4	4,4,4,4	4
5	All 18 + Saline[d]	3	6,7,7	7
6	Irr + CFA[a]	3	6,7,7	7
7	Irr + Saline[d]	2	5,6	5.5
8	P1, P7, P8 + P9 + Saline[e]	2	5,6	5.5
9	Saline Alone	3	6,7,7	7

[a] $RT1^u$ recipients were immunised subcutaneously with 100μg of peptide emulsified in CFA 7 days before receiving an R8 cardiac allograft.

[b] $RT1^u$ animals were injected intravenously with 300μg of peptide in saline 12 days before allografting.

[c] Recipient rats were immunised subcutaneously with a cocktail of all 18 peptides (100μg of each peptide) emulsified in CFA 7 days before cardiac transplantation.

[d] $RT1^u$ animals were primed intravenously with a mixture of all 18 peptides (300μg of each peptide) or an equivalent amount of the irrelevant peptide in saline 12 days before heart grafting.

[e] $RT1^u$ rats were immunised intravenously with a mixture of P1, P7, P8 and P9 (300μg of each peptide) in saline 12 days before challenge with an R8 cardiac allograft.

[f] Animals were assessed daily, and allograft rejection was defined as the complete cessation of myocardial contraction.

5.3.3 *T Cell Proliferation Studies*

I wished to examine whether intravenous allopeptide pre-treatment was able to influence the *in vivo* T cell response to an R8 cardiac allograft. I therefore harvested LNCs from animals 12 days after grafting, and examined their *in vitro* T cell proliferation to individual allopeptides. Figure 5.8 demonstrates that animals immunised with a high dose of the irrelevant peptide display very strong T cell proliferation to the α1 peptide, but not to any of the other allopeptides (Figure 5.8a),

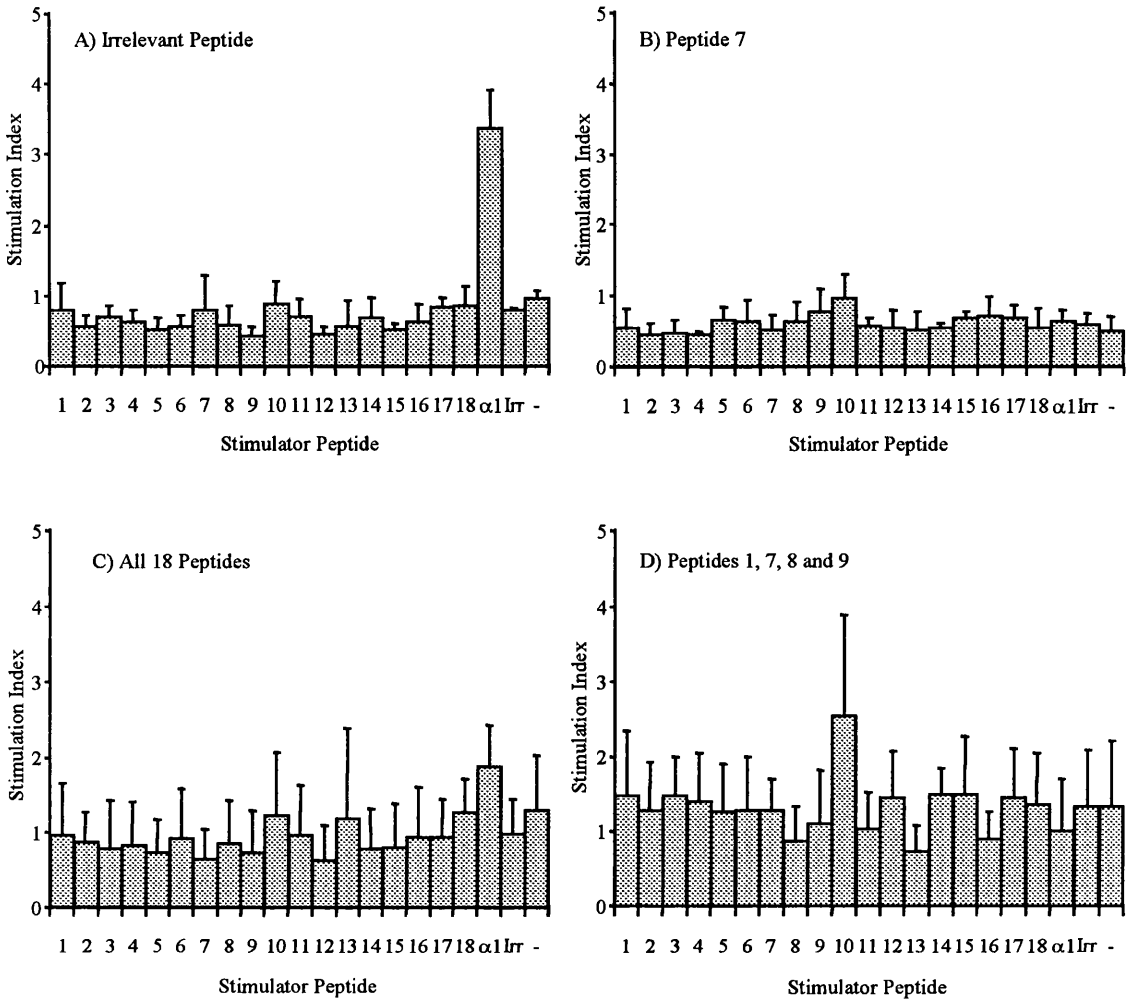

Figure 5.8: *In Vitro* Proliferation of LNCs from RT1^u Rats Immunised with a High Dose of Allopeptide Intravenously and Challenged 12 Days Later with an R8 Cardiac Allograft.

RT1^u rats were immunised intravenously with A) 5.4mgs of the irrelevant peptide, B) 300μg of P7, C) All 18 allopeptides (300μg of each peptide) or D) A mixture of P1, P7, P8 and P9 (300μg of each peptide) in 300μl of saline and challenged 12 days later with an R8 cardiac allograft. LNCs were used in T cell proliferation studies 12 days after transplantation. Cells were cultured for 72hrs with individual allopeptides at 40μg/ml, and pulsed with ^{3}H-Thymidine for an additional 24hrs before harvesting. LNCs from naïve RT1^u animals were cultured in the same manner to obtain background levels of proliferation. Results are the mean and standard deviation of 2-3 animals per group.

which is a similar pattern of proliferation to that observed upon immunisation of $RT1^u$ animals with the intact A^a molecule (Figure 3.7). In comparison, Figure 5.8b demonstrates that priming with P7 results in no T cell proliferation to any of the allopeptides, although slight proliferation to the α1 peptide is seen upon administration of a mixture of all 18 peptides, but the error bars overlap with others on the graph (Figure 5.8c). Interestingly, LNCs from animals immunised with the mixture of P1, P7, P8 and P9 do not display proliferation towards any of the immunising peptides or the α1 peptide, but do display a response to P10, although again, the error bars overlap with others on the graph (Figure 5.8d).

5.4 *Discussion*

It was shown that the $RT1^u$ alloantibody response to an R8 blood transfusion was downregulated following intravenous immunisation with a high dose of allopeptide (Figures 5.1, 5.2 and 5.3). However, downregulation of the IgG2b response was less marked than that of the cytotoxic and IgM alloantibody responses, with P1-priming having no effect on the level of IgG2b production (Figure 5.3b). The reason as to why intravenous priming with P7 as compared to P1 should have a different effect on the downregulation of the IgG2b response is not immediately apparent. The process of isotype switching does, however, appear to be more stringent in its requirement for T cell help (Steele et al 1996), which, as Agarwal et al have suggested (Agarwal et al 1996, Agarwal et al 1997) may be one of the mechanisms by which the dominant response to a single epitope is achieved. Therefore, with specific regards to the R8 to $RT1^u$ rat strain combination, those T cells that are specific for the epitope contained within P7 may contribute to a greater extent to the process of heavy chain class switching than T cells specific for other A^a-derived epitopes. Consequently, tolerising to the dominant epitope, by means of intravenous administration of P7, may be more effective at downregulating the IgG2b response than priming with a sub-dominant peptide, for example P1. In comparison, the IgM response is not as T cell dependent (Steele et al 1996), and this would explain why intravenous administration of either peptide was equally effective at downregulating the IgM response. The experiments performed in this study tend to concur with the findings of Steele et al, in that different levels of T cell help appear to be required for different phases of the antibody response. The same reasoning may also explain why priming with peptide subcutaneously augmented the IgM alloantibody response but had little effect on the IgG2b response (Figure 4.5).

It is interesting to note that the difference in the alloantibody response between control and experimental animals is not as marked when animals are challenged with an R8 cardiac allograft instead of a blood transfusion. This is most likely due to a heart graft representing a greater antigenic stimulus than a blood transfusion, either because it contains a greater number of donor cells, or because its persistence acts as

a continual source of graft antigens for trafficking to regional lymph nodes and the spleen. This greater antigenic stimulus presumably results in more efficient activation of recipient T cells, which may overwhelm the inhibitory effect of intravenous peptide administration.

Examination of graft survival data demonstrates that intravenous administration of peptide does not, even upon immunisation with a mixture of all 18 peptides, prolong allograft survival in comparison to naïve controls. As a possible explanation, at days 4 and 7 after heart grafting, cytotoxic alloantibody, although downregulated, is still detectable in serum samples, and this level may represent a sufficient amount of circulating alloantibody to effect graft rejection.

Examination of the proliferation data obtained following challenge with either an R8 blood transfusion or cardiac allograft, demonstrates that there is no clear pattern of downregulation in the T cell proliferative responses. This again correlates with graft survival data, and suggests that tolerance to P7 is not complete; immunisation with P7 before challenge with an R8 blood transfusion still results in proliferation to P7. Furthermore, in some circumstances, proliferation appears to switch to other areas of the A^a molecule. For example, in Figure 5.8d, the proliferative response after priming with a mixture of P1, P7, P8 and P9 was predominantly directed towards P10. This peptide is derived from the β-sheet of the α2 domain, and is not considered to be in one of the hypervariable regions, although it does differ from the corresponding region of the $RT1^u$ sequence by 3 amino acids (Figures 3.1a and 3.1b). Shirwan's work in the PVG-R8 to PVG-1U strain combination (Shirwan et al 1995) has not explored the effect of peptides derived from this area upon graft rejection. However, Fangmann et al (Fangmann et al 1992a), using the DA ($RT1^a$) to Lewis ($RT1^l$) rat strain combination examined the effects of peptide immunisation on skin graft survival. One of the A^a peptides used in this study encompassed most, but not all, of the sequence of P10, and it is interesting that whilst priming with this peptide did not influence the rejection kinetics of a subsequent DA skin graft, an enhanced anti-A^a alloantibody response was observed. In comparison, it is of interest that earlier experiments in my own study suggested that P10 was not

actually involved in the provision of help for the anti-A^a alloantibody response (see Figure 4.4) which may reflect biological differences in the strain combinations used. However, this does suggest that in the R8 to $RT1^u$ combination, switching of the T cell response to different epitopes may stimulate effector mechanisms other than alloantibody that ultimately result in allograft rejection.

KEY POINTS

A) Evaluating the *in vitro* T cell proliferation responses to A^a-derived allopeptides after challenge with the intact A^a molecule established the presence of a dominant epitope located within the hypervariable region of the α1 domain (the outer surfaces of the α-helix). This epitope corresponds to the 15-mer A^a allopeptide, P7.

B) Priming with individual peptides before challenge with the intact A^a molecule revealed the existence of several additional sub-dominant peptides. These epitopes correspond to P1, P9 and P16. P1 and P9 derive from areas of the A^a molecule that are identical in sequence to the corresponding regions in the A^a molecule, and in essence represent self-$RT1^u$ peptides.

C) Intravenous administration of a high dose (300μg) of the dominant (P7) or sub-dominant (P1) peptides in saline, markedly downregulated both the cytotoxic and IgM alloantibody responses to a subsequent R8 blood transfusion. In addition, P7 was able to downregulate the later IgG2b response. Following intravenous administration of P7, *in vitro* proliferation to this dominant peptide was still observed, suggesting that the alloresponse is not completely abrogated.

D) Following intravenous administration of a high dose of P7, the alloantibody responses were not as markedly downregulated to an R8 cardiac allograft as to an R8 blood transfusion. In addition, pre-treatment with either P7, a mixture of P1, P7, P8 and P9 or all 18 peptides was unable to prolong the survival of subsequent R8 cardiac allografts in comparison to naïve controls. Proliferation data suggest that, despite pre-immunisation, a T cell response to the A^a molecule can still occur, although in some cases the response appears to shift from the dominant epitope to other areas of the A^a molecule.

CHAPTER 6:

DISCUSSION

Organ transplantation in the PVG-R8 (RT1.A^aB/D^uC^u) to PVG-$RT1^u$ (RT1.A^uB/D^uC^u) rat strain combination provides a model in which to examine in detail the complexities of the indirect pathway of allorecognition. As the two strains differ only at an isolated MHC class I locus, allograft rejection can be attributed to the self-restricted recognition of allogeneic epitopes of the disparate class I molecule, A^a (Bradley 1996). The main aim of this thesis was to examine the specific regions of this molecule that are involved in the $RT1^u$ immune response to an A^a-bearing R8 blood transfusion or cardiac allograft. The experiments performed used a series of overlapping synthetic 15-mer peptides spanning the length of the α1 and α2 domains of the A^a molecule. This experimental protocol differs from similar studies using this approach in the same strain combination (Shirwan et al 1995, MacDonald et al 1997, Mhoyan et al 1999), in that I have been able to study the influence of not only dominant epitopes, but also potential sub-dominant and cryptic epitopes on the alloimmune response.

To search for dominant A^a epitopes, $RT1^u$ animals were doubly immunised with the intact A^a molecule in the form of an R8 skin graft and an R8 cardiac allograft. LNCs were then stimulated *in vitro* with individual allopeptides, and T cell proliferation assessed. This approach highlighted a probable dominant epitope contained within the hypervariable region of the α1 domain, as proliferation was predominantly directed at P7 and the α1 peptide, which are derived from this area. It remains questionable however, whether the dominant A^a epitope is incorporated precisely within the sequence comprising P7, as the above proliferation studies consistently showed a stronger response to the α1 peptide than to P7. Furthermore, priming with either P7 or P8 accelerated the allospecific antibody response upon challenge with the intact A^a molecule. It may be therefore, that P7 and P8 each contain a distinct

epitope, or alternatively that the optimal A^a epitope spans the two peptides, and in doing so, encompasses the five overlapping amino acids that they share. Benichou's theory that the indirect alloresponse is generally limited to a single dominant epitope lends support to the latter theory (Benichou et al 1994a). However, Benham and Fabre, through analysis of the fine specificity of the T cell response, have demonstrated the presence of several overlapping epitopes within the hypervariable region of the A^a molecule, which were able to stimulate a polyclonal T cell response (Benham et al 1994). The experimental protocols used in this thesis were unable to distinguish between these two possibilities, and to examine which is the case, other protocols would have to be employed. For example, one could use a series of peptides overlapping by single amino acid residues spanning the region in question and perform studies similar to those in this thesis (e.g. (Benham et al 1994). Alternatively, it may be possible to elute and sequence peptides from MHC molecules of $RT1^u$ animals immunised with the intact A^a molecule.

These findings raise questions regarding the mechanisms by which epitope dominance occurs. In the R8 to $RT1^u$ rat strain combination, graft rejection is predominantly mediated by alloantibody, and is critically dependent upon recipient CD4 T cells for the generation of this response (Gracie et al 1990, Morton et al 1993). Therefore, limitation of the alloimmune response to a single T_h cell determinant presumably occurs through regulation of either or both of the T and B cell responses. This regulation appears to be an active phenomenon, as peptide-priming experiments demonstrated that multiple epitopes could potentially accelerate the anti-A^a alloantibody response.

A possible mechanism by which epitope dominance may evolve, is through biasing of the T cell repertoire towards epitopes derived from the hypervariable regions of the parent molecule (Gould et al 1999). In this manner, allospecific B cells presenting a dominant epitope may receive earlier, more efficient T cell help, thereby resulting in their preferential activation and proliferation. Moreover, this process may actively downregulate the T cell response to other peptide epitopes, as it has been observed that cognate T_h cell - B cell interactions rapidly (within 1-2hrs)

result in the downregulation of other MHC class II-peptide complexes on the surface of a B cell (Constant 1999). This effectively diminishes the frequency at which other A^a-derived epitopes are presented by B cells, making it less likely that T cells specific for these additional epitopes will become involved in the anti-A^a alloimmune response. This concept may also explain the ability of pre-immunisation with a sub-dominant peptide in an immunogenic fashion to stimulate an accelerated alloantibody response against the intact donor antigen, as it may augment the T cell response to that peptide to such an extent that it can compete successfully with the naturally stronger T cell response to the dominant epitope.

In addition to the dominant epitope, other "sub-dominant" epitopes may have the potential to influence the immune response to the A^a molecule. This was examined using two different experimental approaches. Firstly, animals were subcutaneously immunised with allopeptide in CFA and their subsequent alloantibody and *in vitro* T cell proliferative responses examined. Only P1, P7 and the α1 peptide were able to stimulate an *in vitro* T cell proliferative response, whereas P16 was additionally able to stimulate an anti-peptide antibody response. Secondly, $RT1^u$ animals were pre-immunised with allopeptide before receiving an R8 blood transfusion, and the development of the cytotoxic alloantibody response examined. This experiment revealed that two further peptides, P1 and P9 were able to evoke help for an accelerated anti-A^a cytotoxic alloantibody response upon challenge with the intact A^a molecule. These experiments suggest that the $RT1^u$ T cell repertoire can recognise epitopes derived from peptides P1, P9 and P16, because, as previously discussed, the generation of an antibody response to either an A^a allopeptide or the intact A^a molecule requires cognate help from T cells specific for the A^a peptide epitope presented by a B cell. Achieving a positive antibody read-out is, however, dependent upon presentation of a specific epitope by a B cell, and consequently, these assays are only an indirect measure of the T cell response. It was surprising therefore that this approach revealed a greater number of potential A^a epitopes than standard T cell proliferation assays. Nevertheless, I believe that this technique of epitope mapping is the most accurate, since it reflects the main effector mechanism of graft rejection in this strain combination, that of alloantibody. Moreover, the

validity of this approach was subsequently confirmed by pre-immunising $RT1^u$ animals with allopeptide, and examining their effect upon cardiac allograft survival.

Regarding the assays used to detect antibody, it is interesting that there was an apparent discrepancy in the response to P1 and P9 depending on whether the anti-peptide antibody response or the antibody response to the intact A^a molecule was examined. There are two possible explanations for the different results achieved using each assay. Firstly, the administration of a blood transfusion after pre-immunisation with allopeptide represents a secondary challenge with A^a antigen. However, animals only received a single injection of allopeptide during the course of the anti-peptide antibody assay. It is therefore possible that the responses to the self-peptides P1 and P9 were too weak during the primary response (i.e. following peptide immunisation), and only became evident following secondary challenge with the A^a molecule, in a manner analogous to the detection of the dominant epitope only after double immunisation of $RT1^u$ animals. To test this hypothesis, one could measure the anti-peptide antibody response following secondary challenge with peptide or the intact A^a molecule. This study was not performed in this thesis, but it is notable that Fangmann et al did not observe an antibody response to a peptide derived from the same area of the A^a molecule as P9 in Lewis rats following secondary *in vivo* stimulation with the peptide in question (Fangmann et al 1992a).

An alternative explanation for these findings may reside in the differential capacity of B cells to present antigenic epitopes, depending on whether antigen is encountered as an intact molecule or as peptide fragments. It has been suggested that B cells are the class of APC mainly responsible for the presentation of complex protein antigens, whereas dendritic cells favour the presentation of peptide fragments (Constant 1999). Therefore, that immunisation with both P1 and P9 failed to stimulate an anti-peptide antibody response, but was able to affect the antibody response upon challenge with the intact A^a molecule may be the result of their preferential internalisation and presentation by dendritic cells rather than by B cells.

It may be therefore, that accurate sub-dominant epitope mapping will only be achieved if an optimum method of analysis is used, which may well differ with

different strain combinations, depending upon the individual characteristics of a particular model's alloimmune response. It is important however that accurate mapping of the sub-dominant epitopes involved in the indirect T cell response is achieved, particularly for the efficient design of peptide-based tolerogenic strategies. In the R8 to $RT1^u$ rat strain combination, monitoring the alloantibody response to the intact A^a molecule is, in effect, a functional *in vivo* read-out of the mechanisms responsible for allograft rejection. Although this approach may not apply to other strain combinations, especially those in which rejection is not alloantibody-mediated, it has been demonstrated in the current study that peptides derived from the non-hypervariable regions of an allo-MHC molecule may potentially be as important in the indirect alloresponse as those peptides derived from hypervariable regions. For example, the amino acid sequences in the A^a and A^u molecules that correspond to P1 and P9 are identical, yet priming $RT1^u$ animals with these peptides had as great an effect on the cytotoxic alloantibody response to and the rejection kinetics of A^a-bearing allografts as priming with peptides derived from the hypervariable region of the $\alpha 2$ domain, namely P15 and P16. That self-peptides are able to stimulate an alloimmune response is not unprecedented and concur with the findings of Fedoseyeva et al (Fedoseyeva et al 1996) who observed the activation of autoreactive T cells following alloimmunisation.

Other experimental techniques may be used to directly measure the T cell response to individual allopeptides, for example, assays examining cell-mediated cytotoxicity and DTH responses. However, a cell-mediated cytotoxicity assay would reflect the recipient CD8 T cell response towards the allogeneic A^a MHC class I molecule, and it is unlikely that such an assay could be used to measure the CD4 T cell response to allogeneic A^a-derived allopeptides. Certainly, attempts within this laboratory have failed to detect an anti-A^a cytotoxic T cell response following challenge of $RT1^u$ rats with an A^a-bearing graft (e.g. (Bradley et al 1992). In support of this, CD8 T cell depletion of $RT1^u$ recipients does not prevent rejection of subsequent R8 allografts (Morton et al 1993, Pettigrew et al 1998). DTH assays may have provided a more accurate assessment of immunogenic peptides than simple T cell proliferation assays. However, this approach would still have been of limited value, as it fails to

provide any indication as to the responsiveness of the main effector mechanism of graft rejection in this model, that of alloantibody.

My experiments appear to concur with the observations of Agarwal et al (Agarwal et al 1996, Agarwal et al 1997) and Steele et al (Steele et al 1996), which demonstrate that the requirement for T cell help alters as the alloantibody response progresses. Specifically, the process of heavy chain isotype class switching from IgM to IgG appears to require a greater level of T cell help than the initial IgM response. For example, subcutaneous administration of allopeptide was able to accelerate the kinetics of the initial IgM response, but the effect on the IgG2b response was only minimal. This restriction of the T cell response at the time of isotype class switching may be one of the mechanisms responsible for focusing of the alloimmune response onto a dominant epitope. If so, one would expect that the emergence of a dominant epitope would not occur immediately, but would be delayed until nearer the time of class switching. *In vitro* T cell proliferation assays were not performed this early in the current study, however, it has been recently documented by Benichou et al (Benichou et al 1999) that the initial indirect T cell response is polyspecific, with only 10-30% of activated T cells directed towards the dominant epitope. That the indirect recognition of alloantigen is a dynamic process, with different recipient T cell clones activated at different stages of the alloimmune response, may have important repercussions upon the design of peptide-based tolerogenic strategies. For example, if multiple donor epitopes are involved in the initial indirect T cell response, then this may decrease the effectiveness of using only a limited number of donor epitopes in the tolerogenic strategy. Conversely, as the T cell response focuses, this process may either break, or make redundant, the tolerance that has been achieved to any one particular epitope.

Nevertheless, it appears that targeting the indirect pathway of allorecognition with allopeptides to promote antigen-specific non-responsiveness is a promising strategy. It is unlikely however, that one could predict and incorporate into the initial tolerising inoculum all the conceivable donor epitopes that are involved in the acute alloimmune response, for, as demonstrated in this thesis, even sequences that are

identical between donor and recipient are potentially stimulatory. Moreover, as the alloimmune response progresses, different antigen processing pathways may become more prevalent. For example, internalisation of complexes of donor antigen bound to secreted alloantibody, through the B cell Fc receptor, results in a different spectrum of B cell-presented epitopes than occurs following initial internalisation of unbound alloantigen through the B cell Ig receptor (Watts 1997). The stimulation of additional recipient T cell clones by the presentation of these further epitopes may result in the alloimmune response either spreading or becoming re-activated (in the case of self-peptide sequences) to previously non-stimulatory sequences of the donor antigen. Mechanisms such as these may explain the process of epitope spreading that occurs during chronic allograft rejection (Ciubotariu et al 1998, Suciu-Foca et al 1998) and highlight the difficulty in accurately predicting which of the donor epitopes are involved in the alloimmune response.

The key unresolved issue that will therefore determine the clinical applicability of such tolerisation protocols that incorporate limited stretches of donor antigen, is how a recipient's immune system will respond, not to the sequences administered in a tolerogenic fashion, but to the additional donor epitopes that will be presented upon processing of the graft antigens. Two dichotomous outcomes can be envisaged. Firstly, tolerance may spread by the process of linked epitope suppression to encompass the additionally presented epitopes, or secondly, tolerance may be broken by the presentation of additional donor epitopes in an immunogenic fashion, thus initiating a compensatory alloimmune response that could effect rejection. Several factors may influence whether the residual graft antigens are recognised in a tolerogenic or stimulatory fashion, but the balance between epitope spreading and tolerance is likely to be mainly dependent upon the features of the donor sequence that is used in the initial inoculum. Specifically, different results may be achieved depending on the area of the donor MHC molecule from which the tolerising sequence is derived and upon the amount of the donor sequence, in terms of amino acid sequence, that is administered. For example, Mhoyan et al observed that peptides derived from the hypervariable regions of the RT1.A^a molecule were more effective at inducing intrathymic tolerance to a subsequent A^a-bearing allograft than

peptides derived from less polymorphic areas of the molecule (Mhoyan et al 1997). Similarly, it was reported that the use of a combination of peptides was more effective that when used singly (Shirwan et al 1997a).

Why peptides derived from the hypervariable regions of an alloMHC molecule should be more effective at inducing linked epitope suppression has not been fully assessed. Peptides from the hypervariable region do, however, appear more likely to contain dominant epitopes, which are presumably presented by recipient APC at a higher frequency than other epitopes during the indirect response to an alloantigen. In addition, it has been suggested that for linked epitope suppression to occur, downregulation of the T cell response to additional donor epitopes requires their co-presentation with the initial "suppressor epitope" on the same APC (Davies et al 1996, Wong et al 1997). One can therefore postulate that achieving tolerance with peptides derived from the hypervariable region will be more effective at inducing linked epitope suppression than other epitopes because it is less likely that, upon processing of graft antigens, the residual donor epitopes will be presented by recipient APC without concurrent presentation of the suppressor epitope. For the same reason, I expected that the use of sub-dominant epitopes would be more effective at inducing linked epitope suppression than cryptic epitopes. Consequently, I hoped that thorough mapping of the dominant and sub-dominant epitopes, by using a functional *in vivo* read-out, would permit a more rational design of effective peptide-based tolerogenic strategies. My results suggest that this approach was successful in part, as judged by the ability of intravenous priming with donor peptide to downregulate the alloantibody responses to an R8 blood transfusion and, to a lesser extent, an R8 cardiac allograft. The results obtained following administration of P1 were particularly interesting, as they suggest that priming with a sub-dominant epitope can result in linked epitope suppression that is extended to the naturally dominant epitope.

Intravenous administration of high doses of allopeptide to $RT1^u$ rats was not successful in prolonging allograft survival, despite partially downregulating alloantibody responses and, in some cases resulting in the shifting of the T cell

proliferative response to epitopes other than those administered in the initial inoculum. This suggests that although the response to the dominant epitope could be partially inhibited, the alloimmune response compensated by reactivity, rather than tolerance, spreading to the residual graft antigens. For example, immunisation with a combination of the dominant and sub-dominant peptides, P1, P7, P8 and P9, was associated with proliferation to P10 following heart grafting. It is notable however, that subcutaneous administration of P10 was not associated with an accelerated alloantibody response upon challenge with the intact A^a molecule. One could hypothesise that this epitope may instead be involved in the development of either a cytotoxic T cell response or a DTH-like response to the graft.

Despite the failure of intravenous pre-immunisation with peptide to induce prolonged allograft survival, this does not necessarily imply that the principle of peptide-based tolerogenic strategies to achieve tolerance through linked epitope suppression is flawed. It is possible that prolonged graft survival would have resulted from the administration of P7 or a different combination of peptides, either at a higher dose, or at different time intervals with respect to allografting. For example, the amount of peptide administered was chosen on a somewhat arbitrary basis, using the work of Shirwan as a guide (Shirwan et al 1997b). Ideally however, the optimum amount of peptide to be administered would have been calculated by means of dosing experiments, but even then, discrepancies in the proliferation data and between blood transfused and heart grafted animals may have prevented conclusive findings.

In summary, this piece of work suggests that although the $RT1^u$ alloimmune response focuses upon the first hypervariable region of the donor $RT1.A^a$ class I MHC molecule following immunisation of $RT1^u$ rats with an A^a-bearing blood transfusion or cardiac allograft, several other areas of this molecule contain epitopes that have the potential to be involved in the indirect alloimmune response. However, it may be difficult to accurately predict these additional donor epitopes, as they may derive from unexpected areas of the alloMHC molecule, even those regions that are identical in amino acid sequence to the corresponding regions of the recipient MHC

molecule. Moreover, it appears that the ability to accurately map these sub-dominant epitopes will depend upon the use of an assay that most accurately reflects the *in vivo* alloimmune response in a particular strain combination. The attempts in this study to achieve allograft tolerance by pre-immunisation with either the dominant or sub-dominant epitopes in a tolerogenic fashion were unsuccessful. It is not clear however, whether this was due to an inability to completely downregulate the T cell response to the administered peptide, or whether the process of linked epitope suppression was not powerful enough prevent the development of a further immune response to the remaining graft antigens.

The resolution of this question has important clinical applications, because the relative propensity for tolerance, rather than reactivity to spread to the residual graft antigens will largely dictate the success of peptide-based tolerogenic strategies. For example, if tolerance does tend to spread to encompass other alloantigenic epitopes, then this would permit the use of relatively limited stretches of the donor antigen to induce non-responsiveness. Moreover, such a situation would also allow the recipient to receive a wider range of mismatched allografts by using key sequences of a single donor alloantigen to induce linked epitope suppression. My results however, are inconclusive, due in part to the relatively crude nature of the *in vitro* T cell proliferation assay used, which may not accurately represent the *in vivo* T cell response to a foreign antigen (Nevala et al 1997). To determine the true nature of T cell specificity, it may be possible to use a more sensitive read-out of the *in vivo* T cell response. One such approach may be to use limiting dilution analyses (LDA), whereby single cells are plated into individual wells, and their antigen specificity examined (Sharrock et al 1990). Alternatively, using the sensitive enzyme-linked immunospot (ELISPOT) assay, it may be possible to analyse the specificities of the indirect T cell response as early as the fifth day post-transplant as documented by Benichou et al (Benichou et al 1999). This assay measures the T cell response indirectly by using antibodies to detect cytokines secreted by T cells during *in vitro* culture. It appears to be more sensitive than standard LDA, and offers further advantages in that it is able to indicate the pattern of cytokine polarisation for the response to a given epitope, and moreover, is able to detect T cells that release

cytokines but that do not necessarily proliferate. The use of high affinity dimers of molecularly engineered MHC class II complexes may however be the most effective means of assessing the CD4 T cell response to a given epitope (Lebowitz et al 1999). These would be designed to incorporate a specific bound peptide, in this case an A^a-derived allopeptide, into a bivalent MHC class II ($RT1.B^u/RT1.D^u$) structure. Such complexes would bind to the relevant $RT1^u$ CD4 T cell clones with sufficient stability to permit flow cytometrical analysis. This technique would therefore specifically examine the indirect CD4 T cell response, and could be used at any time-point during the alloimmune response. This approach is analogous to the use of tetrameric MHC class I complexes, which have been successfully used to analyse in more detail the *in vivo* CD8 T cell response than can be achieved using LDA (Altman et al 1996, Gutgemann et al 1998, Wilson et al 1998).

In conclusion, this thesis provides new information on the indirect T cell response to alloantigen and specifically how it is able to provide help for the development of an alloantibody response. Although the relative role of alloantibody has to date been generally overlooked as a mechanism for graft rejection, it's importance in acute and, in particular, chronic allograft rejection is now becoming apparent, as judged by the recent modification of the Banff criteria for alloantibody-mediated rejection as is applicable to renal allografts (Racusen et al 1999). My results further suggest that using synthetic peptides derived from sequences of the donor MHC molecule to downregulate the alloantibody response may be a feasible approach in the attempt to prolong allograft survival.

CHAPTER 7:

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