INDIRECT T CELL ALLORECOGNITION AND ALLOANTIBODY-MEDIATED REJECTION OF MHC CLASS I-DISPARATE HEART GRAFTS.

By

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A thesis submitted to the University of Glasgow towards the degree of Doctor of Medicine

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Abstract

The T cell allorecognition pathways responsible for the rejection of MHC class I disparate allografts remain poorly defined. The respective contributions of the 'direct' recognition of the allogeneic class I molecule by recipient CD8 T cells, and the 'indirect' recognition of processed allo-class I MHC peptide fragments by recipient CD4 T cells, requires clarification. Recent studies in the rat have established a role for T cell dependent antibody. MHC class I disparate PVG.R8 ($RT1.A^a$) heart grafts are rejected acutely in naïve, and hyperacutely in sensitised, $PVG.RT1^u$ recipients by CD4 T cell dependent alloantibody. This thesis explores the T cell allorecognition pathways responsible and demonstrates that direct injection of plasmid DNA encoding a truncated, water-soluble sequence of the $RT1.A^a$ heavy chain (pcmu-tA^a) results in accelerated rejection of PVG.R8 heart grafts (MST 2 days). Pcmu-tA^a injection did not generate of an allocytotoxic T cell response, but an anti-A^a IgG2b cytotoxic alloantibody response developed. That T cell recognition of the class I MHC alloantigen was restricted to the indirect pathway was confirmed by CD4 T cell depletion, which abrogated the alloantibody response and resulted in prolonged graft survival, rather than accelerated rejection. By comparison CD8 T cell depletion had no discernible effect. Priming CD4 T cells for indirect allorecogntion by the administration of synthetic 15-mer allopeptides spanning the $\alpha 1$ and $\alpha 2$ domains of the $RT1.A^a$ antigen did not stimulate an alloantibody response against the intact A^a antigen but the antibody response to a subsequent PVG.R8 heart graft was accelerated. Graft rejection was, however, only modestly accelerated (MST 4 days). These results suggest that soluble class I MHC and allopeptides are equally efficient at priming CD4 T cells by the indirect pathway, but that soluble class I MHC is a more effective immunogen, not because it activates an additional subset of directly restricted T cells, but because its tertiary protein structure provides the appropriate B cell epitopes for the development of humoral immunity against the graft. This is analogous to the provision of cytotoxic T cell help by indirectly restricted T cells and the confirmation that the indirect

pathway may be responsible for the development of effector mechanisms directed against intact MHC on donor cells provides the first demonstration of its role in effecting accelerated rejection. These results further imply that the potentially tolerogenic effects conferred by the ability of soluble donor class I MHC to induce apoptosis in the alloreactive CD8 cytotoxic T cell population may be masked by a detrimental indirect CD4 T cell response.

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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, Mr. G. Pettigrew, and Professor JA Bradley. The experimental procedures described in this thesis were undertaken solely by the author, with the exception of the preparation of paraffin sections, which was performed by the Department of Pathology.

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 Chapter 3 were presented orally at the British Transplantation's Society in 1997, and at the Fifth International Basic Science Symposium in 1997:

"Antibody-Mediated accelerated rejection following immunisation with RT1.A^a α chain DNA. Evidence that T cells providing B cell help have been primed by the indirect pathway."
G. Pettigrew, E. Lovegrove, J. Maclean, E.M. Bolton, J.A. Bradley.

Some of the results described in Chapter 3 were incorporated into the publication:

"Indirect T cell allorecognition and alloantibody-mediated rejection of MHC Class I-disparate heart grafts"
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The 'Sir Roy Calne Young Investigator's Award' was awarded for this paper, and the results were further presented at the British Transplantation Society's Annual Meeting, 2000.

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

ABBREVIATIONS

APC	Antigen Presenting Cell
ATP	Adenosine Triphosphate
BCR	B Cell Receptor
β_2M	β_2-Microglobulin
BSA	Bovine Serum Albumin
CD	Cytoplasmic Domain
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	Concavalin A
CO_2	Carbon Dioxide
cpm	Counts Per Minute
^{51}Cr	Chromium 51
CsA	Cyclosporine A
C-Terminal	Carboxy Terminal
C-Terminus	Carboxy Terminus
CTL	Cytotoxic T Lymphocyte
^{o}C	Degrees Celsius
DNA	Deoxyribonucleic Acid
DTH	Delayed Type Hypersensitivity
EE	Early Endosome
ELISA	Enzyme-Linked Immunosorbant Assay
ER	Endoplasmic Reticulum
ERp57	Endoplasmic Reticulum Protein 57
Fas	Fibrobalst Associated Antigen
FCS	Foetal Calf Serum
FITC	Fluorosine Isothiocyanate
Fmoc	9-Fluorenylmethoxycarbonyl
g	Grams
HBSS	Hanks Buffered Salt Solution

H + E Staining	Haematoxylin and Eosin Staining
HI	Heat Inactivated
HLA	Human Leukocyte Antigen
HPLC	High Pressure Liquid Chromatography
Hrs	Hours
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-12	Interleukin-12
Il-2	Interleukin-2
Il-2R	Interleukin-2 Receptor
IP_3	1,4,5 Inositol Triphosphate
Ir	Immune Response
IRF	Interferon Regulatory Factors
ISRE	Interferon Stimulated Response Element
IT	Intrathymic
IVC	Inferior Vena Cava
kb	Kilobases
kDa	Kilo Dalton
kg	Kilograms
L	Immunoglobulin Light Chain
LE	Late Endosome
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
μCi	Micro Curie
μg	Microgram(s)
μl	Microlitre(s)
NaCl	Sodium Chloride
NK	Natural Killer cell
N-Terminal	Amino Terminal
PA28	Proteasome Activator 28
PBA	PBS/0.2%BSA/0.1% sodium azide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PTK	Protein Tyrosine Kinase
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
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
WF	Wistar Furth

CHAPTER ONE

INTRODUCTION

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CHAPTER ONE

INTRODUCTION

1.1 Overview

This thesis' principal experiment involves the transfer of DNA encoding a major histocompatibility complex (MHC) class I alloantigen in a model of rat allograft rejection. This experimental system was designed to examine several aspects of the alloimmune response; the possible tolerogenic effect of soluble allo-MHC, the significance of the alloantibody response, and chiefly, the role of the 'indirect' pathway of allorecognition in initiating accelerated graft rejection.

The differences between the indirect pathway of allorecognition and the conventional 'direct' pathway pertain to the critical initial stages of the alloimmune response. T cell activation through the two pathways may occur simultaneously, and elucidating their respective contributions to graft rejection is obviously important, particularly with regards the design of future tolerogenic strategies. The principles of direct allorecognition were perceived in the 1950s, for they are simply an extension of the seminal experiments that established graft rejection as an immunological process. Indirect allorecognition has, by contrast, become prominent only over the last decade. In order to directly compare the two pathways of alloantigen presentation, one has to consider not only the historical data concerning the elucidation of the cellular basis of graft rejection, but also the subsequent discoveries of the structure of MHC, and of the nature of antigen processing and presentation, which were fundamental to the development of the concepts of indirect allorecognition.

1.2 Historical Perspective

1.2.1 The Discovery of the Major Histocompatibility Antigens

The elucidation of the nature of the major histocompatibility complex (MHC) was initiated by Gorer's observation that the serological reaction between the red cells of different mouse strains could be mapped to a single dominant gene (1). He subsequently demonstrated that the product of this gene, 'antigen II', was equally critical for tumour graft rejection; the eventual loss of a 'Strong A' strain's susceptibility to a sarcoma cell-line, upon repeated back-crossing with a resistant C57 strain, correlated with the acquisition of the C57 allele for antigen II (2). In conjunction with Snell, Gorer was able to extend these observations by studying newly developed 'congenic' strains, which had been established using backcrossing techniques. They found in all the strains studied that a similar antigenic locus was responsible for tumour cell rejection, but that each strain exhibited a different allele at this locus (3). They thus established the first major histocompatibility complex, now known as MHC class I, and presciently suggested that it possessed a 'long series of alleles'.

It took over 20 years to establish that another group of 'lymphocyte-defined' antigens were largely responsible for stimulating proliferation in mixed leukocyte culture (4-6). These antigens mapped to the Ir region and were subsequently established as a second form of MHC antigen, the MHC class II antigens (7, 8).

1.2.2 The Elucidation of the Effector Mechanisms of Graft Rejection

Tumour cell rejection in Gorer's experiments was associated with the development of antibody against the tumour cell (2). This antibody was able to agglutinate or lyse other cells *in vitro* that displayed the same 'antigen II' haplotype as the tumour cells and Gorer's experiments therefore provided a mechanism through which the recognition of a histocompatibility antigen could result in rejection. This humoral effector mechanism was uncontested for a decade until Medawar, as a result of studying the rejection of skin grafts in humans (9), and outbred rabbits (10-12) illustrated two principles that have governed transplant immunology to this day:

1. Allograft rejection is an actively acquired immunological phenomenon, with second set skin grafts of the same phenotype as the donor undergoing significantly more rapid lymphocyte infiltration and rejection.

2. A cellular, rather than humoral effector mechanism governs graft rejection.

The latter statement was initially based on the observation that rejection correlated with lymphocyte infiltration into the graft, rather than the presence of anti-donor antibodies. The importance of cellular over humoral mechanisms of graft rejection was confirmed by the demonstration that immunity could be adoptively transferred into naïve animals by transferring lymphocytes from animals primed with donor antigen.

It has since become appreciated that the small lymphocyte can effect graft rejection through two mechanisms. Firstly, by infiltrating the graft, small lymphocytes can release the necessary inflammatory mediators responsible for the recruitment and activation of an effector macrophage population which, as noted by Brent, is analogous to a delayed type hypersensitivity (DTH) response. He was able to reproduce its typical features of neutrophil and monocyte infiltration, by the subcutaneous injection of donor spleen cells into animals previously primed with a donor skin graft (13, 14). A second cellular effector mechanism was proposed upon the observation that lymphocytes recovered from recipients that had already rejected a graft were able to directly lyse donor cells *in vitro* (15-17). This effect could not be reproduced using lymphocytes from naïve donors, suggesting that these cytotoxic lymphocytes developed *in vivo* and as such, represented a powerful mechanism by which graft cells could be destroyed.

The development of the mixed leukocyte culture to enable the generation of cytotoxic lymphocytes has also provided an *in vitro* model for dissecting the mechanics of alloreactivity. The demonstration of cognate interactions between cultured cells (18) and of the intrinsic involvement of the H-2 complex in this interaction (19), resulted in the eventual elucidation of the nature of antigen presenting cells (APCs) (20), and also of MHC-restriction (21-23). In short, the use of mixed leukocyte culture clarified the integral components of the allo-

immune response; that graft rejection was initiated as a result of recipient T cell recognition of allo-MHC on the surface of donor APCs.

By the 1970s it had been established that an allogeneic graft was destroyed through a combination of three basic mechanisms:

1. A cytotoxic T cell response.

2. A DTH-like response due to the activation of recipient T cells within the graft.

3. An antibody-mediated response.

The earlier concept of alloantibody-mediated graft destruction had been largely discounted following the elucidation of the cellular mechanisms of graft rejection and the demonstration that alloantibody could enhance graft survival (24). However, alloantibody regained prominence upon the demonstration that the presence of alloantibody correlated with the development of hyperacute rejection (25).

1.3 The Major Histocompatibility Complex

1.3.1 Class I and Class II MHC Structure

Klein (26) proposed the general categorisation of MHC into class I and class II antigens upon the discovery that MHC could be structurally separated into two forms, and that this division correlated with the previous functional definition into either serologically-defined or lymphocyte-defined antigens. Class I and class II MHC molecules are members of the immunoglobulin (Ig) superfamily (see (27) for review). They are membrane glycoproteins, which have evolved by mutation and tandem duplication of the Ig domain gene and have similar structures. Both have four surface Ig domains, a transmembrane domain, and a cytoplasmic domain (28) (see Figure 1.1). Of the four surface Ig domains, the pair adjacent to the cell membrane are typical V-region-like Ig domains. In comparison, the outermost pair have a sheet and helical structure, such that, of seven β □strands, the first four fold to form a β-pleated sheet and the remaining three form a long α-helix. The two membrane distal domains are aligned face to face, resulting in the sheet strands forming a single continuous anti-parallel β-pleated sheet.

The structural components of the extracellular domains differ in the MHC class I and class II antigens. In MHC class I antigens, the α1 and α2 domains of the 44kDa α chain form the membrane-distal domains. The α-chain also contains a third domain, α3, which forms one of the membrane-proximal domains. A smaller 12kDa conserved molecule, β_2-microglobulin, forms the final membrane-proximal domain (see Figure 1.1B). The class II structure is assembled from a pair of non-identical protein chains, a 32kDa α chain and a 28kDa β chain. Each of these chains is composed of two domains; a membrane proximal Ig-like domain and a membrane distal domain that has a β-pleated sheet and α-helical-like structure. Both the α and β chains of MHC class II molecules contain transmembrane and cytoplasmic domains, whereas these are present in only the α chain of an MHC class I molecule (see Figure 1.1A)

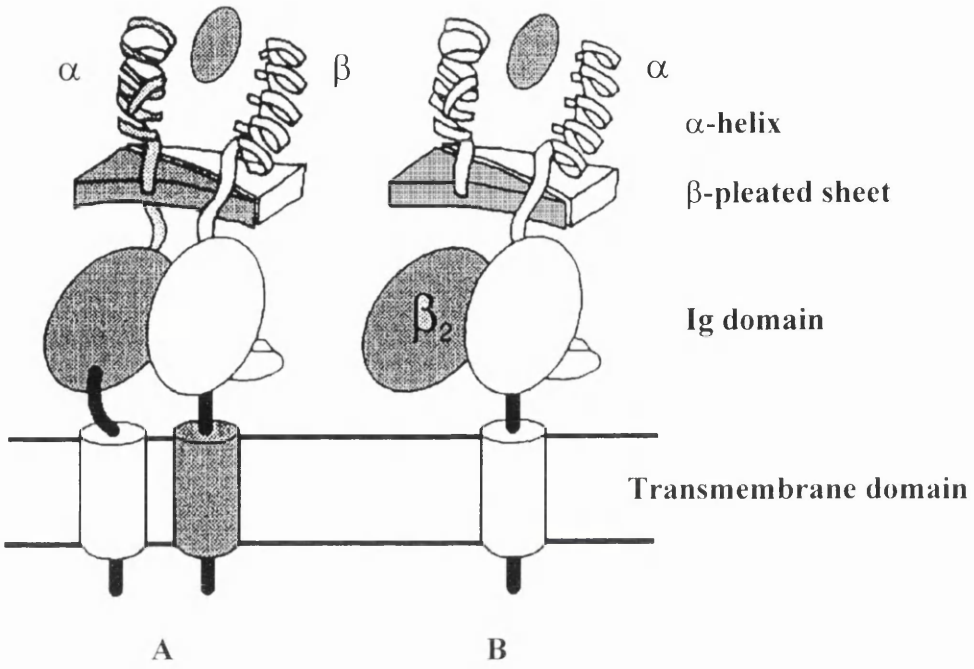


Figure 1.1 Schematic representation of the MHC structure.

A) MHC class II.
B) MHC class I.

β_2 represents the β_2-microglobulin light chain. Adapted from Halloran (28).

Although it was appreciated that the MHC was responsible for presenting peptides (29, 30), Bjorkman's crystallographic elucidation of the fine three-dimensional structure of MHC class I (31, 32) established that the α1 and α2 domains were arranged such that they formed a groove for peptide binding, with the β-pleated sheets contributing the floor, and the α-helices the side walls, of the groove. More recently, the crystallographic structure of MHC class II antigens has also been established (33, 34). Class II antigens have a generally similar three-dimensional structure to class I antigens, with the peptide binding groove formed as an interchain dimer of the α1 and β1 domains in an analogous fashion to the α1 and α2 domains of the class I structure. In contrast to the MHC class I structure, the peptide-binding cleft of class II antigens is open at either end. This difference is due to the nature of the amino acids found at either end of the peptide binding cleft. Class I antigens tend to have bulky amino acids, such as tryptophan in these positions, whereas the corresponding amino acids in class II structures are commonly those with smaller side chains, such as asparagine, which do not block

the ends of the cleft. These differences account for the ability of MHC class II molecules to bind longer peptides than class I molecules, as peptides are able to protrude from either end of the cleft.

1.3.2 Organisation of the Rat MHC (RT1)

The rat MHC, RT1, is approximately 3200 kilobases (3 centimorgans) long and is located on the short arm of chromosome 20 (35). Figure 1.2A depicts the genetic map as deduced through serological, biochemical and phenotypical analysis. Figure 1.2B represents the physical map of RT1, as obtained by using sequencing and restriction fragment length polymorphism (RFLP). (36). The MHC can be broadly divided into three sequential regions, I, II and III. The murine MHC differs from other species studied, in that it has undergone a major translocation event, which has resulted in the class II and class III loci being located between the major class I loci.

1.3.2.1 Class I MHC loci

Classical class I MHC antigens (Ia) are characterised by their polymorphism, their capture and binding of peptide, and their ability to elicit an allo-cytotoxic and an allo-humoral response. Other class I loci, the so-called non-classical, or Ib antigens, have been recognised and their products are frequently serologically undetectable (see (37, 38) for review). Those that are expressed are generally monomorphic and these antigens do not appear to have strong antigen presenting functions.

Classical MHC Class I Antigens (Ia)

The functional Ia antigens of the rat MHC are located in the RT1.A region. The degree of polymorphism in this region is much less marked than in other species. This is due to the presence of only one locus for the classical antigens within this region (39-41), in comparison to other species that usually express between two and four loci. The number of alleles within this locus is similarly reduced and has been estimated serologically at twelve (42, 43).

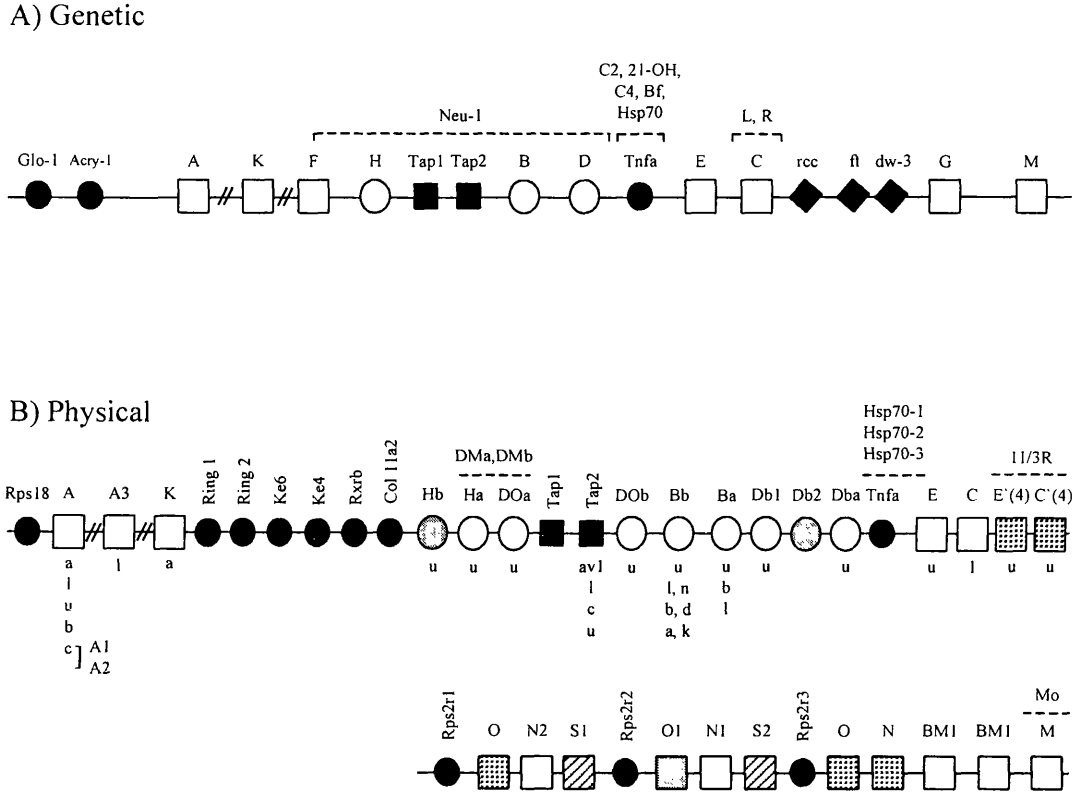


Figure 1.2 The current status of the genetic and physical maps of the rat major histocompatibility complex.

The genetic map (A) is based on data from serological, biochemical and phenotypical studies. The physical map (B) is based upon sequencing and RFLP data. The symbols E`(4) and C`(4) indicate four E-like and four C-like loci, respectively. Abridged from Gill (36)

- Class I MHC loci
- Loci detected by PCR or hybridisation analysis, but not fully sequenced
- Known pseudogenes
- S loci
- Class II MHC loci
- Other loci

To date nine alleles have been sequenced; RT1.A^a (39), RT1.A^n (44, 45), RT1.A^b (46), RT1.A^u (41), RT1.A^l (47, 48), RT1.A^c (40), and more recently, RT1.A^o, RT1.A^f, and RT1.A^g (45). The RT1.A^c (40), RT1.A^o and RT1.A^f (45) haplotypes are unusual, as sequencing reveals that each contains two distinct class I antigens, presumably because a second class I locus is present within the RT1.A region, as is the case for example with RT1.A^c (RT1.$A1^c$ and RT1.$A2^c$) (40).

Although other Ia loci are present within the rat MHC, they do not appear to have a strong function as antigen presenting molecules and are frequently mono- or oligo-morphic. They behave, at most, as minor transplantation antigens. For example, the classical MHC class I region additionally contains the antigens RT1.K (Pa) and RT1.F. Only one serotype of the Pa antigen has been sequenced (49), but three different alleles are responsible for differing levels of its expression (50), and these are inherited according to simple Mendelian genetics (51). Pa is thought to function as an immunoglobulin transporter during pregnancy. The RT1.F antigen is similarly monomorphic (52), but its function is unknown.

Non-Classical MHC Class I Antigens (Ib)

The remainder of the class I genes, which have been estimated to be as many as 60 in the rat (53), map to the RT1.E/C/M, non-classical region. They perform a wide range of functions. Many are simply pseudogenes without an identifiable gene product, and either provide a reservoir of class I-like genes for the diversification of the classical class I products through recombinant events (54), or perhaps represent classical antigens which have been superseded during the evolution of the MHC. Non-classical antigens are unable to bind as wide a diversity of peptides as the classical antigens. Several have been identified in humans and the mice that can bind specific ligands. They may be pivotal in the response against certain bacteria, viruses and heat shock proteins (hsp) (55, 56). Functional, non-classical class I antigens may however be more revalent in the rat as a means of compensating for the otherwise limited polymorphism at the classical locus. Leong et al (57), for example, have recently described an RT1.U antigen which maps to the RT1.E/C/M region and is closely related phylogenetically to the classical antigens. It is serologically detectable, elicits a

cytotoxic T cell response, and possesses typical residues at the conserved sites required for peptide binding.

1.3.2.2 Class II MHC loci

The gene density within the MHC class II region is relatively low, approximately one gene per 40 kilobases, and large stretches have been sequenced without detecting recognisable genes. Recombination events within such low-density areas are relatively rare, and mapping has confirmed the strikingly similar basic organisation of the region in the human, mouse, and rat (58) (see Figure 1.3). The rat has two functional classical class II loci, RT1.B and RT1.D, which correspond to the human DQ and DR antigens respectively. The DR antigen is amongst the most polymorphic of the human MHC antigens. In comparison, the rat B and D antigens apparently exhibit limited polymorphism, in that there are only eleven serologically defined class II antigens (a-d, f, h, k-n, and u) (59). Nucleotide sequencing of the D locus confirms the presence of relatively few alleles; the RT1.Dα locus carries a virtually identical allele for every rat strain studied, and only 6 different alleles have been discovered for the RT1.Dβ locus (60). However, genetic sequencing suggests that the RT1.B region is more polymorphic, with at least nine RT1.Bβ alleles (61) and six RT1.Bα alleles (62) identified. This discrepancy in the degree of polymorphism as established by genetic, compared to serological techniques has yet to be reconciled. Only one pair of functional α and β genes are expressed at the B and D loci, and this again contrasts with the human DR antigen, which can have as many as four functional β chains (63). In humans, a third class II locus, DP, exists. The equivalent loci in the rat and in the mouse, RT1.H and H-2P respectively, do not express functional antigens as their β chain components are pseudogenes (64-67).

Within the RT1 class II region, additional sequences code for two non-classical antigens. The DM antigen, which is similarly placed in the human (named HLA-DM) and mouse genome (named H-2M), appears to have a universal role in the exchange of the CLIP peptide for antigenic peptide (68). RT1.DO is also a non-classical, monomorphic class II antigen (69). It was initially discovered in the HLA as an α-chain gene DN and a β chain gene DO. Subsequent studies on the

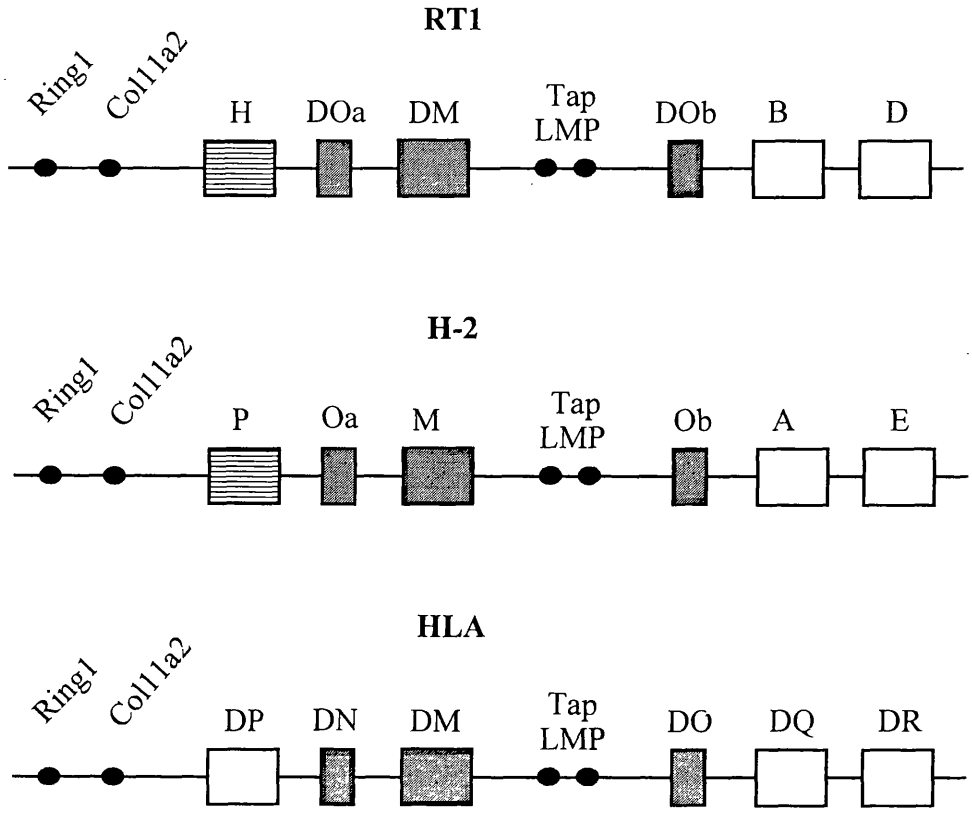


Figure 1.3 Comparative organisation of the class II major histocompatibility region of the rat (RT1), mouse (H-2), and human (HLA).

Classical class II loci

Pseudogenes

Non-classical loci

analogous mouse antigen, H-2O, revealed that these chains could combine into a functional protein (70, 71), although tissue distribution is restricted to B cells and epithelial cells of the thymic medulla (72). The DO antigen modulates peptide loading of class II molecules in the endocytic pathway (see section 1.4.3.5). The DOa and DOb alleles are separated by sequences for TAP-A and TAP-B, and LMP-2 and LMP-7 (73) whose products are integral components of the class I antigen processing pathway.

1.3.2.3 Class III MHC loci

Unlike the class I and class II regions, the products of the class III loci do not appear to have a common evolutionary origin. They consequently perform an eclectic range of functions, but these are generally connected with the immune response. It may be that mutation to incorporate their genetic sequences into the MHC was associated with an evolutionary advantage. Products identified to date include the complement factors, C2, C4, Bf (74, 75) and several soluble proteins such as TNF-α and hsp70 (76, 77).

1.3 3 Genetic Organisation of the Rat Class I and Class II MHC Antigens.

For eukaryotic genes, the genetic coding sequence is divided into a series of sections known as exons, which are separated by non-coding sequences called introns. The boundaries between introns and exons are marked by specific nucleotide sequences, frequently AGGTAAGT and TCNCAGG. For members of the Ig superfamily, exons generally correspond to the domains of the protein. The first exon contains the message for the leader sequence, including the signal peptide. This guides the nascent peptide through the endoplasmic reticulum and is cleaved before final maturation.

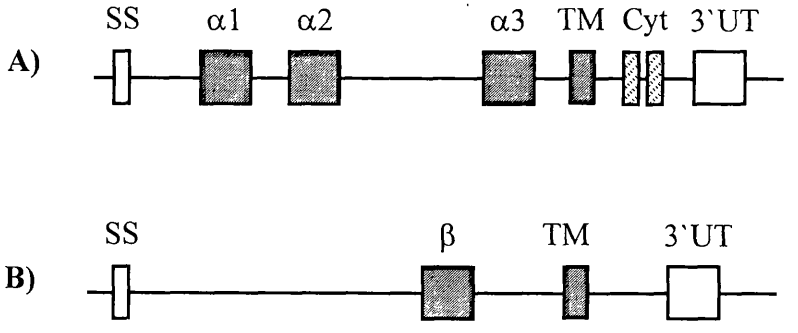


Figure 1.4 Exon-intron organisation of the heavy and light chains of class I MHC genes.

A) α-heavy chain of a class I MHC gene
B) β_2-microglobulin light chain.

SS -signal sequence
TM -transmembrane domain
Cyt -cytoplasmic tail
3'UT -3' untranslated region

MHC class I genes typically span 4000 to 5000 bases and contain eight exons (Figure 1.4A), with two exons coding for the cytoplasmic tail, and the final eighth exon untranslated. The gene for the non-polymorphic β_2-microglobulin molecule is composed of four exons, although the majority of the actual coding sequences originate from the second exon (Figure 1.4B). MHC class II genes are similarly arranged. Only one exon, however, codes for the α chain transmembrane and cytoplasmic domains, whereas separate exons code for each in the β chain gene. There is an additional, non-expressed β chain gene at each of the major class II loci, which is located to the left of the B region and in the middle of the D region (78) (Figure 1.5).

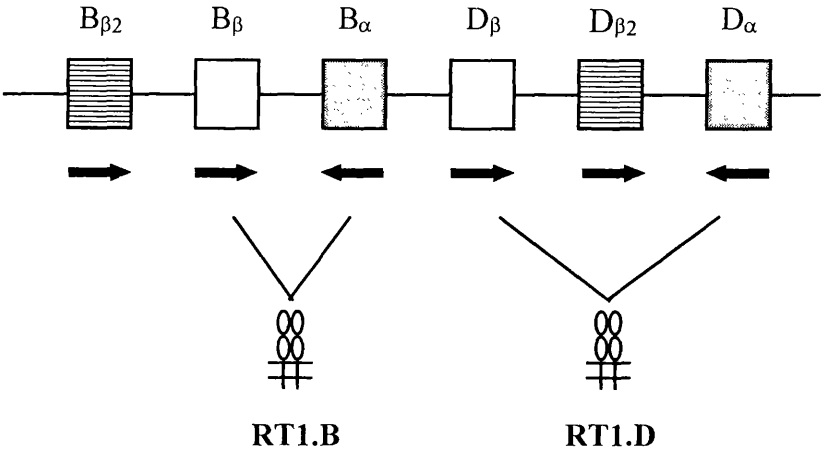


Figure 1.5 Genomic organisation of the class II alleles of the rat MHC. Arrows indicate the direction of transcription.

α alleles

β alleles

Pseudogenes

1.3.4 MHC Gene Transcription

1.3.4.1 Transcription Machinery

As with most eukaryotic genes, MHC sequences typically contain the conserved TATA and CCAAT elements in their upstream promoter (Figure 1.6). The TATA box, which is situated approximately 30 bases upstream of the initiation of transcription, acts as the core promoter, to which the basal transcription initiation complex binds. This consists of at least five factors, and transcription is initiated upon binding of RNA polymerase II to these factors (see (79) for review). Termination of transcription occurs at a variable distance (frequently hundreds or thousands of bases) downstream of the termination codon. The nascent mRNA sequence is modified in three ways. Firstly, a methylated guanine residue is attached to the 5` end of the molecule, at a specific 'cap' site. Secondly, a poly adenosine tail is added to the 3` end of the molecule by a specific polymerase, which is triggered by the AAUAAA signal in the 3` non-coding region. The nascent mRNA finally undergoes endo- or exonucleotyic cleavage to remove the introns, followed by exon splicing to form the mature mRNA sequence.

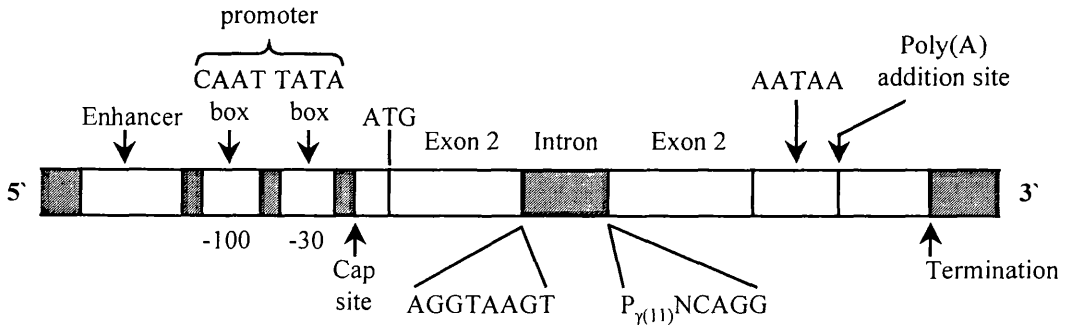


Figure 1.6 Transcriptional machinery for a typical eukaryotic gene.

1.3.4.2 The Regulation of transcription

In addition to the TATA and CCAAT elements, there are a number of cis-acting enhancer sequences within the upstream promoter, whose features are conserved and specific for each class of MHC gene. The binding of various regulatory proteins to these enhancers alters the rate at which the polymerase complex transcribes the upstream message. Four enhancer elements have been identified within the class I promoter region; the enhancer A element, the interferon stimulated response element (ISRE), site α, and the enhancer B element (Figure 1.7A) (80). The enhancer A element is a possible ligand for the binding of the five proteins of the NF-κB family (81). The binding of interferon regulatory factors (IRF) 1 and 2 to the ISRE site results in increased and decreased rates of transcription respectively. This provides a mechanism by which IFN-γ can regulate transcription, since the levels of IRF are IFN-γ sensitive (82). The ligands for the site α are unclear, but occupancy of this cis-acting, regulatory element appears to be necessary for ISRE-induced transactivation (83). Finally, the enhancer B element represents an inverted CCAAT box, and is an essential component of class I activation (84). The clustering of these enhancer areas within the class I promoter suggests that they act in a synergistic fashion upon class I transcription.

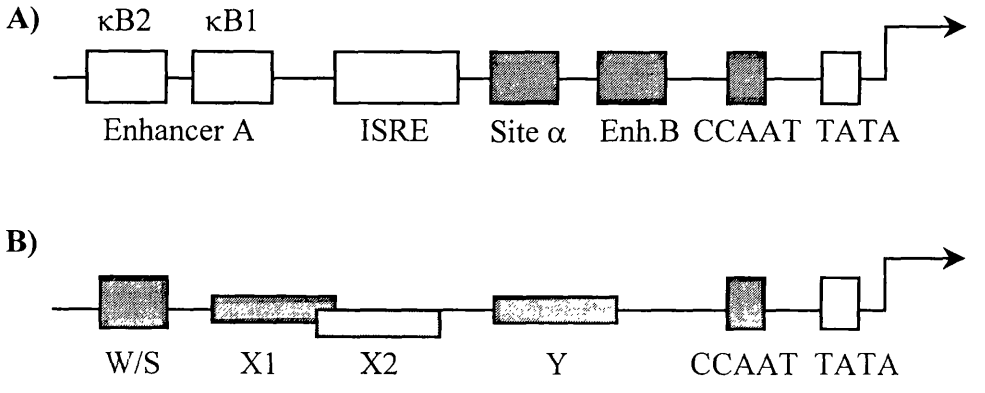


Figure 1.7 Promoter structures of MHC class I and class II genes.

A) MHC class I promoter structure.
B) MHC class II promoter structure.

For MHC class II genes, the upstream promoter elements include W/S, X, and Y boxes (Figure 1.7B) (see (85) for review). The X box consists of X1 and X2 halves. Five members of the 'RFX' family of transcription factors (RFX1-RFX5) interact with the X1 box, although only RFX5 has been observed to induce class II transcription. RFX5 binds to the box, as part of a sub-unit of an RFX complex, which also contains an RFX-associated protein (RFXAP) and a p41 protein, whose identity is unknown. Although several protein families have been identified that can bind to the X2 box, only the X2 binding protein (X2BP) is crucial to class II transactivation. The W/S box has a similar sequence to the X1 box, and also binds the RFX complex. Precise spacing of the W/S box in relation to the X box is required for class II promoter activity. The Y box is an inverted CCAAT box that binds, amongst other proteins, NF-Y. The above proteins do not work independently, but collectively form a protein complex, which acts as a basic regulatory module on the W/S-X-Y region (86). Binding of this complex is not sufficient for class II transcription. This requires the additional presence of the class II transactivator protein (CIITA), which acts as a co-activator by binding to the regulatory module at the RFX5 subunit (87). CIITA is expressed constitutively only within antigen presenting cells.

The nucleotide sequences of the enhancer boxes are not conserved, but vary according to the individual MHC class II gene. Consequently, the enhancer boxes of different class II genes bind their respective components (RFX, X2B and NF-Y) of the regulatory module with different affinities. Nevertheless, as a complete unit the regulatory module appears to bind the class II enhancer region with a constant affinity as synergistic RFX-X2BP-X box complexes can be formed on the X-box regions of all MHC class II genes *in vitro* (86, 88). This permits the transcription of α and β chains to be regulated in a co-ordinated fashion.

1.4 Antigen Processing and Presentation

1.4.1 MHC Restriction and Antigen Processing

The MHC was initially discovered through its ability to initiate an alloimmune response. It was subsequently realised that, more importantly, the interaction of *self*-MHC with the TCR was fundamental to any specific immune response. This was prompted by the observation that *in vitro* T cell proliferative responses to antigen required an additional population of cells, commonly macrophages (89, 90). Rosenthal subsequently noted that the macrophage and lymphocyte populations had to display the same immune response (Ir) genes (20, 91). It was not appreciated that this experiment demonstrated the principles of MHC-restriction, as at that time, the nature of the Ir genes was unknown. MHC-restriction was first clearly demonstrated by Zingernagel (21-23), who noted that virally infected fibroblasts could only be lysed by primed T cells that displayed the same MHC class I phenotype. Simultaneously, David and Sachs established that the Ir proteins represented a second class of MHC antigen (7, 92). The following year Erb and Kappler (93-95) reported a similar restriction phenomenon for MHC class II. Subsequently, Cantor demonstrated that T cells could be divided serologically into cytotoxic (T_c) and helper (T_h) subsets (96, 97), and in turn, that each subset was specific for a different class of MHC. Thus the principles of T cell activation were established: CD4 helper T cells recognise antigen presented by APCs in the context of MHC class II, whereas CD8 cytotoxic T cells respond to antigen presented by MHC class I.

Knowledge of the APC was still in its' infancy, and a decade passed before Ziegler and Unanue demonstrated that disrupting the lysosomal function of APCs prevented MHC class II-restricted antigen presentation (98, 99). It was concluded that in such cells antigen was modified, or 'processed' before its presentation. Towsend et al subsequently confirmed that this also applied to MHC class I-restricted antigen presentation (100-102). Understanding of the class I and class II processing pathways has increased tremendously over the last decade.

1.4.2 Class I MHC antigen processing

The peptides that are ultimately presented by MHC class I molecules are generated in the cytosol, by a complex known as the proteosome. The association of peptide with nascent MHC class I heterodimers occurs in the endoplasmic reticulum (ER), with the transfer of peptides from the cytosol to the ER achieved with the aid of the transporter associated with processing (TAP).

1.4.2.1 The Proteosome Complex and Antigen Degradation

The Regulation of Proteolysis

Peptide fragments are continuously required to stabilise MHC class I molecules, and peptides eluted from surface MHC molecules are generally derived from self-protein, rather than foreign antigen (103). The generation of self-peptides for MHC class I loading requires regulation, otherwise essential self-proteins would be needlessly destroyed. The exact mechanisms of this regulation are as yet unknown. Yewdell has suggested that proteins that are misfolded, due to defective ribosomal synthesis, are more susceptible to protease-digestion (104). Otherwise, certain sequences of a protein may make it more susceptible to digestion. For example, the presence of either PEST regions (105) (areas rich in proline, glutamine, serine and threonine), or destruction boxes (106) promote degradation.

The commonest means by which proteins are targeted to the proteosome is through the conjugation of ubiquitin to the internal lysine residues of the proteins. Several enzymes catalyse this reaction and they add additional ubiquitin molecules to the lysine residues of already-bound ubiquitin molecules. It is unclear how polyubiquitination destabilises protein, but it probably unfolds the tertiary structure of the protein and provides a signal for uptake by the proteosome (107).

The Proteosome Complex

The proteosome consists of a barrel-shaped 20s core, which lyses proteins that associate with class I MHC molecules (108). In simple archaebacterium, its crystal structure reveals a stack of four rings (109). Each ring contains seven subunits, which are type α in the outer rings and β in the inner rings. The inner rings additionally contain the proteolytic elements. In higher eukaryotes additional subunits, such as a PA28 activator, a PA700 subunit (110) and a 19s ATPase complex (111) make up a larger 26s proteosome complex (112). The PA28 activator is inducible by IFN-γ and consists of a hexamer of αβ subunits that attaches as a cap to both ends of the 20s proteosome. It increases the spectrum of peptides that are generated, partly by increasing cleavage after hydrophobic amino acid residues (113), but also by rendering the substrate protein susceptible to protease activity at two sites concurrently, resulting in double peptide cleavage (114).

In higher eukaryotes only three of the β subunits in each β7 ring are active. Each of these possesses distinct proteolytic activities; for example β5 has a chymotrypsin-like activity, whereas β2 resembles trypsin. IFN-γ, however, induces the expression of three additional β subunits, MECL-1, LMP2 and LMP7, which compete respectively with the constitutively expressed β2, β7 and β5 subunits. Their incorporation results in an immuno-proteosome, which alters the spectrum of peptides that are generated (115-117). In particular, LMP7 increases cleavage after hydrophobic or basic residues, which may improve the efficiency with which the resulting peptides bind to nascent MHC molecules. The nucleotide sequence of LMP2 and LMP7 are situated within the MHC class II region of the genome.

It has generally been assumed that the proteosome strictly regulates proteolysis to ensure that the generated peptides are 8-9 amino acids in length. However, Kisselev recently demonstrated that the peptide products of archeabacterial proteosome varied widely in length with only 15% falling within the 7-9 amino acid residue length (118, 119). Neither is the proteosome complex indispensable.

Mammalian cells can adapt *in vitro* to its loss by the over-expression of another large proteosome particle (120).

1.4.2.2 The TAP Transporter

Once appropriately sized peptides have been generated by the proteosome, they pass into the ER. This process is controlled by a heterodimeric transporter complex referred to as the transporter associated with processing (TAP). The TAP complex is comprised of two subunits, TAP-1 and TAP-2, which are encoded by genes in the MHC class II region. Each TAP subunit has an N-terminal hydrophobic region with up to eight transmembrane domains. This allows it to straddle the membrane of the ER. The peptide-binding site is located on the C-terminal end of the hydrophobic segments of both subunits, adjacent to the hydrophilic cytosolic domain (121, 122). It is likely that peptides are both bound by their N- and C- terminal regions, with looping of the central portion of the peptide (123). This permits the binding and transport of peptides as long as 40 amino acids across the ER membrane (124), but peptides of 8-13 residues generally bind most efficiently (125). Although TAP is the main transporter, subsidiary ones exist (126).

There are two TAP haplotypes in the rat, A and B (127-129) which demonstrate linkage disequilibrium with the classical class I antigen (130). The differences in the peptides that they transport are profound; in RT1.A^a bearing hosts that normally express TAP-A, the A^a antigen becomes immunogenic if it is linked instead to TAP-B (131). Genetic mapping and subsequent cloning of the rat transporters (130, 131) have identified that the type A and B haplotypes relate to the TAP-2 locus. Alleles at this locus exhibit polymorphism, but the alleles of one haplotype can be distinguished from the other on the characteristics of 25 amino acid residues in the N-terminal two-thirds of the chain (132, 133). TAP-2B is similar to mouse TAP-2, in that it has a high affinity for peptides with a hydrophobic C-terminal amino acid residue. In comparison, TAP-2A is akin to the human transporter and is much more permissive, binding peptides regardless of their C-terminal amino acid residue. It is unclear what evolutionary advantage the persistence of TAP-2B provides, as its substitution with TAP-2A does not impair peptide loading of MHC class I molecules (134). There is no apparent functional polymorphism present in either the mouse or the human (125, 135, 136).

1.4.2.3 Assembly of the Peptide-MHC Class I Complex

The stability of the MHC class I heterodimer is conferred by the bound peptide. Without an appropriately bound peptide, nascent MHC molecules do not reach the cell surface, but instead undergo degradation within the ER (137, 138). Assembly of the MHC-peptide complex requires a series of protein mediators, or chaperones. Initially, class I heavy chains associate with calnexin. This is an ER-retained transmembrane protein, which aids the appropriate folding and the formation of disulphide bonds in the heavy chains (139). β_2-microglobulin then binds to the heavy chain, and this catalyses, at least in humans, the exchange of calnexin for its soluble homologue, calreticulum (140). Two further chaperones subsequently become incorporated into the class I complex, the recently discovered ERP57 (141-143), and a 48kDa protein, tapasin. Tapasin is a transmembrane protein belonging to the immunoglobulin superfamily, and is encoded in the human and murine class II MHC regions immediately centromeric to the class II region (144). There is no redundancy within the ER for tapasin and it has at least two important functions (145). Primarily, it enables the MHC complex to bind to the TAP transporter, and as many as four tapasin-MHC complexes can bind to one TAP dimer (146). This multimeric complex may enhance the efficiency of peptide loading, by increasing the availability of different MHC molecules. Tapasin also directly stabilises empty class I molecules, possibly by binding to the $\alpha 1$ and $\alpha 2$ domains of the nascent molecule. This is an analogous property to that of HLA-DM in MHC class II peptide loading, and is independent of its association with TAP (147). Upon peptide binding, all auxiliary molecules are released from the class I molecule and the mature class I complex is exported to the cell surface via the golgi and trans-golgi network, and from there follows the 'default' pathway of vesicular traffic to the cell surface (148).

1.4.2.4 Presentation of Exogenous Peptide

Although, the cytosolic proteosome generally degrades endogenous proteins (either self or viral), it can also sample exogenous substrates. APCs in particular are able to transfer antigens from class II-processing organelles to the cytosol for proteosomal lysis (149-151). Additionally, MHC class I can either re-circulate from the cell surface into the phagosomal pathway, or perhaps is targeted to the endocytic pathway by linking to the invariant chain signal (152).

1.4.3 MHC Class II Antigen Processing

The MHC class II processing pathway presents extracellularly derived antigens to CD4 T cells. Nascent class II heterodimers are bound to an invariant chain (Ii), whose signal sequence directs the class II complex from the ER to the endosomal compartments that process engulfed antigen. There, the Ii is lysed into smaller peptide fragments, and the DM antigen catalyses their exchange for antigenic peptide. Mature MHC class II-peptide complexes travel to the cell surface by mechanisms that are poorly understood.

1.4.3.1 Processing of Exogenous Antigen

APCs use several mechanisms to internalise extracellular antigen into their highly developed endosomal and lysosomal compartments; receptor mediated endocytosis (153), phagocytosis, and macropinocytosis. The relative contribution of each of these mechanisms varies according to the class of APC (154). B cells, for example, generally recognise antigen through its binding to the Ig surface receptors. The Ig receptors are associated on the cell surface with clathrin coated pits that internalise antigen to the endosomal pathways. Such receptor-mediated endocytosis permits B cells to process and present antigen, specific for their surface immunoglobulin receptors, at 10^3 to 10^4 times lower concentration than cells which internalise antigen non-specifically (155, 156).

In comparison, macrophages and dendritic cells capture antigen by either phagocytosis or macropinocytosis. Phagocytosis is initiated by the binding of Ig-derived peptides to surface receptors. This activates the cell membrane to form pseudopods that initially engulf the bound particle and then internalise it by detaching from the membrane as phagosomes. Macropinocytosis (cell drinking) generally occurs at clathrin coated pits, but unlike those involved in receptor-mediated uptake, the pits are of a larger size, which permits sampling of a greater volume of the extracellular milieu. The relative ability of an APC to capture antigen equates to the overall efficiency with which that antigen is presented (157).

APCs contain a complex arrangement of distinct endosomal compartments, through which internalised antigen is trafficked sequentially. The first structures receiving endocytosed material are early endosomes (EE). These are less acidic than later

endosomes, which limits their proteolytic potential. EEs are specifically concerned with the recycling of plasma membrane proteins to the cell membrane. Transfer occurs via small vesicles that emanate from the EE tubule (158). Antigens that are not recycled are subsequently transported to late endosomes (LE). LEs characteristically display numerous small membrane vesicles in their lumens and develop from the vacuolar parts of the EE network. They gradually mature into lysosomes (159), by progressively accumulating internal membranes, dense waste material and specific cell surface markers, such as lysosome-associated membrane protein (LAMP).

These changes correlate with the development of protease activity. Protein antigen is degraded by a combination of endoproteases, which hydrolyse internal amide bonds of proteins, and exopeptidases, which hydrolyse one or two amino acids from either end of the target peptides. The enzymes are characterised according to the particular 'active-site' amino acid residue of the target protein that they recognise. There appear to be three major endoproteases; the aspartate protease, cathepsin D, and the cysteine proteases, cathepsins L and S (160). Two further cathepsins, B and H, have limited endoproteolytic activity, but their function is mostly vestigial (161).

1.4.3.2 Trafficking of Nascent MHC Class II Dimers through the Endoplasmic Reticulum

After translation, MHC class II molecules and the Ii are, in similar fashion to MHC class I molecules, co-translationally inserted into the ER, due to the presence of a C-terminal signal sequence (162). Chaperones such as calnexin (163, 164) retain class II molecules in the ER until they are properly folded and associated with the Ii. The association of MHC class II and the Ii occurs through the binding of the class II-associated invariant chain peptide (CLIP) portion of the Ii to the MHC class II peptide binding groove (164-168). This renders the groove inaccessible to peptides present within the ER (see Figure 1.8).

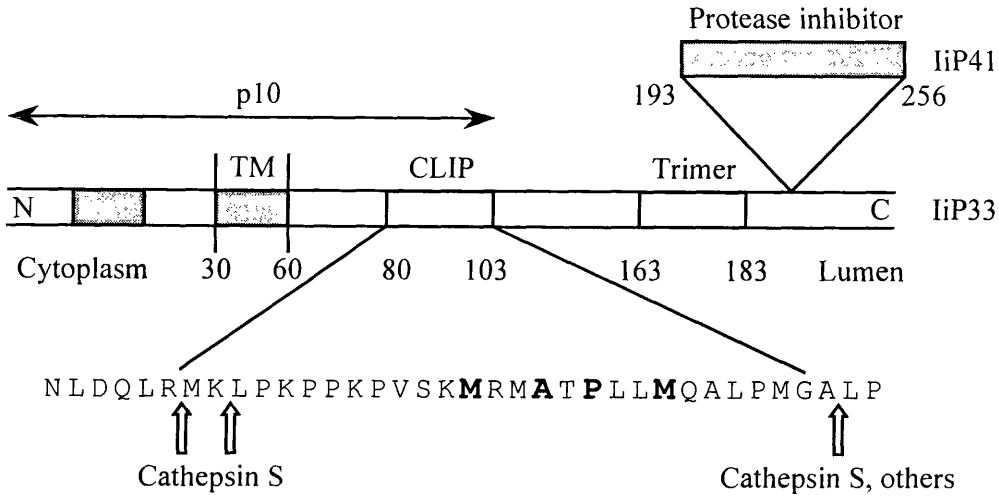


Figure 1.8 Structure of the invariant chain.

The human P31 isoform is depicted. Important structural features include a cytoplasmic sequence involved in endosomal trafficking (shaded), a transmembrane domain (TM), the class II associated invariant chain peptide (CLIP), and a trimerisation domain (trimer). In addition, a protease inhibitor domain occurs in certain isoforms, e.g. P41. Arrows designate probable cleavage sites of the CLIP protein by cathepsins. The amino acids in bold type are responsible for anchoring the invariant chain to the class II peptide-binding groove. Adapted from Chapman (160).

Trimerisation of Ii chains, as a result of an interaction at the C-termini (169, 170), and possibly at the transmembrane trimerisation sequences (165), subsequently occurs, resulting in the formation of nonameric MHC class II-Ii complexes (see Figures 1.8 and 1.9). Proteins within the ER are normally expressed at the cell surface after transfer through the golgi and trans-golgi network, but nonameric MHC class II complexes are diverted into the endosomal network by di-leucine based targeting signals in the Ii chain cytoplasmic tail (171-173). This targeting does not involve cell surface recycling, and trimerisation of the Ii substantially increases the efficacy of the signal sequence (174).

1.4.3.3 Entry into the Endosomal Pathways

In professional APCs, MHC class II accumulates in endocytic structures called MHC class II compartments (MIICs) (175). This is presumably their entry point into the endocytic pathway as the Ii, which is degraded quickly, is also first detectable in this compartment (176, 177). MIIC probably represent the immature LEs found in non-professional APCs. Certainly, they contain typical lysosomal membrane proteins and

enzymes (178), and in comparison, the transferrin receptor, whose presence characterises EEs, is not present. Recent immunoelectron microscopy has confirmed the similarities between the two types of vesicle (179). MIICs mature in stages, from a multivesicular structure, to an intermediate structure, containing both vesicles and membrane sheets, and finally to a multilaminar vesicle, which contains membrane sheets only.

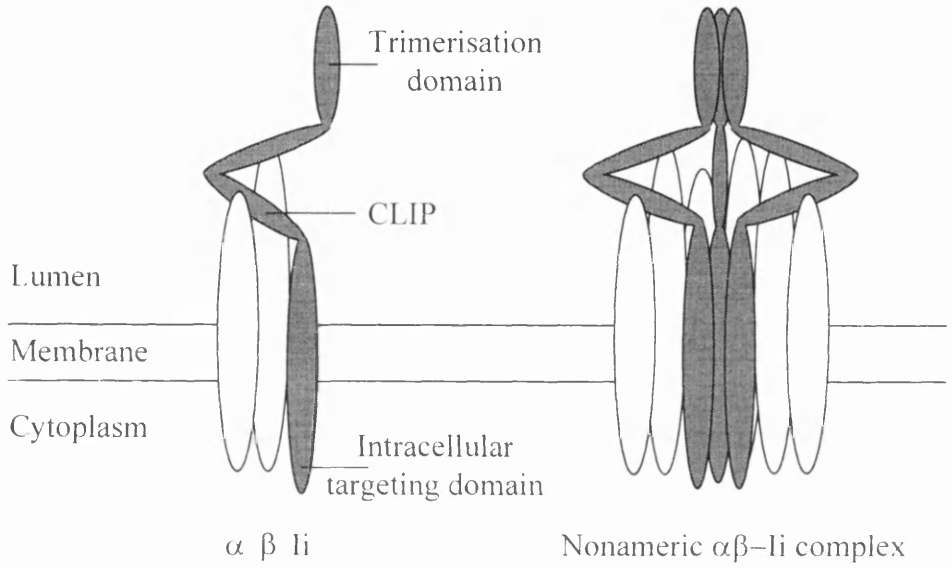


Figure 1.9 Interaction of the invariant chain with class II MHC.

The invariant chain (shaded) associates with MHC class II αβ heterodimer (unshaded). The left panel shows a complex of one Ii molecule and a nascent class II molecule, whereas the right panel shows trimer association through the trimerisation and transmembrane domains to form nonameric complexes. Adapted from Pieters (180).

Mellman and others however, have proposed that class II complexes enter the endocytic pathway at the EE stage (163, 181, 182), and are incorporated in so-called class II-containing vesicles (CIIVs) (177, 181, 183, 184). The significance of these vesicles is uncertain, for they appear to be morphologically distinct from other endocytic compartments, in that they do not contain Ii. The class II molecules within these vesicles may have however entered via recycling from the cell membrane (185), rather than directly from the ER. Different compartments may moreover be more relevant in different types of APCs.

Whatever the exact point of entry of MHC class II molecules into the endocytic pathway, the associated Ii is degraded rapidly in distinct steps to generate successive fragments of 22, 18 and 12kDa (186, 187) (see Figure 1.8). Both cathepsins S and L are responsible for Ii degradation, with the former perhaps more important (188). Degradation begins at the lumenal C-terminal domain, and by disrupting the trimerisation sequence, separates the invariant chains to leave individual MHC class II dimer-Ii complexes. The 12kDa Ii fragment that remains after the final cleavage contains the CLIP peptide (see Figure 1.8).

1.4.3.4 MHC Class II Peptide Loading

The exact site of antigenic peptide exchange for the CLIP fragment is uncertain, but probably occurs in MIICs (177, 182, 186, 189-191). Exchange is generally dependent on the HLA-DM (DM) antigen (183, 192, 193) (analogous to H-2M in the mouse), which is found in the later MIICs (179, 184, 194-196). DM molecules reach this compartment independently of the Ii, as a result of a tyrosine-based signal sequence in the cytoplasmic tail of the β chain (194, 197), possibly by binding to the internal clathrin-associated adapter protein complexes, AP1, AP2 and AP3 (198-203).

The DM antigen is non-polymorphic and non-classical in that its' peptide-binding groove is closed (204). Mutant human cell lines defective in HLA-DM can only generate MHC molecules containing CLIP peptides (205), and similar results have recently been obtained *in vivo* using H-2M-deficient mice (206-208). By binding to an MHC class II molecule, DM catalyses peptide exchange by stabilising empty MHC class II dimers, thus preventing their degradation, particularly in acidic compartments (209). This permits sampling of putative peptides, so that only those with high enough binding affinity for the class II dimers are ultimately chosen, while those with low affinity rapidly dissociate (193, 210-212). A single HLA-DM molecule facilitates serial loading of MHC class II molecules, attaining a turnover of 3-12 CLIP complexes a minute (213).

MHC class II peptide loading can occur in the absence of DM in several situations. For example, certain class II antigens can release CLIP independently (214). This ability has been ascribed, in the case of RT1.A^k, to an extremely low affinity for the

CLIP fragment (215). Certain compartments, notably EEs, lack the DM antigen, but are still able to load class II complexes with peptide, possibly because of a reduced affinity for CLIP in their less acidic environment (216).

Once peptide has bound with sufficiently high affinity to a nascent MHC class II molecule, DM dissociates, and the mature class II complex is transported to the cell surface. This may occur by several mechanisms, most probably by either fusion of the multivesicular MIICs with the plasma membrane (217), or via vesicles detaching from tubular extensions of early MIICs (218).

The class II peptide complexes that reach the cell membrane do not necessarily remain static, but can recycle into the EEs (185), particularly those complexes in which the CLIP fragment has remained bound (219). Recycled class II complexes return to the cell membrane directly via small vesicles emanating from the EE tubules (158) and do not enter the major class II loading compartments.

1.4.3.5 Regulation of MHC Class II Processing

The successful presentation of exogenous antigens depends upon the concurrent regulation of several processes. The availability of DM, the efficiency with which the Ii is degraded, and the ability to process antigen into suitable peptides within the lysosome, may all influence peptide presentation by MHC class II. DM is generally expressed at limiting levels within the APC (220), and nascent class II dimers may therefore compete for its' binding; DM-catalysed loading of DR2-CLIP, for example, was considerably slowed when DR3-CLIP complexes were added (213). Additionally, different APCs contain different isoforms of Ii, which have different functions. One such isoform, IiP41 contains a 64 amino acid, cysteine-rich domain. This has strong homology to the repetitive segment in thyroglobulin (221, 222), and is expressed in 5% of B cells, and in 50% of dendritic cells (223). The additional domain probably acts as an inhibitor of cysteine proteases, such as cathepsin L (224, 225), thus preventing degradation of the CLIP complex past the 10kDa stage (226) (see Figure 1.8). This presumably influences the spectrum of peptides that are bound to, and ultimately presented by, MHC class II molecules, as the ability of certain peptides to displace CLIP may vary depending on the extent to which CLIP has been degraded.

As mentioned in section 1.3.2.2, the DO antigen is expressed in B cells and in the thymic medulla. It is a non-classical class II antigen, and appears to antagonise the function of DM, for which it has a strong binding affinity (227). It may therefore be an important modulator of MHC class II-restricted antigen processing.

1.4.3.6 Presentation of Endogenously-Derived Antigens by Class II MHC

Cytosolically derived peptides (i.e. endogenous peptides) are normally processed by the MHC class I pathway but can, under certain conditions, enter the MHC class II pathway. One mechanism by which this may occur is through internalisation of membrane proteins into the endocytic pathway. However, although MHC class I-derived peptides are naturally associated with surface MHC class II (103, 228), there is no direct evidence that internalised membrane proteins undergo degradation (229). Cytosolic antigens may alternatively be sampled by the process of autophagy, whereby a cisterna, presumably of ER origin, randomly engulfs a portion of cytoplasm and matures into an autophagic vacuole that subsequently fuses with the LE (230). Cytosolic proteins can also be translocated across the MIIC membrane by a receptor mediated pump requiring ATP and heat shock proteins (231).

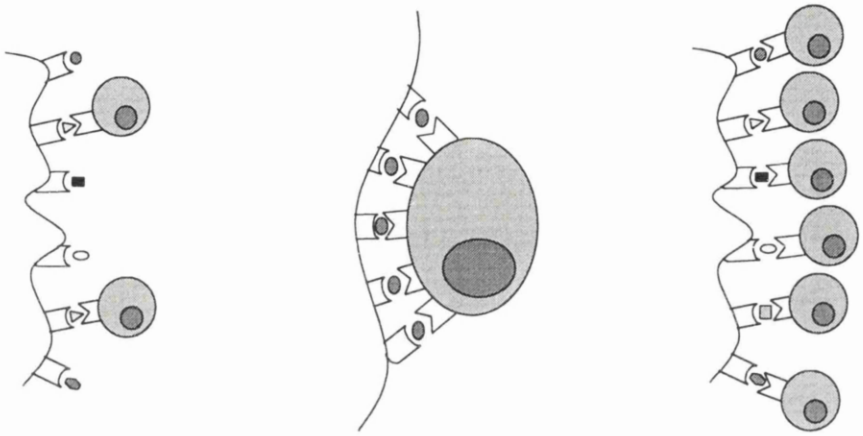
1.5 Allorecognition

Allograft rejection was conventionally perceived as the result of the 'direct' T cell recognition of allogeneic MHC molecules on the surface of donor cells. However, the realisation that MHC-restricted peptide presentation was equally relevant to the thymic selection of the T cell repertoire (232) resulted in the re-evaluation of the principles of allorecognition. Specifically, it was necessary to explain not only how T cells selected on the basis of self MHC-restriction were able to recognise allogeneic MHC, but also why such a large precursor frequency of T cells was able to do so. Two possible explanations have been suggested. Firstly, if one assumes that an allogeneic MHC molecule mimics the complex of an antigenic peptide bound to self-MHC, then every allogeneic molecule on the surface of a donor antigen-presenting cell is a potential determinant for T cell activation. This represents a much higher 'determinant density' (233) than occurs upon the processing of nominal antigen, wherein only approximately 0.1 to 1% of the surface MHC molecules express the relevant peptide (see Figures 1.10A and 1.10B). An alternative explanation for the strength of the alloimmune response is provided by the ability of a single MHC molecule to bind many different peptides; each combination of the MHC molecule with a different peptide represents a distinct binary complex that may be recognised by a different T cell clone (234) (see Figure 1.10C). Experimental evidence suggests that the 'high determinant density' and 'multiple binary complex' theories both contribute to the process of allorecognition (235).

1.5.1 The Occurrence of Indirect Allorecognition during Graft Rejection

Elucidation of the role of MHC molecules in the presentation of nominal antigen led Lechler and Bachelor (236) to question whether the self-restricted recognition of alloantigen could contribute to graft rejection. This was based on their observation that kidney allografts depleted of interstitial dendritic cells were still rejected, albeit more slowly than grafts with their full complement of cells. They argued that because dendritic cells provided additional costimulatory signals, they were uniquely capable of activating recipient alloreactive T cells. In their absence T cell activation could still occur, through a second route in which donor MHC is internalised, processed, and presented by recipient APCs prior to expression at the cell surface as peptide fragments bound to recipient MHC class II. Further evidence to support this

'indirect pathway' was provided by Sherwood (237). She noted that mouse skin grafts were rejected with accelerated second-set kinetics if recipients additionally received splenocytes from syngeneic animals previously primed with donor strain splenocytes (237). Since neither intact donor antigen nor sensitised recipient T cells were transferred, recognition of donor antigen (as presented by the transferred APCs) was restricted to the indirect pathway, providing *in vivo* functional evidence as to its role in effecting acute graft rejection.



A. Nominal Antigen

B. High Determinant Density

C. Multiple Binary Complexes

Figure 1.10 Proposed models of alloreactivity.

Figure A depicts the recognition of a self-restricted nominal antigen, which is generally presented by no more than 100 of the MHC molecules on the surface of an APC. In comparison, the allogeneic MHC molecules on the surface of a donor APC either represent a much higher frequency of T cell determinants (Figure B) or stimulate a polyclonal T cell response (Figure C).

Direct evidence for the recognition of processed graft antigens was provided by Benichou (238). He noted that 10 days after the transplantation of mouse skin grafts, recipient T cells proliferated *in vitro* to the polymorphic region of the donor class II MHC molecule (238). Similarly, 10 days after transplanting Lewis ($RT1^l$) rats with DA ($RT1^a$) renal allografts, Fangmann demonstrated T cell proliferation to the hypervariable regions of the donor class I MHC, but only if the recipients had additionally been primed with a donor skin graft 4-6 weeks prior to receiving the kidney graft (239). In a different rat strain combination, WF ($RT1^u$) to Lewis ($RT1^l$), self-restricted T cells specific for donor class II peptide were detected following cardiac allografting. These T cells were $CD4^+$ and could be detected as early as three

days post-transplant (240, 241). Studies on human recipients of kidney grafts have now also revealed the presence of recipient T cells reactive against donor MHC peptide (242).

1.5.2 The Role of Indirect Allorecognition in Initiating Graft Rejection

In Sherwood's experiments, T cells activated through indirect recognition of donor antigen appeared an important mediator of graft rejection since sensitising animals to this pathway resulted in accelerated rejection (237). However, intact donor MHC was detectable as part of the innoculum and it is possible that T cells were additionally activated via the direct pathway. An alternative approach to assess how the indirect pathway contributes to graft rejection is to immunise with the denatured chains or peptide fragments of donor MHC, so that only the indirect pathway is primed. Such studies have generally resulted in either no difference (243), or in only a slight decrease in the survival times of subsequent grafts (244-248). Thus, despite the evidence that indirect T cell activation occurs during allograft rejection, it may not actively contribute to the process of graft rejection.

In comparison, pre-immunisation with intact allo-MHC molecules on the surface of donor cells results in accelerated rejection with second-set rejection kinetics (245, 246). For example, Shirwan et al (245), reported that immunisation with peptides derived from the hypervariable region of the donor RT1.A^a molecule, resulted in a minimal acceleration in the rejection of a subsequent A^a-bearing heart graft (five days for experimental animals compared with six days for controls). Immunisation with irradiated donor splenocytes however, shortened graft survival to just one day. He attributed the rapid rejection of the latter group to the ability of intact allo-MHC, as presented by donor splenocytes, to activate an additional subset of directly restricted T cells.

One can however argue, because priming with the intact allogeneic MHC molecule also provides a source for recipient APCs to generate allopeptide fragments, that the indirect pathway is simultaneously activated. If so, the effector mechanisms responsible for accelerated rejection following priming with donor cells may not be exclusively due to priming of the direct pathway, but rather to the synergy afforded by the concurrent priming of T cells through both the direct and indirect pathways.

As to why directly and indirectly primed T cells may have a synergistic effect on graft rejection is perhaps explained by consideration of the nature of their respective target determinants. It is simple to envisage that T cells that directly recognise an allogeneic MHC molecule are also able to mount an efficient cytolytic response against donor graft cells bearing that antigen. In comparison, because indirectly activated T cells are restricted to processed donor peptides presented in the context of self-MHC, they are unable to recognise, and consequently directly damage, graft cells bearing the intact allo-MHC determinants. One way they could counter this is by acting as helper cells for the development of effector mechanisms mediated by directly restricted T cells.

To establish the contribution of the indirect pathway to allograft rejection, it is therefore necessary to re-evaluate those effector mechanisms traditionally considered to be mediated by directly restricted T cells (delayed-type hypersensitivity, lymphocytotoxicity and alloantibody responses), to assess the extent that their development is influenced by T cell activation via the indirect pathway.

1.5.2.1 Delayed Type Hypersensitivity (DTH) Responses

Although the cognitive phase of a DTH response involves the specific recognition of foreign antigen by host T cells (usually $CD4^+$), the effector phase represents an innate immune response in which activated macrophages perpetuate an inflammatory response that damages tissue non-specifically. A DTH response therefore represents a potential mechanism by which the infiltration of indirectly activated T cells, despite not recognising the antigenic determinants expressed on donor cells, could still effect graft damage. This is supported experimentally by the observation that the passive transfer, into Lewis ($RT1^l$) rats, of syngeneic T cell clones that are specific for allopeptide derived from the class II MHC ($RT1.D\beta^u$) of the Wistar Furth rat strain (WF, $RT1^u$) augments the DTH response that occurs upon subcutaneous injection of both $RT1.D\beta^u$ peptide, and more especially, WF splenocytes (249-251). Indirect T cell activation within the graft is dependent upon the initial infiltration and presentation of donor antigen by recipient APC. This process takes several days and the indirectly restricted DTH response is therefore unlikely to be a major effector of acute graft rejection.

1.5.2.2 The Provision of T Cell Help for Allocytotoxicity and Alloantibody responses.

Indirectly restricted T cells could provide help for the development of either an allocytotoxic or an alloantibody response. In general, CD4 T cell help is necessary for the development of both of these responses, although the exact mechanics of this help, specifically the nature and degree of physical linkage that occurs between the helper and effector cell, has yet to be determined. It is possible that by secreting soluble cytokine factors, the helper cell provides sufficient help by simply being in close proximity, rather than by physically binding to, the effector cell (see Figure 1.11A). Such a model however, is unregulated and potentially damaging, as theoretically any activated CD4 T cell could provide help for the effector cell regardless of the antigenic determinants for which they are specific.

The provision of T cell help could be more strictly regulated if physical linkage between the helper T cell and the effector cell was instead necessary. Linkage could occur through two mechanisms. Firstly, the helper and effector cell may interact indirectly through a common APC (see Figure 1.11B). This would require the APC to present determinants that both the effector and helper cells recognise. Since these determinants would not necessarily be the same for each cell population, the helper cell would not recognise determinants that the effector cell presents. Nevertheless, the increased expression of costimulatory ligands on the surface of the donor cell, which its interaction with the helper T cell induces, would ultimately increase the activation state of the effector cell (252). A second form of linked help may occur through a direct *cognate* interaction between the class II MHC/peptide complex of the effector cell and the TCR of the helper cell (see Figure 1.11C). This arrangement may be especially advantageous in that T cell help would be restricted to T cells specific for the donor peptide fragments (as presented by the effector cell). By ensuring such specificity, the response may be more tightly regulated. Although fewer cells can participate in the provision of linked, as compared to unlinked, help, effector cell activation may nevertheless be unimpeded, due to additional signalling through costimulatory ligands.

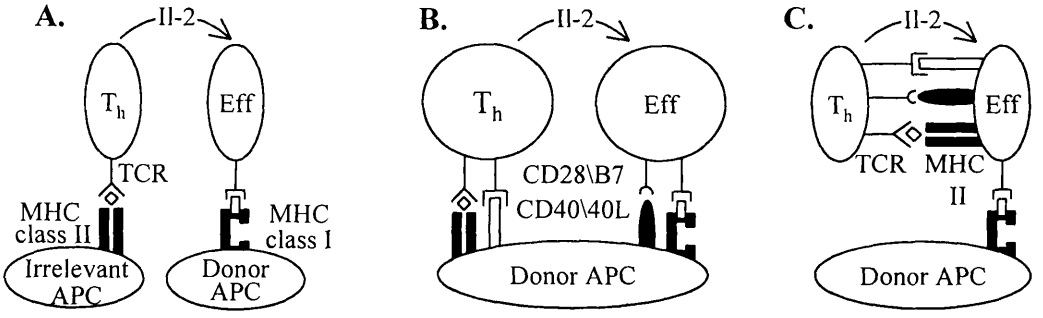


Figure 1.11 Diagrammatic representation of the varying degrees of physical linkage between helper (T_h) and allo-specific effector cells (Eff).

Figure A Physical proximity only. The helper T cell is restricted for a third party irrelevant antigen; there is no determinant sharing.

Figure B Physical linkage through third party linked determinants. The helper T cell interaction with its determinant on the surface of the donor APC increases the latter's expression of costimulatory ligands (CD28/B7, CD40/40L). Their increased expression results in more efficient activation of the alloreactive effector cell (252).

Figure C Cognate linkage through the MHC/peptide of the effector cell and the TCR of the helper cell. Note that the provision of cognate help for the allospecific effector cells can only be provided by T cells primed through the indirect pathway, and such helper cells will not be able to recognise the intact allogeneic determinants on the surface of the donor APC.

With specific regards to the alloimmune response, the mode of activation of the helper T cell population is fundamentally different for each of the above mechanisms of linked help. Using the cytotoxic CD8 T cell response as an example, cognate help (Figure 1.11C) is dependent upon the cytotoxic T cell additionally functioning as an APC and presenting the target donor antigen as peptide fragments. Such help would be limited to indirectly primed CD4 T cells that recognise self-restricted allopeptide fragments. In the alternative 3 cell cluster model (Figure 1.11B), dual linkage with the APC requires the APC to display the appropriate allogeneic class I and class II determinants for simultaneous recognition by recipient cytotoxic effector T cells and CD4 helper T cells. Since donor APC fulfil this criteria, this represents a mechanism by which directly restricted CD4 T cells can provide help. Elucidating the pathways by which T cell help is normally provided for the development of allocytotoxicity and alloantibody responses is therefore fundamental to establishing the relative contributions of the direct and indirect pathways to graft rejection.

1.5.2.3 The Cytotoxic CD8 T Cell Response

Although the cytotoxic T lymphocyte (CTL) response is a major component of the alloimmune response, the precise nature of the T cell help that is required for its' development is poorly defined. CTL depend upon the presence of Il-2 for their maturation and CD4 T cells generally provide this (96, 97, 253, 254). Although *in vitro* studies suggest that there is no absolute requirement for linkage between the T_h and the T_c (255-258), *in vivo* experiments contradict this (259, 260).

Both the three-cell cluster and the cognate T_h/T_c interaction hypotheses (Figures 1.11B and 1.11C) appear capable of providing help for the development of an allocytotoxic response. In support of the former, Mitchison and O'Malley (261) demonstrated that target cells were more effectively lysed if they expressed the cytotoxic and helper determinants simultaneously (non-cognate linkage). They concluded that a three-cell cluster involving the donor APC, the CD4 T_h cell, and the CD8 T_c cell was sufficient to induce cytolysis (Figure 1.11B). Shared MHC-restriction of the T_h and T_c subsets did not increase cell lysis, indicating that help provided through a cognate interaction with the CD4 T_h cell (Figure 1.11C) was unlikely to contribute to the development of CD8 T_c cell response.

Rosenberg reported similar results *in vivo* (262). In certain mouse strains, class I disparate skin grafts were still rejected after CD8 T cell depletion of the recipients. A subset of CD8 T cells resistant to the depleting antibody mediated graft rejection, and their development was dependent upon help provided by CD4 T cells. CD4 T cell activation was contingent upon the expression of additional helper determinants, which could be completely unrelated to the lymphocytotoxic target. These helper determinants had to be expressed in conjunction with the cytotoxic determinants, but could be co-expressed on a second 'inducer' graft (263). Similarly, the examination of a class I disparate strain combination, in which skin grafts were accepted permanently, revealed self-restricted CD4 T_h cell proliferation *in vitro* to the donor MHC class I antigen (264, 265). These results suggest that help provided through the indirect T cell recognition of the disparate class I antigen was insufficient to stimulate rejection and that the development of an effective cytotoxic response required the expression of additional helper determinants.

Rosenberg's studies were performed on strains in which CD8 T cells produced their own T cell help and were able to reject skin grafts autonomously (266-269). The lack of dependence upon the helper CD4 T cell subset may reflect deficiencies in the class II antigen processing pathways, preventing CD4 T cell activation through the indirect pathway. Similarly, whereas human CD8 T cells express MHC class II (270), the situation in the mouse remains undefined (271, 272). The above experiments may therefore not accurately reflect the extent to which indirectly primed CD4 T cells can provide cytotoxic CD8 T cell help. In the rat, for example, *in vitro* CD8 T cell proliferation, and the rejection of skin and vascularised grafts, are more heavily reliant upon CD4 T cells (273-276).

Evidence to support the cognate model of CD4 T cell help for cytotoxic T cell development was initially provided by the observation that certain accessory cells involved in the generation of an allocytotoxic response displayed self-MHC class II-restriction (265, 277, 278). Auchincloss recently validated these findings, as he noted that CD8 T cell-depleted mice rejected MHC class II-deficient skin grafts normally (279, 280). The absence of donor class II prevented CD4 T cell activation via the direct pathway and CD4 T cell help, necessary for the generation of CD8 effector cells, was instead provided through the recognition of processed donor class I antigen, as presented by recipient APCs. Lee and Auchincloss proposed the incorporation of the recipient APC, specifically a recipient B cell, into a four-cell cluster model, which still permitted linkage for regulatory purposes (Figure 1.12A). He did not address the nature of the interaction of the self-restricted CD4 T_h cell with the T_c cell, but linkage could equally be provided by assuming that the TCR of the indirectly primed CD4 T cell interacts with MHC class II of the CD8 T cell in a cognate fashion (Figure 1.12B) (281). Allocytotoxic CD8 T cell effector mechanisms have been traditionally viewed as typifying the direct T cell response, but Lee's results suggest that the development of effector mechanisms may instead rely on directly and indirectly activated T cells cooperating in a synergistic fashion.

Both directly and indirectly activated T cells appear capable of providing help for the development of an allocytotoxic T cell response. The relative contribution of the indirect pathway is still a matter of conjecture, and will remain so until the nature of

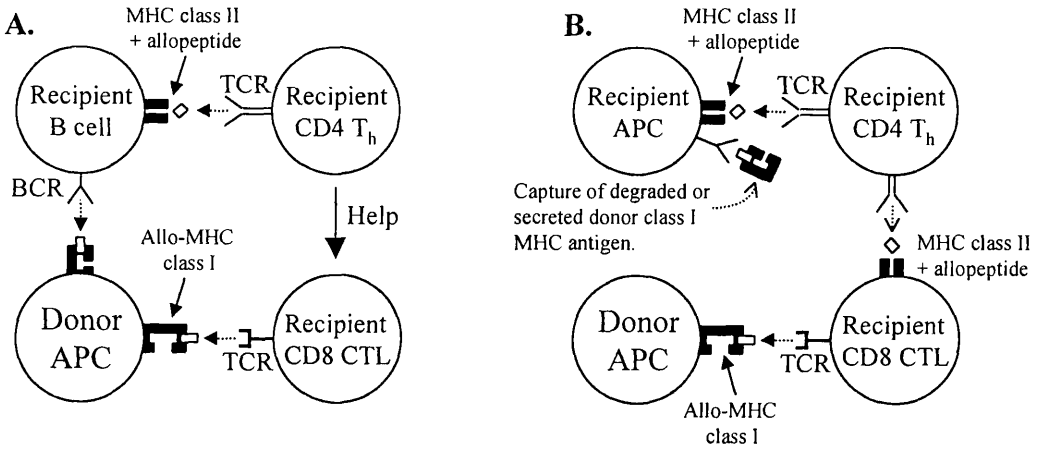


Figure 1.12 The provision of indirect CD4 T cell help for allo-cytotoxic T lymphocytes.

CD4 T cells that are activated through the indirect pathway provide help for the generation of an allo-cytotoxic CD8 T cell response through a four cell cluster (280) with the recipient APC most likely a B cell (Figure A). Regulatory linkage could also occur by assuming a cognate interaction between the class II MHC/peptide complex of the CD8 T cell and the TCR of the CD4 T helper cell (Figure B) (281).

the interaction between CD8 and CD4 T cells can be firmly established. It seems unlikely, given the strength of the direct response, that indirectly primed T cells would preferentially provide cytotoxic T cell help. That is, unless their ability to provide cognate help is, in terms of CD8 T cell activation, inherently advantageous. Recent evidence regarding 'cross-priming' of the CD8 T cell response to exogenous antigens, instead suggests that CD4 T cells provide help by modulating the APC, rather than the CD8 T cell (252, 282, 283) (Figure 1.13). This obviously requires the APC to express the helper and cytotoxic determinants simultaneously and is analogous to directly activated T cells providing non-cognate help by a three-cell cluster, as was originally suggested by Mitchison and O'Malley (261).

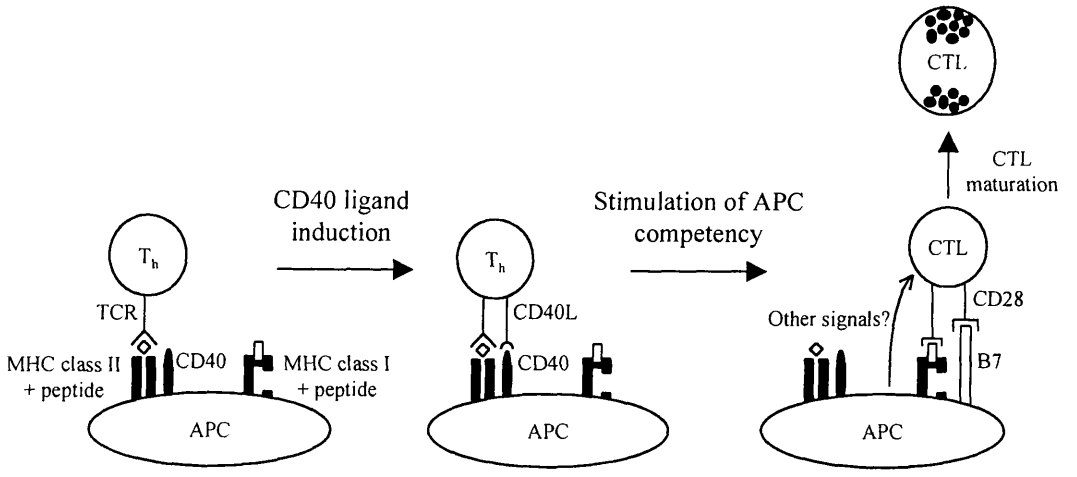


Figure 1.13 The provision of T cell help for cross-reactive CTL responses.

The CD4 T cell provides help to the cytotoxic effector cell, by indirectly augmenting costimulatory molecules on the APC (282). This requires binding to the APC (left hand figures). For an alloimmune response in which the target APC is of donor origin, only allo-restricted CD4 T cells that directly recognise the allogeneic class II MHC, rather than those which are primed indirectly for self-restricted allopeptide, can fulfil this role (261).

1.5.2.4 The Alloantibody Response

The provision of T cell help for antibody production to nominal protein antigen is more defined. The transformation of 'immature' B cells into Ig-secreting activated plasma cells requires cognate help from T cells (284-287). CD4 T cells are similarly required for the development of an alloantibody response (288), and since alloreactive B cells will present donor allo-peptide, one would expect that only indirectly primed T cells would be capable of providing the necessary help. Confirmation that indirectly primed T cells are involved in the alloantibody response was initially established by Bradley and colleagues (289, 290). In an MHC class I-disparate rat renal allograft model, PVG-R8 (RT1.A^a) to PVG-RT1^u (RT1.A^u), rejection was dependent upon CD4 T cells and, in contrast to Rosenberg's findings (263), CD8 T cells were superfluous. Rejection was alloantibody mediated for passive transfer of hyperimmune serum was able to accelerate or restore rejection in naïve and nude rats respectively (289). In this model it is unlikely that CD4 T cell activation occurs through direct recognition of the disparate class I A^a antigen, but instead is contingent upon recipient APC processing the donor class I antigen and

presenting it as peptide fragments in conjunction with self MHC class II. The exclusion of CD4 T cell activation via the direct pathway implies that indirectly restricted CD4 T cells are alone responsible for the development of an alloantibody response, through the provision of cognate B cell help. More recent experiments by MacDonald et al (246) have again highlighted that the recognition of processed donor allopeptide is integral to the rejection response in this strain combination, for subcutaneous injection of synthetic peptides derived from the hypervariable regions of the donor A^a molecule prior to grafting accelerated the rejection of subsequent A^a-bearing cardiac allografts. Furthermore, the administration of these peptides in a tolerogenic fashion, by targeting them to the resting recipient B cell population, downregulated the subsequent alloantibody response to the intact A^a antigen.

Fabre and colleagues have similarly found, in the DA ($RT1^a$) to Lewis ($RT1^l$) rat strain combination, that allopeptide priming can accelerate the subsequent alloantibody response to the intact donor MHC molecule (244, 247). This difference was most marked when interstitial dendritic cell-free grafts were used as donor organs (291). In this experiment, priming with peptide markedly accelerated both the alloantibody response and the kinetics of allograft rejection, seemingly confirming the importance of the indirect pathway in the provision of B cell help. However, the early alloantibody response was dependent upon the presence of donor dendritic cells, which, as professional APCs, are capable of initiating a strong direct CD4 T cell alloresponse. Kelly et al further analysed this possibility that B cell help was provided by directly-restricted CD4 T cells (292). In PVG ($RT1^c$) rats, the allo-class I A^a antigen could only promote an antibody response when it was encountered on the surface of donor cells, in this case a vascularised allograft, and not when administered as a purified molecule. Furthermore, the MHC class I alloantibody response could be abrogated by the presence of antibodies reactive against the donor class II MHC molecule. Kelly concluded that T cells recognising the allogeneic class II MHC directly were responsible for providing help for the early alloantibody response to a vascularised graft, in a manner similar to the three cell cluster model that Mitchison and O'Malley had suggested governed the allocytotoxic T cell response (261). This concept is not unprecedented; earlier work with non-MHC antigens confirmed that activated T cells could provide non-cognate B cell help, although this help was contact dependent (293-295).

The discrepancies between Fabre's results and those of Bradley's laboratory may be a result of analysing different aspects of an inherently complicated alloantibody response, which involves the separate processes of antigen induced activation, proliferation, antibody secretion and isotype switching. As the levels of T cell help for each of these processes may differ, so too may the relative requirement for help provided through either directly or indirectly restricted CD4 T cells. For example, Steele and Auchincloss (296) have noted that MHC class II-deficient mouse skin grafts are still rejected with a similar IgM and IgG response to the residual donor antigens as to controls receiving normal grafts. The absence of donor MHC class II restricts CD4 T cell activation to the indirect pathway and suggests, in concurrence with Bradley (297), that priming this pathway results in the development of a normal alloantibody response. In comparison, grafting skin from an unmodified donor to recipients deficient in MHC class II (but with a normal CD4 T cell repertoire), produced only a moderate titre of cytotoxic antibody, which was solely of the IgM isotype (296). In this latter model CD4 T cell activation is restricted to the direct pathway and Auchincloss concluded that the non-cognate B cell help provided by directly activated T cells is sufficient to initiate the primary IgM alloantibody response, but is insufficient to induce heavy chain class switching to IgG isotypes.

Fabre and Auchincloss' data can therefore be reconciled by assuming that the antibody response in Fabre's model was exclusively of the IgM class (in his experiments the isotype response was not analysed), for even an IgM-restricted alloantibody response (direct CD4 T cell help) will activate complement and may consequently be as detrimental to graft survival as a more differentiated IgG response (indirect CD4 T cell help). Two issues nevertheless require clarification. Firstly, as suggested by Fabre, is it only help from directly activated T cells that is responsible for the early alloantibody response? Secondly, as suggested by Auchincloss, is the capability to induce Ig heavy chain class switching restricted to only the indirectly primed subset of T_h cells?

With regards to the nature of T cell help for the initial alloantibody response, Bradley's results suggest that this can be adequately provided by indirectly restricted CD4 T cells (246, 289). The alloantibody response to the disparate MHC class I antigen in the PVG.R8 to PVG.RT1^u strain combination is governed by indirectly

primed T_h cells, yet it is detectable as early as four days after a vascularised cardiac allograft (Lovegrove et al, submitted). Certainly, within a few hours of encountering antigen, B cells can process and present it to T cells (298, 299).

One can also hypothesise that the required presence of donor dendritic cells to instigate an early alloantibody response simply reflects the migratory capabilities of dendritic cells, rather than a dependence on T cell activation via the direct pathway. Activation of B cells in a primary response occurs in T cell areas of the spleen (300) and dendritic cells may be uniquely proficient at trafficking antigen to this area (301). The migration of donor dendritic cells could therefore be responsible for an early alloantibody response, irrespective of whether alloantigen is recognised within the spleen predominantly via the direct or indirect pathways.

Finally, the three-cell cluster theory (292) was based on the observation that PVG rats mounted a much stronger alloantibody response to the A^a antigen when it was encountered as part of a completely mismatched graft. The indirect CD4 T cell response to this antigen, as judged by the inability to develop antibody against its purified form, is however weak. This may reflect strain-specific abnormalities in either antigen processing or the CD4 T cell repertoire and may not be typical of the general immune response to allo-MHC in other strains or species. In other words, the indirect CD4 T cell response to MHC class I alloantigens may generally be sufficient to provide B cell help without the requirement for additional helper allo-determinants.

With regards to Auchincloss' hypothesis, that T cell help for Ig heavy chain class switching can only be provided by a cognate interaction with indirectly restricted CD4 T cells, recent advances in the understanding of the regulatory signals that govern the antibody isotype switch for nominal antigens, offer some insight. Rao's group (302, 303) have confirmed that, as opposed to the initial IgM response, the class switch from IgM to IgG demands a higher frequency of primed T_h cells. This may reflect a stringent requirement for an interaction between CD40 of the B cell and CD40L of the T cell for isotype switching (304-307). In turn, the T cell CD40L is upregulated by signalling via the TCR, as occurs through a cognate interaction with the MHC complex of a B cell, (308). This supports Auchincloss' findings of the requirement for indirectly primed T cells to instigate class switching. However, there

is also *in vitro* evidence of CD40L-induced class switching in the absence of TCR signalling (309, 310). In addition, Steele's work was performed on a mouse skin graft model, and his findings may not apply to vascularised grafts, for the kinetics of the alloantibody response are much slower following skin grafting. This perhaps reflects an inherent bias for allo-activation to occur through the indirect pathway (281).

In summary, results to date suggest that either of the above mechanism for T_h cell activation can provide B cell help. The relative contribution of each are likely to depend upon the particular characteristics of a given alloimmune response, but such characteristics remain poorly defined.

1.5.2.5 Targeting the Indirect Pathway to Achieve Tolerance

The use of tolerogenic protocols that specifically target the indirect pathway, provide an alternative means to study its' role in effecting acute allograft rejection. Sayegh initially noted that feeding Lewis ($RT1^l$) rats with allopeptide derived from WF ($RT1^u$) MHC class II, subsequently reduced the DTH response not only to the peptides, but also to intact donor splenocytes (311). More significantly, in the same strain combination, intrathymic injection of the same peptides resulted in prolonged cardiac allograft survival (312). Similar results with MHC class I allopeptide, but with the use of concomitant sub-therapeutic immunosuppression, have been more recently reported for the WF to ACI ($RT1^a$) and the PVG.R8 ($RT1^a$) to PVG.$RT1^u$ ($RT1^u$) rat strain combinations (313-315). These results are startling for several reasons. The allopeptides administered were derived from only a single MHC molecule of the donor rat strain and yet had profound effects upon the survival of fully mismatched allografts. Furthermore, the synthetic peptides used represented a comparatively small percentage of the total allo-MHC sequence, and since the design of these peptides was somewhat arbitrary, they may not even be representative of the sequences that are presented upon processing of the donor MHC antigen by the recipient. Nevertheless, such studies not only provide the strongest evidence to date as to the importance of the indirect pathway of allorecognition in the process of graft rejection, they also suggest that tolerogenic strategies targeting this pathway may be clinically advantageous.

1.6 Aims of Thesis. The Use of Soluble Class I Allo-MHC

1.6.1 Introduction

In the class I-disparate rat model of allograft rejection, PVG.R8 to PVG.RT1^u, the assumption that CD4 T cell activation is dependent upon recognition of the processed donor RT1.A^a alloantigen, implies that the indirect pathway of allorecognition is solely responsible for graft rejection (297). Alternative explanations that rely on the direct pathway of allorecognition can however be postulated (297). Firstly, it is possible that the recombinant event that established the donor R8 strain translated not only the MHC class I locus, but also altered as yet undefined neighbouring antigens, such that the antigenic disparity between the two rat strains is not strictly limited to the RT1.A locus. Such putative additional antigenic disparities may be able to activate T_h cells directly, in an analogous fashion to the rejection of skin grafts in certain class I-disparate mouse skin allograft models (262). Alternatively, recipient CD4 T cells may recognise the disparate class I alloantigen directly. Although this appears to violate the basic laws of MHC-restriction, exceptional CD4 T cell clones have been described that are lytic towards MHC class I positive target cells (316, 317).

One way to test the hypothesis that recipient CD4 T cells are activated *directly* in the R8 to RT1^u strain combination is to examine the effect that priming RT1^u rats with purified donor RT1.A^a antigen has upon the survival of subsequent R8 allografts. If this mode of immunisation has the same effect on graft survival as pre-immunisation with intact donor R8 cells, then one can reasonably exclude additional antigenic discrepancies as having a major influence on graft rejection. Sensitisation of RT1^u rats with intact donor R8 cells in the form of a skin graft (246), an R8 blood transfusion (Lovegrove et al, manuscript in preparation) or subcutaneous injection of irradiated R8 splenocytes (245) leads to prompt rejection of subsequent R8 cardiac allografts (within one to two days of grafting). However, the effect of pre-immunisation with purified RT1.A^a antigen is generally much more modest. For example, the administration of synthetic A^a antigen, either as complete heavy chain protein (248) or as allopeptides derived from the hypervariable regions of the A^a molecule (245, 246), accelerates graft rejection by two days at most.

Shirwan has similarly noted, in the same rat strain combination, that priming with donor cells results in a much more rapid rejection of a subsequent heart graft than priming with synthetic donor antigen (245). He too postulated that the accelerated rejection associated with donor cell priming is the result of the activation of an additional subset of T cells via the direct pathway of allorecognition. It is notable in the above experiments, however, that the synthetic A^a antigen that was administered, either as misfolded heavy chain or as peptide fragments, did not display the same tertiary and quaternary conformations as the complete membrane-bound RT1.A^a MHC class I antigen. Consequently, in contrast to pre-immunisation with donor R8 cells, prior exposure to the synthetic A^a antigens did not result in the development of an alloantibody response able to cross-react with intact A^a molecules on the cell surface of subsequent R8 allografts (245, 246). Rejection in PVG.R8 to PVG.$RT1^u$ rat strain combination is alloantibody-mediated (289, 290, 318). Pre-immunisation with intact A^a antigen, as on the surface of donor cells, may therefore cause rapid graft rejection simply because it displays the appropriate cross-reactive B cell epitopes to efficiently prime humoral immunity, rather than to any fundamental differences in the mode of T cell activation.

In summary, evidence to date does not permit definite conclusions as to the pathways of CD4 T helper cell allorecognition that initiate graft rejection in the PVG.R8 to PVG.$RT1^u$ rat strain combination, and in particular, whether indirectly restricted CD4 T cells are responsible for the accelerated rejection that results following priming with intact donor R8 cells. To clarify this, an experimental system was designed whereby T cell activation was restricted to the indirect pathway, but in which B cells could be simultaneously activated against the intact donor RT1.A^a antigen. I reasoned, as discussed in section 1.6.4, that the use of soluble RT1.A^a antigen would achieve this objective. Soluble MHC class I alloantigen can however exert potent effects upon recipient CD8 T cells, which may complicate interpretation of the CD4 T cell response. For example, activated CD8 T cells can influence the pattern of cytokine secretion of CD4 T_h cells (319) and can, under certain circumstances, provide help to B cells (320). Conversely, soluble MHC class I alloantigen can prevent the development of a cytotoxic CD8 T cell response by inducing apoptosis in alloreactive CD8 T cells (321). In using soluble MHC class I alloantigen to assess the influence of the indirect CD4 T cell response, one must

therefore initially consider its direct effects, either inhibitory or stimulatory, on the cytotoxic CD8 T cell population.

1.6.2 The Tolerogenic Properties of Soluble Class I Allo-MHC

Liver allografts are accepted more readily than other solid organ grafts, possibly because they are associated with the release of tolerogenic donor antigens (322). The search for these donor antigens resulted in the discovery that HLA could exist as a soluble serum factor. Van Rood and colleagues initially documented that soluble HLA could be detected in normal human serum (323), Kamada et al subsequently confirming the presence of donor MHC in the serum of liver recipients (324). Further studies suggested that soluble HLA was present in serum in three distinct biochemical forms (325). The largest, a 44kDa molecule, retains the transmembrane region and cytoplasmic tail, while these were absent in the smallest 35-37kDa molecule. The intermediate, 40kDa molecule lacks the transmembrane segment and is probably produced by alternate splicing (326, 327).

Shed soluble donor MHC was therefore proposed as the tolerogenic agent responsible for the relative ease with which liver allografts are accepted. This has not, however, been conclusively demonstrated experimentally, as several groups have evaluated the effects of the administration of purified soluble class I antigen and the results are contradictory. Sumimoto and Kamada (328) initially reported that the kinetics with which PVG (RT1.A^c) rats rejected DA (RT1.A^a-bearing) cardiac allografts were slowed by the concurrent continuous infusion of naïve DA rat serum. This effect was dependent upon the presence of the donor A^a antigen, since the administration of serum that had had the class I antigen removed by affinity chromatography did not prolong graft survival. Foster et al (329) performed similar experiments, but additionally examined the effects of the various physical forms of the RT1.A^l antigen. When the A^l antigen was administered to DA recipients as purified membrane-bound molecules, incorporated into protein micelles to form multivalent complexes, Lewis (A^l-bearing) renal allografts were accepted indefinitely. However, a water-soluble preparation of the purified RT1.A^l antigen was only able to prolong graft survival when administered in conjunction with a sub-therapeutic dose of cyclosporin.

In contrast, neither Priestley (330) nor Spencer (331) were able to reproduce the tolerogenic effect of soluble donor class I in their rat allograft models. Even in combination with a sub-therapeutic dose of cyclosporin, the intravenous administration of RT1.A^a antigen did not prolong the survival of either DA hearts in PVG recipients (331) or DA kidney grafts in PVG or Lewis recipients (330).

The discrepancies in the above studies reflect a limited understanding of the mechanisms responsible for the tolerogenic effects of soluble allo-MHC. Potential mechanisms involve binding to the TCR, or an interaction with other receptors (332), or downregulation of the indirect T cell response (333). The most likely mechanism of T cell tolerance is through interaction of the soluble MHC class I with the TCR of CD8 T cells. The ability of soluble MHC class I to prevent cytotoxic T cell development was first documented in the early 1990's (334, 335). This coincided with the publication of the two-signal model hypothesis for T cell activation (336), whose principles could explain the tolerogenicity of soluble MHC. Specifically, soluble MHC, because it is not expressed upon a cell membrane, is unable to provide the second costimulatory signal required by T cells for activation, and that in its absence, the provision of signal one alone induces an anergic state in T cells.

This hypothesis does not however explain why larger aggregated forms of soluble class I alloantigen are more tolerogenic than shorter water-soluble versions (329). *In vitro* models of T cell activation suggest that this is due to the ability of multivalent soluble MHC aggregates to stably bind to several TCRs simultaneously on the surface of a CD8 T cell (321, 337-339). For example, McCluskey et al initially demonstrated that the molecularly engineered soluble counterpart of the MHC class I antigen, H-2D^d/Q10^b was only able to stimulate Il-2 release from a T cell hybridoma when in a multivalent state (337). More recently, Dal Porto and colleagues genetically engineered a divalent MHC class I molecule by inserting the DNA sequence encoding the extracellular domains of H-2K^d into the 5` variable domain of a murine γ1 gene (339). This chimeric protein inhibited *in vitro* lysis of H-2K^b-bearing target cells when administered in nanomolar concentrations. Likewise, Abastado et al produced a recombinant divalent soluble H-2K^d antigen-peptide complex by using a specific anti-H-2K^d antibody to link the α3 domains. Whereas

the unlinked monomers were unable to stimulate the relevant T cell hybridoma, dimers triggered the T cell hybridoma to secrete Il-2, and this was followed by an unresponsive state during which T cells were refractory to further stimulation (338). Zavazava and Kronke recently extended these observations and have established a mechanism by which polyvalent soluble antigen can prevent CD8 T cell activation. They reported that T cells that interact with hydrophobic soluble MHC, which is capable of forming multimers, undergo apoptosis as a result of Fas ligand upregulation (321). Shorter, hydrophilic, soluble MHC derived from the cytosol, and consequently unable to form aggregates, was less than 50% as efficient at inducing apoptosis. Cell death was prevented by the concurrent ligation of the T cell with CD28, and this explains why a third preparation of the class I antigen, which was derived from fragments of cell membrane, and therefore more likely to contain associated costimulatory molecules, did not induce apoptosis.

1.6.3 Can Soluble MHC Class I Activate CD8 T Cells Directly?

Recent evidence has qualified the two-signal model of T cell activation. *In vitro* experiments suggest that the costimulatory requirement for T cell activation, particularly for CD8 T cells, is relative, rather than absolute (340-346). For example, Viola and Lanzavecchia (340) have demonstrated that CD4 T cell activation requires a defined number of signalling events to occur through the surface TCR. In the absence of costimulation, this threshold value is 8000, whereas concurrent costimulation lowers this value to 1500. CD4 T cell activation (as measured by calcium influx) also occurred, in the absence of costimulation, upon incubation with soluble class II tetrameric complexes (347, 348). By using a similar approach, Wang has recently confirmed the ability of soluble class I MHC tetramers to activate CD8 T cells without additional signalling (349). Pardigon and Luxembourg (344, 345) have also verified that CD8 T cells can also be fully activated in the absence of a second signal, albeit with an efficiency of at least 20 times less than when costimulation is available (344). Although the studies employing class I and class II tetramers (347-349) noted that soluble, monovalent MHC did not induce T cell activation, in a different model using T cell hybridomas as responders, full activation was achieved using soluble MHC in monvalent form monovalent soluble class I

MHC did induce full activation of a T cell hybridoma, but unlike activation induced by class I dimers, CD8 co-receptor expression by the hybridoma was required (350).

Results from *in vitro* experiments cannot necessarily be extrapolated to *in vivo* conditions, for in the above experiments the absence of costimulation may have been compensated for by either the addition of Il-2 (344, 346), or by immobilising the MHC class I protein into a non-physiological state by binding to plates (341, 347, 348). Neither of these practices were used in Wang's study (349), which is probably the most representative of the probable *in vivo* effects of soluble class I antigen. In his study, only tetrameric class I complexes could achieve CD8 T cell activation *in vitro*, and similar multivalent complexes are likely to be required to achieve the necessary *in vivo* cross-linking of the TCR.

1.6.4 The use of movalent soluble MHC class I alloantigen.

One can postulate from the above experiments that the recipient CD8 T cell response to the *in vivo* administration of soluble MHC class I alloantigen will range from either no activation, to partial activation (anergy/inhibition), or to full activation, depending on whether monovalent, divalent, or multivalent complexes, are administered. As discussed in section 1.6.1, this thesis attempts, by administering soluble donor class I alloantigen, to explore the mechanisms resposible for accelerated rejection, and specifically, to assess whether the requirement for priming with donor cells reflects solely the presence of the appropriate B cell epitopes for the development of humoral alloimmunity, or whether direct T cell activation through recognition of determinants on the surface of donor cells additionally contributes. The principal tenet concerning the use of soluble class I allo-MHC is therfore that T cell activation occurs only through the indirect recognition of its processed fragments. The administration of soluble MHC class I alloantigen as a monomer that does not associate into multivalent complexes would be expected to fulfil this requirement.

To manufacture monovalent soluble MHC class I it is necessary to excise the transmembrane and cytoplasmic domains, since these are hydrophobic and responsible for self-aggregation. Soluble allo-MHC manipulated in this way has been used *in vivo* and the theoretical constraint that it would not result in CD8 T cell

activation appears valid (351-354). For example, Geissler et al have reported that although intramuscular injection with a plasmid DNA vector encoding a membrane-bound form of the RT1.A^a molecule resulted in an anti-A^a cytotoxic T cell response, no such response was detected upon immunisation with a construct encoding a soluble version lacking the transmembrane and cytoplasmic domains (351). The same constructs were subsequently used *ex vivo* to transfect Lewis ($RT1^l$) hepatocytes (352). Lewis rats injected into the portal vein with syngeneic hepatocytes that additionally expressed the membrane bound RT1.A^a antigen demonstrated accelerated liver graft rejection, and showed CTL sensitisation. In contrast, injection of the soluble construct resulted in prolonged graft survival and was associated with a diminished anti-A^a cytotoxic T cell response. Furthermore, administration of the soluble MHC construct was able to prevent the immune activation and accelerated rejection that resulted from injection with the vector encoding the membrane-bound form of the antigen (353, 354).

Although these latter results suggest that monomeric soluble class I alloantigen may, as opposed to having no discernible effect, inhibit the direct CD8 T cell alloresponse, this does not preclude its use to explore the indirect CD4 T cell response in a model of accelerated, humoral rejection. It in fact permits exploration of its potential to down-regulate the cytotoxic CD8 T cell response as a subsidiary objective.

1.7 Summary and Objectives

The contribution of the indirect pathway to allograft rejection remains unclear, but it is probably as great as that of the direct pathway, particularly with respect to the provision of T cell help for the development of an alloantibody response. Although Kelly and Fabre (292) have suggested that the early, acute alloantibody response is dependent upon the provision of help from T cells activated exclusively via the direct pathway, antibody depletion studies suggest that in the MHC class I disparate PVG.R8 to PVG.RT1^{u} rat strain combination, the alloantibody response mediating rejection is dependent upon CD4 T cell activation (290, 297, 355). Although this most plausibly occurs as a result of the indirect recognition of processed donor A^{a} antigen, it is impossible to exclude that, since pre-immunisation of RT1^{u} rats with intact R8 cells accelerates the rejection of a subsequent R8 allograft to a greater degree than priming with purified forms of the A^{a} antigen (245, 246, 289), CD4 T_h cells either respond to additional alloantigens expressed by donor APCs, or recognise the A^{a} alloantigen directly upon the surface of donor APC (297). Clarification of this issue has obvious implications for the understanding of the T cell recognition pathways involved in the rejection of class I disparate allografts. It also has a wider bearing on the nature of T-B cell collaborations during the development of an alloantibody response, since the allorecognition pathway of CD4 T helper cells (indirect or direct) dictates whether T cell help for B cells is provided by cognate (antigen-specific) or non-cognate (antigen-non-specific) cell-cell collaboration (292, 296)

This thesis attempts, through the use of a soluble form of the donor RT1.A^{a} antigen, to explore more fully the role of indirectly primed T cells in the provision of help for the development of an alloantibody response, and in particular, to examine the ability of indirectly restricted T cells to effect accelerated humoral rejection. The three-dimensional structure of soluble MHC class I contains the appropriate B cell epitopes to generate an antibody response that would cross-react with the RT1.A^{a} antigen as expressed on the surface of donor graft cells. Additionally, because it lacks both costimulatory ligands and, when administered in a monovalent form, the ability to induce TCR cross-linking, it is highly unlikely to result in the direct activation of either recipient CD4 or CD8 T cells. It is more contentious whether in

this form the soluble class I antigen would actively inhibit the recipient CD8 T cell response, but since CD8 T cell depletion does not effect rejection in the R8 to $RT1^u$ strain combination (289), this will not influence the interpretation of the results.

The soluble MHC was manufactured using a molecular-based approach as this enabled the genetic sequence of the full-length $RT1.A^a$ antigen to be altered efficiently, such that a pure preparation of monovalent soluble class I antigen at physiological concentrations was assured.

CHAPTER TWO

CLONING AND EXPRESSION OF RT1.A^a HEAVY CHAIN ENCODING PLASMID VECTORS

CHAPTER TWO

CLONING AND EXPRESSION OF RT1.A^a HEAVY CHAIN ENCODING PLASMID VECTORS

2.1 Introduction

In certain animal models the administration of soluble class I alloantigen tolerises the alloreactive CD8 T cell population, but in others no effect is observed (328-331). There are several possible explanations for these discrepancies. The first concerns the mode of administration of the soluble class I alloantigen, for soluble MHC molecules have a short half-life (1.5 to 2.5 hours) (331). Physiological concentrations are perhaps therefore achieved only by its' administration as a continuous infusion, as in Sumimoto's experiments (328), rather than as a bolus injection, as favoured by Priestley and Fabre (330, 331). The method employed to purify the class I MHC (328) is equally important and results obtained using donor serum as a source of antigen may have been influenced by the presence of additional immunosuppressive serum factors (356, 357). Similarly, the protocol employed to purify the class I alloantigen will influence the precise physical conformation of the recovered MHC class I complexes, and in particular will dictate the relative concentrations of multivalent and monovalent complexes, that are recovered. These have markedly different effects on the CD8 T cell response (329).

To overcome the inconsistencies associated with purifying the RT1.A^a antigen directly from the appropriate rat strain, I chose instead to administer it as a DNA-based plasmid vaccine. This approach ensures the absence of contaminating inhibitory factors, and permits the formation of a recombinant molecule that is lacking the transmembrane and cytoplasmic domains of the heavy chain. Without these domains, only monovalent complexes will be expressed, which, as discussed in section 1.6.4, are least likely to activate recipient CD8 T cells.

Plasmids are double stranded circular DNA molecules that range in size from 1kb to more than 20kb and are found in a variety of bacterial species. They replicate and are inherited independently of the bacterial chromosome, but rely on protein

encoded by the host for their replication and transcription. Plasmid-encoded genes are frequently advantageous to the bacterial host and, for example, confer resistance to antibiotics, and the ability to degrade sugars and aromatic compounds.

The use of plasmids as mammalian expression vectors is dependent upon the presence of prokaryotic sequences that facilitate the propagation of the vector in bacteria, and eukaryotic transcription units that control the expression of the insert within the mammalian cell. The prokaryotic sequences include a replicon; an origin of replication together with cis-acting control elements. Replicons dictate the extent to which plasmids replicate within the host and therefore the number of plasmids per cell (copy number). Plasmid vectors typically encode an ampicillin-resistance gene, enabling selection of successfully transformed bacteria. The essential eukaryotic modules consist of a promoter element, to mediate transcription of the insert within the mammalian cell, and signals required for the efficient 3` polyadenylation of the transcript. In commercially available plasmid vectors the promoter/enhancer region is typically derived from viruses, as these have a broader host range and are active in a wider variety of tissues. These include the human cytomegalovirus immediate/early promoter (358), the Rous sarcoma virus (359) and the SV40 early promoter (360). Post transcriptional modification of the nascent mRNA transcript, such as polyadenylation, is essential for subsequent translation. Polyadenylation signals consist of a highly conserved 6-nucleotide sequence, AAUAAA, together with GU or U-rich sequences further downstream. The expression of certain mammalian genes is further increased by the presence of an intron sequence which provides functional splice donor and acceptor sites. These are frequently included in the vector design.

Plasmid vectors have been used to transfect mammalian cells *in vitro* for two decades. Their *in vivo* effectiveness at stimulating a T and B cell response has only recently been demonstrated (361, 362) and the plasmid characteristics that govern the nature and strength of the immune response remain poorly understood. This chapter describes the design of several RT1.A^a expressing plasmids, and the subsequent comparison of their ability to achieve *in vitro* and *in vivo* expression of the RT1.A^a antigen.

2.2 Results

2.2.1 Cloning of the RT1.A^a Heavy Chain Sequence into Plasmid Vectors

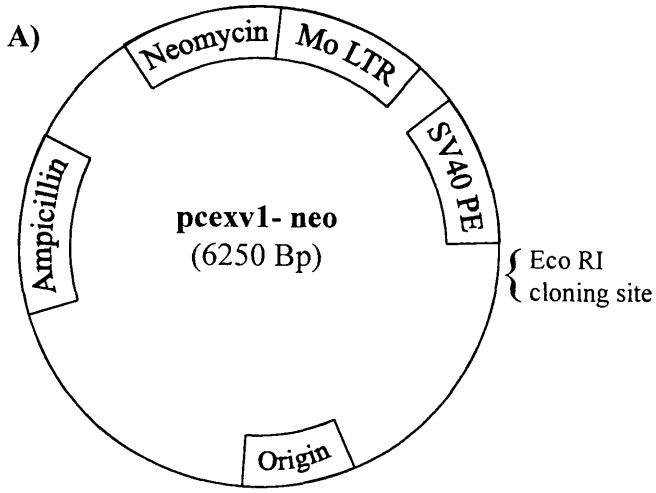
Figure 2.1 depicts the genetic sequences of the two forms of RT1.A^a α-chain that were kindly gifted by Simon Powis (Welcome Trust Building, University of Dundee, UK.). The full-length version is as described by Rada (39) and includes transmembrane and cytoplasmic domains. The soluble version was created by mutating the original full-length sequence to include a stop codon, followed by a Xba I restriction site, after the methionine residue at position 284 in the transmembrane region. The full-length and truncated sequences were inserted into the pcexv1-neo vector (pcexv-A^a and pcexv-tA^a, respectively), which contains an SV40 early promoter upstream of the Eco RI cloning site, and an additional neomycin-resistance gene for the purposes of stable cell transfection (Figure 2.2A).

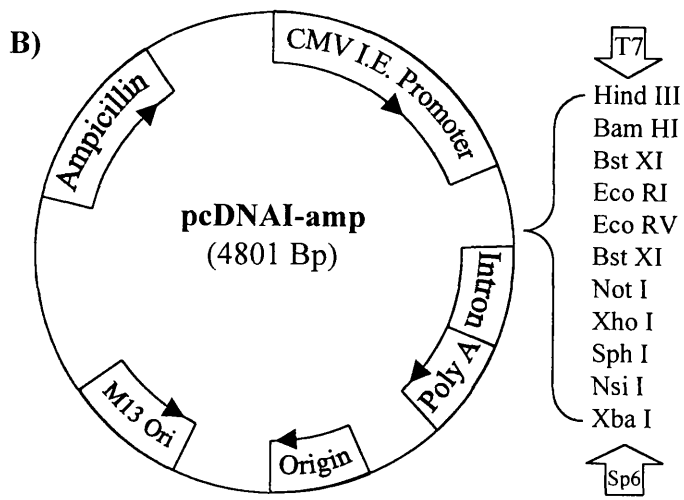
Although the SV40 promoter is an eukaryotic promoter and can theoretically drive expression of the class I constructs upon intramuscular injection, the large size of the pcexv1-neo vector is likely to substantially decrease the efficiency of *in vivo* expression. Consequently, the class I sequences were subcloned into several commercial expression vectors which offered characteristics more conducive to intramuscular expression. The pcDNAI-amp plasmid (Invitrogen, Carlsbarg, California) (Figure 2.2B) contains a polyadenylation sequence and a CMV early-late viral promoter which is generally the most effective commercially available promoter (358). It is also smaller than the pcexv1-neo vector and has a high copy bacterial origin of replication, which permits easier purification. The pCI-neo vector (Promega, Madison, USA) (Figure 2.2C) has similar features to the pcDNAI-amp plasmid but additionally contains a chimeric intron site upstream of the cloning site, comprising a 5` donor site from the first intron of the human β-globin gene, and a 3` acceptor site from the intron of the sequence for the variable region of the immunoglobulin heavy chain. Chimeric intron sites can improve transcription of the insert by as much as 20-fold (363-365). However, the pCI-neo vector is larger in size as it also contains a neomycin resistance gene, which is unnecessary for the purposes of *in vivo*

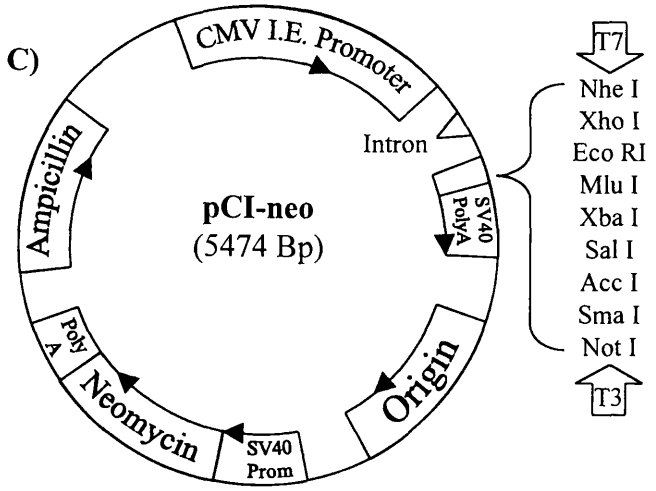
Figure 2.1 The nucleotide sequence of the MHC class I RT1.A^a α-chain.

The start and stop codons, the poly-adenylation signal, and the restriction digest sites involved in the subcloning procedures are shown in bold. The signal sequence is shown in italics (nucleotides 10-81). The soluble version was created by mutating the original sequence to incorporate the nucleotides for a stop codon and a Xba I digest site (taatctagacg) at position 934 (the methionine amino-acid residue at position 284 of the transmembrane region). Eco RI restriction sites are present at either end of the sequence for the purposes of cloning into the pcexv1-neo and pcDNAI-amp expression vectors. The sequence for the soluble antigen was subcloned into the pcmu-IV vector at the Not I and Xba I restriction sites, whereas cloning into the pCI-neo vector was achieved by using the Eco RI and the Xba I sites for the soluble antigen and the Eco R1 and PVU II sites for the full-length antigen.


Eco RI
start
Not I
taatcta gacg
stop
Pvu II
Poly A-
signal
Eco RI


A)
Neomycin
Mo LTR
SV40 PE
Ampicillin
pcexv1- neo
(6250 Bp)
Origin
Eco RI
cloning site


B)
CMV I.E. Promoter
Ampicillin
pcDNAI-amp
(4801 Bp)
Intron
Poly A
M13 Ori
Origin
T7
Hind III
Bam HI
Bst XI
Eco RI
Eco RV
Bst XI
Not I
Xho I
Sph I
Nsi I
Xba I
Sp6


C)
CMV I.E. Promoter
Intron
SV40 PolyA
Ampicillin
pCI-neo
(5474 Bp)
Poly A
Neomycin
SV40 Prom
Origin
T7
Nhe I
Xho I
Eco RI
Mlu I
Xba I
Sal I
Acc I
Sma I
Not I
T3

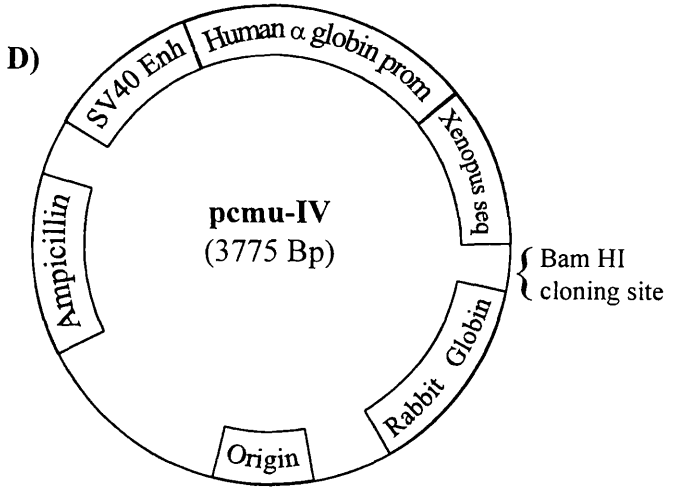

D)
SV40 Enh
Human α globin prom
Xenopus seq
Ampicillin
pcmu-IV
(3775 Bp)
Rabbit Globin
Origin
Bam HI
cloning site

Figure 2.2 Plasmid expression vectors (opposite).

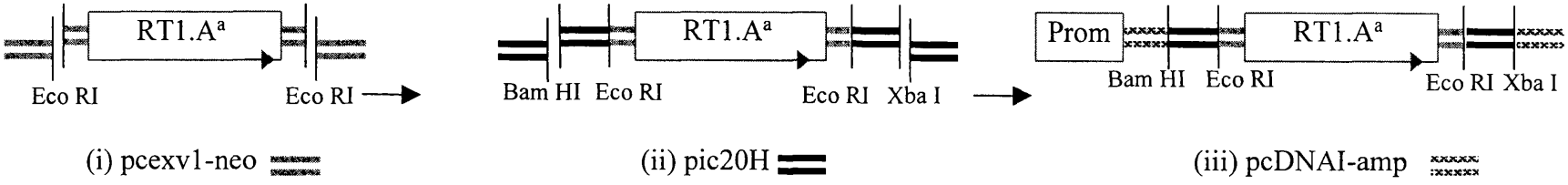
The RT1.A^a sequences were kindly provided by S. Powis (Wellcome Trust Building, University of Dundee, UK), inserted into the Eco RI cloning site of the pcexv1-neo vector, upstream of an SV40 promoter/enhancer (A). Pcexv1-neo includes a neomycin resistance gene under the control of a eukaryotic promoter. This permits stable transfection of transfected cell lines, but does not aid *in vivo* intramuscular expression. The commercially available vectors pcDNAI-amp (Invitrogen, Carlsbarg, California) (B) and pCI-neo (Promega, Madison, USA) (C) incorporate a polycloning site with multiple restriction digest sites, situated downstream of the CMV immediate/early promoter. Expression of the insert is optimised by the presence of an intron acceptor site. In addition the pCI-neo plasmid contains a neomycin resistance sequence. The pcmu-IV vector was provided by E. Joly (Babraham Institute, Cambridge, UK) (D). The RT1.A^a sequences were inserted into the pcmu-IV vector at the Bam HI cloning site, under the control of a promoter complex consisting of an SV40 enhancer, a human α-globin promoter and a xenopus capping sequence (see text for details). The 3` non-coding rabbit globin sequence provides an intron acceptor site and a polyadenylation sequence. All plasmids contain an origin of replication (origin) and an ampicillin sequence which permits expansion and selection in bacterial hosts.

intramuscular injection. Finally, Etienne Joly (Babraham Institute, Cambridge, UK) kindly gifted the full-length RT1.A^a sequence which had been cloned into the pcmu-IV vector (366) Within this vector (Figure 2.2D), a human α-globin promoter and an SV40 enhancer control expression of the inserted MHC class I sequence. The stability of the mRNA is enhanced by the xenopus sequence, which adds an untranslated 5` cap (367). The untranslated 3` end of the rabbit β-globin gene provides an intron acceptor site and the polyadenylation signal (368). This vector is not commercially available but this promoter and enhancer complex achieve high levels of expression of the downstream class I inserts (Etienne Joly, personal communication).

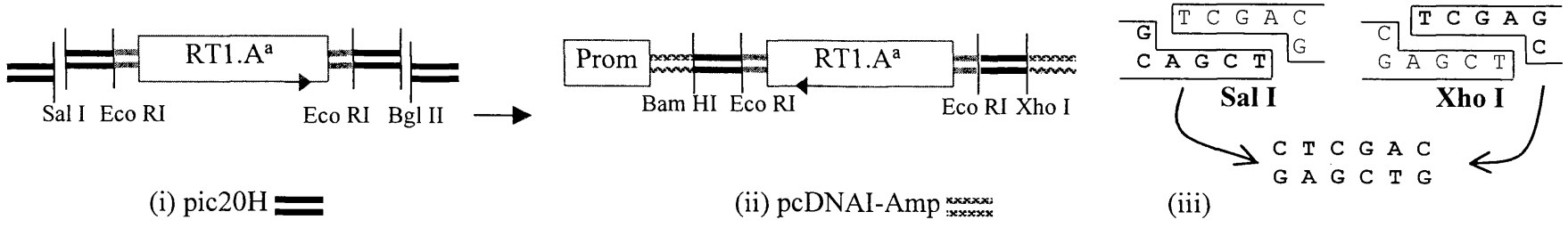
The class I sequences were subcloned into the pcDNAI-amp vector in two stages (Figure 2.3A). The full-length (A^a) and soluble RT1.A^a (tA^a) heavy chain sequences were initially excised from the pcexv1-neo vector and reinserted into the pic20H cloning vector (Invitrogen, Carlsbarg, California) by digesting with Eco RI restriction enzyme (pic20H/A^a and pic20H/tA^a, respectively). The sequences were then excised from the pic20H constructs by doubly digesting at the Bam HI and Xba I restriction sites, and religating into the pcDNAI-amp vector at the same sites (the RT1.A^a-encoding pcDNAI-amp vectors are hereafter referred to as pcdna-A^a and pcdna-tA^a) . The Bam HI and Xba I restriction digests cleave DNA to leave overhanging non-complementary ends, which increases the efficiency of the ligation reaction by ensuring that the pcDNAI-amp can only reform into double-stranded circular DNA by the inclusion of the class I sequence in the correct orientation. Control constructs were also created, in which the class I sequences were inserted in an anti-sense direction, firstly by doubly digesting the pic-20H/A^a construct with Bgl II and Sal I restriction enzymes, and then by cloning into the pcDNAI-amp vector at the Bam HI and Xba I sites (Figure 2.3B)

To create the pCI-neo class I constructs (pcineo-A^a and pcineo-tA^a), Eco RI/Xba I and Eco RI/Pvu II digests were performed to excise the truncated and full-length RT1.A^a sequences respectively from the pcexv1-class I vectors. The full-length sequence was inserted into the pCI-neo vector at the same digest sites, but the truncated sequence was inserted into the Eco RI and Sma I cloning sites

Figure 2.3 Diagrams of sequential cloning of RT1.A^a sequence into pcDNAI-amp in sense (A) and antisense (B) orientations.



A) The RT1.A^a sequence was excised from the pcexv1-neo vector (i) and inserted into the pic20H cloning vector by digestion at the Eco RI site (ii). It was subsequently transferred into the pcDNAI-amp vector by digestion at the Bam HI and Xba I restriction sites (iii). Digestion with two enzymes leaves non-complementary ends in the parent vector, ensuring that the RT1.A^a sequence was inserted in the correct orientation, with the 5` end immediately downstream of the promoter (Prom).



B) Subcloning of the RT1.A^a sequence into the pcDNAI-amp vector in an antisense orientation. The pic20H plasmid was again used as an intermediary cloning vector by inserting the RT1.A^a sequence at the Eco RI restriction site. The RT1.A^a sequence was then excised from the pic20H vector and inserted, antisense, into the pcDNAI-amp vector by ligating the 5` Sal I digest into the 3` Xho I site, and the 3` Bgl II digest into 5` Bam HI site (i and ii). Ligation of either a Sal I or Bgl II digested insert into, respectively, Xho I or Bam HI digested parent vectors is still possible, for although these enzymes recognise different restriction enzyme sequences, the overhanging ends created upon digest are complementary (iii). Note that the religated segment is no longer recognised by either of the restriction enzymes (iii).

(an Sma I digested end can religate onto a Pvu II digest as they are both class I restriction enzymes that cleave to leave blunt, rather than overhanging, ends).

To insert the sequence for the truncated, soluble version of the RT1.A^a sequence into the pcmu-IV vector, the pcmu-A^a plasmid was digested at the Xba I and Not I sites to excise the full-length sequence. The pcmu-IV vector was then re-circularised by religating in the presence of the cDNA sequence for the truncated RT1.A^a antigen, which had been excised from the pcexv-tA^a vector using similar restriction digests. Empty control, pcmu-IV, plasmid was created by excising the full-length A^a insert at the Bam HI restriction site (Figure 2.4). Figure 2.5 depicts the series of DNA agarose gels that were performed in the creation and purification of the class I MHC containing pcmu-IV vectors.

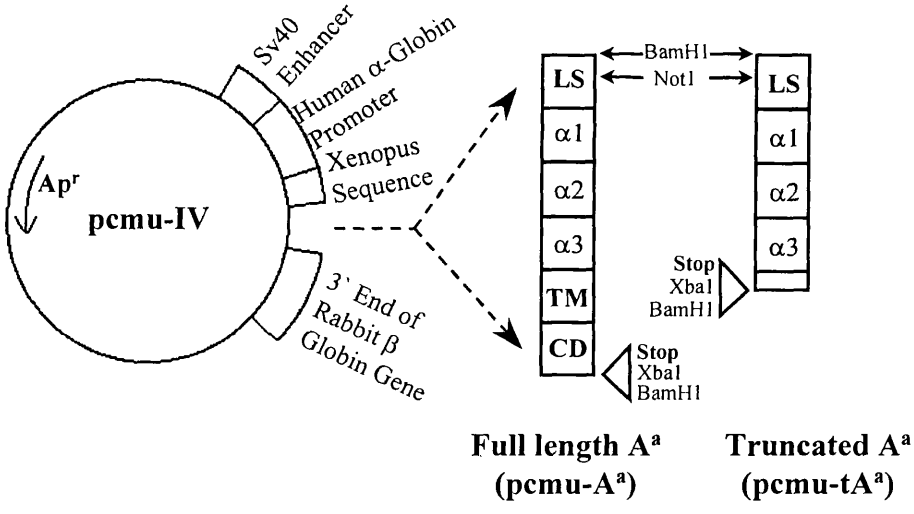
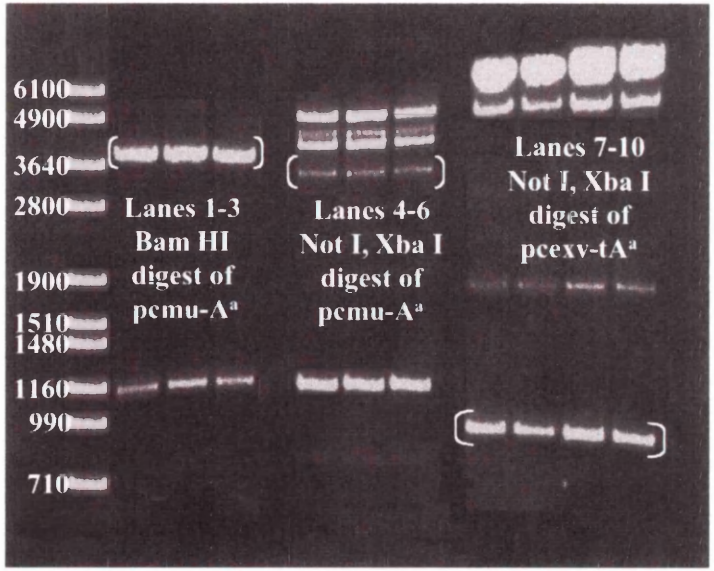


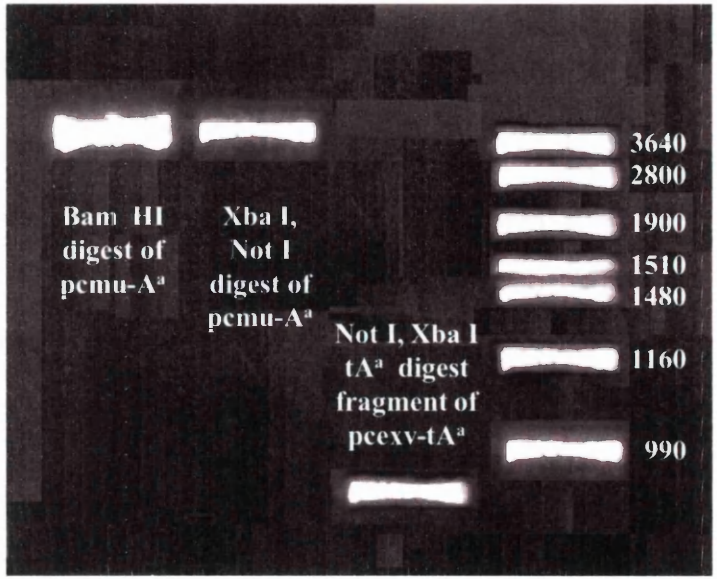
Figure 2.4 Diagram of pcmu vector and RT1.A^a class I MHC inserts.

The pcmu-tA^a vector was created by excising the full-length RT1.A^a sequence from the pcmu-A^a vector at the Not I and Xba I sites and inserting a similarly digested sequence for the truncated version (from pcexv-tA^a) at the same sites. The first 14 amino acids of the leader sequence (LS), upstream of the Not I site, are provided by the corresponding region of the mouse class I (H2-D^b) sequence. The Xba I site directly downstream of the stop codon of the full-length sequence is not present in the original description of the RT1.A^a sequence (39) but had been previously incorporated by mutagenesis. The truncated A^a sequence encodes a soluble form of the class I α-chain resulting from the inclusion of a stop codon at nucleotide 934 in the transmembrane region (TM), thereby excising the cytoplasmic domain (CD).

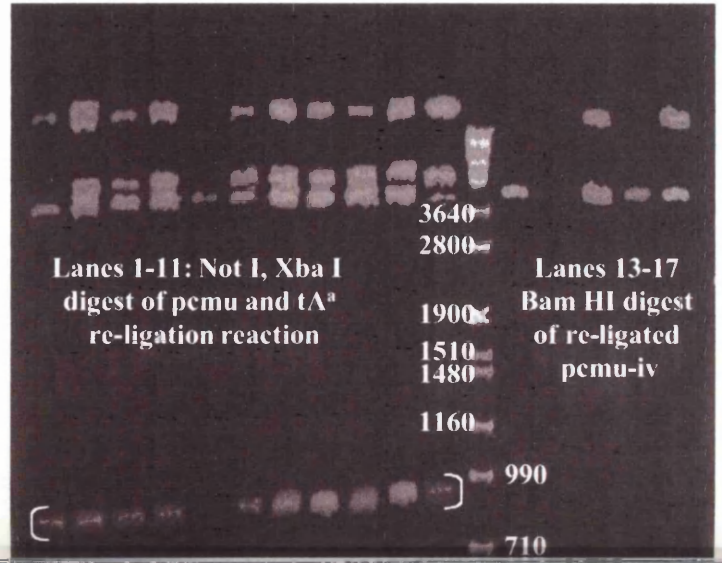
A)


6100
4900
3640
2800
1900
1510
1480
1160
990
710
Lanes 1-3
Bam HI
digest of
pcmu-A^a
Lanes 4-6
Not I, Xba I
digest of
pcmu-A^a
Lanes 7-10
Not I, Xba I
digest of
pcexv-tA^a

B)


Bam HI
digest of
pcmu-A^a
Xba I,
Not I
digest of
pcmu-A^a
Not I, Xba I
tA^a digest
fragment of
pcexv-tA^a
3640
2800
1900
1510
1480
1160
990

C)


Lanes 1-11: Not I, Xba I
digest of pcmu and tA^a
re-ligation reaction
3640
2800
1900
1510
1480
1160
990
710
Lanes 13-17
Bam HI digest
of re-ligated
pcmu-iv

D)

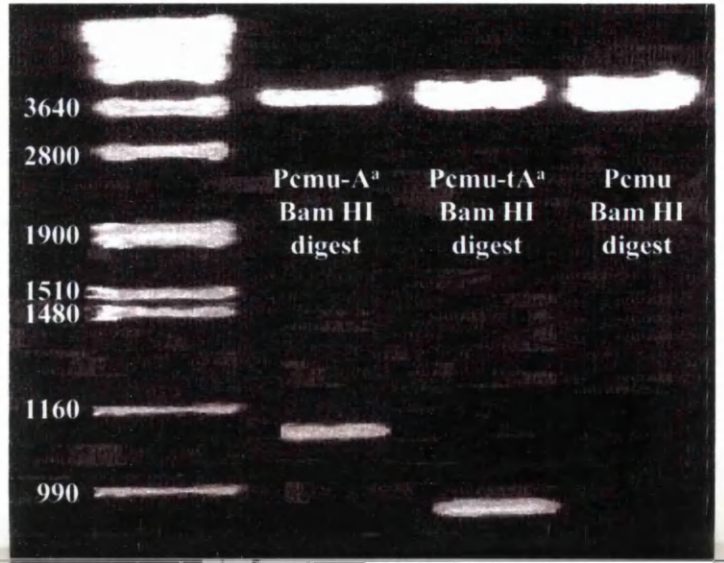

3640
2800
1900
1510
1480
1160
990
Pcmu-A^a
Bam HI
digest
Pcmu-tA^a
Bam HI
digest
Pcmu
Bam HI
digest

Figure 2.5. Creation of pcmu-IV vector constructs.

A) The pcmu-A^a vector (see text) was digested with either Bam HI (lanes 1-3) or with Xba I and Not I (lanes 4-6) and the products run on a 1% agarose gel. The bands approximating to the 1160 MW marker represent the excised full length heavy chain of the RT1.A^a sequence. The simultaneous Xba I and Not I digest has been incomplete; the larger bands represent either non-linearised plasmid or plasmid that has been digested at only one of the sites, leaving the RT1.A^a sequence attached, whereas completely digested plasmid (MW 3600n) is represented within the brackets. Lanes 7-10 depict the Xba I and Not I digest of the pcexv-tA^a vector. The cDNA sequence for the truncated RT1.A^a antigen lacks the transmembrane and cytoplasmic domains present in the full-length sequence and therefore the band for the soluble RT1.A^a sequence is smaller (890n vs 1120n). The bands within brackets were excised from the gel and purified using Promega Wizard DNA clean-up kits (Promega). The digests are run on triplicate wells because at least 1 μg of plasmid was digested for cloning purposes, which would overload a single well.

B) A sample of each of the clean-up products was re-run on a further agarose gel in order to confirm the purity of the samples and to permit quantitation using 'geldoc' software (Biorad, Hemel Hempstead, UK).

C) Ligation of the Not I/Xba I digested soluble RT1.A^a sequence into the pcmu-IV vector was detected by the ability to confer ampicillin resistance to successfully transformed DH5α E. coli bacteria. Individual colonies were selected and expanded overnight, and the plasmid purified and digested with Not I/Xba I. This reveals the appropriately sized fragment (900 nucleotides) for the truncated RT1.A^a fragment (lanes 1-11, within brackets). The larger bands of >4000 nucleotides again represent dimers and trimers of the undigested parent vector. Lanes 13-17 depict repeat Bam HI digests of the empty pcmu-IV vector, confirming that no class I inserts are present.

D) The pcmu-tA^a plasmid corresponding to the digest in lane 9 (Gel C) and the pcmu-IV plasmid corresponding to lane 13 (Gel C) were chosen. The original bacterial colonies from which they were derived were re-expanded, purified using 'Qiagen' columns (Qiagen, Crawley, UK), and repeat digests performed. This confirms appropriately sized class I inserts and these bacterial clones were used for all subsequent plasmid purification.

2.2.2 *In Vitro Expression of the RT1.A^a Plasmid Constructs*

Expression of MHC class I by the vector constructs was assessed by lipid-mediated, cell line transfection. Despite optimisation of transfection conditions (see section 2.5.2.2), there was neither expression of the RT1.A^a antigen on the cell surface nor in the cell culture supernatant following transient transfection of the rat myoblast cell line, L6, with the initial class I constructs, pcexv-A^a and pcexv-tA^a (data not shown). Transient transfection, in which the plasmid does not integrate into the host genome, is a relatively inefficient, short-lived process. Transfection was therefore repeated but successfully transfected cells, which co-express neomycin resistance, were selected by growing in geneticin (Sigma-Aldrich, Poole, UK). This permits incorporation of the plasmid into the genome, resulting in a population of stably transfected cells, in which every cell expresses the plasmid product. Only 2-3 cells per flask were initially resistant to geneticin, confirming that the efficiency with which the L6 cell line is transiently transfected is poor. Flow cytometric analysis of the stably transfected cell line confirmed that only the full-length, and not the truncated, RT1.A^a molecule was expressed on the cell surface (Figure 2.6). Conversely, soluble RT1.A^a, as detected by an A^a-specific ELISA, was present in the supernatant of cells transfected with the truncated, but not the full-length, A^a molecule (Figure 2.7). Cells transfected with the control 'empty' vector expressed neither membrane-bound, nor water-soluble RT1.A^a protein (Figures 2.6 and 2.7). The L6 cell line was stably transfected on four occasions with identical results. Flow cytometric analysis of the pcexv-A^a transfectants invariably resulted in a bifid graph (Figure 2.6C), suggesting that two cell populations were present. The level of fluorescence in the left-hand population was no higher than the control line and this probably represents cells that have lost their plasmid on cell division.

To confirm that cloning of the RT1.A^a heavy chain into the pcDNAI-amp, pCI-neo and pcmu-IV vectors resulted in the translation and expression of a detectable cell surface protein, transient transfection of the L6 cell line was performed. Again flow cytometric analysis of the transfected cells did not reveal cell surface expression of the RT1.A^a protein. As the pcDNAI-amp and pcmu-IV vectors don't encode a selectable marker, stable transfection was not possible and

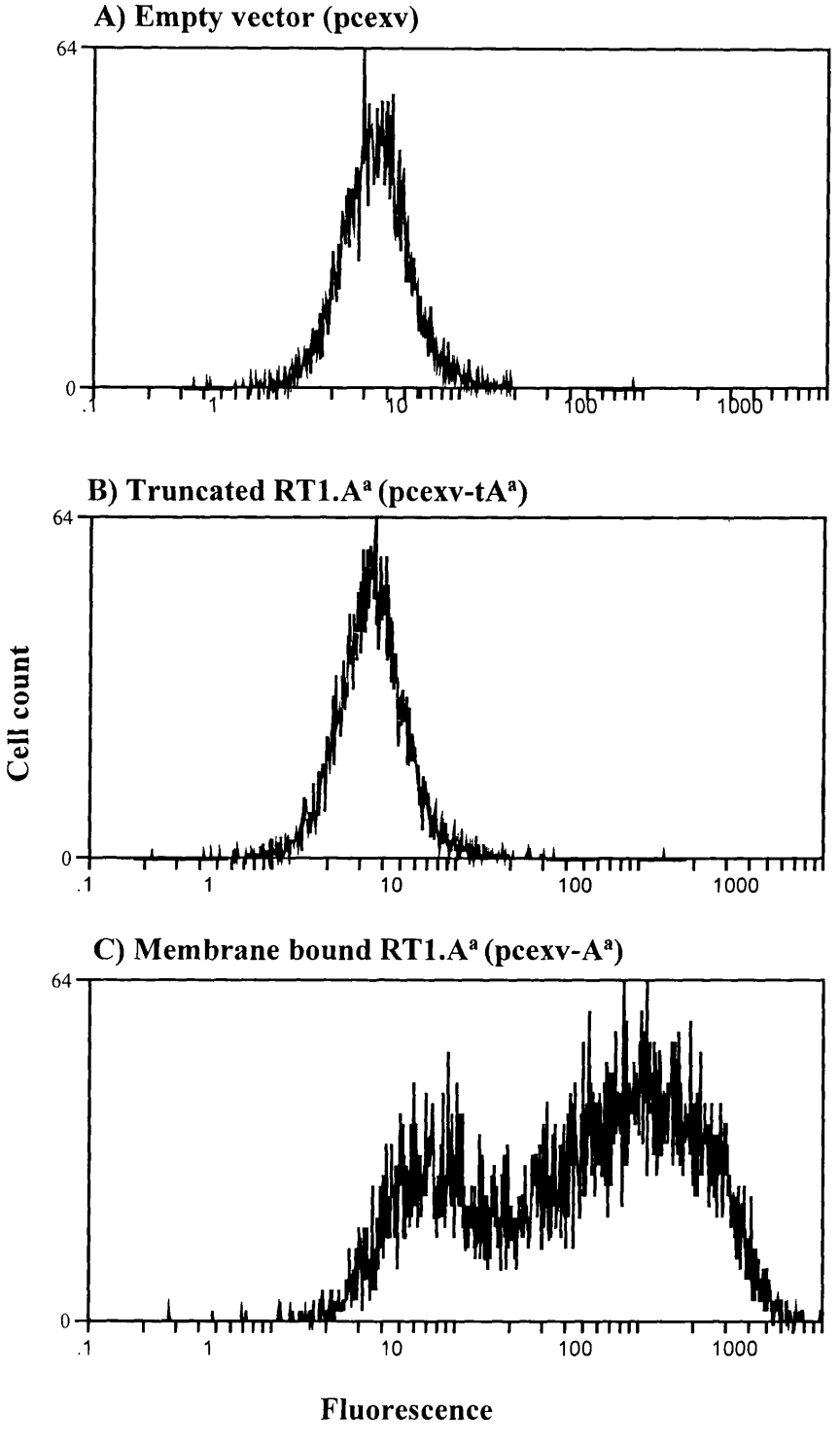


Figure 2.6 Cell surface expression of RT1.A^a MHC class I.

The rat myoblast cell line L6 was stably transfected empty plasmid, pcexv (A), or plasmid encoding either the truncated (water-soluble) RT1.A^a molecule, pcexv-tA^a (B), or the full-length, (membrane-bound) RT1.A^a molecule, pcexv-A^a (C). Flow cytometric analysis was performed after incubation of the cells with FITC-conjugated MN4-91-6 monoclonal antibody (mAb), which labels a polymorphic determinant of RT1.A^a.

subsequent studies were performed using a human embryonal cell line, 293, which is transfected with approximately 90% efficiency using liposome-mediated transfer. Flow cytometric analysis of 293 cells that had been transiently transfected with the vectors encoding the full-length RT1.A^a heavy chain confirmed cell surface expression for all of the constructs (Figure 2.8 A-D). Transfection with the constructs encoding the truncated, water-soluble RT1.A^a antigen resulted in cell surface expression similar to control non-transfected cells (Figures 2.8E and 2.8F). The pcdna-A^a, pcineo-A^a and pcexv-A^a vectors were all associated with similar levels of RT1.A^a expression (Figure 2.9), which is unsurprising given the similarities in their promoter regions. Expression following transfection with the pcmu-A^a vector was significantly higher (Figure 2.9).

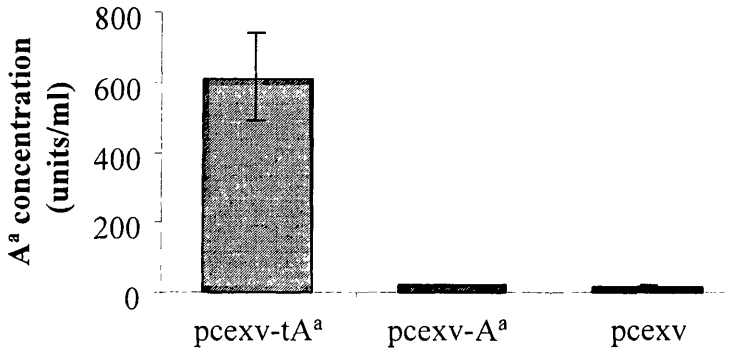
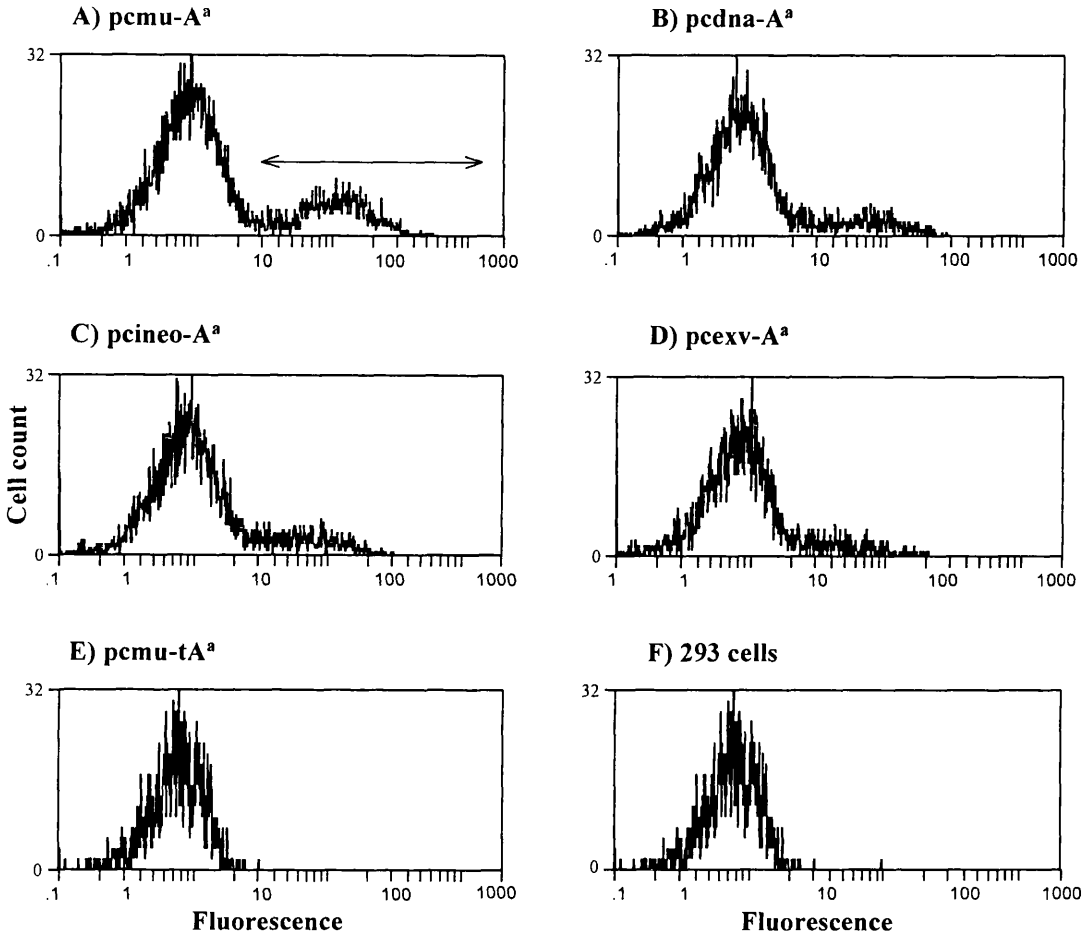


Figure 2.7 Soluble RT1.A^a protein in stable L6 culture supernatant.

Rat myoblast L6 cells were stably transfected with either the full-length RT1.A^a construct (pcexv-A^a), the truncated RT1.A^a construct (pcexv-tA^a), or empty plasmid (pcexv). Undiluted samples of cell culture supernatant were assayed for the presence of soluble RT1.A^a protein by ELISA, using MN4-91-6 mAb as the capture antibody and biotinylated OX18 mAb for detection. A detergent lysis of ACI splenocytes was used as a standard; the undiluted sample was arbitrarily assigned a concentration of 1000 units/ml. From serial dilutions of the standard, a standard curve was constructed using Biorad software and the concentration of the test samples calculated. The results represent the mean and standard deviation of a minimum of four experiments.

An RT1.A^a specific ELISA was performed to confirm the presence of soluble RT1.A^a protein in the cell culture supernatant of the transiently transfected 293 cells (Figure 2.10). There were significantly higher levels of RT1.A^a protein in the supernatant from cell lines that had been transfected with the vectors encoding the truncated, water-soluble RT1.A^a class I sequence than in supernatant from the cell line transfected with the empty control vector. There was no detectable RT1.A^a protein in the supernatant from cells transfected with vectors encoding the full-

length RT1.A^a sequence (Figure 2.10, levels for pcmu-A^a depicted as example). Of the vectors encoding the truncated A^a antigen, the pcmu-tA^a plasmid was associated with the highest mean level of expression, but this difference did not



reach statistical significance (Figure 2.10).

Figure 2.8 Cell surface expression of the RT1.A^a MHC class I antigen.

The full-length RT1.A^a heavy chain sequence was cloned into the pcmu-IV, the pcDNAI-amp, the pCI-neo, and the pcexv1-neo vectors (as described in section 2.2) to form respectively, pcmu-A^a, pcdna-A^a, pcineo-A^a and pcexv-A^a. The truncated RT1.A^a sequence was also inserted into the pcmu-IV vector (pcmu-tA^a). The human embryonal kidney cell line 293 was transiently transfected with these constructs and flow cytometric analysis performed 48 hours later, by labelling with the FITC–conjugated MN4-91-6 mAb. The diagrams are representative of a minimum of three experiments. The pcmu-A^a plasmid consistently resulted in higher levels of RT1.A^a expression (A). Of the truncated RT1.A^a constructs only the pcmu-tA^a result is shown (E). Similar results were achieved with the remainder. Quantitative comparison of the levels of expression associated with each of the class I constructs was performed by gating separately on the transfected cell population, as depicted in the arrowed population in Figure A, and calculating its mean channel fluorescence (Figure 2.9).

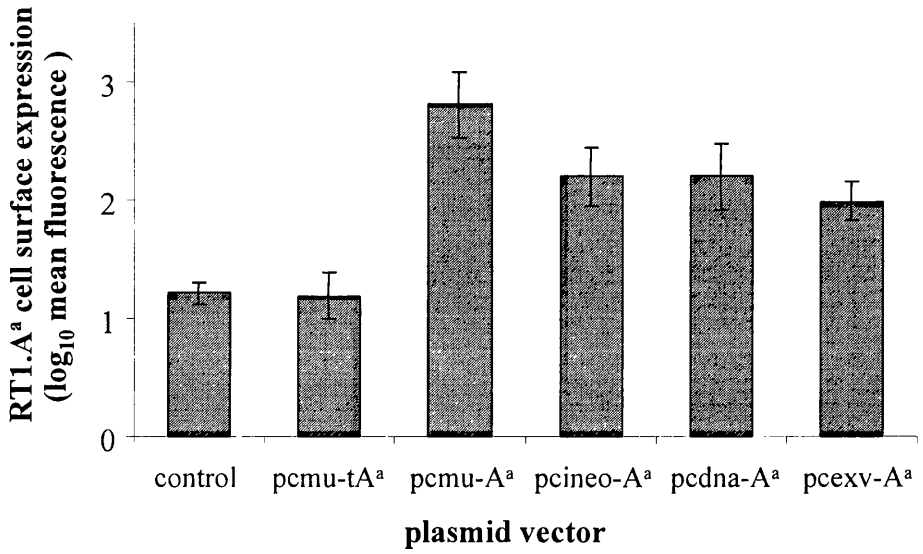


Figure 2.9 Quantitative comparison of cell surface RT1.A^a expression

293 cells were transiently transfected with the vectors encoding the RT1.A^a class I antigen using liposome-mediated transfection. 48 hours later cell surface expression of the RT1.A^a antigen was quantified by labelling cells with FITC-conjugated MN4-91-6 mAb and using flow cytometric analysis to calculate the mean channel fluoresence of the separately-gated transfected cell population (Figure 2.8). Values on the y-axis represent the mean channel fluorescence of this cell population. One way analysis of variation confirms the presence of separate populations (ratio of between group to within group variation: 35.7, $p < 0.001$). Smilarly, there was no statistical difference between the levels of RT1.A^a expression in control, non-transfected cells and cells transfected with pcmu-tA^a vector, which encoded the truncated (water soluble) version of the RT1.A^a antigen (Mean +/- SD: 1.56 +/- 0.19 vs. 1.49 +/- 0.094). There was no detectable surface RT1.A^a expression following transfection with any of the plasmid vectors that encoded the truncated A^a cDNA (data not shown). The pcineo-A^a, pcdna-A^a and pcexv-A^a vectors achieved comparable levels of A^a class I expression (Mean +/- SD: 2.2 +/- 0.24, 2.2 +/- 0.28, 1.99 +/- 0.17), but this was significantly higher than the control cell line (95% CI for the difference in means; 0.22 to 1.05; $p < 0.001$). The pcmu-A^a vector induced the greatest levels of RT1.A^a expression (2.8 +/- 0.27), which was significantly greater than the levels associated with the other class I-expressing constructs (95% CI for the difference in means; 0.15 to 1.06, p=0.003)

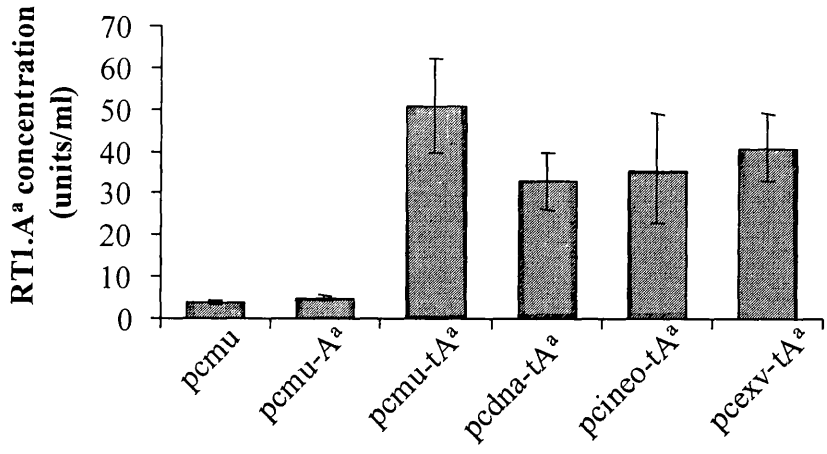


Figure 2.10 Soluble RT1.A^a protein expression following 293 cell line transfection.

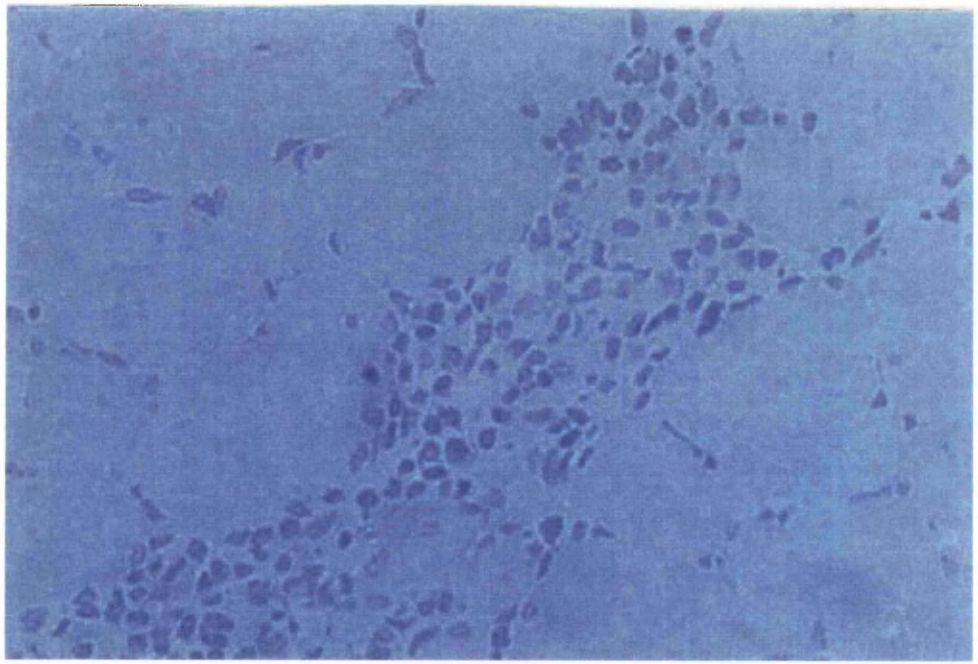
Human kidney embryonal 293 cells were transiently transfected with RT1.A^a class I vector constructs and undiluted cell culture supernatant analysed for the presence of the soluble RT1.A^a protein 48 hours later by A^a specific ELISA. The results represent the mean and standard deviation of four experiments. A one way analysis of variance confirms that significantly different populations are present (ratio of between group to within group variation: 22.86, $p<0.001$). Cell lines transfected with the vectors encoding the truncated RT1.A^a sequence secrete significantly more RT1.A^a antigen into the cell culture supernatant than either the control, non-transfected lines (95% CI for the difference of the means = 27 to 67 units/ml, $p=0.002$), or lines transfected with the full-length RT1.A^a sequence (results for only the pcmu-A^a vector are shown). Although the mean secretion of RT1.A^a antigen was highest for the pcmu-tA^a vector, this was not significantly different when compared to the other vectors encoding the soluble version of the A^a class I antigen (cf. pcineo-tA^a; 95% CI for the difference in the means = -4.3 to 35.3; $p=0.246$).

2.2.3 In Vivo Expression of the RT1.A^a Plasmid Constructs

2.2.3.1 Local Expression of the RT1.A^a Antigen

Intramuscular expression of the RT1.A^a antigen was assessed by histological examination of the tibialis anterior muscle five days after PVG.RT1^u rats were injected with 100μg of the pcmu-IV vector encoding either the full-length or truncated RT1.A^a cDNA sequence. Three days prior to plasmid injection, the muscle was injected with 400μl of 0.5% Bupivicaine (1-butyl-N[2, 6-dimethyl phenyl] 2-piperidine-carboxamide) to induce myocyte regeneration, and thereby increase the efficiency of gene transfer. Figure 2.11A depicts the typical histological appearance following bupivicaine injection. Areas of muscle regeneration containing numerous small myocytes are present and are arranged in

A



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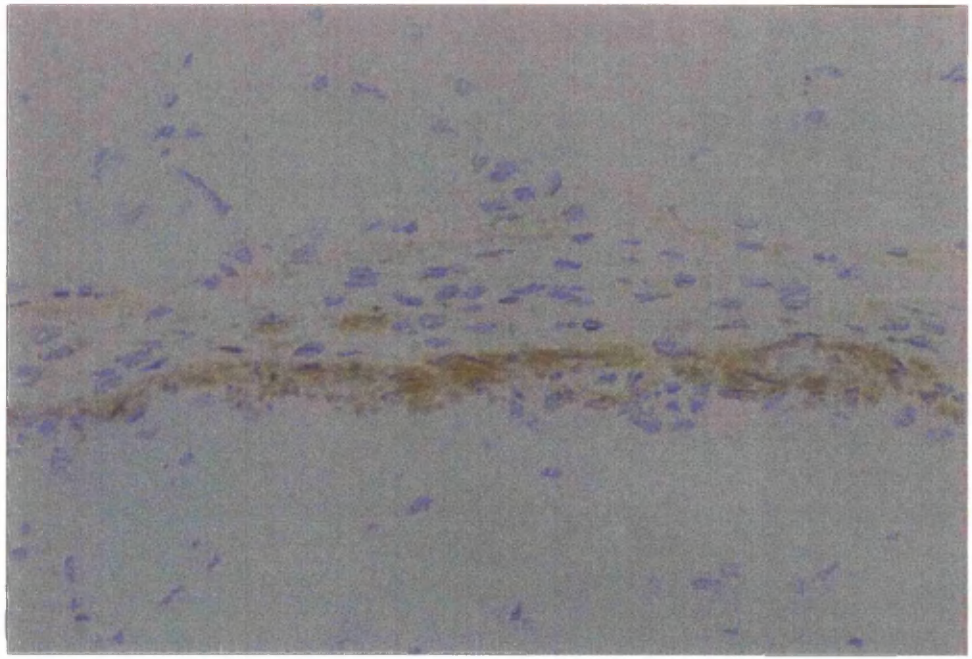

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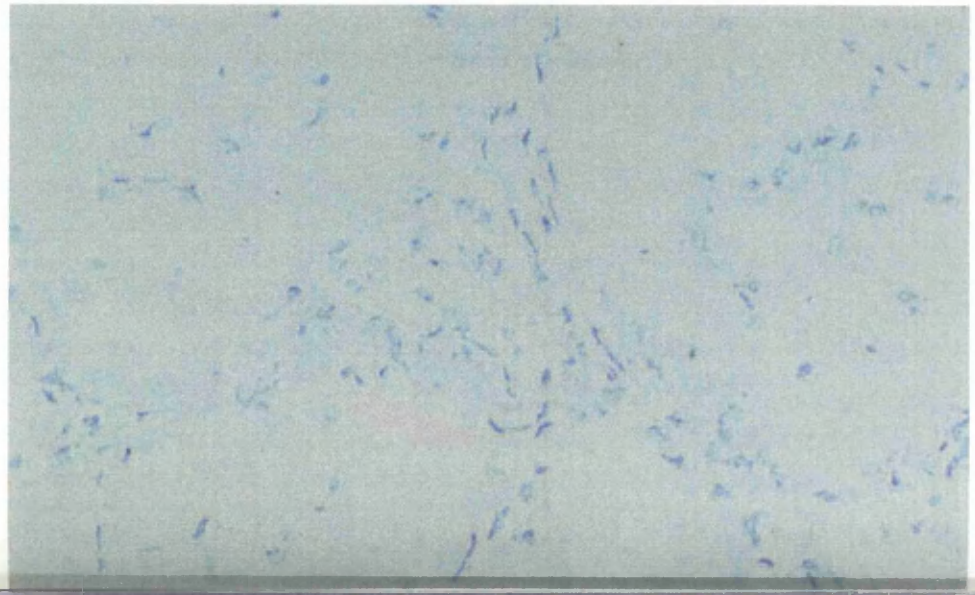

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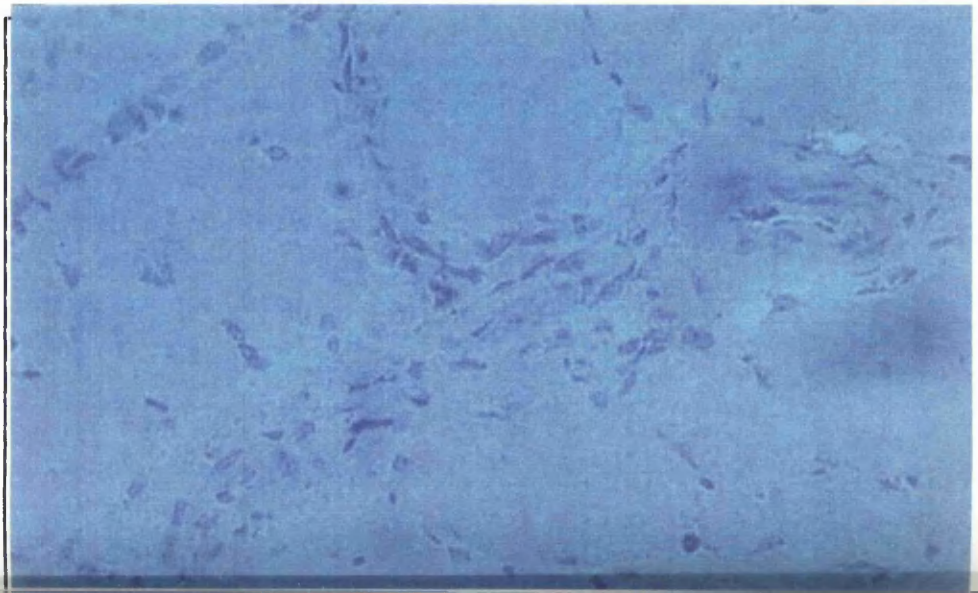

Figure 2.11 Histological confirmation of intramuscular RT1.A^a expression following injection with A^a-encoding pcmu vectors.

Immunoperoxidase staining with RT1.A^a specific MN4-91-6 mAb was performed on cryostat sections that had been prepared from the tibialis anterior muscle of PVG.RT1^u animals. The muscle had been injected eight days prior to harvest with 400µl of 0.5% bupivicaine (1-butyl-N[2, 6-dimethyl phenyl] 2-piperidine-carboxamide) and three days later with 100µg of either 'empty' pcmu-IV or plasmid encoding the RT1.A^a heavy chain (pcmu-A^a).

A) Muscle injected with bupivicaine only. A typical tract of bupivicaine-induced regeneration containing numerous small myocytes is demonstrated. Normal larger myoblasts surround these tracts.

B) Muscle injected with pcmu-A^a plasmid. Peroxidase staining (brown) with MN4-91-6 mAb reveals expression of RT1.A^a alloantigen confined to the areas of regeneration.

C) MN4-91-6 mAb staining of muscle from PVG.RT1^u rats that had been injected with 'empty' control plasmid.

D) Control isotype (ESH8) mAb staining of pcmu-A^a injected muscle.

tracts, consistent with injection sites. These tracts are surrounded by normal, non-regenerating muscle that contains larger myoblasts.

Following injection with the pcmu-IV construct encoding the full-length A^a sequence (pcmu-A^a), peroxidase staining with MN4-91-6 mAb revealed that expression was confined to areas of regeneration (Figure 2.11B). No staining was present in muscle that had been injected with the control empty pcmu-IV vector (Figure 2.11C). Antibody against third party RT1.A antigen, which would have been the optimum control isotype antibody, was not available. Instead, the muscle was stained with the mouse IgG2a mAb, ESH8, which is directed against human factor VIII (Scottish Antibody Production Unit, Law, Scotland, UK). No expression was discernible (Figure 2.11D).

Muscle tissue, which had been injected with the vector encoding the truncated version of the RT1.A^a heavy chain sequence (pcmu-tA^a), stained weakly upon labelling with MN4-91-6, with the results neither reliable nor convincingly higher than the levels of background staining (data not shown). Presumably the lack of cell surface tethering of the product of this plasmid prevented accurate fixation and staining.

2.2.3.2 *Alloantibody Response to the A^a Product of the Pcmu Vectors.*

Expression of the RT1.A^a antigen following intramuscular injection of plasmid was further confirmed by assessing the development of an anti-A^a alloantibody response. PVG.$RT1^u$ hosts injected intramuscularly on day 0 with the pcmu-IV vector encoding either the full-length (pcmu-A^a) or truncated (pcmu-tA^a) class I constructs developed a lymphocytotoxic alloantibody response, which could be detected by day 10 and was still present on day 26 following the plasmid injection (Figure 2.12). Injection of the control, empty pcmu-IV vector did not generate an alloantibody response. Although the alloantibody response to the product of the pcmu-A^a vector displayed similar kinetics to the response against the pcmu-tA^a vector, at all time points the magnitude of the response to the full-length RT1.A^a antigen was smaller (Figure 2.12). Similar findings have been reported for plasmids that code for the retained or secreted forms of both the hepatitis C (369) and ovalbumin antigens (370). Although the genetic sequence for the soluble

RT1.A^a protein lacks the transmembrane and cytoplasmic domains, the rate of translation of its product will be similar to that of the full-length antigen. The quantitative difference in the antibody response presumably reflects greater bioavailability of the water-soluble RT1.A^a antigen at the site of primary B cell activation within the T cell areas of secondary lymphoid tissues.

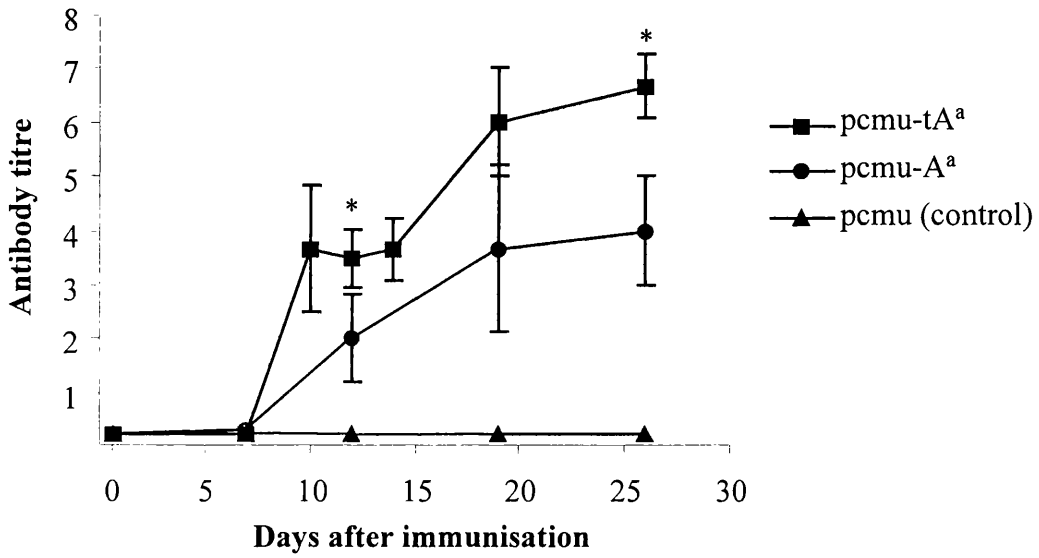

Figure 2.12 Circulating lymphocytotoxic RT1.A^a alloantibody response.

PVG.RT1^u rats were immunised with the pcmu-IV vector encoding either the full-length (pcmu-A^a) or truncated (pcmu-tA^a) nucleotide sequence of RT1.A^a. Control animals were immunised with empty pcmu-IV plasmid. Animals received 400µl of 0.5% bupivicaine intramuscularly followed 3 and 8 days later, by injection of 200µg of plasmid. Lymphocytotoxic antibodies in serum samples were detected by their ability to lyse ^{51}Cr-labelled, Con A-transformed splenic blasts in the presence of guinea pig complement. Results are expressed as the mean and standard deviations of antibody titre (last dilution of serum that gave >=20% cytotoxicity) of a minimum of three animals. Values on the abscissa represent the time elapsed in days from the first injection of plasmid.
* $p < 0.05$, Mann-Whitney U test (2-tailed).

The alloantibody response to the product of the pcmu-tA^a vector consisted of an early IgM response that was detectable by day seven, but heavy chain class switching, to the IgG2b isotype exclusively, was evident by day 10 (Figure 2.13). This confirms that plasmid injection results in an efficient T and B cell response to the RT1.A^a antigen since isotype switching is particularly T helper cell dependent (296, 303). The kinetics of the IgG2b response closely matched those of the allocytotoxic response (cf. Figure 2.12 to Figure 2.14). This is unsurprising,

for in the rat, the IgG2b antibody subclass is the most effective mediator of complement-dependent lysis (371). The magnitude of the IgG2b response to the pcmu-tA^a vector was significantly higher than to the pcmu-A^a vector (Figure 2.14). No other isotype response was detected at later time points. No antibody was detectable in control animals (data not shown).

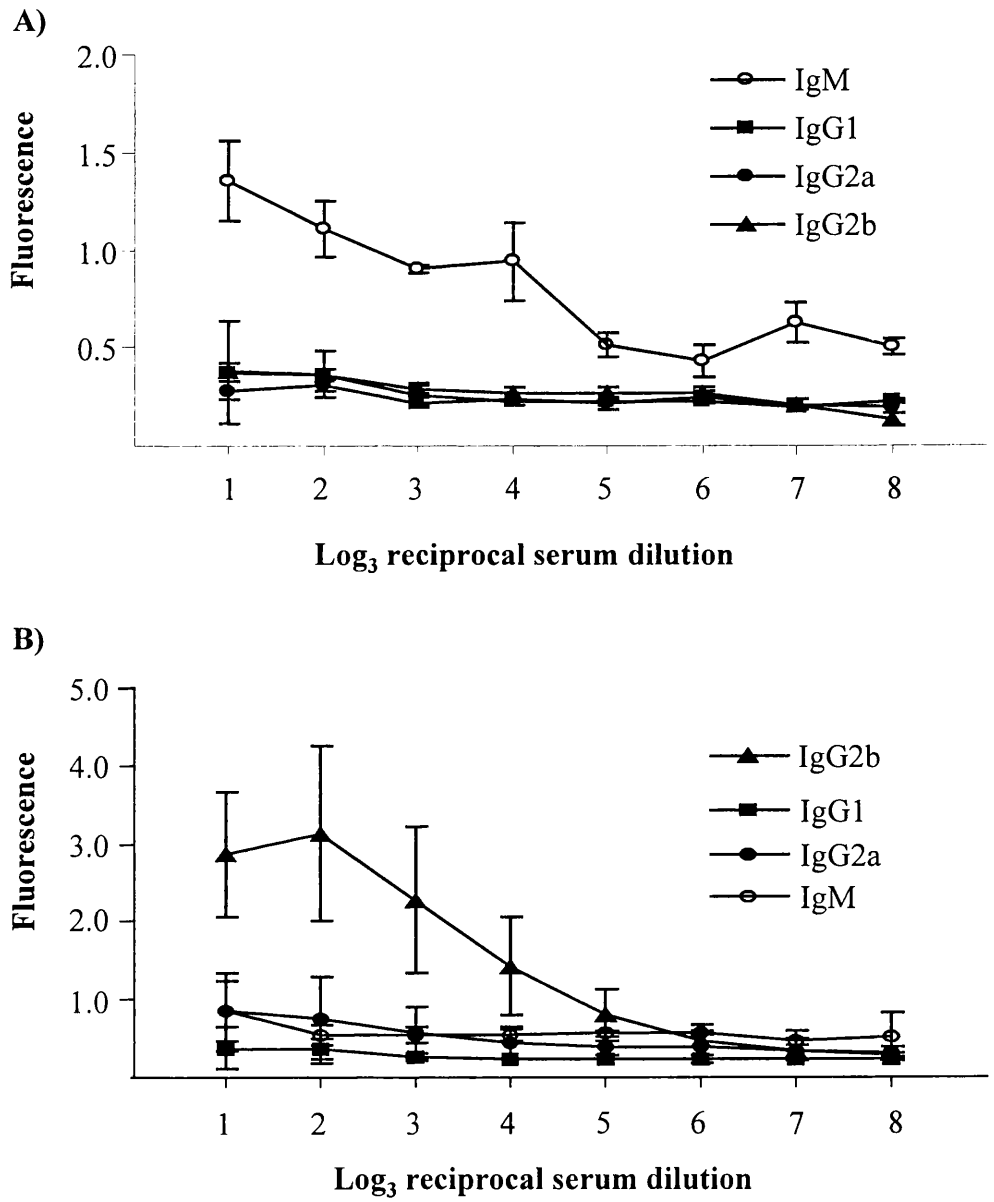

Figure 2.13 Ig class and subclass of the anti-RT1.A^a antibody response.

PVG.RT1^u rats were immunised with 400µl of 0.5% bupivicaine intramuscularly followed 3 and 8 days later, by injection of 200µg of pcmu-tA^a. 7 days (Figure A) and 12 days (Figure B) later, sera were analysed for anti-A^a alloantibody by flow cytometric analysis, using PVG.R8 LNCs as targets and FITC-conjugated mouse anti-rat Ig subclass-specific mAb. Results (mean channel fluorescence) are shown as the mean and SD for a minimum of three animals.

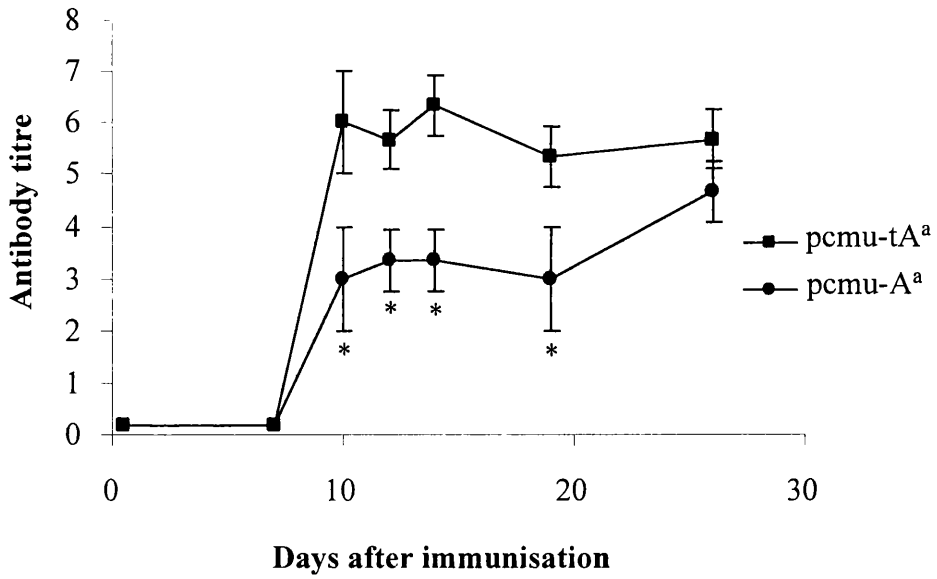

Figure 2.14 The allospecific IgG2b response following intramuscular injection with RT1.A^a encoding pcmu vectors.

PVG.RT1^u rats were immunised with 400μg of the pcmu-IV vector coding for either the full-length RT1.A^a cDNA sequence (pcmu-A^a) or the truncated soluble version (pcmu-tA^a). Serum samples taken on days 10, 12, 14, 19 and 26 after immunisation were assessed for IgG2b content by flow cytometry using naïve PVG-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 (level of fluorescence observed with cells and antibody alone). Control animals that received the empty pcmu-IV vector did not generate an anti-A^a IgG2b response (data not shown). Results represent the mean and standard deviations of 3 animals per group. Statistical analysis was performed using the Mann-Whitney U test; * $p < 0.05$.

2.3 Discussion

The cDNA sequence for the truncated water-soluble version of the RT1.A^a antigen was created by inserting a stop codon after the α3 domain of the heavy chain, as initially described by Bjorkman (31). The transmembrane and cytoplasmic domains are responsible for tethering the RT1.A^a antigen to the cell membrane and their absence is equally advantageous in preventing aggregation of the soluble protein into multivalent complexes.

Transient *in vitro* transfection of the L6 rat myoblast cell line with either the full-length or truncated versions of the RT1.A^a antigen was unsuccessful and stable transfection was required. Expression in the rat myoblast cell line is important, as the *in vitro* model it represents most closely correlates with the proposed *in vivo* use of the plasmid. Equally, stable transfection provides the most strenuous examination of the potential for the soluble version of the RT1.A^a antigen to be expressed, even transiently, on the cell surface. If this were to occur, the central tenet of the experimental model, that the soluble A^a construct is unable to activate T cells directly, would be invalidated. Flow cytometric analysis of L6 cells stably transfected with the soluble A^a construct revealed levels of cell-surface fluorescence identical to the control line (Figure 2.6B).

Subsequent *in vitro* expression experiments were performed on the human kidney embryonal cell line, 293, which is transiently transfected with much greater efficiency. Transfection with the pcmu-A^a vector resulted in a significantly higher level of cell-surface expression of the RT1.A^a antigen than was associated with the other A^a-encoding vectors (Figure 2.9). Similarly, transfection with the pcmu-tA^a vector achieved greater levels of secreted soluble RT1.A^a antigen, though this did not reach statistical significance (Figure 2.10). The reason why the pcmu-IV vector was more efficient at expressing RT1.A^a protein than the commercially-available vectors (which contain generally powerful viral promoters and enhancers) is unclear; it presumably relates to the small size of the pcmu-IV vector and to an intrinsic ability of the human α-globin promoter to preferentially induce expression of MHC inserts.

The quantities of plasmid required to assay the *in vivo* expression of each of the RT1.A^a constructs makes such an approach impractical and although the 293 cell line is of human derivation, it is reasonable to assume that A^a encoding pcmu-IV vectors would achieve the highest levels of expression of the RT1.A^a antigen in rat hosts. The pcmu-IV constructs were therefore chosen for subsequent *in vivo* experiments, although their small copy number is disadvantageous in that greater quantities of bacterial culture are required to purify a unit of plasmid and this potentially increases the concentration of bacterial contaminants. For this reason all plasmid that was used *in vivo* was prepared using Qiagen kits (Qiagen, Crawley, UK) that incorporated an Endotoxin Removal Buffer (section 2.5.1.5)

The *in vivo* use of DNA vectors generally involves either their incorporation into cationic lipids (see (372) for review), or the injection of the naked DNA intramuscularly. The latter technique results in the uptake of only picograms of the plasmid (373, 374), possibly through the T tubules and caveolae of skeletal muscle (375). Expression can be increased by pre-injection of the muscle with either hypertonic solutions (376) or chemicals that stimulate muscle regeneration, such as bupivicaine (377). Plasmid DNA does not replicate in injected muscle cells, but can persist for years as an episome. Myoblasts do not, however, express costimulatory signals and one could consequently argue that their manipulation to express antigenic proteins would result in T cell tolerance, rather than stimulation (235). Nevertheless, intramuscular plasmid vaccination induces strong humoral and cellular immune responses (361, 362). It is now apparent that T cell activation following naked DNA injection intramuscularly is at least partly due to antigen presentation by dendritic, rather than muscle, cells (378). Dendritic cells are able to phagocytose protein antigen after it has been expressed by muscle cells, but are also capable of internalising and subsequently transcribing the plasmid DNA (379). Dendritic cells are professional APC and in theory only limited expression of the antigen would be sufficient to result in a powerful T cell response. Moreover, plasmid DNA of bacterial origin contains, unlike eukaryotic DNA, unmethylated CpG motifs, which have a strong adjuvant effect, promoting dendritic cell maturation, and migration to lymphoid tissue (380, 381).

A consequence of the presentation of injected DNA antigen by dendritic cells is the preferential development of a Th1-biased T cell response (382). Il-12 is produced by the dendritic cells in response to unmethylated motifs present within the plasmid DNA. This in turn induces IFN-γ production and creates the appropriate cytokine milieu to polarise towards a Th1 response (383). The class and subclass of the antibody response is influenced by the nature of the T cell help (see (384) for review). In the rat a Th2 response favours the development of IgG1 and IgG2a antibody subclasses, whereas the provision of help by Th1 cells results in switching to the IgG2b and IgG2c subclasses (385). These observations may explain why the anti-A^a response in PVG.RT1^u animals following injection of the A^a-encoding plasmid was of the IgG2b subclass (the IgG2c response was not assayed). This is in contrast to previous work in Bradley's laboratory; PVG.RT1^u animals that received either a PVG.R8 blood transfusion (246) or a kidney graft (386) developed an IgG response in which the levels of the subclass isotypes were comparable.

McPherson et al have recently demonstrated that dendritic cells are able to internalise and present intact protein antigen to B cells in conjunction with the necessary costimulatory signals to stimulate isotype switching (301, 387). This results in an abbreviated IgM response. The isotype response to the products of the plasmid vectors is similarly abbreviated, supporting the concept that the immune response to the plasmid vectors is the result of dendritic cell presentation. For example, the IgM response to the A^a antigen following pcmu-tA^a injection was detectable only on day 7 and had switched to an IgG2b response by day 10. In comparison, the IgM response to an R8 blood transfusion is detectable from day 7 to day 14 (Lovegrove E., Ph.D. thesis).

Theorerical justification for using soluble class I alloantigen is reliant on it sharing similar B cell epitopes as the naturally-occuring antigen on the surface of donor PVG.R8 cells, such that a cross-reactive antibody response can develop. The formation of a stable, properly folded MHC class I molecule requires the presence of heavy chain, β_2-microglobulin (β_2M) light chain and bound peptide, but the injected plasmid encoded the heavy chain only. Expression of intact A^a antigen following plasmid injection is therefore reliant on the incorporation of

endogenous β_2M and peptide. The β_2M subunit is highly conserved, as confirmed by the expression of RT1.A^a protein with the appropriate quaternary structure in human cells that were transfected with the heavy chain only. The RT1.A^a antigen, as expressed by plasmid-transfected PVG.$RT1^u$ muscle cells, is however, likely to present a different spectrum of peptide than the equivalent naturally-occuring antigen due to differences in both the pool of cytosolic proteins and in the nature of the TAP haplotype (130). Crucially, despite the differences in β_2M and in the spectrum of bound peptide, the alloantibody assays as used in these studies rely on the use of donor PVG.R8 target cells, and these confirm that the antibody response to the A^a-encoding pcmu-IV vectors cross-reacts with the wild-type RT1.A^a antigen.

2.4 Summary

Several plasmid constructs that expressed the full-length and truncated versions of the RT1.A^a antigen were created. These differed in size and in the nature of the promoter/enhancer regions that controlled expression of the class I inserts. *In vitro* transfection studies confirmed that the constructs expressed either the membrane-bound or soluble RT1.A^a antigens with 100% purity and that the pcmu-IV vector achieved the highest levels of expression. The RT1.A^a antigen was detected by immunohistochemical staining of the muscle of animals that had been injected with the pcmu-A^a construct. Both RT1.A^a encoding pcmu-IV vectors resulted in a strong lymphocytotoxic alloantibody response with heavy chain class switching predominantly to a Th1 isotype subclass. The antibody response to the transgenic RT1.A^a molecule cross-reacts with the wild type antigen. Confirmation of the shared nature of the B cell epitopes is essential in fulfilling the theoretical requirements of the model.

2.5 Materials and Methods

2.5.1 Construction and Purification of the RT1.A^a Heavy Chain Encoding Vectors.

2.5.1.1 Restriction Digests

Restriction digests were performed using enzymes from Amersham Pharmacia Biotech Ltd (Bucks, UK). A common buffer, 'One-Phor-All Buffer-plus', applies to every enzyme, and this simplifies the use of two enzymes simultaneously. Generally, 0.5 to 1μg of target DNA in a total volume of 20μl was digested with one unit of enzyme at 37°C for 2-3 hours. The digest products were separated by agarose gel electrophoresis.

2.5.1.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for purifying DNA and visualising the products of restriction digests. Gels were prepared and run according to the method of Aaij and Borst (388) with modifications by Sambrook (389) as follows: Agarose (0.8-1.5% (w/v)) was dissolved in TAE buffer (40mM Tris-base, 0.2% (v/v) glacial acetic acid, 1mM EDTA, pH 8.0) by heating to 100°C. The solution was cooled to 60°C with gentle stirring to ensure even heat distribution and the gel poured around the required comb. The gel was left to set for 30 minutes at room temperature and then submerged in TAE buffer. Samples were mixed in a 6:1 ratio with DNA gel loading buffer (40% (w/v) sucrose, 0.01% (w/v) bromophenol blue) and volume equivalents of no more than 0.5μg DNA added to each well of the gel. The gels were run horizontally using a Stratagene Minigel system (Cambridge, Cambridgeshire, UK) at 100V until the bromophenol blue tracking dye had advanced sufficiently down the gel. After staining with 1% ethidium bromide (Sigma-Aldrich Ltd , Dorset, UK), the gels were visualised on a 'Biorad Gel Doc 1000' UV transilluminator (Biorad, Hemel Hempstead, UK) and analysed using associated Biorad 'Molecular Analyst Software'. Molecular weight markers VII (0.37 to 8.0 kbp), or VIII (0.67 to 1.114 kbp) (Boehringer Mannheim, Boehringer, Germany) were used to distinguish fragment sizes.

2.5.1.3 Subcloning

The appropriate digest products were eluted from the agarose gel by using either 'Geneclean II' (BIO 101, La Jolla, California) or 'Wizard DNA Clean-Up' (Promega, Southampton, UK) purification kits. Although the specific protocols for these kits differ, they rely on the addition of a high osmolar buffer solution to dissolve the DNA from the agarose gel block, while simultaneously inducing the preferential binding of the DNA to silica particles. The liquid phase containing the impurities is then exchanged with wash buffer, before the DNA is finally eluted from the silica with either TE buffer or water. The final products were re-run on an agarose gel, enabling quantitation of the recovered DNA.

Cloning of DNA inserts was generally designed so that the parent vector was digested with two restriction enzymes simultaneously, resulting in non-cohesive termini that could only be re-ligated by the addition of an insert with reciprocal digests at either end. This increases the efficiency of the ligation reaction; as much as 80% of the linearised vector will be converted to monomeric plasmid/insert DNA chimeras (389). Ligation involves the formation of new bonds between the phosphate residues located at the 5` termini of double-stranded DNA and adjacent 3`-hydroxyl moieties and is catalysed by the addition of T4 DNA ligase (Gibco BRL Life technologies, Paisley, UK). Calculation of the vector to insert DNA ratio was performed according to the equation:

$$\frac{\text{Vector (ng) x size of insert (kbp)}}{\text{Size of vector (kbp)}} \times \frac{\text{Moles of insert}}{\text{Moles of vector}} = \text{Insert (ng)}$$

Generally, 100ng of vector was ligated using 1 Weiss unit of T4 DNA ligase in ligase buffer (66mM Tris-HCl, 5mM magnesium chloride, 1mM dithioreitol, 10mM ATP, pH 7.5) in a volume of 10µl, at a temperature of 16°C overnight. A control ligation reaction without the DNA insert was invariably performed.

2.5.1.4 Bacterial Transformation

Successful re-ligation of the plasmid vectors and subsequent transformation into *E. coli* bacteria confers ampicillin-resistance, due to the inclusion of the gene-sequence for β-lactamase within the plasmid vector. Before *E. coli* bacteria could be transformed with plasmid DNA they were made competent with $CaCl_2$ treatment, as first described by Cohen (390). Briefly, under aseptic conditions, a freshly grown colony was transferred from an agar plate into 100ml of Luria broth (Gibco BRL, Life Technologies) and incubated for 3 hours at 37°C, ensuring that the number of viable cells were not greater than 10^8/ml. The bacteria were then cooled and resuspended in 0.1M $CaCl_2$, and a volume equivalent to no more than 50ng of the re-ligated DNA added. Following incubation on ice for 30 minutes the tubes were rapidly heat-shocked at 42°C. 800μl of broth was added and the mixture plated on ampicillin-containing agar plates and left overnight at 37°C. *E. coli* cells that had been successfully transformed grew as single colonies on the plate. Controls included competent bacteria that received either 50ng of standard preparation of plasmid DNA, or no plasmid. The *E. coli* strain, DH5α, was chosen for transformation as this grows rapidly, contains minimal amounts of carbohydrate, and does not exhibit significant levels of endonuclease activity (391).

2.5.1.5 Plasmid Purification

Miniprep

A freshly grown colony of transformed DH5α *E. coli* bacteria was transferred to 10mls of Luria Broth (Gibco BRL), containing ampicillin at 100μg/ml (Penbritin, SmithKline Beecham, UK) and expanded overnight in a shaking incubator at 37°C. After centrifugation at 4000rpm for 4mins, plasmid was extracted from the bacterial pellet by either performing a boiling miniprep (392) or by using a commercially available Wizard Plus Minipreps DNA Purification System (Promega). The purified plasmid DNA was resuspended in TE buffer.

Maxiprep

For *in vivo* animal use, larger quantities of plasmid were purified using a Qiagen Plasmid MegaKit, incorporating Endotoxin Removal Buffer (Qiagen, Crawley, UK). This purification procedure is based on the optimised alkaline lysis method of Birnboim and Doly (393), and involves the selective capture of plasmid DNA in anion-exchange columns in 1M salt solution, pH 7. Plasmid DNA is recovered from the column by eluting with 1.25M NaCl/Tris buffer at pH 8.0. The presence of endotoxin in standard DNA preparations may influence *in vivo* responses to plasmid DNA (394) and typical endotoxin levels in DNA purified using the endotoxin removal buffer are <50 endotoxin units per mg of DNA (395).

The pcmu-IV vector has a low copy number, which complicates its purification from *E. coli* cells, since the larger volumes of bacterial culture that are required per unit of plasmid purified increase the concentration of bacterial contaminants. Plasmid yield was improved by adding chloramphenicol (180mg/ml)(Pharmacia and Upjohn, Surrey, UK) to the bacterial culture at mid-log phase. The bacteriostatic effect of chloramphenicol increases the copy number of plasmid per bacterial cell. To optimise its use, chloramphenicol was added at various times to 10ml miniprep cultures of the pcmu-A^a vector, and plasmid purified using the Wizard Plus minipreps, as described above. Purified DNA was quantified against standards by agarose gel electrophoresis, using Biorad software (section 2.5.1.2). The highest yield of plasmid was achieved when the chloramphenicol was added 3 to 4 hours after culturing was commenced, when the optical density at wavelength 600nm was approximately 0.35 (Figures 2.15A and 2.15B).

2.5.2. In Vitro Expression of the Products of RT1.A^a-Encoding Plasmid

2.5.2.1 Cell Lines

A rat thigh muscle cell line, L6 (396) and a human embryonal kidney line, 293 (397) (both from ECACC, Salisbury, UK) were used. L6 cells were grown as a monolayer in Dulbecco's modified eagle medium (DMEM) (Gibco BRL), supplemented with 2mM glutamine (Gibco BRL) and 10% foetal calf serum (FCS) (Sigma Biosciences, Dorset, UK). They were subcultured when semi-confluent to a seeding density of 1.5 – 2.0 x 10^3 cells/cm^2 using trypsin/EDTA (Gibco BRL); 5% CO_2; 37°C. 293 cells were grown as a monolayer in Minimal

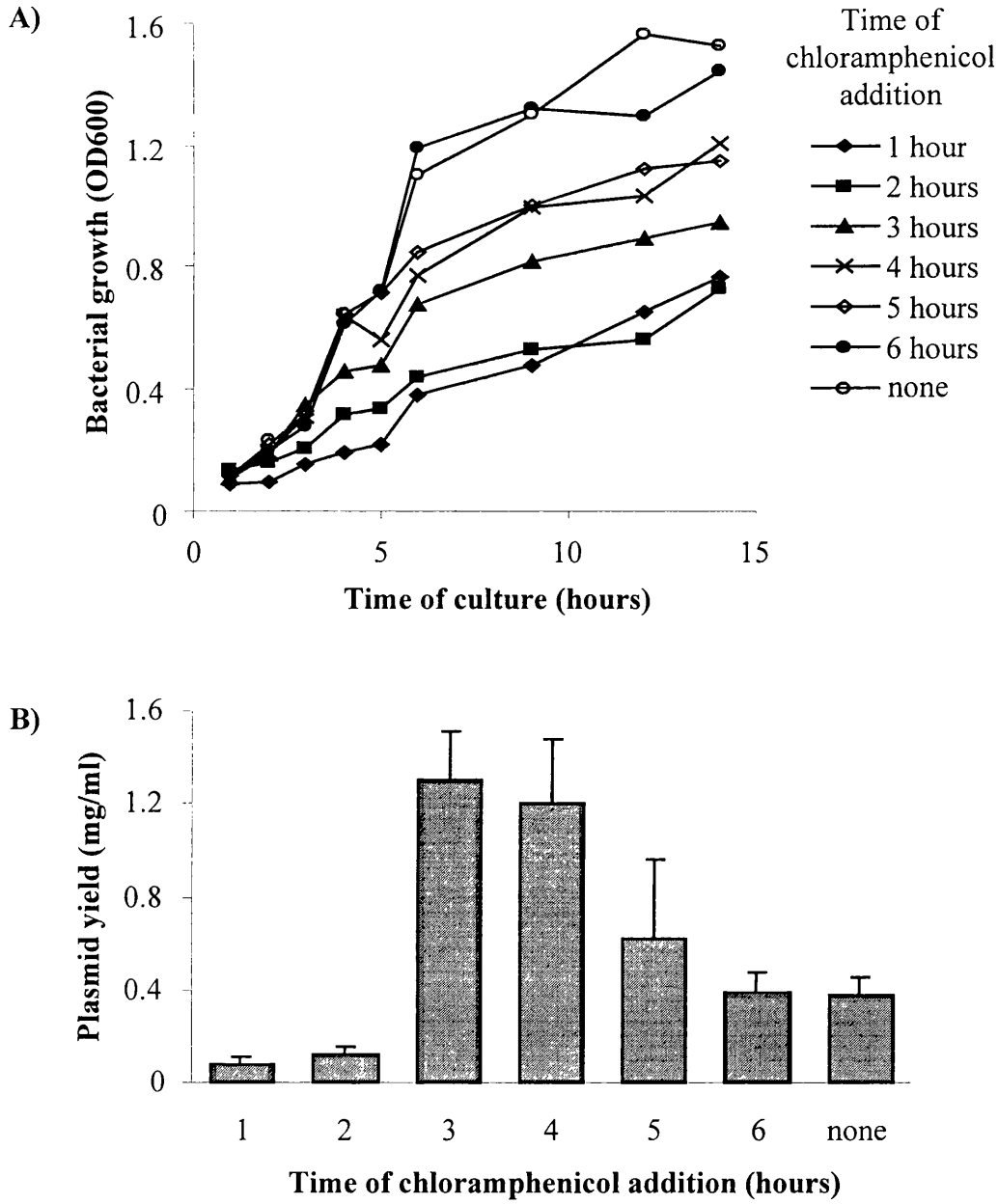

Figure 2.15 Optimisation of conditions for bacterial culture.

100µl of freshly grown pcmu-A[a] transformed *E. coli* bacteria were transferred into a series of 10ml aliquots of Luria Broth and expanded in a shaking incubator at 37°C, 200rpm. A single aliquot of 1.8mg of chloramphenicol was added at different time points (from 0 to 6 hours) after culture initiation and bacterial growth quantified by serial spectrophotometric analysis at 600nm.

A) Bacterial growth as measured at 600nm.

B) After 16 hours of culture, plasmid was purified using Promega Wizard Minipreps, linearised by digestion with Xba I, and quantified against standards on gel electrophoresis, using Biorad software. The graph represents the mean and standard deviation of each experiment performed in triplicate.

Essential Medium (MEM) with Earle's salts (Gibco BRL), supplemented with 2mM glutamine and 10% FCS. Cells were subcultured when 90% confluent and seeded at 3 – 5 x 10^3 cells/cm^2 using 0.25% trypsin.

2.5.2.2 Cell Transfection

Transfection of the rat myoblast cell line, L6 and the human embryonal kidney cell line, 293 was performed using DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate) (Boehringer Mannheim). DOTAP is a cationic liposome-mediated transfection agent that forms stable complexes with negatively charged DNA. These complexes are able to adhere to the cell surface, fuse with the cell membrane and release DNA into the cytoplasm. The DNA:DOTAP ratio that achieved optimum transfection was established by initially transfecting the L6 cell line with the plasmid, pcDNA-CAT. (Invitrogen, UK). The product of this plasmid expresses chloramphenicol acetyl transferase (CAT), which was quantified using a specific ELISA (Boehringer Mannheim). Optimum transfection of L6 cells in a 25cm^2 flask was achieved with a mixture of 5μg of DNA and 25μl of DOTAP (Figure 2.16). Similar data was obtained for transfection of 293 cells (data not shown). Cells were re-plated 24 hours prior to transfection in order to achieve a cell density of 70% at the time of transfection. For stable transfection the number of cells re-plated was reduced to achieve a cell density of 50% at the time of transfection. The DNA/DOTAP mixture was added to the cell culture medium and was replaced with fresh medium after 4 hours in culture. Transient expression of the product was assayed after a further 72 hours in culture. For stable transfection, geneticin (Sigma-Aldrich) was added, which in pilot experiments inhibited cell division at a concentration of 30mg/ml (data not shown).

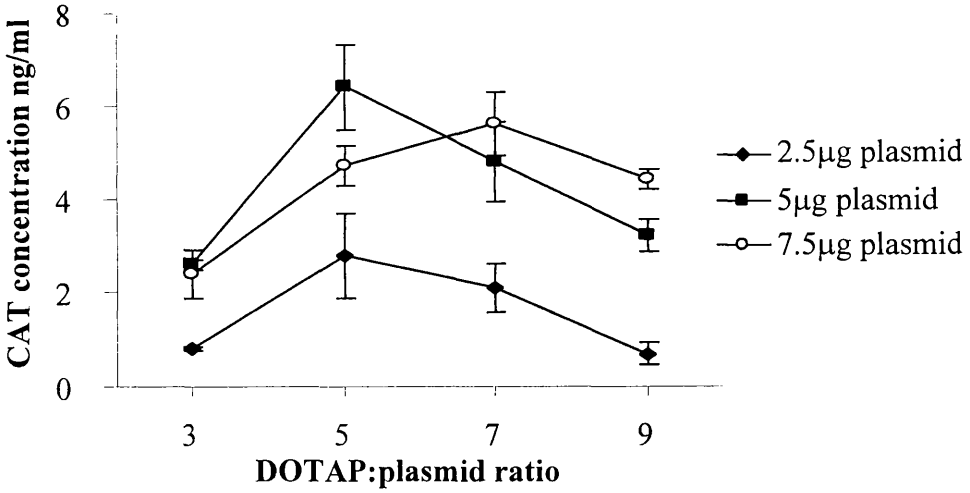

Figure 2.16 Optimising DOTAP transfection conditions

Optimum DNA and DOTAP concentrations for transfection of the L6 line were established by transfecting 1 x 10^6 cells with the pcDNA-CAT plasmid (Invitrogen), which expresses the reporter protein, chloramphenicol acetyl transferase (CAT). The expression of CAT was quantified 48 hours after transfection using a CAT-specific ELISA (Boehringer, Germany). The value on the x-axis represents the concentration of DOTAP used. It is expressed relative to the plasmid concentration; the amount of DOTAP added in µg is the factor of the value on the x-axis and the plasmid concentration. Points represent the mean and standard deviation of three experiments.

2.5.2.3 Monoclonal Antibodies

The mouse monoclonal antibodies (mAb), MN4-91-6, specific for a polymorphic determinant of the rat RT1.A^a MHC class I molecule (398), and OX18, which recognises a monomorphic determinant of the RT1.A MHC class I molecule (399) were used to detect expression of the product of the class I encoding plasmid vectors. These antibodies were produced by intraperitoneal injection of the appropriate hybridoma cell line into pristane-primed Balb/c mice (Harlan, UK) to produce ascites, from which IgG was purified by protein A column chromatography (ProSep, Fisher Scientific, Loughborough, UK). The concentration was determined by UV spectrophotometry. Biotinylation of the OX18 was performed by the N-hydroxysuccinimide method using a commercially available kit (Boehringer Mannheim) followed by overnight dialysis against PBS at 4°C. The mouse IgG2a mAb, ESH8, which is directed against human factor VIII (Scottish Antibody Production Unit, Law, UK), was used as isotype control (400).

2.5.2.4 Flow Cytometric Analysis of RT1.A^a Cell Surface Expression

Cell surface expression of the RT1.A^a antigen was quantified by flow cytometric analysis. 5 x 10^5 transfected cells were washed in washing medium (2.5.3.2), resuspended in 100µl of PBS/0.2% BSA/0.1% sodium azide (PBA) and incubated for 30 mins at 4°C with FITC-conjugated MN4-91-6 mAb. The cells were then washed twice, resuspended in PBS and flow cytometry performed using a Coulter Epics XL flow cytometer (Coulter Ltd., Luton, UK) with associated software. To compare the levels of RT1.A^a expression induced by the different vectors, the transfected cell population was gated and the mean channel fluorescence of this sub-population noted. A one-way analysis of variation was performed to confirm that the levels of RT1.A^a expression induced by the various class I constructs represented different statistical populations. Modified T tests according to the Bonferroni method were then performed to interpret the significance of the variation for each specific construct.

2.5.2.5 Development of the ELISA against Soluble RT1.A^a

An RT1.A^a specific ELISA was developed according to the protocol described by Pockley AG et al. (401), with minor modifications. A control standard was prepared from the detergent lysates of rat ACI spleen (RT1.A) and was arbitrarily assigned a concentration of a 1000 units/ml. For the assay a 96 well, Immulon IV microtitre plate (Dynatech Laboratories, Billinghurst, UK) was bound overnight at 4°C with 100µl of MN4-91-6 (5µg/ml) in carbonate/bicarbonate buffer, pH 9.6. The plate was washed with 0.05% Tween in PBS (washing buffer), and non-specific binding blocked by incubation with 10% BSA for one hour at 37°C. A series of tripling dilutions of the RT1.A^a standard in cell culture medium were prepared. 100µl of the standards or test samples (neat cell culture supernatant from the RT1.A^a transfected L6/293 cell lines) were added to the wells of the plates incubated for one hour at 37°C. Following washing, bound soluble class I was detected by the addition to each well of 100µl biotinylated OX18 (5µg/ml) in PBS/0.2%BSA. After incubation for one hour at 37°C plates were washed and a 100µl of a 1:1000 dilution of extravidin/peroxidase conjugate (Sigma) in PBS/0.2%BSA added to each well and incubated for a further hour at 37°C. Plates

were re-washed and 100µl of TMB (KPL Ltd., Guildford UK) substrate added. The reaction was stopped by the addition of 100µl of 0.2M H_2O_4 and the absorbance determined at 450nm using a Dynatech MR500 plate reader and associated 'Biorad' software (Dynatech Lab, Billinghurst, UK). A standard curve for the dilutions of the standards was constructed, from which the concentrations of the test samples were calculated. The presence of statistically distinct populations was assessed by performing a one-way analysis of variation. Intergroup variation was calculated using modified T tests (Bonferroni method).

Initial results were limited by high backgrounds. A variety of blocking agents were tested, 10% FCS, 10% BSA, 5% 'marvel' (Premier Bands Ltd, UK) or 'Megga-block III' (Bionostics), and the latter was consistently associated with lowest background values (data not shown). However, acceptable background values (OD450 <0.1) were only achieved when commercially-obtained biotinylated OX18 (Cedarlane Laboratories Ltd, Ontario, Canada) was used, suggesting that there had been non-specific biotin-binding associated with the biotinylated OX-18 batch that had been prepared in the laboratory.

2.5.3. *In Vivo Expression of the Products of RT1.A^a Encoding Plasmids*

2.5.3.1 Animals.

PVG.RT1^u (A^{u}B/D^{u}C^u) congenic rats and PVG.R8 (A^{a}B/D^{u}C^u) recombinant rats, whose derivation is cross-referenced elsewhere (402), were obtained from Harlan Ltd., (Bicester, Oxon, UK). DA (RT1^{av1}), PVG (RT1^c), and Lewis (RT1^l) rats were obtained from the same source. All animals were maintained under standard animal house conditions at the University of Glasgow Central Research Facility and were used when 8-12 weeks old.

2.5.3.2 Tissue Culture Medium

Washing Medium

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

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.

2.5.3.3 Cell preparations

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.

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 with trypan blue exclusion.

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 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°C in 5% CO_2.

2.5.3.4 Plasmid Administration

Gene transfer of the DNA sequences encoding the RT1.A^a heavy chain was achieved by directly injecting plasmid DNA into the skeletal muscle of adult rats (374). To induce regeneration of plasmid DNA into skeletal muscle and thereby increase the efficiency of gene transfer (403, 404), 400μl of 0.5% Bupivicaine (1-butyl-N[2, 6-dimethyl phenyl] 2-piperidine-carboxamide) (Astra, King's Langley, UK) was first injected into each tibialis anterior muscle using a 28-gauge needle. Three and eight days later, 200μg of pcmu-IV encoding either the full-length or truncated nucleotide sequence of the RT1.A^a heavy chain was injected into each tibialis anterior muscle. As control, empty pcmu-IV plasmid was administered. The amount of plasmid injected was decided empirically, based upon the equivalent concentrations that achieved T cell activation in mice (362).

2.5.3.5 *Detection of Cytotoxic Alloantibody*

The lymphocytotoxic RT1.A^a alloantibody response was determined in serum samples from modified PVG.RT1^u rats by assessing the ability to lyse target ^{51}Cr-labelled donor strain PVG.R8 ConA-transformed splenoblasts (section 2.5.3.3) in the presence of guinea pig complement. Briefly, PVG.R8 splenocytes were adjusted to a concentration of 2 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°C in 5% CO_2. The target cells were then suspended in 1ml of serum-free culture medium and incubated with 5.0MBq ^{51}Na-Chromate (CJS11, Amersham International plc.) for

90 minutes in a water-bath at 37°C with regular agitation. Cells were washed once with washing medium (section 2.5.3.2), 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 triply 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 37^0C 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).

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

$$\frac{\text{Experimental Release} - \text{Spontaneous Release}}{\text{Maximum Release} - \text{Spontaneous Release.}} \quad \text{X } 100$$

The serial antibody response was assessed by calculating, for a given time-point, the mean and SD of the lowest antibody titres that resulted in a cytotoxicity of >=20%. This was an arbitrary cut-off value, chosen on the grounds that background levels were invariably less than 10%. Statistical analysis on these time points was performed by Mann-Whitney U tests (2-tailed).

2.5.3.6 *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 (2.5.3.3) at a concentration of 1 x 10^6 cells/ml in PBS/0.2% BSA/0.1% sodium azide (Sigma).

Serum samples were triply 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°C in the dark. Plates were washed twice as before, and cells re-suspended in 100µl of PBA.

IgM and IgG2b alloantibodies were detected using a Coulter Epics XL flow cytometer (Coulter Ltd, Luton, UK) with associated software. The serial antibody response was assessed by calculating, for a given time-point, the mean and SD of the lowest antibody titres that resulted in a mean channel fluorescence greater than twice background. Statistical analysis on these time points was performed by Mann-Whitney U tests (2-tailed).

2.5.3.7 *Preparation of Cryostat Sections*

Muscle samples from rats that had been injected with the RT1.A^a encoding pcmu vectors were harvested upon sacrifice of the animals. Preservation of muscle tissue was achieved using an isopentane freezing bath. The muscle was dissected and placed transversely on a cork card and embedded in OCT compound (Tissue-Tek, BDH Ltd). This was then frozen by placing in isopentane that had been cooled to freezing point in liquid nitrogen. Samples were stored at –70°C. 5µm

crytostat sections were cut at –20°C onto gelatinised multispot slides (C.A. Hendley, Essex, UK)

2.5.3.8 *Immunoperoxidase staining*

Sections were fixed in acetone at room temperature for 10 minutes and then washed in PBS. Excess moisture was dried from around each section, then the slides were incubated for 5 minutes with 0.6% hydrogen peroxide in methanol to quench endogenous peroxidase activity. The sections were washed in PBS and then treated with avidin solution (0.01%) (Sigma) for 5 minutes, followed by a biotin solution (0.001%) (Sigma) for a further 5 minutes. This blocks endogenous avidin-binding activity (405). Slides were rewashed, excess moisture removed, then 50μl (10μg/ml) of primary biotinylated monoclonal antibody (either MN4-9-16 or OX18) applied to each section. Slides were incubated for 45 minutes at room temperature in a humidified chamber. Slides were thoroughly washed then incubated for 30 minutes with StreptABCComplex (Dako, Glostrup, Denmark), a solution containing biotinylated horseradish peroxidase and streptavidin that binds peroxidase to the primary antibody at a higher ratio than conventional, peroxidase-conjugated secondary antibodies, thereby increasing the detection sensitivity. Slides were washed and incubated for 5 minutes with 0.6 mg/ml 3,3'-diaminobenzidine tetra-hydrochloride (peroxidase substrate) (Sigma) plus 0.01% hydrogen peroxide. Slides were re-washed, counterstained in Harris' haematoxylin for one second, dehydrated and cleared in aclohols and xylene, then mounted in DPX (BDH Ltd., Poole. UK).

CHAPTER THREE

THE INFLUENCE OF A^a-PRIMING ON THE ALLOANTIBODY RESPONSE TO, AND THE REJECTION OF, CLASS I MHC DISPARATE HEART GRAFTS.

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CHAPTER THREE

THE INFLUENCE OF A^a-PRIMING ON THE ALLOANTIBODY RESPONSE TO, AND THE REJECTION OF, CLASS I MHC DISPARATE HEART GRAFTS

3.1 Introduction

Several mechanisms have been proposed to explain the rejection of MHC class I disparate grafts by CD4 T cells (406). These differ fundamentally as to whether CD4 T cell activation is restricted to the recognition of processed allo-class I MHC peptide as presented by recipient APC and consequently, whether the indirect pathway of allorecognition is solely responsible for effecting acute graft rejection. For example, recipient congenic PVG.RT1^u (A^uB/D^uC^u) rats differ from donor recombinant PVG.R8 (A^aB/D^uC^u) rats at the classical class I RT1.A locus only, but rejection is CD4 T cell dependent (289, 290). Although Bradley has proposed that CD4 T cells are indirectly restricted and effect rejection through the provision of B cell help for the development of an alloantibody response, the possibility that CD4 T cells instead recognise antigen directly on the surface of donor cells has not been excluded (297). As discussed in section 1.6.1, recent evidence from Shirwan supports the hypothesis that T cells directly restricted for antigenic determinants on the surface of PVG.R8 cells are pivotal in effecting rejection. He noted that immunising PVG.1U (PVG.RT1^u) rats with peptides corresponding to the hypervariable region of the donor RT1.A^a antigen, which in theory achieved maximal stimulation of the indirect pathway, achieved only a modest acceleration in the rejection of subsequent PVG.R8 heart grafts, whereas immunising with intact donor cells resulted in rejection within 48 hours (245).

Clarification of the mechanisms responsible for accelerated rejection in the PVG.R8 to PVG.RT1^u strain combination are of obvious importance in the understanding of the T cell allorecognition pathways involved in the rejection of MHC class I disparate grafts. It is equally relevant to the nature of T-B cell collaboration during alloantibody production to MHC class I, since the allorecognition pathway of CD4 T helper cells (indirect or direct) dictates whether

T cell help for B cells is provided by cognate (antigen-specific) or non-cognate (antigen-non-specific) cell-cell collaboration (292, 296). The experiments in this chapter report the effects that priming PVG.RT1^{u} rats with donor RT1.A^{a} antigen have on the subsequent rejection of PVG.R8 hearts. Different forms of the RT1.A^{a} antigen were administered; a series of 15-mer allopeptides corresponding to the α1 and α2 domains of the RT1.A^{a} antigen, PVG.R8 skin grafts, or pcmu-IV plasmids encoding the RT1.A^{a} heavy chain (either the full-length or the truncated, water-soluble form). These antigens were chosen on their respective ability to prime the direct or the indirect T cell response and on their display of the appropriate conformational B cell epitopes to stimulate an alloantibody response directed against intact RT1.A^{a} antigens on donor graft cells. Immunising with the different forms of the RT1.A^{a} antigen resulted in marked differences in the kinetics of rejection of subsequent PVG.R8 heart grafts, but as will be discussed, these differences relate primarily to the presence of cross-reactive B cell epitopes, rather than to any intrinsic difference in the mode of helper CD4 T cell activation.

3.2 Results

3.2.1 The Alloantibody Response to Priming with RT1.A^a antigen

PVG.RT1^u rats were immunised with a full-thickness PVG.R8 skin graft, or 900μg of pooled A^a peptide corresponding to the α1 and α2 domains of the RT1.A^a antigen (see section 3.5.3), or 400μg of pcmu-IV plasmid (see section 3.5.4) encoding either the full-length (pcmu-A^a) or truncated (pcmu-tA^a) sequence for the RT1.A^a heavy chain. Serum was sampled 12 days later and the presence of anti-A^a allocytotoxic alloantibody detected by assaying against chromium-labelled PVG.R8 lymphoblasts in the presence of guinea-pig complement (Figures 3.1 and 3.2). PVG.RT1^u recipients sensitised by the application of a full thickness PVG.R8 skin graft developed high levels of circulating cytotoxic anti-A^a alloantibody (Figure 3.1). In contrast, immunising PVG.RT1^u rats with synthetic allopeptides corresponding to the hypervariable regions of the A^a MHC class I molecule did not stimulate the development of cytotoxic antibody recognising intact RT1.A^a on donor cells (Figure 3.1). Concurrent experiments in our laboratory have demonstrated that an antibody response can be generated to several of the individual allopeptides, confirming their immunogenicity (Lovegrove E, submitted) but, as Benham has also demonstrated (291), anti-peptide antibody does not cross-react with the intact RT1.A^a antigen. The product of the pcmu-tA^a vector also stimulated a strong allocytotoxic alloantibody response, and this was of the same magnitude as the antibody response that developed following PVG.R8 skin grafting (Figure 3.2). The magnitude of the antibody response to the product of the pcmu-A^a vector was, as noted in section 2.3.2.2, lower than to the product of the pcmu-tA^a vector (Figure 3.2).

3.2.2 The Kinetics of Heart Graft Rejection following Priming with RT1.A^a Antigen

PVG.RT1^u rats were primed with an R8 skin graft, or with RT1.A^a peptide, or with the RT1.A^a-encoding pcmu-IV plasmids, twelve days before receiving a PVG.R8 heterotropic cardiac allograft. PVG.RT1^u rats respond strongly to RT1.A^a class I MHC alloantigen for, as shown in Table 3.1 (group 1), naïve animals rejected A^a-bearing PVG.R8 heart grafts rapidly (MST 7 days). Prior

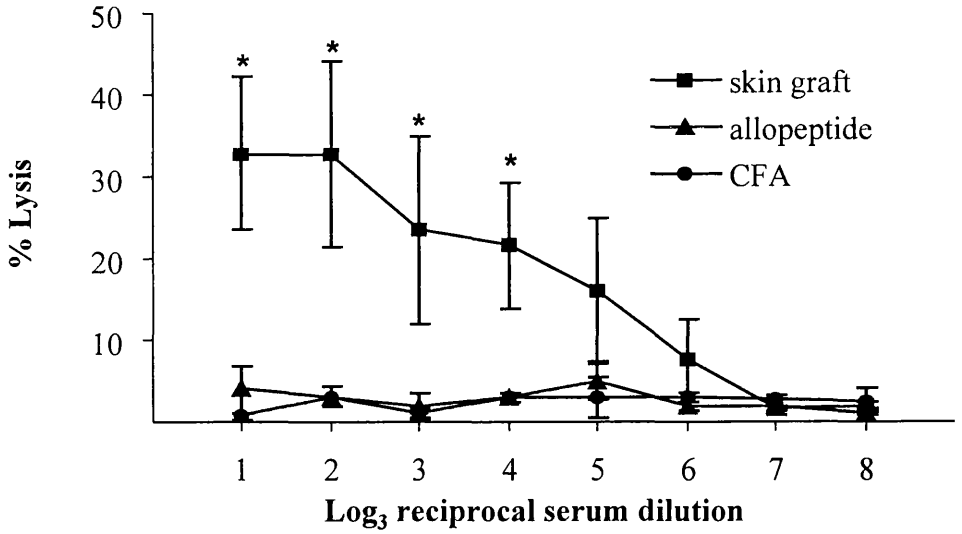


Figure 3.1 Cytotoxic alloantibody response upon RT1.A^a immunisation.

The circulating lymphocytotoxic RT1.A^a antibody response of PVG.RT1^u rats was assessed 12 days after application of a full thickness PVG.R8 skin graft or immunisation with A^a allopeptide. Allopeptide-immunised animals received 900µg of pooled A^a peptides emulsified in CFA and given subcutaneously into each hind footpad. Control animals received adjuvant alone. Sera were assayed against ^{51}Cr-labelled PVG.R8 ConA blasts in the presence of guinea-pig complement (section 2.5.3.5). Values shown are the mean and SD of four animals per group. * Skin graft vs. allopeptide, $p<0.02$, Mann-Whitney U test (2-tailed).

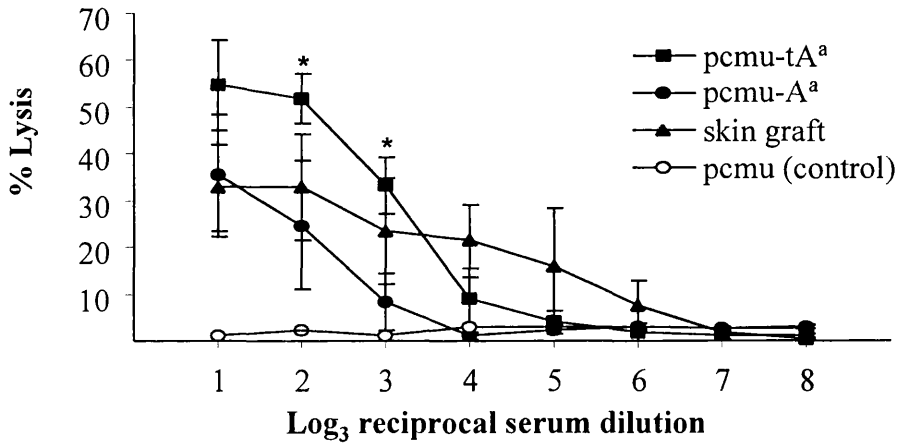


Figure 3.2 Cytotoxic alloantibody response to A^a encoding pcmu-IV vectors.

The circulating lymphocytotoxic RT1.A^a antibody response of PVG.RT1^u rats was assessed 12 days after immunisation with pcmu-IV plasmid vectors encoding either the full-length RT1.A^a antigen (pcmu-A^a) or the truncated, water soluble version (pcmu-tA^a). Control animals were immunised with empty pcmu-IV plasmid. Animals received 400µl of 0.5% Bupivicaine intramuscularly followed 3 and 8 days later, by injection of 200µg of plasmid. Values shown are the mean and SD of four animals per group. Shown also for comparison is the cytotoxic antibody response in animals sensitised 12 days earlier with an R8 skin graft (data from Figure 3.1). * Pcmu-tAa vs. pcmu-A^a $p<0.02$, Mann-Whitney U test (2-tailed).

exposure to the A^a antigen is effective at inducing accelerated rejection as PVG.RT1^u recipients that were sensitised by the application of a full-thickness PVG.R8 skin graft, 12 days before heart transplantation, consistently rejected PVG.R8 heart grafts within 1 day of transplantation (Table 3.1, group 2).

Immunisation of PVG.RT1^u rats with the synthetic allopeptides was less effective at promoting accelerated rejection of A^a-bearing heart grafts (Table 3.1, group 4). Compared to control animals immunised with CFA only or with irrelevant peptide emulsified in CFA, rejection times were faster (MST 4 vs. 6.5 days, $p<0.02$), but heart graft rejection in allopeptide-primed animals was still markedly slower than that observed in animals primed by a PVG.R8 skin graft ($p<0.02$). These findings are consistent with a recent study by MacDonald, who instead studied the effects of immunising with individual allopeptides (246). She noted that priming PVG.RT1^u rats subcutaneously with an emulsion of CFA and a single allopeptide corresponding to the hypervariable regions of either the α1 (amino acids 57-80) or the α2 domains (amino acids 143-163) of the RT1.A^a molecule achieved a similar modest acceleration in the rejection of PVG.R8 hearts (MST 5 vs. 6 days).

Table 3.1. Rejection of RT1.A^a class I disparate PVG.R8 heart grafts by PVG.RT1^u recipients.

Group	Immunisation protocol	n[a]	Heart graft Survival (days[b])	MST (days[c])
1[d]	None	4	6,7,7,7	7
2[d]	PVG.R8 skin graft[e]	5	1,1,1,1,2	1
3[d]	CFA[f]	4	6,6,7,8	6.5
4[d]	CFA/peptide[f]	5	4,4,4,5,5	4
5	CFA/irrelevant peptide[f]	3	6,7,7	7

[a] Number of animals in group.
[b] Grafts were assessed by daily palpation, and rejection was defined as complete cessation of myocardial contraction.
[c] Median survival time.
[d] Groups 1 vs. 2, groups 2 vs. 4, and groups 3 vs. 4, $p<0.02$, Mann-Whitney U test.
[e] Animals were immunised by the application of a full-thickness PVG.R8 skin graft 12 days before heart transplantation. Skin grafts were rejected on days 6 or 7.
[f] Animals were immunised with 900μg of pooled A^a allopeptides (50mg of each 15-mer), emulsified in CFA, and given s.c. into each hind footpad 12 days before heart transplantation. Control animals received either adjuvant alone (group 3) or 100μg of irrelevant peptide emulsified in CFA (group 5).

To assess whether the difference in the rejection kinetics between skin-grafted and peptide-immunised animals was due to more effective priming of the humoral immune response, rather than to differences in the T cell activation pathways, the rejection kinetics of R8 heart grafts were assessed in PVG.RT1^u rats, which had been immunised 12 days earlier with the plasmid vectors encoding the sequence for either the full-length or truncated RT1.A^a antigen. All animals rejected PVG.R8 heart grafts rapidly, with similar kinetics to those animals that had received a skin graft (Table 3.2, groups 1 and 2 and cf. Table 3.1 group 2). Although animals immunised with the truncated RT1.A^a antigen rejected their grafts slightly more quickly than those immunised with the full-length antigen (group 2 vs group 1), this difference was not significant (MST 1.5 vs. 2 days, p = 0.49). Control recipients immunised with the 'empty' pcmu-IV plasmid rejected PVG.R8 heart grafts at the same rate as naïve PVG.RT1^u recipients (cf. Table 3.1 group 1 and Table 3.2 group 3). Immunising with the plasmid encoding the truncated RT1.A^a antigen did not effect the rejection of third party PVG.RT1^c heart grafts (Table 3.2, group 4).

Table 3.2. Rejection of RT1.A^a class I disparate PVG.R8 heart grafts by PVG.RT1^u recipients after immunisation with DNA encoding RT1.A^a antigen.

Group	Immunisation protocol	n[a]	Heart graft Survival (days)[b]	MST (days)[c]
1[d]	pcmu-A^a [(e)]	5	1,2,2,2,3	2
2[d]	pcmu-tA^a [(e)]	7	1,1,1,2,2,2,2	1.5
3[d]	pcmu (control)[e]	4	6,7,7,7,	7
4	pcmu-tA^a [(e)]	3	5,6,7 (PVG.RT1^c) [(e)]	6
5	Immune serum[f]	3	5,6,6	6

[a] Number of animals in group.
[b] Grafts were assessed by daily palpation, and rejection was defined as complete cessation of myocardial contraction.
[c] Median survival time.
[d] Groups 1 vs. 3, p <0.02. Groups 2 vs. 3, p<0.01. Groups 1 vs. 2, p=0.49
[e] PVG.RT1^u rats were immunised with 400μg pcmu-IV plasmid encoding full-length, membrane bound A^a class I MHC (pcmu-A^a), truncated A^a class I MHC (pcmu-tA^a), or empty plasmid control (pcmu). 12 days later, rats received a heterotopic PVG.R8 (or third party PVG.RT1^c) cardiac allograft.
[f] 1.5mls of immune serum (harvested from PVG.RT1^u animals 12 days after injection with 400μg of pcmu-tA^a plasmid) was administered to unmodified PVG.RT1^u recipients on days 0, 1, and 2 relative to a PVG.R8 cardiac allograft.

3.2.3 *The Anti-A^a Antibody Response to an R8 cardiac Allograft following Priming with RT1.A^a Antigen*

Although immunisation with A^a allopeptide failed to stimulate an alloantibody response to the intact RT1.A^a molecule (Figure 3.1), class I allo-peptide priming has been previously noted to accelerate the subsequent alloantibody response to the intact class I molecule, as displayed by a vascularised allograft (244, 247, 291). To assess whether the more rapid rejection in peptide primed animals was the consequence of the development of a more rapid anti-A^a antibody response, sera from PVG.$RT1^u$ rats primed with the RT1.A^a antigen were sampled for the presence of cytotoxic alloantibody four days after R8 heart grafting. Initial experiments demonstrated that sera from rats that had developed an anti-A^a antibody response following priming with either an R8 skin graft or the plasmid encoding the truncated RT1.A^a heavy chain (pcmu-tA^a) unsurprisingly contained higher levels of alloantibody four days after, rather than at the time of, secondary challenge with an R8 cardiac allograft (Figure 3.3).

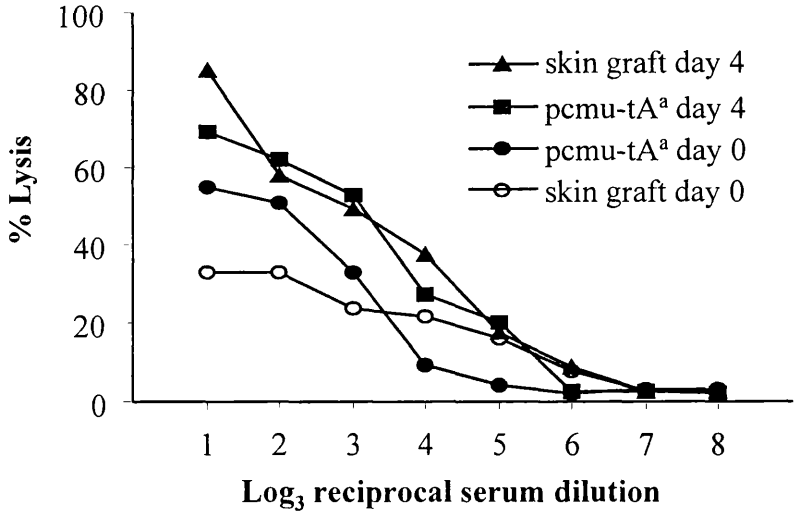


Figure 3.3 Progression of the anti-A^a antibody response in RT1.A^a-primed PVG.$RT1^u$ rats following PVG.R8 heart transplantation.

Animals received either a full thickness PVG.R8 skin graft or were injected intramuscularly with 400μg of plasmid encoding the truncated RT1.A^a antigen (pcmu-tA^a). Heart transplantation was performed 12 days later. Sera were sampled for cytotoxic anti-A^a antibody at the time of transplantation (day 0) and four days later (day 4). Samples at day 0 correspond to the data depicted in Figure 3.2. Results shown are the mean of four animals per group.

More importantly, the anti-A^a alloantibody response developed more rapidly in those animals that had been primed with the allopeptide mixture than in those animals primed with adjuvant only (Figure 3.4). Peptide immunisation therefore appears to accelerate rejection by priming T cells to provide cognate help for the generation of a humoral immune response directed against intact RT1.A^a antigen on the surface of donor graft cells.

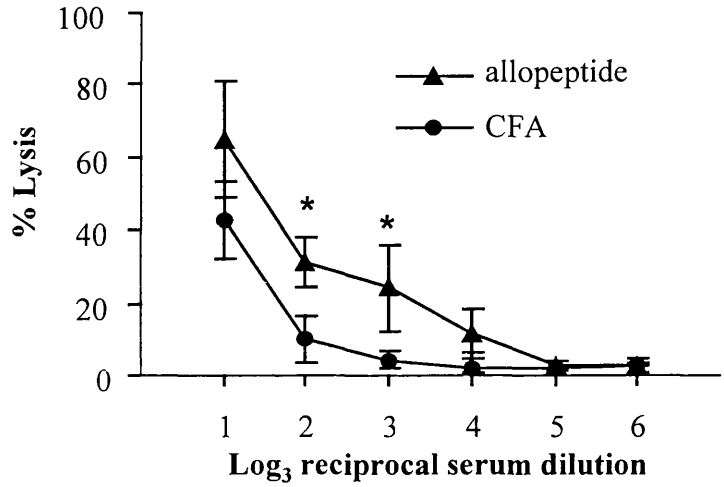


Figure 3.4 Cytotoxic anti-A^a antibody response of PVG.RT1^u rats to an R8 cardiac allograft following allopeptide immunisation

Recipients were pre-immunised with either 900μg of pooled A^a allopeptides emulsified in CFA, or CFA alone, 12 days before heart transplantation. Serum was sampled four days after cardiac transplantation and analysed for the presence of cytotoxic alloantibody. Results shown are the mean and SD of four animals per group.
* $p < 0.05$, Mann-Whitney U test (2-tailed).

3.2.4 The Cytotoxic CD8 T cell Response to RT1.A^a Antigen

As discussed in section 1.6.4, the plasmid construct for the truncated version of the RT1.A^a heavy chain (pcmu-tA^a) was designed to ensure that its' soluble class I protein product would not engender a direct CD8 T cell response. The assumption that the full-length RT1.A^a product of the pcmu-A^a vector would be expressed by professional recipient APC (378, 379, 407) implies, by comparison, that an anti-A^a cytotoxic CD8 T cell response may develop. To assess the cytotoxic CD8 T cell response, spleen cells were obtained from PVG.RT1^u rats 12 days after immunisation with either pcmu-A^a or pcmu-tA^a and assayed against ^{51}Cr-labelled PVG.R8 Con A blasts. Splenocytes harvested from RT1^u recipients 12 days after

PVG.R8 skin grafting, were used as a positive control. Although lymphoid cells from PVG.RT1^u recipients primed with PVG.R8 allografts do not generally display very high levels of *in vitro* cytotoxic T cell activity (290), spleen cells from animals grafted with PVG.R8 skin showed significant levels of CTL activity (Figure 3.5). CTL activity was also detected in animals immunised with the full-length A^a vector, pcmu-A^a, but crucially, the levels of cytotoxicity in animals immunised with the truncated A^a vector, pcmu-tA^a, were similar to control animals immunised with the empty vector (Figure 3.5).

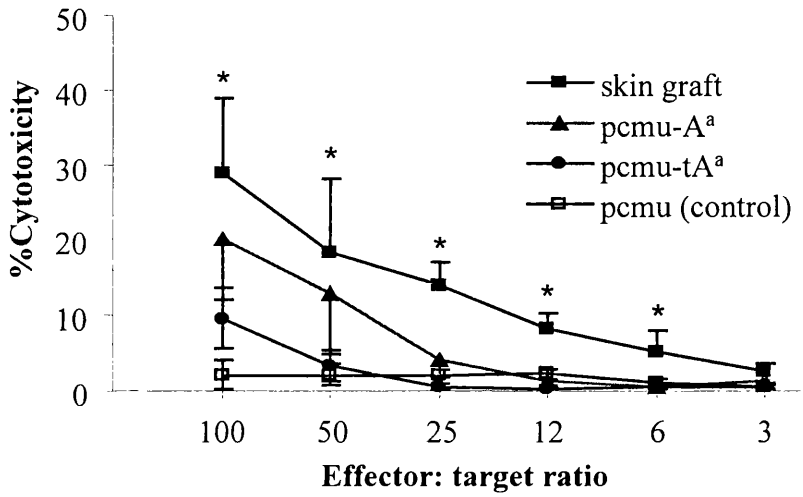


Figure 3.5 Analysis of the CTL response to RT1.A^a alloantigen

PVG.RT1^u rats were grafted with PVG.R8 skin or immunised with 400μg of the plasmid vectors encoding the full-length RT1.A^a heavy chain (pcmu-A^a), the truncated heavy chain (pcmu-tA^a), or empty control plasmid (pcmu). The animals were sacrificed after 12 days and spleen cells were assayed against ^{51}Cr-labelled R8 Con A blast target cells in a 6-hour cytotoxicity assay. Values shown are the mean and SD of three animals per group. This experiment was repeated on two occasions with similar results.
* Skin graft vs. pcmu-tA^a groups, $p<0.02$, Mann-Whitney U test, 2-tailed.

Additional experiments were performed in which LNC obtained from PVG.RT1^u rats immunised as above were further stimulated *in vitro* for 5 days with irradiated fully allogeneic (RT1^a) spleen cells (Figure 3.6). The secondary *in vitro* challenge with RT1.A^a antigen permits a more rigorous assessment of the ability of DNA encoding soluble A^a to induce a CTL response. It also provides a means of examining whether immunisation with soluble MHC class I results, by inducing apoptosis in alloreactive CD8 T cells (321), in inhibition of the cytotoxic T cell response. After *in vitro* stimulation, the level of CTL activity observed in LNC

from animals primed with pcmu-tA^a were comparable to those seen in T cells from control animals immunised with empty plasmid (Figure 3.6), suggesting that neither augmentation nor inhibition of the cytotoxic CD8 T cell response had occurred.

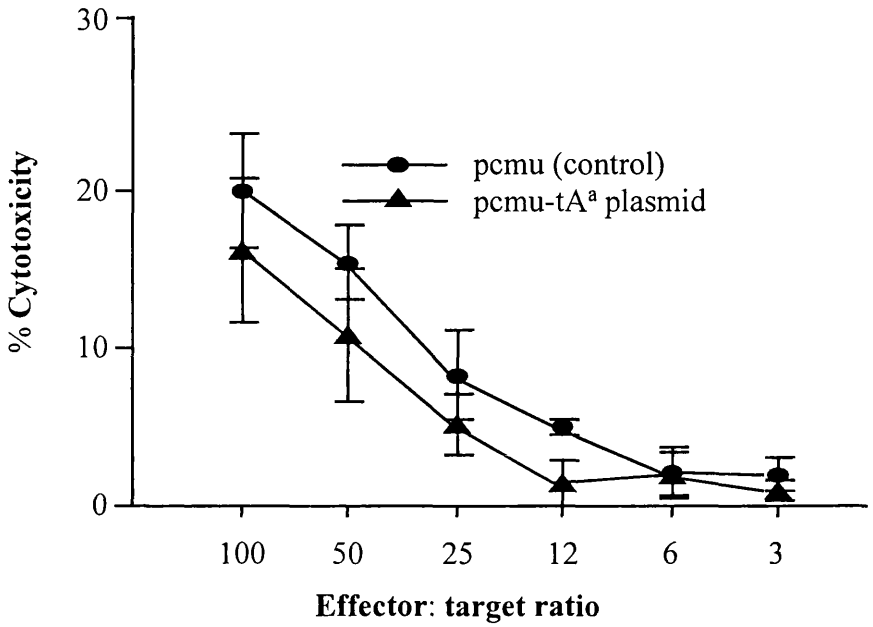


Figure 3.6 The CTL response following *in vivo* and *in vitro* challenge with RT1.A^a antigen.

LNC were obtained from PVG.RT1^u rats 12 days after immunisation with 400μg of pcmu-tA^a (or pcmu control plasmid) and used as responders (4 x 10^7 in 20ml) in a bulk MLR. Stimulators were fully allogeneic (RT1^a), irradiated (20Gy) spleen cells (2 x 10^7). After 5 days of *in vitro* stimulation, responders were tested for their ability to lyse ^{51}Cr-labelled PVG.R8 Con A blast targets in a 6-hour cytotoxicity assay. Values shown are the mean and SD of three animals per group. There was minimal lysis of third-party Lewis (RT1^l) lymphoblast target cells (data not shown).

3.2.5 *Elucidation of the Effector Mechanisms Responsible for Accelerated Rejection Following pcmu-tA^a Priming*

The lack of a discernible cytotoxic CD8 T cell response following immunisation with the pcmu-tA^a vector provides indirect evidence that its' soluble RT1.A^a product accelerates the kinetics of rejection of subsequent PVG.R8 heart grafts by priming humoral immunity. To confirm this, 1.5ml of immune serum (harvested from PVG.RT1^u animals 12 days after injection with the pcmu-tA^a plasmid) was administered to naïve PVG.RT1^u animals at the time of a PVG.R8 cardiac allograft, and everyday thereafter. A similar protocol using hyperimmune serum from PVG.RT1^u rats challenged with a PVG.R8 skin graft has been previously demonstrated to accelerate the rejection of R8 heart grafts to within 24 hours (246). In this instance, rejection times were not significantly different than control animals (Table 3.2 group 5). A limitation in the quantity of available immune serum prevented the administration of more than 3 doses to each rat. In two of the recipients the hearts beat appreciably weaker directly after the administration of the immune serum and perhaps increasing the total volume of serum that was administered may have accelerated rejection.

By comparison, histological examination of the rejected hearts was suggestive of humoral rejection (Figures 3.7 and 3.8). Control unmodified PVG.RT1^u recipients of PVG.R8 hearts reject their grafts on day 7 (Table 3.1 group 1). Low power examination of the rejected hearts (Figure 3.7A) reveals a marked cellular infiltrate, consistent with diffuse cellular rejection. High power views confirm that myocytes are separated by a severe infiltration of mononuclear cells (Figure 3.7B), consisting of lymphocytes and macrophages. There are multiple scattered foci of myocardial fibre necrosis (Figure 3.7B) and thrombus is present within the lumen (Figure 3.7A), indicating severe rejection. There is also a dense endocardial and subendocardial monocyte infiltrate (Figure 3.7D). Priming PVG.RT1^u recipients with the plasmid encoding the truncated RT1.A^a heavy chain resulted in rapid rejection of a PVG.R8 heart graft (MST 1.5 days, Table 3.2 group 3). Histological examination of hearts that were removed at the time of rejection is consistent with humoral rejection (Figure 3.8). At low power magnification, there is little cellular infiltrate but the atrial and ventricular orifices are occluded with

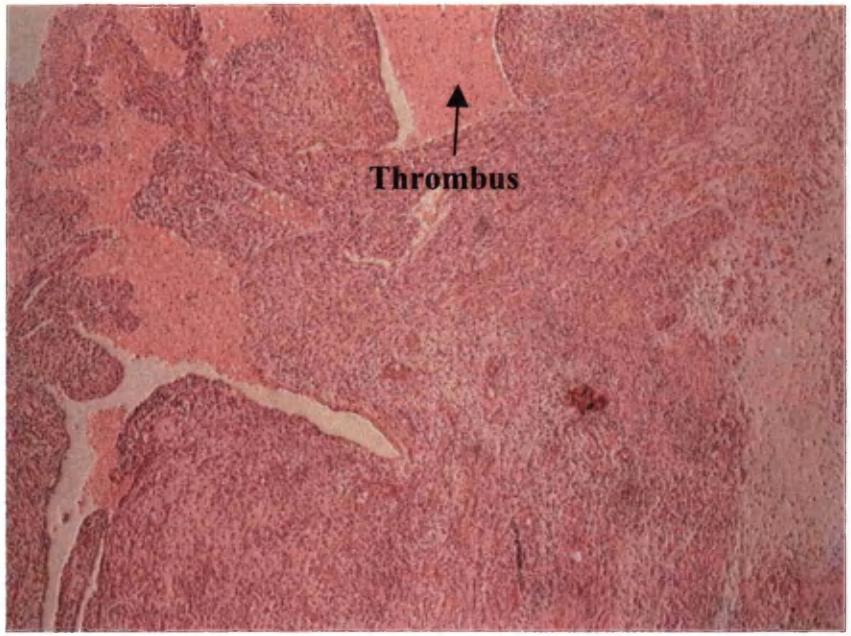
thrombus indicating that the heart had stopped beating (Figure 3.8A). Although the ultrastructure of the heart muscle appears relatively normal, high power magnification (Figure 3.8B) reveals that virtually every myocyte has lost its striatal pattern (c.f. control muscle, Figure 3.8D). This is the earliest indicator of myocyte death. Other features consistent with humoral rejection include haemorrhage into the muscle fibres (Figure 3.8B) and extravasation of neutrophils from capillaries (Figure 3.8C). Sections of heart grafts from PVG.RT1^{u} rats that had initially been primed with an R8 skin graft revealed similar findings (data not shown).

3.2.6 T cell Depletion Studies

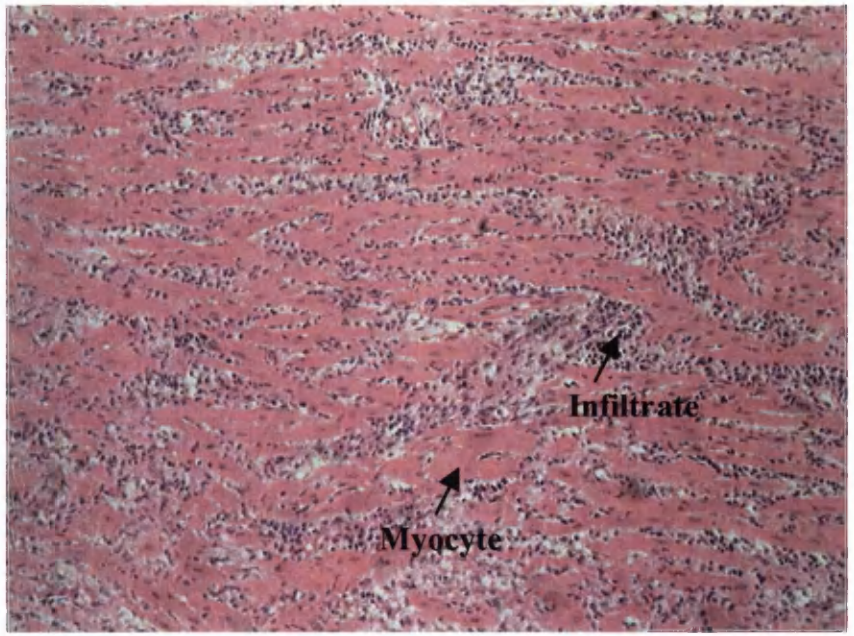
To determine whether the ability of immunisation with pcmu-tA^{a} to promote accelerated rejection of PVG.R8 heart grafts was CD4, and not CD8, T cell-dependent, pcmu-tA^{a} plasmid-immunised animals were simultaneously treated with mAbs directed against T cell subsets. As shown in Table 3.3, the rapid rejection associated with immunisation with pcmu-tA^{a} was not prevented by treatment with the anti-CD8 mAb, MRC OX8, either at the time of plasmid injection or at the time of the heart graft (MST 1 day, groups 3 and 4 respectively). Treatment with OX8 mAb results in >99% CD8 T cell depletion (355) as determined by flow cytometric analysis (data not shown). Confirmation that this phenotypic depletion corresponded to an *in vivo* depletion of functional cytotoxic T cells was provided by the observation that LNC obtained from PVG.RT1^{u} rats during the first week after *in vivo* treatment with OX8 mAb, and that had undergone culture *in vitro* for 5 days against fully allogeneic RT1.A^{a} irradiated splenic stimulators (as described in the legend to Figure 3.5) did not develop a measurable CTL response (<10% cytotoxicity at an effector: target cell ratio of 100:1). In comparison high levels of cytotoxicity were detected using LNC from unmodified PVG.RT1^{u} rats (>50% cytotoxicity at an effector: target cell ratio of 100:1).

In vivo treatment of pcmu-tA^{a}-immunised PVG.RT1^{u} rats with the anti-CD4 mAb MRC OX38 not only prevented accelerated graft rejection, but extended graft survival beyond that observed in naïve PVG.RT1^{u} recipients (MST 13 days, Table 3.3, group 5). MRC-OX38 treatment results in approximately 50% CD4 T cell

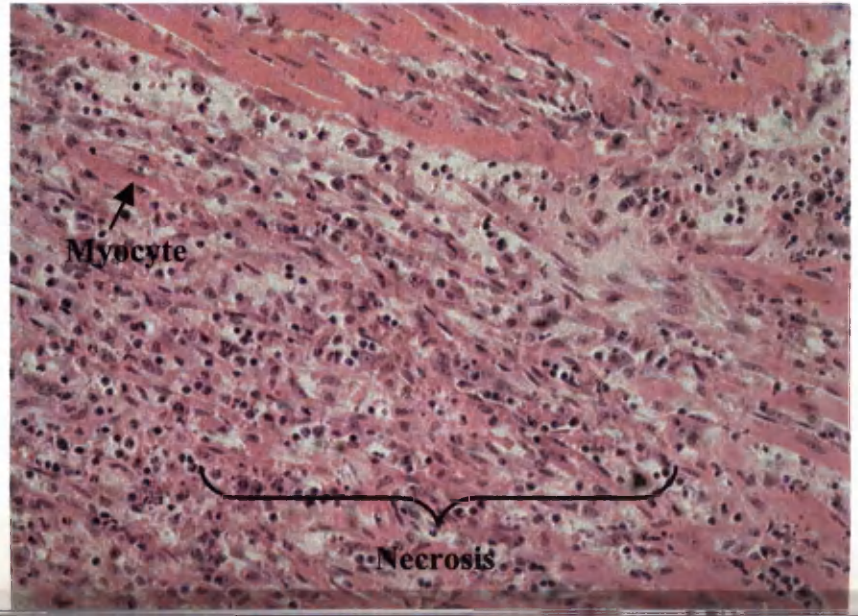
A)


Thrombus

B)


Infiltrate
Myocyte

C)


Myocyte
Necrosis

D)

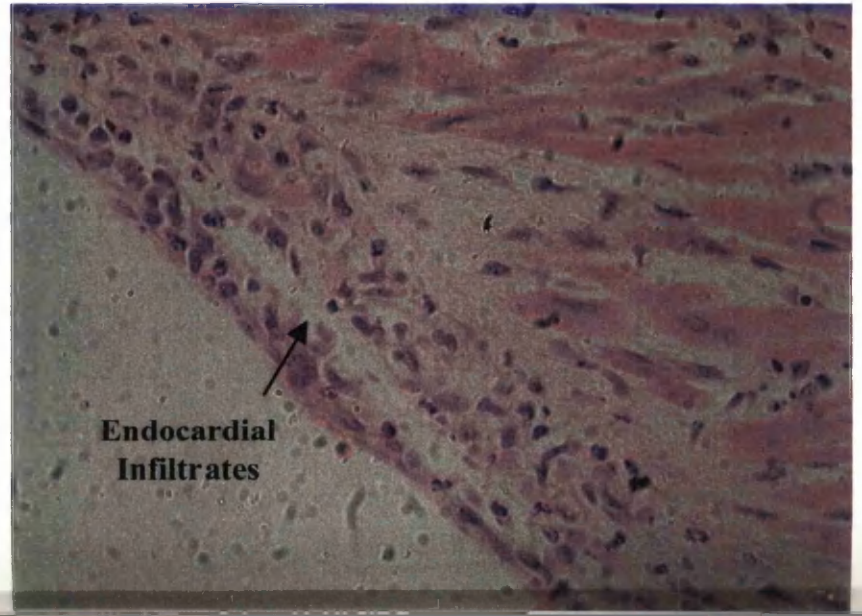

Endocardial
Infiltrates

Figure 3.7 Histological examination of PVG.R8 hearts after transplantation and rejection in naïve PVG.RT1^u recipients.

Unmodified PVG.RT1^u rats were transplanted with heterotropic PVG.R8 heart grafts. These were removed upon rejection (MST 7 days, Table 3.1), fixed, sectioned and stained in haematoxylin and eosin.

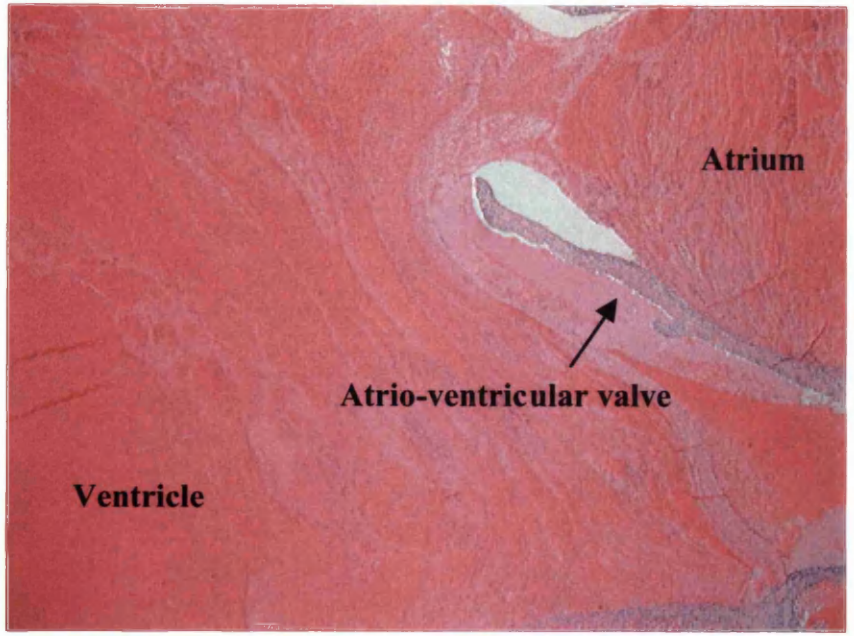
Figure A Low power (x2 magnification) view depicting generalised cellular infiltrate of the atrium. Thrombus is present within the lumen.

Figure B High power (x40 magnification) view. Severe mononuclear infiltrate between myocyte fibres is present.

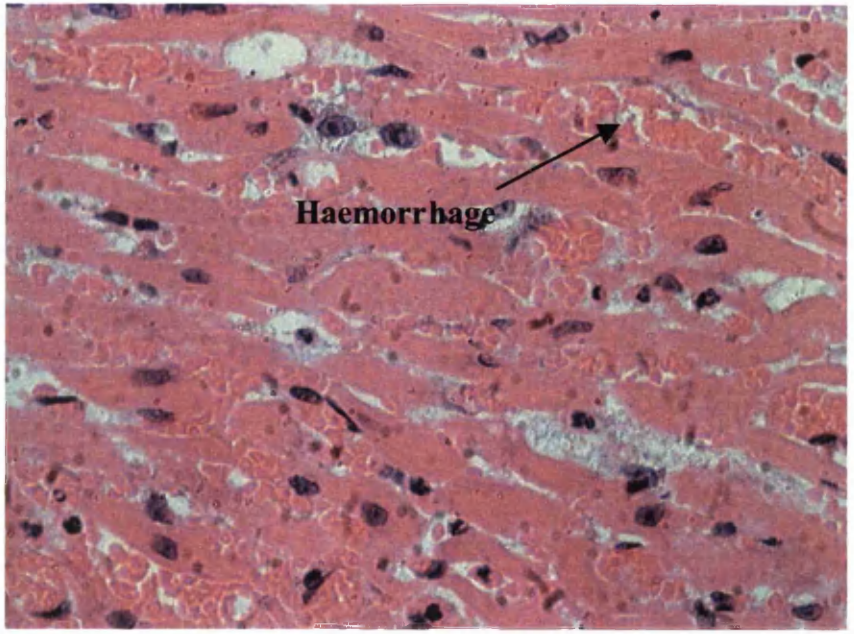
Figure C High power view of an area of necrosis containing isolated degenerating myocyte fibres (marked).

Figure D Demonstration of heavy endocardial and sub-endocardial monocyte infiltrate.

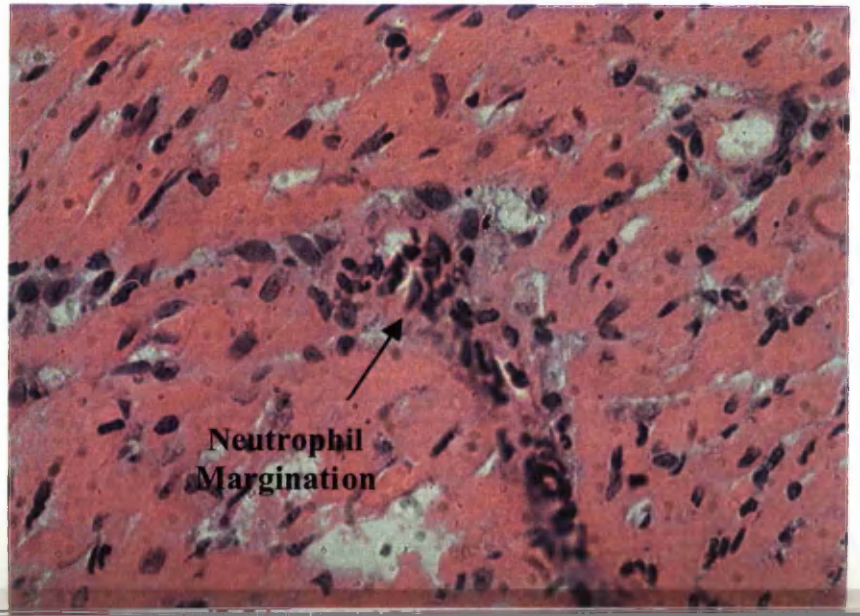
A)


Atrium
Atrio-ventricular valve
Ventricle

B)


Haemorrhage

C)


Neutrophil
Margination

D)

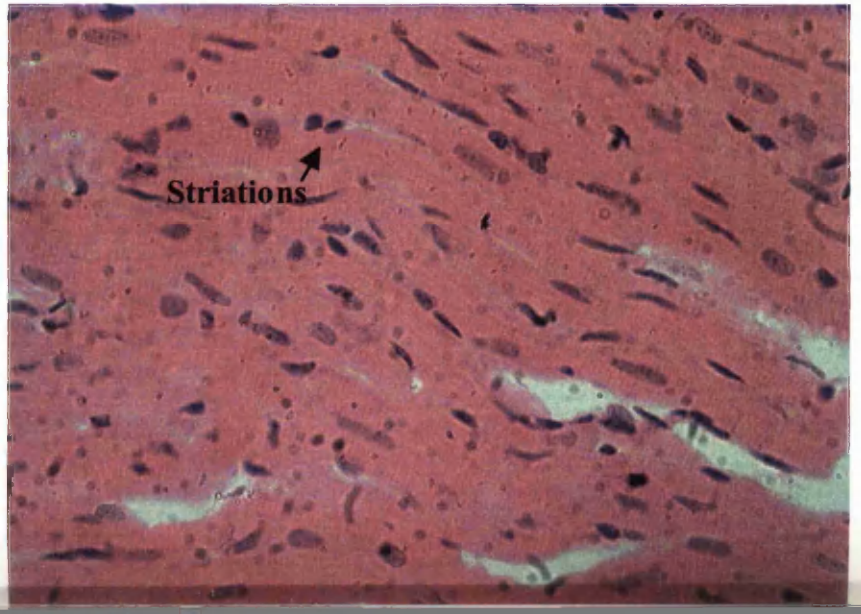

Striations

Figure 3.8 Histological examination of PVG.R8 hearts after transplantation and rejection in pcmu-tA^a-primed PVG.RT1^u recipients.

PVG.RT1^u rats were injected with 400μg of plasmid encoding the truncated RT1.A^a heavy chain (pcmu-tA^a), and 12 days later received a heterotropic PVG.R8 heart graft. Grafts were removed upon rejection (MST 1.5 days, Table 3.3), fixed, sectioned and stained in haematoxylin and eosin.

Figure A Low power (x2 magnification) view depicting thrombus completely filling the atrium and ventricle.

Figure B High power (x40 magnification) view depicting heamorrhage between muscle fibres.

Figure C High power view of ventricular arteriole. Neutrophils (characterised by their polymorphic, multilobed nuclei) are marginating through the wall, in response to the dead ventricular muscle tissue.

Figure D High power view of the ventricular muscle fibres of a normal, non-transplanted heart. The muscle fibres display numerous striations, which are absent in the fibres depicted in Figure B and Figure C. Loss of muscle striations is the earliest recognisable feature of muscle death.

depletion (289) and it is likely that recovery of the residual CD4 T cell population was responsible for eventual rejection of the heart grafts.

The ability of the mAb treatments to influence heart graft survival in pcmu-tA^a primed animals closely mirrors their effect on the development of a cytotoxic RT1.A^a Ab response. The administration of anti-CD4 mAb completely abrogated the early alloantibody response, whereas there was no discernible difference, as compared to control isotype antibody-injected injected animals, in the cytotoxic alloantibody response following CD8 T cell depletion (Figure 3.9).

Table 3.3. Rejection of R8 heart grafts following T cell subset depletion of pcmu-tA^a plasmid primed PVG.RT1^u recipients.

Group[d]	DNA vaccination[a]	mAb Treatment	n	Heart graft survival (days)	MST (days)
1	pcmu (control)	-	4	6,7,7,7	7
2	pcmu-tA^a	-	7	1,1,1,2,2,2,2	1.5
3	pcmu-tA^a	Anti-CD8[b]	4	1,1,1,1	1
4	pcmu-tA^a	Anti-CD8[c]	3	1,1,1	1
5	pcmu-tA^a	Anti-CD4[c]	4	8,12,14,17	13

[a] PVG.RT1^u rats were immunised with 400 μg pcmu-IV plasmid encoding either a truncated A^a class I MHC (pcmu-tA^a), or empty plasmid as control. Twelve days after the first DNA injection, rats received a heterotopic PVG.R8 cardiac allograft.

[b] Animals in group 3 received 2mg anti-CD8 mAb (OX8) on days –1, 0, and 1 relative to heart transplantation.

[c] Animals in groups 4 and 5 were treated with anti-CD8 (2mg OX8 on days –1, 0 and 1) or anti-CD4 (5mg OX38 on days –3, 0, 3, 6, and 9) relative to plasmid injection.

[d] Group 1 vs. group 5, $p < 0.02$. Group 2 vs. group 5, $p < 0.02$.

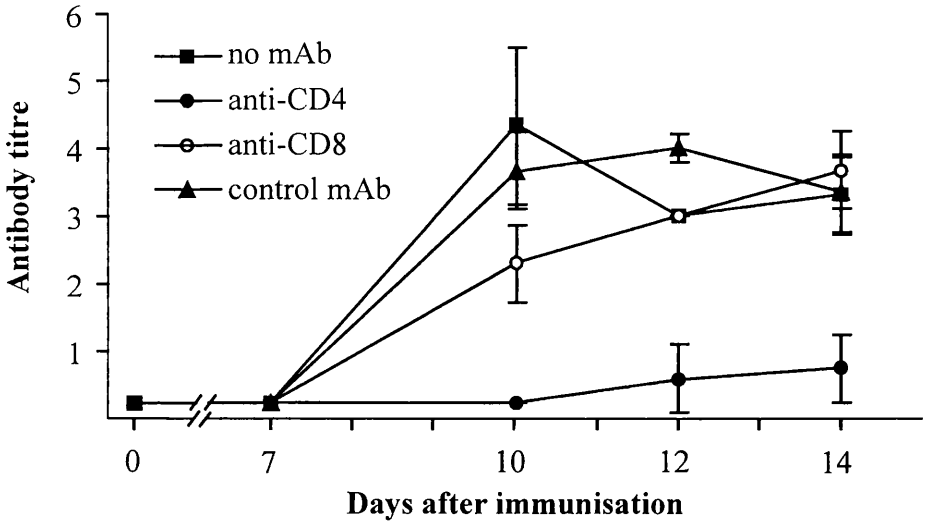


Figure 3.9 Lymphocytotoxic anti-A^a alloantibody response in pcmu-tA^a immunised PVG.RT1^u rats following T cell subset depletion.
PVG.RT1^u rats were immunised with 400μg pcmu-tA^a, and treated with anti-CD4 (5mg OX38 (or isotype control, ESH8) given i.p. on days –3, 0, 3, 6, and 9 relative to plasmid injection) or anti-CD8 antibody (2mg OX8 on days –1, 0, and 1). Levels of cytotoxic antibody were assessed by assaying sera (four animals per group) against ^{51}Cr-labelled PVG.R8 Con A blasts in the presence of guinea pig complement. Results are expressed as mean and SD of antibody titre (last dilution of serum that gave > 20% cytotoxicity).

3.2.7 Immunisation of Lewis Rats (RT1^l) with RT1.A^a-Encoding pcmu-IV Vectors

The Lewis rat strain (RT1^l) is a high responder towards the RT1.A^a antigen; completely mismatched DA (RT1^a) hearts are rejected rapidly and priming with donor strain blood results in accelerated, rather than prolonged, graft survival. To examine whether priming with soluble class I allo-MHC resulted in a similar acceleration in the rejection of A^a-bearing heart grafts as in the PVG.RT1^u strain, Lewis rats were immunised with 400μg of the A^a-encoding vectors (pcmu-A^a and pcmu-tA^a) and 12 days later transplanted with a DA (RT1^a) cardiac allograft. Animals immunised with either the plasmid encoding the full-length RT1.A^a antigen, pcmu-A^a, or the soluble version, pcmu-tA^a, rejected their grafts only modestly more rapidly than control animals injected with the 'empty' plasmid (Table 3.4). Rejection times in the group immunised with pcmu-A^a were slightly more rapid than in the group immunised with pcmu-tA^a, but this difference was not statistically significant (MST 4.5 vs. 5, $p = 0.286$). The alloantibody response did not, however, correlate with rejection, since the antibody response in the pcmu-A^a animals was minimal, whereas a significant titre developed in the pcmu-tA^a immunised animals (Figure 3.10).

Table 3.4 Rejection of DA heart grafts by Lewis recipients after immunisation with DNA encoding A^a class I MHC

Group	Immunisation protocol	n	Heart graft Survival (days)	MST (days)
1[a]	pcmu (control)	4	6,6,7,7	6.5[b]
2[a]	pcmu-A^a	4	4,4,5,5	4.5[b]
3[a]	pcmu-tA^a	4	5,5,5,6	5[b]

[a] Lewis animals were injected with 400μg of the pcmu plasmid vectors encoding either the full-length RT1.A^a antigen (pcmu-A^a) or the truncated, water-soluble version (pcmu-tA^a). Control animals were immunised with empty pcmu plasmid. Twelve days after plasmid immunisation, rats received a heterotopic DA cardiac allograft. Grafts were assessed by daily palpation and rejection was defined as complete cessation of myocardial contraction.

[b] Group 1 vs. group 2 and 3, $p < 0.03$ and $p = 0.09$, respectively.
Group 2 vs. group 3, $p = 0.286$.

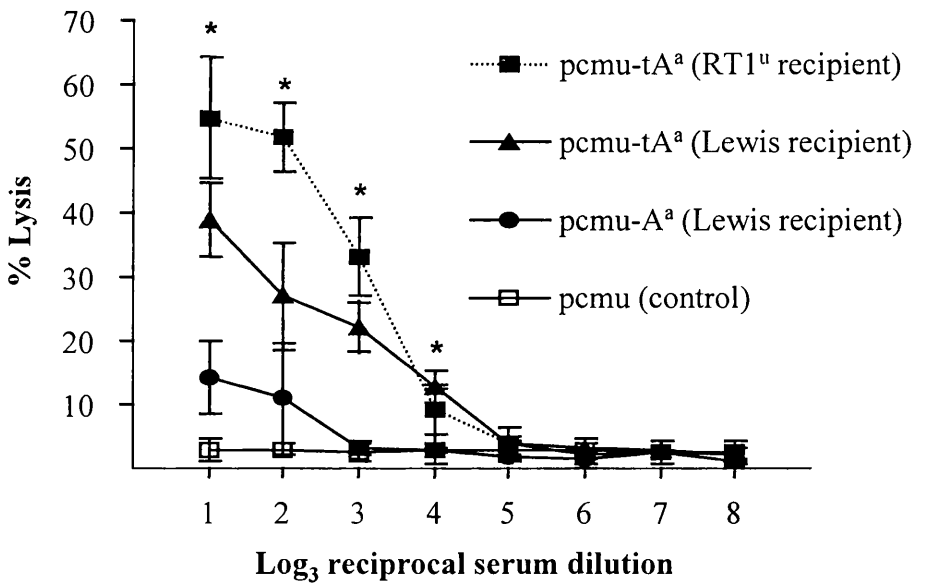


Figure 3.10 Development of the cytotoxic anti-A^a alloantibody response in Lewis rat recipients.

Lewis animals were injected with 400μg of the pcmu plasmid vectors encoding either the full-length RT1.A^a antigen (pcmu-A^a) or the truncated, water-soluble version (pcmu-tA^a). Control animals were immunised with empty pcmu plasmid. 12 days after plasmid immunisation, the presence of serum cytotoxic anti-A^a antigen was measured by the ability to lyse ^{51}Cr-labelled DA (RT1^a) Con A splenoblasts in the presence of guinea pig complement. Values shown are the mean and SD of four animals per group. Shown also for comparison is the cytotoxic Ab response in RT1^u animals (dashed) sensitised 12 days earlier by injection of the pcmu-tA^a vector (from Figure 3.2). * Pcmu-tAa (Lewis recipient) vs. pcmu-A^a (Lewis recipient), $p<0.02$, Mann-Whitney U test, 2-tailed.

3.3 Discussion

PVG.RT1^u animals primed with an R8 skin graft rejected a subsequent R8 heart graft rapidly (Table 3.1). This was associated with the development of a strong anti-A^a cytotoxic alloantibody response. Animals primed with allopeptide did not generate an anti-A^a antibody response, and grafts were rejected only modestly more rapidly than controls (Figure 3.1, Table 3.1). Since the passive transfer of hyperimmune serum into naïve PVG.RT1^u recipients results in the rejection of an R8 heart graft within 24 hours (246), the most likely explanation for the difference in the rejection times between skin-grafted and peptide-primed animals is that the intact RT1.A^a antigen on the surface of donor R8 skin cells provides the appropriate conformational epitopes for the generation of an antibody response that cross-reacts with the subsequent heart graft.

The antibody response to alloantigen is, however, T helper cell dependent, and the indirect CD4 T cell recognition of certain class I alloantigens does not provide sufficient help for the generation of an effective alloantibody response; the expression of additional T helper cell determinants on donor graft cells are required (292). The ability of an R8 skin graft, rather than allopeptide immunisation, to prime the development of an alloantibody response responsible for accelerated heart graft rejection may therefore not only reflect the presence of the appropriate B cell epitopes, but may also be due to more efficient helper T cell activation.

A number of reasons may be postulated as to why skin grafting generates more efficient T cell help. Firstly, the intact RT1.A^a antigen, as presented by an R8 skin graft, may be processed to generate more optimal A^a-peptide T helper cell determinants than occurs upon immunising with the allopeptide mixture. In an attempt to encompass all potential determinants involved in the indirect response to the RT1.A^a antigen, a series of 18 peptides that spanned the α1 and α2 domains were administered. Sequential peptides however, differed by five, rather than one amino acid, and it is it is conceivable that the optimum T cell epitope is not contained within any individual immunising peptide but instead corresponds to an amino acid sequence that spans two consecutive peptides. Nevertheless, all studies that have

examined the effects of allopeptide immunisation to date (244-246, 291) have consistently reported a similarly modest acceleration in vascularised allograft rejection. In particular, PVG.RT1^{u} rats immunised with the full-length RT1.A^{a} heavy chain protein, which presumably did result in optimal peptide epitope presentation to T helper cells, accelerated rejection of PVG.R8 heart grafts to day 4 (248). The allopeptide mixture used in this thesis achieved a similar acceleration in rejection, suggesting that maximal stimulation of the indirect pathway was achieved. This is further supported by the observation that similar peptides have been targeted to abrogate the anti-A^{a} antibody response (246). Further refinement of the administered peptides would be consequently unlikely to alter the kinetics of graft rejection and the difference in rejection times between peptide-primed and skin-grafted animals is therefore the result of activation of either T cells via other allorecognition pathways or alternative effector mechanisms.

One such alternative allorecognition pathway by which skin grafting may generate more efficient T cell help for alloantibody production is through activation of a CD8 T cell subset that directly recognises the intact class I RT1.A^{a} antigen. This is equally unlikely, for although there are examples whereby the CD8 T cell response can directly influence the antibody response (319, 320), in the model employed in this thesis, CD8 T cell depletion influences neither the alloantibody response nor the kinetics of heart graft rejection (355).

A more difficult explanation to refute is that skin grafting results in CD4 T cell activation via the direct pathway. Possible antigenic determinants for direct CD4 T cell recognition are either the intact donor RT1.A^{a} antigen or additional disparities carried over in the recombinant event that created the PVG.R8 strain (297). The recognition of the donor class I antigen by recipient CD4 T cells appears to break the rules of MHC restriction, but exceptional CD4 T cell clones that recognise class I MHC have been described (316, 317). Similarly, although there is no direct evidence to confirm that additional antigenic discrepancies influence CD4 T cell activation in this strain combination, in an analogous class I disparate mouse model, additional, undefined antigens are responsible for the activation of T cells which provide help for the generation of an anti-alloMHC class I cytotoxic T cell response (263).

If the above hypothesis, that accelerated rejection is a manifestation of direct T cell allorecognition, is correct, it has important implications for the nature of T-B cell collaboration, for it provides support for the three-cell cluster model (292). According to this model, the direct allorecognition pathway dominates the initial alloimmune response and is uniquely responsible for the provision of help for the early alloantibody response. Moreover, since directly activated CD4 T cells are able to recognise intact donor antigen on the graft, they could theoretically additionally effect rejection through either a direct cytotoxic response, or through the initiation of a DTH response.

Immunising with the plasmid vector encoding the full-length membrane bound RT1.A^a antigen resulted in the development of an anti-A^a antibody response and subsequent heart grafts were rejected in a similar time scale to those animals that had initially been primed with a skin graft (Tables 3.1 and 3.2). By administering only the appropriate nucleotide sequence, a DNA-based approach ensures that the RT1.A^a antigen is expressed with complete purity, and this result therefore obviates the need to invoke the recognition of additional putative alloantigens as being responsible for accelerated rejection. It does not preclude direct T cell recognition of the RT1.A^a antigen influencing the alloantibody response for, as discussed in section 2.3, professional APCs can internalise the pcmu-A^a vector and express the RT1.A^a antigen on their cell membrane, in conjunction with appropriate costimulatory molecules.

The influence that the vector encoding the soluble class I alloantigen has on the alloantibody response is the most critical result. T cell recognition of the product of the pcmu-tA^a vector is expected to be restricted to the indirect pathway. This hypothesis is the principal tenet governing the design and interpretation of the experiments described in this thesis and requires further consideration. It is based on the two signal model for T cell activation (336); that naïve T cell activation requires not only an interaction with the appropriate MHC/peptide complex via the T cell receptor, but also signalling through costimulatory receptors. In the absence of costimulation, an interaction with the TCR results in partial activation, or anergy. Since the costimulatory signals are provided by professional APC the two-signal model provides a means of preventing potentially deleterious

responses developing to peripheral self-antigens. Although soluble MHC, released from transfected cells *in vivo*, could interact directly with the TCR of alloreactive T cells, the absence of costimulatory signals delivered by APC would in theory prevent T cell activation.

It has more recently been suggested, however, that the costimulatory requirement for T cell activation, particularly for CD8 T cells, is relative rather than absolute (340-345). Viola and Lanzavecchia (340) have demonstrated that *in vitro* CD4 T cell activation requires a defined number of signal events to occur through the surface T cell receptor. In the absence of costimulation, this threshold value is 8000, whereas concurrent costimulation lowers this value to 1500. Several recent studies have also verified that CD8 T cells can be activated in the absence of a second signal (344-346, 408), albeit with an efficiency of at least 20 times less than when costimulation is present (344). Similarly, Delon (350) has demonstrated that Ca^{2+} mobilisation in CD8 T cells can be triggered with soluble peptide/MHC class I complexes in the absence of a costimulatory signal. These *in vitro* studies may nevertheless not accurately represent *in vivo* CD8 T cell activation. Delon's studies examined calcium influx rather than differentiation of the CD8 T cells into effector cells (350), and in the other studies the absence of CD4 T cell help was compensated for by either the addition of Il-2 (344, 346) or by immobilisation of the class I protein by binding to plates (345, 408). Immobilisation effectively increases the ligand density of the class I MHC complex, and this presumably results in more effective cross-linking of the TCR and CD8 co-receptor complex. Recent evidence suggests that to achieve similar cross-linking *in vivo,* physiological soluble MHC class I (i.e. non-immobilised) has to be aggregated into multivalent complexes; whereas tetrameric class I MHC resulted in T cell activation (348, 349), monovalent and divalent complexes were non-stimulatory (348).

The exact physical conformation of the soluble class I MHC product used in this thesis was not formally assessed and should have ideally been verified by SDS-PAGE analysis under non-reducing conditions. Nevertheless, it was constructed by excising the hydrophobic transmembrane and cytoplasmic domains of the full-length RT1.A^a heavy chain. In their absence it is likely that soluble MHC class I

resulting from gene transfer is released in monomeric form and consequently unable to activate T cells directly. This assumption receives strong support from the analysis of the CTL responses (Figures 3.5 and 3.6) and from the inability of CD8 T cell depletion to influence either the alloantibody response to, or the rejection kinetics of, heart grafts (Figure 3.9 and Table 3.3). RT1.A^a plasmid constructs have also been assessed in Lewis rats by Geissler (351), who similarly demonstrated that a cytotoxic T cell response developed following vaccination with the vector encoding the full-length heavy chain, but not with the soluble version. More recently, the transfer of hepatocytes that were transfected with the soluble antigen was associated with downregulation of the cytotoxic T cell response (352-354).

In summary, T cell recognition for either the allopeptide mixture or the soluble class I MHC product of the pcmu-tA^a vector is restricted to the indirect pathway. This infers that soluble MHC class I alloantigen is a more effective immunogen solely because its tertiary protein structure provides the conformational B cell epitopes necessary for generation of pre-transplant antibodies directed against target graft cells expressing intact allogeneic MHC class I. My results therefore offer an alternative explanation to that proposed by Shirwan (245) as to why priming with donor R8 splenocytes results in rapid rejection; that, in an analogous fashion to priming with soluble class I alloantigen, the immunogenicity of donor cells resides in their ability to prime humoral immunity that may nevertheless be solely dependent on help from indirectly-restricted CD4 T cells. Shirwan did not assess the anti-A^a antibody response that followed splenocyte infusion, but the results from my experiments and others (246, 290, 355) suggest that a strong lymphocytotoxic alloantibody response would have developed.

The corollary, that the accelerated rejection is mediated by humoral, rather than cellular mechanisms, is confirmed by the histological examination of the rejected hearts, but was not supported by the experiments involving passive transfer of immune serum. This discrepancy can be explained by the observation that plasmid-primed animals frequently did not reject their heart grafts until day two. The levels of pre-formed anti-A^a antibody were presumably not enough to cause immediate rejection, and if so, the transfer of a 1.5ml sample of this serum could

hardly be expected to be any more effective. In two of the rats, however, the hearts beat perceptively less strongly following hyperimmune serum administration, but had recovered by the next day. This approach may have induced accelerated rejection if serum had been taken from $RT1^u$ animals that had been administered several pcmu-tA^a plasmid injections, such that a secondary alloantibody response was sampled.

Implicit to the hypothesis that T cell help for the development of an anti-A^a antibody response is restricted to the indirect pathway is of course the assumption that the alloantibody response is T cell dependent. By using MHC class II deficient recipients, Steele has demonstrated that a limited IgM alloantibody response can develop in the absence of help from recipient CD4 T cells, but that isotype switching required cognate T cell help (296). Agarwal has similarly documented that the process of isotype switching is determined by T cell availability (302, 303). Nevertheless, an alloantibody response restricted to the IgM isotype can still theoretically activate complement to the extent necessary to compromise graft survival. The results from my experiments, that an IgG2b response developed to the soluble $RT1.A^a$ protein and, most importantly, that CD4 T cell depletion abrogated the alloantibody response (Figure 3.9) confirms the CD4 T cell-dependent nature of the alloantibody response. Anti-CD4 T cell treatment results in depletion of approximately only 50% of peripheral CD4 T cells (289) and the eventual rejection of heart grafts and the development of an alloantibody response presumably reflects recovery of the residual population. In contrast anti-CD8 treatment resulted in profound CD8 T cell depletion (~99%) but had no discernible effect on graft survival.

Whereas rapid rejection (within 1 to 2 days) is generally considered to be antibody mediated, the mechanisms responsible for the modest acceleration following allopeptide priming were not assessed. Peptide immunisation accelerated the kinetics of the antibody response to the heart graft (Figure 3.3). This suggests that peptide primed T cells (recognising the same peptide/self MHC class II complex as presented by $RT1.A^a$-specific B cells) provide cognate B cell help for the generation of an alloantibody response, and that the availability of T cell help is the rate limiting factor in the development of this response. Although the more

rapid cytotoxic alloantibody response following peptide priming (first detectable by day four after heart grafting) correlated with the kinetics of accelerated rejection (MST day 4, Table 3.1) histological examination of the rejected hearts was not performed.

Nevertheless, a feature of the experimental work of this thesis is how closely the alloantibody response to the RT1.A^a antigen corresponds to the rejection of PVG.R8 heart grafts, whether in the accelerated rejection following pcmu-tA^a plasmid priming, or in the delayed rejection following anti-CD4 mAb treatment. Consequently, rejection in unmodified, and not just peptide-primed recipients, is possibly alloantibody-mediated. This has been previously suggested, based on experiments involving the passive transfer of immune serum (289), and more recently, on the observation that B cell tolerance is involved in the prolongation of graft survival (409). If so, the histological appearances of cellular rejection in PVG.R8 heart grafts from unmodified PVG.$RT1^u$ recipients (Figure 3.7) are simply a marker for activation of the alloimmune response, but are not representative of the alloantibody response that is main effector mechanism responsible for rejection. Certainly, the presence of cellular infiltrates within a graft does not necessarily correlate with rejection (410). However, the possibility that indirectly primed CD4 T cells may effect the rejection of PVG.R8 grafts in PVG.$RT1^u$ recipients through other mechanisms, such as the initiation of a DTH response within the graft (249-251), has not been excluded.

The alloantibody response to the product of the vector encoding the truncated RT1.A^a heavy chain (pcmu-tA^a) was of a similar magnitude in Lewis and PVG.$RT1^u$ rats (Figure 3.10), but the kinetics of graft rejection were markedly different (Table 3.2 vs. Table 3.4). Knechtle et al have also administered RT1.A^a encoding plasmid to Lewis recipients and have found a similarly modest acceleration in the rejection of A^a-bearing heart grafts (411). The difference in rejection times is surprising, for the passive transfer of anti-A^a hyperimmune serum to Lewis recipients has been previously documented to result in hyperacute rejection of DA ($RT1^a$) hearts (412). In this latter study hyperimmune serum was prepared by performing three serial skin grafts and 6mls of serum was administered, which presumably represents a much greater antibody titre than was

achieved with the protocol for plasmid injection used in my experiments. A possible explanation therefore for the differences between plasmid-primed PVG.RT1^u and Lewis rats in the rejection kinetics of A^a-bearing heart grafts is that for a given alloantibody concentration, the antibody-dependent effector mechanisms are triggered less efficiently in Lewis rats. In support of this hypothesis, complement, which is the most likely mediator through which alloantibody effects rejection, has been demonstrated to have inherently different levels of activity in different rat strains (413). For example, the passive transfer of 1ml hyperimmune serum into the naïve recipient of a high responder strain combination resulted in hyperacute rejection, whereas in a low responder combination (Lewis recipient) led to graft enhancement. In the low responder strain combination, however, hyperacute rejection was restored if guinea pig complement was administered along with the hyperimmune serum. It would be interesting to examine whether the concurrent administration of guinea pig complement to those Lewis recipients that had been primed with the plasmid for the soluble RT1.A^a antigen would result in as rapid a rejection as occurred in the A^a-plasmid primed PVG.RT1^u recipients of PVG.R8 heart grafts.

The results from my experiments and those of Knechtle (411) suggest that the rejection of DA hearts by Lewis recipients is instead effected by the cytotoxic CD8 T cell, rather than by the humoral response. Lewis recipients primed with the plasmid construct for the full-length, membrane bound RT1.A^a antigen failed to develop a strong alloantibody response, yet rejection was if anything more rapid than in those animals that expressed high levels of anti-A^a alloantibody following priming with the plasmid for the truncated antigen (Table 3.4 and Figure 3.10). The cytotoxic CD8 T cell response to the product of the A^a-encoding pcmu plasmid constructs was not assessed but Geissler has used similar A^a plasmids and in his experiments an anti-A^a cytotoxic CD8 T cell response was detectable only in Lewis rats immunised with the plasmid construct for the full-length RT1.A^a antigen (411), and not in those immunised with the truncated version. The hypothesis that the cytotoxic CD8 T cell response is more pivotal in the Lewis rat could be tested by antibody depletion studies and one would expect that, in contrast to the R8-RT1^u strain combination, CD8 T cell depletion would abrogate the accelerated rejection associated with plasmid priming.

3.4 Summary

The physical nature of the RT1.A^a antigen with which PVG.$RT1^u$ rats were immunised significantly influenced the rate of rejection of PVG.R8 heart grafts. Priming with a PVG.R8 skin graft stimulates an IgG2b cytotoxic alloantibody response and resulted in rejection of an R8 heart graft within 24 hours. Priming T cells for indirect recognition with allopeptides corresponding to the $\alpha 1$ and $\alpha 2$ domains of the RT1.A^a heavy chain did not result in the development of an anti-A^a antibody response and R8 hearts were rejected only modestly more rapidly. Crucially, intramuscularly injection with plasmid encoding a water soluble version of the RT1.A^a heavy chain (pcmu-tA^a) was associated with a strong anti-A^a alloantibody response and similar rejection kinetics of PVG.R8 heart grafts as occurred in animals primed with a skin graft. T cell recognition of soluble class I allo-MHC is theoretically restricted to the indirect recognition of allopeptide fragments presented by recipient APC. The absence of a direct CD8 T cell response was confirmed by T cell antibody depletion studies and the lack of *in vitro* cytotoxic killing. Histological examination of the heart grafts from pcmu-tA^a-primed recipients verified that rejection was antibody mediated. These results suggest that soluble class I allo-MHC is a more effective immunogen than allopeptide because it displays the appropriate conformational B cell epitopes for the development of humoral immunity directed against intact MHC on donor cells, and not because of any inherent differences in the nature of helper T cell recognition. Surprisingly, although Lewis animals that had been similarly primed with pcmu-tA^a plasmid developed an equally strong anti-A^a alloantibody response, rejection of A^a-bearing heart grafts was much less rapid. This possibly reflects strain-specific differences in the levels of complement activity.

3.4 Materials and methods

3.5.1 Animals

Animals were purchased and maintained as described in Section 2.5.3.1

3.5.2 Antibodies

MRC OX8 (anti-rat CD8) (254) and MRC OX38 (anti-rat CD4) (414) monoclonal antibodies were used for *in vivo* T cell depletion studies. These were produced by intraperitoneal injection of the appropriate hybridoma cell line into pristane-primed Balb/c mice (Harlan, UK) to produce ascites, from which IgG was purified by protein A column chromatography (ProSep, Fisher Scientific, Loughborough, UK). The antibody concentration was determined by UV spectrophotometry. The OX8 and OX38 mAb treatment regimens used to induce blockade of the CD8 and CD4 T cell subsets, respectively, were based on experience with these Abs in previous studies (290, 355). The mouse IgG2a mAb, ESH8, which is directed against human factor VIII (Scottish Antibody Production Unit, Law, Scotland, UK), was used as an isotype control (400).

3.5.3 Allopeptides and Allopeptide Immunisation

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^a (residue 28 [glycine] to residue 212 [phenylalanine] inclusive, (39)), were purchased from Immune Systems Ltd. (Paignton, UK). A control peptide (designated irrelevant) was also manufactured. This contains the same amino acids as peptide number 7, which corresponds to the hypervariable region of the α1 domain, but in a random order. All peptides were synthesised by FMOC chemistry, purified by HPLC, and assessed by Mass Spectrometry for purity. All peptide preparations were shown to be greater than 80% pure. The peptide sequences are depicted in Table 3.5.

PVG.RT1^u rats were immunised subcutaneously in each hind footpad with a single injection of 900μg of allopeptide (comprising a mixture of 50μg of each of the 18 individual allopeptides), dissolved in 50μl of water and emulsified with a

comparable volume of CFA (Sigma). 100μg of irrelevant peptide emulsified in CFA was administered as control.

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

Table 3.5 The amino acid sequences of the 15-mer synthetic peptides

Peptide sequences begin at amino acid residue number 25, as the amino acids preceding this represent the signal sequence, which is cleaved during final transit of the nascent MHC through the endoplasmic reticulum.

3.5.4 Plasmid Administration

Gene transfer of the DNA sequences encoding the RT1.A^a heavy chain was achieved by directly injecting plasmid DNA into the skeletal muscle of adult rats (374). To induce regeneration of plasmid DNA into skeletal muscle and thereby increase the efficiency of gene transfer (403, 404), 400μl of 0.5% Bupivicaine (1-butyl-N[2, 6-dimethyl phenyl] 2-piperidine-carboxamide) (Astra, King's Langley, UK) was first injected into each tibialis anterior muscle using a 28-gauge needle. Three and eight days later, 200μg of pcmu-IV encoding either the full-length or truncated nucleotide sequence of the RT1.A^a heavy chain was injected into each tibialis anterior muscle. As control, empty pcmu-IV plasmid was administered.

3.5.5 Cardiac Transplantation

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 and the animal exsanguinated. 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, and the aorta, pulmonary artery and great veins were identified. The right superior vena cava (SVC) and IVC were ligated with 5^{o} 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 first division. 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.

Recipient Transplantation

Heterotopic cardiac transplantation was performed using the modified technique of Ono and Lindsey (415). 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^{o} 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, Ethicon Ltd). 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° vicryl sutures (Ethicon Ltd.). Grafts were assessed daily by palpation, and rejection was defined as the complete cessation of myocardial contraction.

3.5.6 Skin Transplantation

Recipients were grafted on the flank with full thickness PVG.R8 skin allografts as previously described (275). Briefly, anaesthetised $RT1^u$ animals were grafted onto the flank with $1cm^2$ full thickness R8 abdominal skin, secured by four 4° sutures (Ethicon Ltd) 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.

3.5.7 Cytotoxic Alloantibody Determination

Lymphocytotoxic antibodies were determined by assaying test serum against ^{51}Cr-labelled R8 ConA blasts in the presence of guinea pig complement as described in section 2.5.3.5.

3.5.8 Cell-Mediated Cytotoxicity Assays

PVG.$RT1^u$ rats were grafted with PVG.R8 skin (Section 3.5.5) or immunised with plasmid vectors (Section 3.5.4) encoding either of the $RT1.A^a$ heavy chain constructs (pcmu-A^a or pcmu-tA^a) or with pcmu-IV (empty vector). After 12 days, purified spleen cells (Section 2.5.3.3) were used as effector cells and assayed against ^{51}Cr-labelled allogeneic R8 Con A blast targets in a standard 6 hour Cr-release assay (416). Briefly, two fold serial dilutions of effector cells were made to give effector: target cell ratios of 100:1, 50:1, 25:1, 12.5:1, 6:1, and 3:1. Equal volumes (75µl) of effector and target cells were mixed in wells of 96 V-well

microtitre plates (Sterilin, Teddington, UK), with each combination set up in triplicate. Spontaneous release was determined using target cells incubated without effector cells, and maximum release was determined using target cells incubated with 10% Triton x 100 (Sigma). The plates were centrifuged briefly, then incubated for 6 hours at 37°C in 5% CO_2 in air. From each well, 75µl of supernatant was harvested and the amount of released isotope determined using a Compugamma counter (LKB, Milton Keynes, UK). Specific ^{51}Cr release was calculated by the formula:

$$\frac{\text{Experimental Release} - \text{Spontaneous Release}}{\text{Maximum Release} - \text{Spontaneous Release.}} \quad X\ 100$$

4 x 10^7 LNC from $RT1^u$ animals immunised with either an R8 skin graft or the truncated $RT1.A^a$ plasmid vector were additionally cultured for a further 5 days *in vitro* against 2 x 10^7 irradiated (20Gy) DA splenic lymphocytes in 20mls tissue culture medium in 25cm^2 tissue culture flasks (Nunclon). The influence of the initial *in vivo* challenge with A^a antigen on the *in vitro* generation of cytotoxic T cells was assessed by assaying against ^{51}Cr-labelled allogeneic Con A blasts as described above.

3.5.9 Histological Examination of Rejected Hearts

Upon rejection, the animals were sacrificed and the hearts excised and preserved in 10% formal saline solution. After embedding in paraffin wax, the grafts were sectioned (5µm), and stained with haemotoxylin and eosin by the Department of Pathology, Western Infirmary, Glasgow.

CHAPTER FOUR

DISCUSSION

Transplant models in which the donor and recipient share either class I or class II MHC loci have been pivotal in the elucidation of the principle that CD8 and CD4 T cells effect rejection through the recognition of MHC class I and class II antigens respectively. The role however, of CD4 T cells in the rejection of class I MHC disparate grafts has not been clarified, as different models have demonstrated that their participation is variable. This ranges from having no involvement (262, 266, 268), to providing essential help for the generation of a cytotoxic CD8 T cell response (275, 417), and to even effecting graft rejection autonomously (269, 290, 418).

The ability of the CD4 T cell population alone to effect rejection of MHC class I disparate grafts remains a particular puzzle and several mechanisms have been proposed (406). These include MHC class II restricted CD4 CTL (418), MHC class I restricted CD4 CTL (316, 419), the activation of eosinophils (420), the initiation of a DTH response within the graft (250), and finally, the provision of B cell help for the development of an alloantibody response (290, 297, 399). In the latter model, Bradley and colleagues proposed that B cell help is provided by self-restricted CD4 T cells that recognise peptide epitopes of the MHC class I alloantigen. Their observations thus highlight the importance of the indirect pathway of allorecognition in effecting graft rejection. Nevertheless the hypothesis that CD4 T cells recognise additional antigenic disparities has not been conclusively disproved (297), and is supported by the observed discrepancies in the rejection kinetics of heart grafts following priming with either allopeptide or donor cells (245). The experiments detailed in this thesis therefore provide conclusive evidence that rejection can occur when T cell activation is restricted to the indirect pathway and demonstrate for the first time that this is equally applicable to the process of accelerated rejection.

Of the proposed mechanisms by which indirectly restricted CD4 T cells mediate allograft rejection autonomously, it is likely that the provision of B cell help is unique in effecting rapid rejection since the generation of alloantibody directed

against intact alloantigen permits donor cells to be targeted specifically. Otherwise, since indirectly restricted CD4 T cells cannot respond to intact donor antigen, they can only damage graft cells through non-specific bystander mechanisms. Priming indirectly-restricted CD4 T cells to accelerate graft rejection through, for example a DTH response, is dependent upon the recipient APC first infiltrating the graft and then processing and presenting donor antigen. Recipient APC infiltration into the graft is likely to take several days and perhaps explains why in all studies to date allopeptide priming results in either no, or in only modest, acceleration of graft rejection (244-246, 248).

CD4 T cells can also effect the rejection of MHC class I disparate grafts by providing help for the development of an alloMHC class I-restricted cytotoxic CD8 T cell response. Although it was initially suggested that the presence of additional helper determinants on the surface of the donor cell was required for CD4 T helper cell activation (the direct pathway) (263), Auchincloss, using MHC class II-deficient donors, has unequivocally demonstrated that effective help can also be provided by indirectly restricted CD4 T cells (279, 280). The provision of such indirect T cell help for priming allocytotoxicity is therefore analogous to the priming of humoral effector mechanisms as described in this thesis, in that an optimum response relies on a synergistic relationship between the direct recognition of intact allogeneic MHC class I by the effector cell (either the CD8 T cell or B cell) and the indirect recognition of the allogeneic MHC class I by the CD4 helper T cell. Auchincloss' (279, 280) and my experiments therefore emphasise that the requirement for the presence of intact allo-MHC to prime potent effector mechanisms does not preclude a pivotal role for the indirect pathway in their development.

The experimental work of this thesis was modelled on the PVG.R8 to PVG.RT1^{u} rat strain combination as this is ideal for studying the T cell allorecognition pathways responsible for the provision of B cell help. It nevertheless remains an anomaly and it is necessary to explain why, in comparison to other MHC class I disparate models, the alloantibody, and not the cytotoxic CD8 T cell, response predominates. The conventionally-held view that CD8 T cells alone were responsible for the rejection of class I disparate grafts was, however, based on

studies examining skin graft rejection in class I mutant mouse strains (262, 263, 266) and this model is no longer considered representative. The CD8 T cell subset in the class I mutant mouse strains secretes Il-2 and is consequently capable of providing endogenous help for the generation of a cytotoxic T cell response. CD8 T cells in most other species, particularly in humans, do not display this property, and the requirement for CD4 T cell activation, at least to provide CD8 T cell help, is more stringent (270). The lack of contribution of the CD4 T cell subset to rejection in the mutant mouse strains is alternatively explained by the observation that the donor and recipient class I alleles differ by only a few amino acids. Processing of the allogeneic class I MHC molecule by recipient APC may not therefore result in the presentation of immunogenic class II restricted peptide epitopes (406).

The amino acid differences between the donor RT1.A^a allele and the recipient RT1.A^u allele are comparatively more extensive. Immunisation with the RT1.A^a antigen results in the development of a self-restricted CD4 T cell response directed against epitopes derived from these differences (E. Lovegrove submitted). In this model indirectly restricted CD4 T cells appear to effect rejection through the provision of B cell, rather than CD8 T cell help. That cytotoxic T cell killing was detected following either skin grafting or immunisation with the plasmid construct for the full-length, membrane-bound RT1.A^a antigen (Figure 3.5) suggests that a CD8 T cell response does occur during allograft rejection, but that its contribution is masked by the strength of the alloantibody-mediated effector mechanisms.

It is nevertheless possible that the predominance of the alloantibody response, rather than being an intrinsic feature of the PVG.R8 strain's immune system, reflects particular characteristics of the DNA-based immunisation protocol, that the adjuvant effect of DNA polarises the T cell response to favour the provision of help for B cells, and not cytotoxic CD8 T cells (382). As discussed in Section 3.3 however, the mechanisms responsible for accelerated rejection in plasmid-immunised Lewis rats appear to centre on the cytotoxic T cell response, rather than the alloantibody response. This suggests that rejection in the R8 strain is antibody-mediated either because of a relative ineffectiveness of the CD8 T cell

response or because of more efficient complement activity (413, 421), and that this is a strain-specific, rather than a plasmid-specific, effect.

There is yet a further explanation as to why the CD4 T cell and alloantibody responses appear so strong in the PVG.R8 to PVG.RT1^{u} strain combination and this concerns the shared nature of the MHC class II antigen. This theoretically permits CD4 T cell help to be generated by a unique 3-cell cluster model, which differs from the original description by Kelly (292), in that CD4 T cells recognising the peptide/class II MHC complex on the surface of the donor APC may provide cognate B cell help (Figure 4.1). This could only occur if the class II MHC on donor cells were to present a similar spectrum of RT1.A^{a} peptide epitopes (derived from endogenous processing) as would be presented by the recipient APC upon exogenous processing of the RT1.A^{a} antigen. Certainly, membrane proteins are recycled into the class II processing pathway (185) and peptides derived from self MHC class I antigen have been eluted from MHC class II (228), but the recombinant PVG.R8 strain in particular may display a preponderance of MHC class II-associated RT1.A^{a} peptides as a result of incompatibilities at the TAP transporter. The RT1.A^{a} antigen has a high binding affinity for peptides with an hydrophobic C-terminal residue, but the TAP2-B haplotype of the PVG.R8 rat is particularly poor at transporting such peptides into the endoplasmic reticulum (130). The resulting surfeit of empty, unstable RT1.A^{a} molecules would undergo degradation in the endoplasmic reticulum and could enter the class II processing pathway either through transfer into the lysosomal system (422) or by re-routing from the cytoplasm (230, 231). The strongest argument against such a cognate 3-cell cluster model is provided by the beneficial, not detrimental, influence that class II MHC sharing has on human allograft survival. This hypothesis could nevertheless be tested by assessing the influence on the alloantibody response that altering, by perhaps incorporating a permissive TAP haplotype, the spectrum of peptides that is presented by donor PVG.R8 class II MHC.

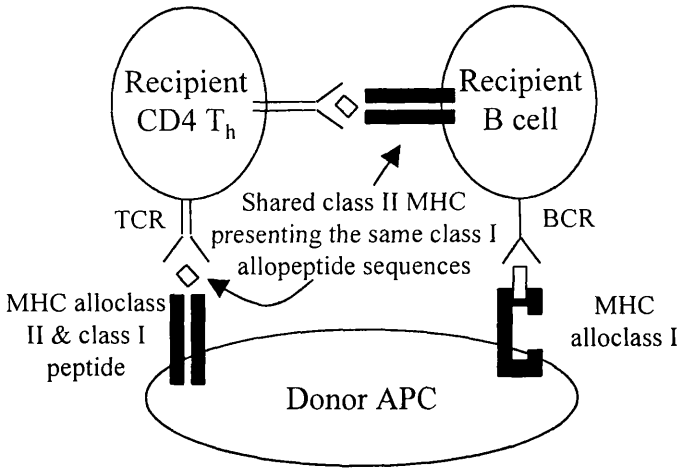


Figure 4.1 Cognate three-cell cluster due to shared class II MHC

The presence of shared MHC class II on the recipient B cell and donor APC permits the formation of, assuming that both cells present similar class I allopeptide, a unique cognate 3 cell cluster that incorporates the recipient CD4 T helper cell (T_h)
BCR B cell receptor.
TCR T cell receptor.

Thus, despite the strong alloantibody response that characterises the rejection of PVG.R8 grafts in PVG.RT1^u hosts being dependent upon CD4 T cell recognition of allo-MHC class I peptide fragments, the possibility that T cell activation occurs through recognition of the shared MHC class II on the surface of donor APC prevents definitive conclusions as to the precise mechanisms by which T cells provide B cell help. Similarly, although the results from my thesis highlight the role of indirect T cell activation in the development of an alloantibody response, they do not necessarily infer that T cell help for the development of the initial alloantibody response to a vascularised allograft is exclusively provided by indirectly restricted CD4 T cells co-operating in a four cell cluster (296). The alternative three-cell cluster model, involving directly-restricted T cells, was proposed to explain why the initial anti-MHC class I alloantibody response was dependant upon the presence of donor MHC class II antigens (281, 292); the two models are distinguished not on their exclusive ability to provide B cell help but rather on the kinetics of the alloantibody response that they are responsible for generating. This difference was not addressed in this thesis because priming with soluble MHC class I alloantigen polarised available T cell help to the indirect pathway and animals were only challenged with a heart graft after the

alloantibody response to the soluble protein had developed. Nevertheless, the kinetics of the alloantibody response to the product of the soluble, pcmu-tA^a vector were similar to the response to a PVG.R8 heart graft (cf. Figures 2.12 and 3.4), which suggests that indirectly restricted CD4 T cells can mediate the development of the early alloantibody response. Uncertainties regarding the rate at which protein is expressed following plasmid DNA injection, however, prevent definitive comparison. It is more reasonable to conclude that indirectly restricted CD4 T cells can effect accelerated rejection, not that they are necessarily responsible for the initial alloantibody response. Additional experiments, such as the use of class II deficient recipients or reconstitution of nude animals with peptide-specific T cell clones, would have to be performed in order to assess the contribution of each model to the development of the primary alloantibody response to a vascularised allograft.

A valid criticism of the experimental work of this thesis is the difference in the physical characteristics of the immunising agents that were administered; i.e. the soluble RT1.A^a protein was administered as a DNA vaccine, whereas the A^a peptides were administered as synthetic protein. They should have ideally been administered either both as DNA vectors or both as recombinant protein. Indirect T cell priming to the RT1.A^a alloantigen could have been achieved using a DNA-based approach by excising the leader signal of the DNA sequence for the RT1.A^a heavy chain. This would prevent trafficking through the endoplasmic reticulum to the cell surface (423), and instead the nascent class I protein would be degraded to peptide fragments within the cell. Likewise heavy chain, β_2M and peptide could have been produced using recombinant techniques and folded *ex vivo* to produce MHC class I alloantigen that displayed the appropriate conformational B cell epitopes (424). The availability of isolated heavy chain would also permit its administration as a means of fully priming the indirect pathway, for it would contain the necessary amino acid sequences for the generation of optimum CD4 T cell epitopes but its tertiary structure would not display the appropriate B cell epitopes for the development of humoral alloimmunity. It is however unlikely, as discussed in section 3.3, that either of these approaches would have altered the results or their interpretation.

One may also contend that the experiments in this thesis are ultimately no more informative than the earlier demonstration that the passive transfer of hyperimmune serum results in accelerated rejection (246, 355), since immunisation with soluble MHC class I alloantigen would be expected to result in the development of an alloantibody response. Surprisingly, the results in this thesis are the first to demonstrate an unequivocal primary alloantibody response to soluble allo-MHC. To date the responses have been undetectable (330, 331), unpredictable (351), or fail to develop until the eighth week (292). Although the effect on graft survival is similar, immunising with soluble protein moreover differs, from the administration of hyperimmune serum, in that it additionally permits examination of the T cell allorecognition pathways that are involved in the provision of B cell help.

The uniquely strong alloantibody response also explains why my experiments are the first to demonstrate accelerated rejection following priming with soluble MHC class I alloantigen. Soluble MHC has in fact generally been proposed as a potent immunoregulatory agent. *In vitro* experiments demonstrate that it exerts its effects by interacting directly with the alloreactive CD8 T cells to block cytotoxic activity and induce apoptosis (321, 333, 338). These effects appear to be dependent on the ability of multimeric forms of MHC class I to cross-link the TCR (321, 333, 338) and earlier conflicting reports on the *in vivo* use of soluble MHC class I alloantigen probably reflect differences in the physical characteristics of the class I alloantigen that was administered (328-331). More recent *in vitro* experiments have, however, demonstrated that monomeric soluble MHC class I can induce apoptosis in alloreactive CD8 T cells, albeit at a concentration five times greater than that required with multimeric forms (321, 425). In addition, the injection of syngeneic hepatocytes expressing monomeric soluble MHC class I alloantigen has now been reported to prolong allograft survival (352). In my experiments injection of the pcmu-tA^a vector had no demonstrable influence on the cytotoxic CD8 T cell subset (Figure 3.6). This is probably a consequence of the monomeric nature of its' product (the hydrophobic transmembrane and cytoplasmic domains have been removed) and of the relatively limited levels of RT1.A^a protein that are released following naked DNA injection, such that effective cross-linking of the TCR of the CD8 T cell did not occur. CD8 T cell inhibition may perhaps have

occurred if an alternative vector system, that achieved higher levels of soluble class I protein expression, had been used. Nevertheless, my results demonstrate that the potential benefits of administering soluble alloMHC class I, even with its dose and valency optimised to achieve maximal inhibition of the direct CD8 T cell response, may be negated by the associated indirect T cell response, which in the presence of intact donor class I MHC may generate powerful effector mechanisms directed against donor graft cells.

In summary, the results from this thesis suggest that the indirect pathway may be more important in effecting graft rejection than is currently appreciated. This may be clinically relevant, for although the indirect pathway has become increasingly prominent over the last decade, it is currently perceived as being more relevant to the process of chronic rejection, because in this situation allorecognition occurs predominantly through the recipient APC. The demonstration that the indirect pathway may be an integral component in the development of effector mechanisms that directly target intact donor antigen suggests that it may be equally pivotal in effecting acute graft rejection. This is especially so, given that the role of alloantibody in effecting acute and chronic rejection has probably been undervalued (426). The recent inclusion of alloantibody-mediated rejection as a separate classification of the Banff criteria for renal allograft rejection (427) suggests that its contribution to clinical transplantation, and consequently, that of the indirect pathway's, will be reassessed. This has practical, and not just theoretical, implications in that tolerogenic strategies specifically targeting the indirect pathway may have a useful clinical role. Intrathymic injection of allopeptide has, in animal models, achieved indefinite survival of completely mismatched grafts (312), suggests that tolerance induced to the indirect pathway may be powerful enough to overcome the influence of T cell activation via the direct pathway. Moreover, only limited donor allopeptide sequences may need to be administered, for the indirect T cell response appears to focus upon only one or two dominant determinants (428). Antigen-specific tolerance remains the ultimate goal of transplantation, because this would avoid the complications of infection and malignancy that are associated with current clinical immunosuppressive protocols. Strategies that target the indirect pathway may represent a novel means of achieving this goal.

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