INDIRECT T CELL ALLORECOGNITION OF DONOR MHC CLASS I ALLOANTIGENS.

by

Adam M. Benham

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Transplantation Biology Unit,
Division of Cell and Molecular Biology,
Institute of Child Health,
University of London,
30, Guilford Street,
London,
WCIN 1EH.

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ABSTRACT

The work in this thesis concerns the role of indirect T cell allorecognition as a component of allograft rejection, using the rat as a model. Previous studies in our laboratory have proven that indirect T cell allorecognition is an important effector mechanism in the rejection of MHC class I mismatched rat skin grafts. I have furthered this work to show that indirect recognition is also an important component of vascularised rat kidney allograft rejection. Interstitial dendritic cell free kidneys from (DAxLEW)F1 donor rats were transplanted into LEW RT1^l recipients that had been previously primed for indirect recognition with DA MHC class I-derived peptides. These animals showed accelerated graft rejection compared with unimmunised controls.

A PVG RTI^{r1} into (PVGxLEW)F1 kidney transplantation model was also investigated, but was found to be unsuitable for unravelling the mechanisms of indirect recognition or for studying tolerance to our donor class I peptides.

The fine specificity of peptide determinants involved in indirect T cell allorecognition in the rat was also investigated. A nested set of 15 amino acid peptides were synthesised; these were derived from a 24 amino acid peptide, P1 (from the DA RT1.A^{av1} MHC class I molecule), known to accelerate rat skin graft rejection. The T and B cell responses to these derivative peptides were observed in the LEW, WAG and PVG rat strains: within the original 24mer, there is more than one T cell epitope towards which the LEW rat mounts a polyclonal T cell response. Furthermore, the number of T cell epitopes within P1 is strain specific.

LEW rats with long surviving allografts, after a 10-14 day course of Cyclosporin A therapy, were assessed for the development of antibodies towards donor DA MHC class I molecules after grafting. Fifteen of 17 rats investigated have no donor MHC class I antibody response. The immune status of some of these rats was examined following P1 priming and was found to vary between individual animals.

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ABBREVIATIONS:

α	alpha
APC	antigen presenting cell
ATP	adenosine triphosphate
ß	beta
$ß_2m$	$ß_2$-microglobulin
BMDDC	bone marrow derived dendritic cell
BSA	bovine serum albumin
BFA	brefeldin A
CAS	concanavalin A supernatant
CD	cluster of differentiation antigen
Con A	concanavalin A
cpm	counts per minute
CsA	cyclosporin A
CTL	cytotoxic T cell
δ	delta
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
ϵ	epsilon
EAE	experimental allergic encephalomyelitis
EBV	epstein barr virus
E. Coli	*escherichia coli*
ER	endoplasmic reticulum
$F(ab')_2$	bivalent fragment of the antigen binding portion of immunoglobulin produced by pepsin cleavage.
FACS	fluorescent activated cell sorter
FCA	freunds complete adjuvant
FCS	fetal calf serum

FDG	fluorescein di-ß-galactoside
FIA	freunds incomplete adjuvant
FISH	fluorescent *in situ* hybridisation
FITC	fluorescein isothiocyanate
g	gram or gravitational acceleration
GPI	glycosyl-phosphatidylinositol
H and E	haematoxylin and eosin
HEL	hen egg lysozyme
hr	hour
HSP	heat shock protein
ICAM	intracellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
IL-R	interleukin receptor
IP3	inositol triphosphate
kD	kilodalton
kg	kilogram
l	litre
LCMV	lymphocytic choriomeningitis virus
LN	lymph node
µCi	microcurie
µg	microgram
µl	microlitre
M	molar
Mab	monoclonal antibody
MBP	myelin basic protein

MBq	megabecquerel
mCi	millicurie
2-ME	2-mercaptoethanol
mg	milligram
MHC	major histocompatibility complex
min	minutes
ml	millilitre
mM	millimolar
NK	natural killer
NP	nucleoprotein
NRS	normal rat serum
OVA	ovalbumin
PBS	phosphate buffered saline
PE	phycoerythrin
PFU	plaque forming unit
PKC	protein kinase C
PVC	polyvinyl chloride
RAM	immunoadsorbent purified rabbit $F(ab')_2$ anti-mouse $F(ab')_2$
RAR	immunoadsorbent purified rabbit $F(ab')_2$ anti-rat $F(ab')_2$
RBC	red blood cell
rpm	revolutions per minute
TBS	tris buffered saline
TCR	T cell receptor
TNF	tumour necrosis factor
UTR	untranslated region
v/v	volume/volume
w/v	weight/volume
X-GAL	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
YAC	yeast artificial chromosome

AMINO ACID SYMBOLS.

Amino Acid	3 letter code	1 letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic Acid	Glu	E
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

CHAPTER 1: INTRODUCTION.

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CHAPTER 1:

INTRODUCTION.

1.1 THE MAJOR HISTOCOMPATIBILITY COMPLEX.

a) GENETICS.

The major histocompatibility complex (MHC) was first discovered through its association with graft rejection in the mouse (Gorer 1936 and 1937) and the region was given the name H-2. Twenty-eight years later, its counterpart, HLA, was discovered in man (Dausset 1958). In the rat, the MHC region is called RT1 (Palm 1964).

The MHC gene cluster can be broadly subdivided into class I and class II genes, that code for proteins involved in immune recognition, and class III genes that code for a variety of proteins involved in the immune response, such as those of the complement cascade (Porter 1985). In man, the MHC gene complex lies on chromosome 6; in the mouse and rat, it is found on chromosomes 17 and 20 respectively. Intensive physical mapping and cloning studies have made the MHC regions of mouse and man amongst the most completely mapped in these species (Trowsdale *et al* 1991). Much of the recent mapping, particularly of the less dense class I area of the MHC, has been accomplished through the use of YAC technology (e.g. Bronson *et al* 1991, Geraghty *et al* 1992b).

In humans, there are three major classical class II loci at the centromeric end of the complex. These are termed DP (formerly SB e.g Trowsdale *et al* 1984), DQ (formerly DC e.g Okada *et al* 1985) and DR (e.g. Spies *et al* 1985), all of which can produce functional α and ß chains. Lying between DP and DQ are a number of genes whose role is different to that of conventional classical class II molecules. These include genes intimately involved with antigen processing, such as those for the TAP transporter (e.g Spies *et al* 1990) and proteasome components (e.g Driscoll

1994), and genes encoded at the DO (Tonelle *et al* 1985) and DM loci (Kelly *et al* 1991a). DM is relatively conserved between man and mouse (Sanderson *et al* 1994) whereas DO is polymorphic between human individuals (Van Endert *et al* 1992). These genes and their possible functions are discussed further in section 1.3

The class II region is followed by a series of class III genes, which include the complement genes C4, Factor B and C2 (Carroll *et al* 1984), tumour necrosis factor (TNF)-A and B (Spies *et al* 1986) and two tandemly linked HSP-70 heat shock proteins (Sargent *et al* 1989). Currently, 4 other HSP-70-like genes have been mapped to different chromosomes in man (Hendershott *et al* 1994). At the telomeric end of the HLA complex are the class I genes B, C (e.g. Bronson *et al* 1991) and A (e.g. Shukla *et al* 1991) which code for the heavy chain of class I molecules and their nomenclature has recently been revised (Bodmer *et al* 1990). It is possible that natural killer cell recognition of T cells is mediated by the HLA-C gene, or one very close to it (Colonna *et al* 1992). In addition, many disease susceptibilities map to the class I region (Geraghty 1993).

In the mouse, the nomenclature of MHC molecules is different and has also recently been rationalised (Klein *et al* 1990): I-A and I-E code for class II genes whereas K, D and L code for class I heavy chains. The mouse is different from man, in that the K region lies centromeric of I-A and I-E. In the rat, the MHC complex is not nearly as well mapped. RT1-A and -C/E loci encode class I genes, with RT1-H, -B, and -D coding for class II genes. The order of the C/E genes is unknown, nor is it certain whether RT1-H expresses functional protein. RT1-A is the only locus demonstrated to restrict cytotoxic T cell responses (Günther and Würst 1984) and the RT1-C product is limited both in its polymorphism and in its distribution, having been found solely on haematopoeitic cells (Stock and Günther 1982). Recently, *in vivo* and *in vitro* experiments in the rat have

demonstrated that the RT1-C region has a crucial controlling role in NK cell allorecognition (Vaage *et al* 1994). The nomenclature of the MHC loci in man, mouse and rat is given in Table 1 and a genetic map of the MHC for these species is provided in Figure 1.

b) NON-CLASSICAL CLASS I GENES.

A number of relatively non-polymorphic class I genes exist, that express protein products in both mouse and man. Their role in immunobiology is uncertain. These genes include HLA-E, -F and -G in man (Koller *et al* 1988, Geraghty *et al* 1990, Geraghty *et al* 1987) and H-2Q, -T and -M in the mouse (e.g Holmes 1989). A number of unexpressed pseudogenes are also found in this region (e.g. Geraghty *et al* 1992a). HLA-G is expressed in the placenta and may have a role in fetal-maternal immunity (Loke and King 1991). In the mouse, expression of the Qa-1 antigen, one of about 24 molecules encoded by H-2T (Hedrick 1992), is upregulated and stabilised after heat shock (Imani and Soloski 1991). This and other H-2T molecules are recognised by gamma-delta T cells (e.g. Vidovic' *et al* 1989), leading to the suggestion that they present antigen to these cells (Bluestone *et al* 1991). The H-2M3 protein appears to be involved in the presentation of N-formylated bacterial peptides (Pamer *et al* 1992, Kurlander *et al* 1992). This was the first demonstration of an immunological role for a non-classical class I molecule. The H-2Q genes of the mouse number between 8 and 10 and most of their products are tethered to the plasma membrane by GPI-linkages (Hedrick 1992). Such molecules can be recognised by cytotoxic T cells, but the presence of presentable peptide remains to be demonstrated. Recently, the non-classical GPI-linked Qa-2 molecule was found to contain peptides that were predominantly nine amino-acids long, but with a stricter ligand specificity for conventional class I molecules (Rötzschke *et al* 1993). A role in the

TABLE 1 (opposite):

Nomenclature of the MHC class I and class II genes for the human, mouse and rat. The table is based on that of Sayegh *et al* 1994.

FIGURE 1 (opposite):

Map of the human MHC (not to scale) showing the relative locations of the MHC class I, class II and class III genes. Other relevant genes in the cluster, such as those involved in antigen processing, are also shown. The map shows the approximate distance in kilobases of the chromosome p21 region encoding these genes, moving left to right from the centromere towards the telomere. Based on Trowsdale *et al* 1991.

TABLE 1:

Species		Class I			Class II		
Human	HLA	A	B	C	DR	DQ	DP
Mouse	H-2	K	D	L	I-E	I-A	-
Rat	RT1	A	E	-	D	B	H

FIGURE 1:
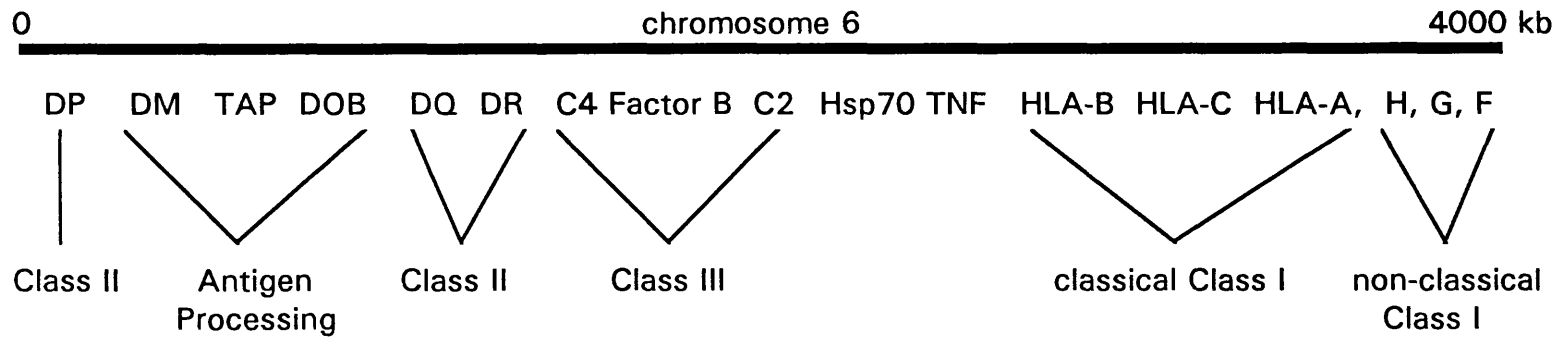
presentation of epithelial pathogen-derived peptides to gamma-delta T cells was proposed.

Soluble class I molecules have been identified in the mouse and in the rat. Q-10 is an example of such a murine protein, where mutation in the transmembrane domain results in a truncated gene product that is not membrane bound and is therefore secreted (Maloy *et al* 1984, Lew *et al* 1986a). In the rat, both a classical RT1.A-like (Spencer and Fabre 1987a) and a non-classical Q-10-like soluble class I molecule have been described (Spencer and Fabre 1987b). Such proteins have also been reported in man (Krangel 1986), but their function is uncertain. The potential of these molecules in transplantation therapy is discussed in section 1.6c.

c) MHC STRUCTURE AND FUNCTION.

Conventional class I molecules are polymorphic, heterodimeric cell surface glycoproteins composed of two chains. The first is a 44kD heavy chain made up of three domains (a_1, a_2 and a_3), each of which is approximately 90 amino acids long. The second is a non-covalently associated 12kD light chain, $ß_2$-microglobulin ($ß_2$m). This non-polymorphic chain is encoded outside the MHC, on chromosome 1 in man. The a_1 and a_2 domains are polymorphic, with regions of hypervariability, whilst the relatively conserved a_3 domain and $ß_2$m lie proximal to the cell membrane. Class II molecules are similar structures, but are made up of heterodimeric 60kD aß chains. Each chain is composed of two domains, a_1 a_2 and $ß_1$ $ß_2$, respectively (Jorgenson *et al* 1992, Germain and Margulies 1993). Both class I and class II molecules function by binding to the T cell receptor and antigen in a trimolecular complex, thus ensuring exquisite molecular specificity (e.g. Ajitkumar *et al* 1988).

A major step forward in immunology came with the elucidation of the structure of the papain-digested, soluble fragment of a human MHC class I

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molecule, that of HLA-A2 (Figure 2), and the simultaneous revelation that it contained antigenic peptide bound within a single groove (Bjorkman *et al* 1987a and b). This was particularly satisfying since it was known that MHC molecules functioned by binding allele-specific peptides (Babbitt *et al* 1985, Buus *et al* 1987), although the nature of the binding site had been unclear.

Other MHC class I crystal structures have subsequently been published (e.g Garrett *et al* 1989, Madden *et al* 1991, Fremont *et al* 1992, Young *et al* 1994) for HLA-Aw68 and HLA-B27, and the murine H-2K^b and H-2D^b molecules respectively. They are all very similar. These structures reveal how a relatively small number of histocompatibility molecules might recognise peptides derived from a vast number of proteins. The a_1 and a_2 domains interact to form an anti-parallel ß-pleated sheet that serves as the base of the groove upon which two a helices sit. The most variable amino acids of the a_1 and a_2 domains are those residues that contribute to the cleft and top of the molecule and therefore play a part in peptide binding and TCR contact.

Using the class I structure as a model, predictions were made for the structure of class II molecules (Brown *et al* 1988) which were shown to be fairly accurate when the first MHC class II structure, that of HLA-DR1, was published last year (Brown *et al* 1993). Subsequent structures of HLA-DR1 complexed with an influenza haemagglutinin peptide at higher resolution (Stern *et al* 1994) and with the bacterial superantigen *S. aureus* enterotoxin B (Jardetzky *et al* 1994) have followed swiftly.

As expected, the class I and class II structures are very similar, but there are important differences. Class I molecules bind orientated, processed peptides of between 8 and 10 residues in the cleft formed by the interaction of the $a1$ and $a2$ domains (Van Bleek and Nathenson 1990, Rötzschke *et al* 1990, Schumacher *et al* 1991, Silver *et al* 1992).

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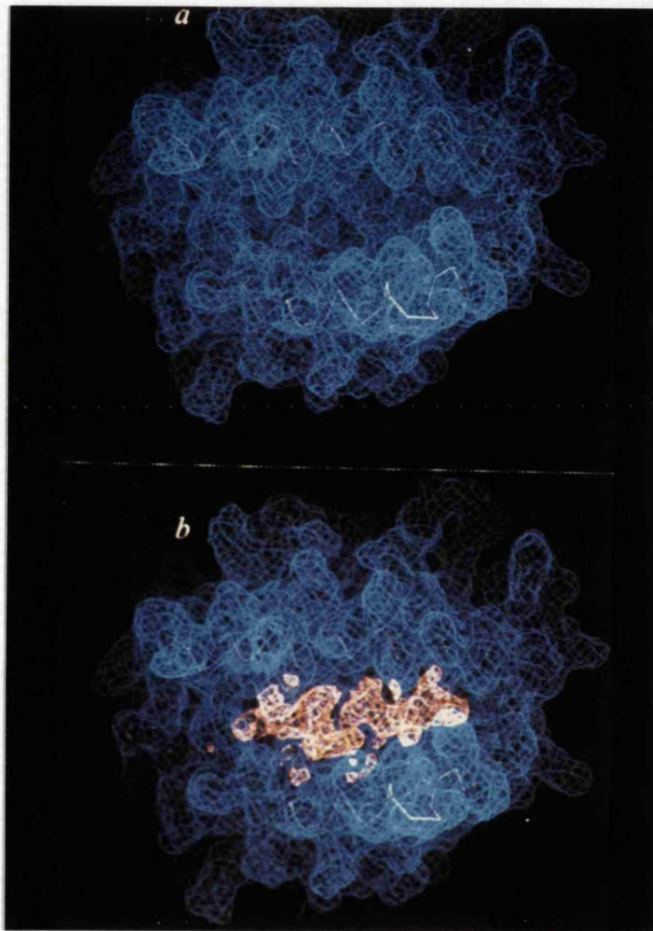
FIGURE 2:

Diagram of the MHC class I molecule without (a) and with (b) peptide in the binding groove. The structures are those of Bjorkman *et al* 1987.

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These frequently include peptides from self proteins, particularly if the cells from which the protein is derived are not infected by virus (Jardetzky *et al* 1991). The groove is closed at either end and longer peptides bulge out in the middle, as was shown for the HLA-Aw68 molecule (Guo *et al* 1992). The structures of five viral proteins associated with the HLA-A2 molecule have also been compared and demonstrate that whilst binding pockets at either end of the molecule are conserved, small changes in peptide bulging at the centre may have a profound influence on antigenicity (Madden *et al* 1993).

Tight peptide binding to class I molecules is regulated both by hydrogen bonding at the N and C termini of the peptide and by 2-3 allele-specific conserved pockets at either end of the groove (Falk *et al* 1991), that allow a wide range of peptides to bind with high affinity and precise T cell specificity (Matsumura *et al* 1992). The HLA-B27 structure in particular has been useful for examining a high resolution image of tight peptide binding and has revealed a conserved arginine anchor residue at position 2 that may be involved in disease association (Madden *et al* 1992). Consensus motifs of subpicomolar quantities of natural peptides eluted from HLA-A2 class I molecules have also been identified using exquisitely sensitive mass spectrometry techniques (Hunt *et al* 1992a).

The class II peptide binding cleft is open ended and the α helices lining the groove slightly less restrictive, allowing a heterogeneous set of longer peptides (average size 15-18 amino acids) to bind in extended conformations. Class II molecules appear to bind 'promiscuous' peptides (Sinigaglia *et al* 1988) and nested peptides derived from the same precursors (Rudensky *et al* 1992). In the class II groove, there are more conserved pockets than for class I, and these are spaced, rather than clustered, to allow elongated binding. This was apparent from the structure

of a single haemagglutinin peptide bound to HLA-DR1 (Stern *et al* 1994). It is held in an extended, twisted conformation by twelve hydrogen bonds between the peptide backbone and the class II cleft, with a third of its surface accessible for binding with the TCR. Five of the thirteen residues of this peptide are sequestered by pockets in the binding site. The open nature of the class II cleft means that binding motifs have been hard to identify when compared with those of class I binding peptides.

Naturally processed peptides bound to murine I-A^d class II molecules have been sequenced and found to originate exclusively from secretory or membrane proteins from the APC itself (Hunt *et al* 1992b). Similarly, peptides bound to purified HLA-DR molecules, obtained from EBV-transformed B cells, have been studied by acid extraction (Chicz *et al* 1993). Many of these peptides were nested sets and were between 10 and 34 residues in length. They were predominantly derived from endogenenous proteins that had encountered the endocytic pathway, especially other HLA molecules. Of the eluted exogenous peptides, some were particularly promiscuous and were able to bind to all the DR alleles investigated.

Though *in vitro* studies have found peptides of 9-10 residues capable of binding class II molecules (Rothbard and Gefter 1991), this study demonstrates that longer peptides may be physiologically relevant and protected from further degradation, since only 27 of the 201 sequenced peptides had fewer than 15 residues. This in depth study supplemented previous work that sequenced peptides from I-E^b and I-A^b mouse B cell lymphomas and found them to be truncated variants between 13 and 17 amino acids long (Rudensky *et al* 1991a and b). In this investigation, however, only a few distinct peptide species were found.

The crystal structure of HLA-DR1 occurred as a dimer in each of the derived structures, giving rise to speculation that T cell signalling may be enhanced by dimerization and that positive and negative selection in the

thymus could be partly controlled by dimer formation (Ploegh and Benaroch 1993). As yet, there is no biochemical evidence that dimerisation occurs *in vivo*.

With conventional antigens, tertiary changes caused by peptide binding, the different sequences of bound peptides and the accessible parts of the MHC molecule all contribute to immunogenicity (e.g. Silver *et al* 1992, Fremont *et al* 1992). Certainly, modifying one residue of an ovalbumin peptide binding to the H-2K^b class I molecule can alter T cell recognition without changing the class I-peptide conformation (Chen *et al* 1993). The situation is different with a superantigen, however. Such a molecule, usually from a retrovirus or bacterium, binds specifically as an intact protein, rather than a peptide fragment, away from conventional MHC/TCR binding sites (Torres *et al* 1993) and can stimulate a large number of T cell clones (e.g Gao *et al* 1989). The N-terminus of the *S. aureus* enterotoxin B protein binds to DR1 a1 and presents a binding site to the TCR that lies above and to the side of the normal MHC peptide-binding groove, thereby blocking it (Jardetzky *et al* 1994). There are few conformational changes as a result of this interaction and the biological effects are probably mediated by unusual TCR-antigen-MHC contacts on the V$_\beta$ chain (Choi *et al* 1991).

1.2 ANTIGEN PRESENTATION.

The immense interest in MHC class I and class II antigens reflects their central importance to antigen processing and presentation, in conventional immune responses and in transplantation, where the structure of MHC molecules themselves are just as relevant as their role in antigen presentation.

Class I molecules are found on the surface of most cells and are generally involved in the presentation of peptides derived from intracellular

pathogens (e.g Chen and Parham 1989) to CD8$^+$ T cells, whose activation results in antigen specific target cell destruction. Class II molecules, however, have a more limited distribution and appear on specialised antigen presenting cells (APCs), which usually also express class I molecules. Class II molecules generally present exogenously derived peptides to CD4$^+$ T cells which can then provide help for a number of immune reactions. Contact between class II and CD4 and with class I and CD8 occurs and is required for activation (Dembic' *et al* 1987, Norment *et al* 1988, Gay *et al* 1987). CD4 (55kD) and CD8 (32-34kD) are cell surface accessory/co-receptor molecules found on about 75% and 25% of peripheral T cells respectively (Parnes 1989); CD8 is normally expressed as a heterodimer, whereas CD4 is a single chain (Littman 1987). In mouse thymocytes, CD8 can also exist as an *αα* homodimer. The structure of their extracellular domains has been determined (Wang *et al* 1990, Ryu *et al* 1990, Brady *et al* 1993, Leahy *et al* 1992), though the complete structure of all four domains of CD4 is still awaited.

Cells with antigen presenting capabilities *in vitro* include B cells (Ashwell *et al* 1984, Rock *et al* 1984, Lanzavecchia 1985), T cells (e.g. Pichler and Wyss-Coray 1994), dendritic cells (Steinman *et al* 1979, Sunshine *et al* 1980), macrophages (e.g. Rosenthal and Shevach 1973, Unanue 1984) and Langerhans cells (Stingl *et al* 1978). Whether all these cells operate as APCs *in vivo* is open to question: even planar lipid membranes bearing class II molecules can present antigen *in vitro* (Watts *et al* 1984, Babbitt *et al* 1985), though in the absence of accessory molecules, T cell non-responsiveness can occur (Quill and Schwartz 1987).

Resting B cells cannot activate resting T cells *in vivo*, as was demonstrated by exploiting chimeric chickens that had host T cells and APCs but donor B cells (Lassila *et al* 1988). These animals had been neonatally treated with cyclophosphamide, a procedure which obliterated

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the stem cells that would subsequently have migrated to the bursa (the specialised avian B cell compartment).

The activation state of the APC, as well as that of the T cell (e.g. Croft *et al* 1992, Ronchese and Hausmann 1993), is an important consideration, as is the fact that *in vitro* studies often utilise immortalised cell line and do not account for spatial restrictions encountered *in vivo*. For example, a recent study demonstrated that potentially self-reactive B cells were unable to access lymphoid follicles, where hypermutation and memory B cell formation occur (Cyster *et al* 1994). Instead, they are competed out by anti-foreign B cells and are condemned to die. Antigen presentation to T cells takes place at different stages of T cell development and at different locations within the body. Primary T cell activation, for example, is restricted to the lymphoid tissue, whereas for secondary T cell responses, pre-primed T cells may be more widely dispersed. In the latter case, any class II-bearing cell may be able to cause activation, whereas in the former case, antigen presentation is restricted to the APCs within the lymphoid tissue (Knight and Stagg 1993).

Antigen structure also affects presentation by B cells. For example, a repetitive form of a viral envelope protein, VSV-G (IND), elicits a good antibody response in mice, whereas the same antigen at the membrane of infected cells is ignored by B cells (Bachmann *et al* 1993).

Naturally, there are exceptions to the generalisations of T cell activation: for example, some $CD8^+$ T cells are class II restricted (e.g. Rahemtulla *et al* 1994). Functional $CD8^-$ MHC class II restricted T cells have also been found in mice lacking CD4 (Locksley *et al* 1993). Similarly, some bacterial antigens can be presented by class I molecules following phagocytosis (Pfeifer *et al* 1993) and some $CD4^+$ cells are class I restricted (Bendelac *et al* 1994). In this latter case, a subset of murine thymocytes exhibit MHC class I dependent positive selection into $CD4^+$ cells (see

section 1.5b) and this process is independent of the CD8$^+$ co-receptor.

1.3 ANTIGEN PROCESSING.

a) PEPTIDE AS ANTIGEN.

Because most cytotoxic T lymphocytes (CTLs) target MHC class I restricted, intracellular proteins, it was for a long time considered that intact viral glycoproteins, expressed on the surface of infected cells, must be the targets for attack. The realisation that cytotoxic T cells recognise degraded forms of antigen (just like their helper counterparts) rather than integral glycoproteins, came with a series of experiments in the mid-1980's. Townsend's group studied a non-glycosylated viral nucleoprotein (NP), that accumulates in the nuclei of infected cells. They used truncated NP proteins that lacked signal sequences, and could therefore not be trafficked to the ER for export, to transfect into murine L cells. CTLs were obtained that recognised 3 discrete NP epitopes, demonstrating that the protein must be degraded intracellularly and transported to the surface for MHC class I restricted survey by some hitherto unknown mechanism (Townsend *et al* 1985). Further experiments showed that these NP epitopes could be defined by small, synthetic peptides that did not necessarily invoke an antibody response, lending weight to the idea that CTLs 'see' short stretches of peptide associated with class I molecules (Townsend *et al* 1986a). Glycosylated, integral membrane proteins are also similarly processed: the influenza haemagglutinin molecule with and without its signal sequence was used to demonstrate that insertion into the plasma membrane had no effect on the determinants displayed to CTLs at the cell surface (Townsend *et al* 1986b). Another group used mouse cells transfected with HLA genes to obtain class I restricted CTLs that recognised human class I as their antigen. Such CTLs could lyse syngeneic, HLA-negative mouse cells if

synthetic HLA peptides were provided (Maryanski *et al* 1986). This was also the first demonstration that allogeneic HLA peptides could bind to intact HLA molecules.

Biological proof that peptide antigens did actually associate with class II and class I molecules was obtained at around the same time. In the former case (Babbitt *et al* 1985), I-A^k molecules from B-cell hybridomas were purified, detergent solubilised and shown to bind a hen egg lysozyme (HEL) peptide known to be involved in presentation. In the latter case (Guillet *et al* 1986), T cells specific for peptides derived from the bacteriophage lamda cI protein were investigated. This group also found that T cell activation could be competitively inhibited by unrelated peptides and this led them to suggest a model of the MHC-peptide complex that was very close to the actual structure.

The mechanisms by which class I and class II molecules bind specific peptides, and the way in which such peptides are generated, are complicated but are becoming clearer (e.g. Germain and Hendrix 1991). In the class I pathway, heavy chain and ß$_2$m synthesis occurs in the endoplasmic reticulum (ER). Stable assembly of heterodimers depends on the presence of presentable antigen (Townsend *et al* 1990, Schumacher *et al* 1990) and subsequent folding may also require other factors such as the p88 chaperone, calnexin (Degen *et al* 1992).

b) **THE PROTEASOME.**

The generation of class I presentable peptides begins in the cytosol and probably involves the proteasome (Goldberg and Rock 1992), a multi-subunit protease two of whose components, LMP 2 and LMP 7, are encoded within the MHC (Glynne *et al* 1991, Ortiz-Navarrete *et al* 1991, Martinez and Monaco 1991, Kelly *et al* 1991b). The role of the proteasome in antigen presentation is open to debate. Some workers have used cells

lacking LMP 2 and 7 to demonstrate unaltered kinetics of antigen presentation (Yewdell *et al* 1994). Others have transfected rat peptide transporter genes (see below) into a cell line that lacks both the transporter and LMP 2 and LMP 7. This was sufficient to restore antigen presentation, indicating that LMP 2 and LMP 7 are not essential for processing (Momburg *et al* 1992). However, this report may be inaccurate, since cells expressing high levels of cell-surface class I were preselected for analysis. This conclusion has therefore been challenged by experiments that utilised knockout mice lacking LMP 7 (Fehling *et al* 1994). These animals express low levels of cell surface class I molecules and present endogenous antigen inefficiently. *In vitro* experiments have shown that the purified 20S proteasome subunit can generate antigenic peptides for class I restricted antigen presentation (Dick *et al* 1994).

Other proteasome purification studies demonstrated that LMP 2 and LMP 7 may have a more subtle effect, by increasing the proteasome's endopeptidic activity towards substrates with hydrophobic or basic residues preceding the cleavage site (Driscoll *et al* 1993). A ubiquitin-dependent aspect to proteolysis is also involved in peptide generation in the class I pathway (Michalek *et al* 1993).

c) THE TAP TRANSPORTER.

Peptide fragments are transported into the ER (Kleijmeer *et al* 1992) by heterodimeric, ATP-dependent MHC-encoded transporters: the TAP1 and TAP2 proteins (Powis *et al* 1991, Kelly *et al* 1992, Spies *et al* 1992, Attaya *et al* 1992). Homologous TAP proteins have been found in man (Trowsdale *et al* 1990, Spies *et al* 1990), mouse (Monaco *et al* 1990) and rat (Deverson *et al* 1990); in this latter animal, polymorphism of TAP 2 results in a different array of allele-specific peptides binding to class I molecules (Powis *et al* 1992). The availability of two TAP deficient cell

lines (first described by Townsend *et al* 1989) has been very valuable in the elucidation of class I-associated antigen presentation: these are the RMA-S TAP 2 deficient mouse cell line and the T2 TAP 1 and TAP 2 deficient human cell line.

Expression of TAP proteins in such mutants demonstrated that the transporter was necessary both for peptide translocation and class I assembly, and that the process was ATP-dependent (Neefjes *et al* 1993). The selectivity and specificity of these proteins has recently been investigated *in vitro* by transfecting human, rat and mouse TAP-deficient cell lines with the relevant TAP genes to reconstitute antigen presenting capacity (Momburg *et al* 1994a): in these transfectants, TAP proteins display a degree of selectivity for the C termini of peptides that they transport and thus influence the profile of peptides presented by class I molecules; the transporter is also size selective (Momburg *et al* 1994b). Experiments in the mouse reach similar conclusions (Schumacher *et al* 1994), with the transporter selecting peptides with a minimum of nine amino acid residues. A peptide efflux mechanism also appears to operate at this stage of antigen processing, maintaining a low peptide concentration in the ER to favour the binding of high affinity peptides to class I molecules.

TAP deficient cell lines have also been used to implicate a second pathway of ER proteolysis in antigen presentation (Henderson *et al* 1992, Wei and Cresswell 1992): class I molecules from these cells contained a smaller array of peptides, with more than nine residues, derived from the signal sequences of ordinary cellular proteins. Furthermore, TAP deficient cells are able to present endogenous, DR1-restricted cytosolic antigen to CD4$^+$ T cells (Malnati *et al* 1992) thus emphasising the flexibility of antigen presentation.

Once a peptide reaches the ER, further trimming may occur before the trimeric ß$_2$m-heavy chain-peptide complex is formed. This process

seems to be mediated by the chaperone calnexin (Ou *et al* 1993), which assists in the assembly of class I molecules before physically associating them to the TAP transporters to facilitate peptide binding (Jackson *et al* 1994, Ortmann *et al* 1994, Suh *et al* 1994). The trimeric complex is then transported to the cell surface via the Golgi apparatus. At this stage, the rate of transport and assembly of loaded complexes can differ, even between different alleles of the same cell line (Neefjes and Ploegh 1988).

d) TRAFFICKING OF MHC/PEPTIDE COMPLEXES.

In the class II pathway (e.g. Cresswell 1994), the assembly and transport of heterodimers is initially guided by the invariant chain (Ii), of which there are a number of different molecular weight species (Teyton and Peterson 1992). Intact Ii can exist as a membrane protein (Koch *et al* 1982), and 2-5% is expressed on the cell surface as a chondroitin sulphate form that may have a role as an accessory molecule (Naujokas *et al* 1993). Cross-linking studies with DR molecules from a B cell lymphoma have shown that Ii and class II *a* and ß heavy chains form a nonameric complex during biosynthesis (Roche *et al* 1991) but the order of assembly is not clear. Once assembled, the nonameric class II-Ii complex is transported through the Golgi to the trans-Golgi reticulum (Romagnoli *et al* 1993). Ii carries a sorting signal at its carboxy terminus (Bakke and Dobberstein 1990, Lotteau *et al* 1990) which routes class II complexes into the endocytic pathway. This region, containing a 25 residue CLIP segment that is often eluted from purified class II molecules, is crucial for class II assembly, transport and peptide acquisition (Romagnoli and Germain 1994). It is at this stage that the class II molecules (bound to Ii derivatives) intersect vesicles containing internalised, degraded antigen (Davidson *et al* 1991). The mechanisms of peptide generation from antigen are unclear, but recent murine *in vitro* studies have implicated the enzyme Cathepsin D in

the generation of presentable peptides (Van Noort and Jacobs 1994). Cathepsin B may be involved in the trimming of fragments generated by Cathepsin D.

Until recently, the precise site at which class II molecules encountered the endosomal pathway was unknown, although class II molecules had been observed in endocytic compartments with lysosomal characteristics (Peters *et al* 1991). However, an exciting new development has been the isolation and characterisation of a specialised endocytic/class II compartment. This was biochemically demonstrated in B cells and was shown to transiently acquire class II molecules that are then loaded with peptides (Amigorena *et al* 1994). A similar compartment was found in a class II-expressing human melanoma cell line in which Ii is degraded whilst class II molecules procure peptide (Tulp *et al* 1994). Antigen-gold labelling studies of human B cells have also confirmed the existence of this discrete class II processing compartment (West *et al* 1994). It should be emphasised that MHC trafficking needs to be studied in a number of different APCs, since it is quite possible that antigen processing can vary between different cell types.

The actual influence that Ii has on the repertoire of peptides that can be presented by class II molecules is uncertain. Experiments, using spleen cells as APCs from H-2^s transgenic mice that were either Ii^o or Ii$^+$, indicated that presentation of epitopes from an endogenous protein, MBP, is reduced or prevented in Ii$^+$ mice but that other epitopes require Ii for their presentation (Bodmer *et al* 1994). Other work (Peterson and Miller 1992) suggests that a minor form of Ii (p41) can expedite antigen presentation.

Some extremely interesting studies using human mutant B cells that lack the non-classical HLA-DMA and DMB genes have shown that these molecules are required for the assembly of conventional class II-peptide complexes and perhaps function as receptors for degraded Ii protein (Morris

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et al 1994, Fling *et al* 1994). This follows previous work on class II processing mutants that demonstrated that HLA-DR molecules bound long, Ii derived peptides (CLIP) that would normally be exchanged for antigenic peptide in a wild type APC (Riberdy *et al* 1992, Sette *et al* 1992). Perhaps non-classical class II molecules can act as CLIP acceptors and peptide carriers during class II processing. Endogenous peptides from MHC related molecules also form the bulk of the material acid-eluted from HLA-DR molecules (Chicz *et al* 1993), hinting at a functional role for MHC molecules in the trafficking of their counterparts or possibly to prevent overstimulation by antigenic peptides when the complexes reach the cell surface.

Proteolytic Ii degradation, rather than peptide loading, determines the rate at which class II molecules pass through the endosomal milieu (Neefjes and Ploegh 1992). The fact that class II molecules associated with peptide are more stable *in vitro* than *in vivo* has given rise to speculation about peptide exchange mechanisms between class II molecules at the cell surface (e.g. Adorini *et al* 1989). However, this is now thought to be unlikely, given that physiological half life measurements of cell surface class II-peptide complexes in human APCs indicate that peptide is irreversibly bound (Lanzavecchia *et al* 1992). It is still possible that some class II molecules are reloaded and recycled after internalisation (Adorini *et al* 1991) though this contribution to antigen presentation is likely to be small.

The distinction between class I and class II processing pathways is not always clear. This has become evident from studies using the drugs chloroquine (a weak base that halts processing via the 'exogenous' class II pathway) and brefeldin A (BFA, which disrupts ER to golgi transport and thus the 'endogenous' pathway). Using these drugs, DR transfected human fibroblasts infected with influenza virus are efficiently lysed by CD4$^+$ cytotoxic T cells. Antigen presentation is not blocked by chloroquine and

the invariant chain is not needed (Sekaly *et al* 1988). Influenza A matrix protein can also be presented by a chloroquine sensitive, BFA insensitive or a chloroquine insensitive, BFA sensitive pathway (Nuchtern *et al* 1990), suggesting the involvement of two pathways, at least for flu-like viruses.

1.4 THE T CELL RESPONSE.

Once MHC-peptide complexes have reached the cell surface, they are available for survey by either cytotoxic or helper T cells and a mere 80-500 complexes can be sufficient for T cell activation (Harding and Unanue 1990, Demotz *et al* 1990). The original observation of 'MHC restriction' i.e. that T cells only recognise antigen presented by self APCs expressing self histocompatibility molecules (Rosenthal and Shevach 1973, Zinkernagel and Doherty 1974, Bevan 1975) can now be explained by the need for a trimeric TCR-MHC-peptide interaction if T cell stimulation is to occur (Davis and Chien 1993). Sequence analysis and molecular modelling of the TCR suggests that its *α* and ß gene products are structurally similar to Ig heavy and light chains (Davis and Bjorkman 1988). It has been proposed that amino acids encoded by the V region (see section 1.4a below) of these genes contact the *α* helices of the MHC molecule whilst the VD and DJ junctional regions interact with peptide bound in the MHC groove (see Figure 3). The elucidation of a crystal structure of the TCR/MHC complex would help clarify matters and would be a great step forward in immunobiology.

TCR contact with the CD4 and CD8 molecules also occurs during APC/T cell communication (e.g. Salter *et al* 1990); indeed, a whole series of adhesion molecules and co-receptors help mediate the binding of these cells and some of these events are discussed below.
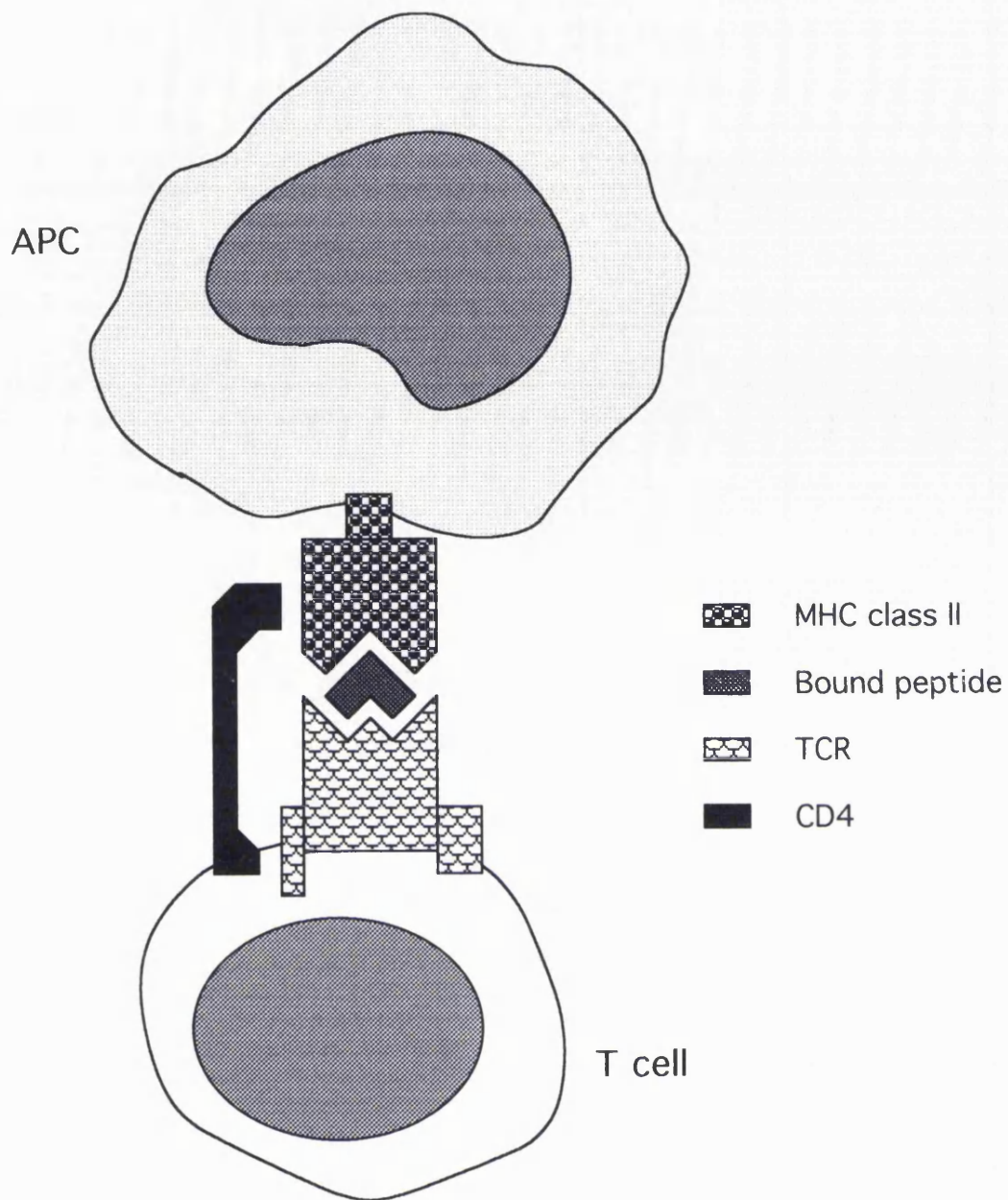
FIGURE 3:

Schematic representation of TCR and MHC molecules in apposition on a T cell and an APC. The MHC independently contacts both the TCR and peptide, as well as the costimulatory molecule CD4. The diagram is based on that of Davis and Bjorkman 1988.

a) THE T CELL RECEPTOR.

The main component involved in antigen specific recognition on the T cell surface is the T cell receptor (TCR). This structure was first identified in the mouse (Allison *et al* 1982, Haskins *et al* 1983), using monoclonal antibodies (Mabs) that blocked T cell clone-specific function, two years after the TCR-associated molecule, CD3, was discovered (Reinherz and Schlossman 1980). CD3 is now known to be composed of δ ϵ and gamma chains as well as (usually) a zeta-zeta dimer (see Figure 4). The TCR exists either as an $\alpha\beta$ or rarer gamma-delta heterodimer whose specificity can evolve through gene rearrangement during somatic mutation.

The human TCR was originally found on both class I and class II restricted T cells (Meuer *et al* 1983 a and b), again by screening for Mabs that specifically prevented antigen induced proliferation of individual T cell clones. The sequences of these two TCRs were different, even though they came from the same donor. This provided the first clue that they were antigen receptors and that somatic mutation was involved in the generation of TCR specificity. The α and β subunits detected in these experiments were disulphide linked membrane glycoproteins of 49 and 43kD, respectively. Subsequent studies showed that malignant T cells at later stages of differentiation could synthesize and express unique epitopes of the $\alpha\beta$ dimer (Acuto *et al* 1983a and b). This also provided evidence that $\alpha\beta$ TCR specificity was clonotypic and could evolve through somatic mutation.

Cell surface expression of the TCR also depends on that of CD3 (Weiss and Stobo 1984) and co-precipitation of the two complexes under certain conditions suggested that CD3 and TCR $\alpha\beta$ are linked at the cell surface (Reinherz *et al* 1983). Cross-linking experiments (Brenner *et al* 1985) later proved this spatial association, with the β chain lying proximal

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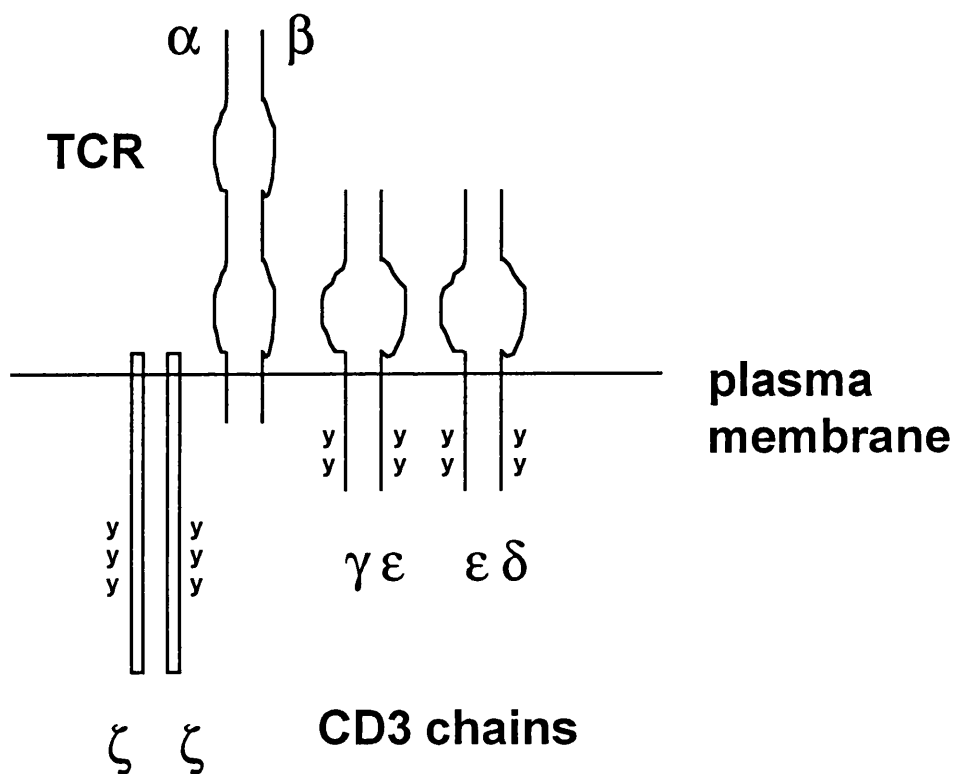
FIGURE 4:

Proposed structure of the TCR with the associated CD3 complex at the plasma membrane. y = potential tyrosine sites for phosphorylation following receptor engagement.

to the CD3 heavy chain. Each TCR subunit associates with an $\epsilon\delta$ or epsilon-gamma dimer, but the stoichiometry is unknown. Attempts to purify TCR $\alpha\beta$ subunits were hampered by difficulties in obtaining enough purified material, though amino-acid sequencing of the 12 N-terminal residues of the ß subunit revealed weak homology to the first framework region of the human lamda light chain V region (Acuto *et al* 1984). Genetic techniques were required to obtain the complete sequences of the α and ß chains in both mouse and man (Saito *et al* 1984a, Chien *et al* 1984 and Patten *et al* 1984). Ironically, the first TCR gene to be genetically isolated in the mouse, the gamma chain, had hitherto been undetected by protein chemistry (Saito *et al* 1984b). It was not until 1986 that Mabs were used to detect a second TCR receptor, gamma-delta (35 and 45kD respectively), on a limited number of murine fetal lymphocytes (Lew *et al* 1986b) and human lymphocytes lacking the $\alpha\beta$ genes (Brenner *et al* 1986). The gamma-delta receptor is also subject to gene rearrangement (Lefranc and Rabbitts 1985) and can be found on dendritic cells in the skin (e.g. Stingl *et al* 1987).

The isolation of TCR cDNA clones permitted their analysis on a genetic level (e.g. Hedrick *et al* 1984a and b, Yangi *et al* 1984) and revealed their sequence homologies with the Ig V, D and J gene sequences. Each α, gamma, ß and δ locus thus has a number of potential V, J and C regions, with the latter two additionally possessing D regions. Functionally rearranged protein is expressed after the occurrence of genetic recombination events that either involve deletion or inversion (e.g. Fujimoto and Yamagishi 1987, Malissen *et al* 1986). Diversity is generated within the V gene itself and at the VJ or VDJ borders (see Figure 5).

The α and δ genes are encoded together at one locus in the germline configuration, and the δ segment must be deleted before productive α
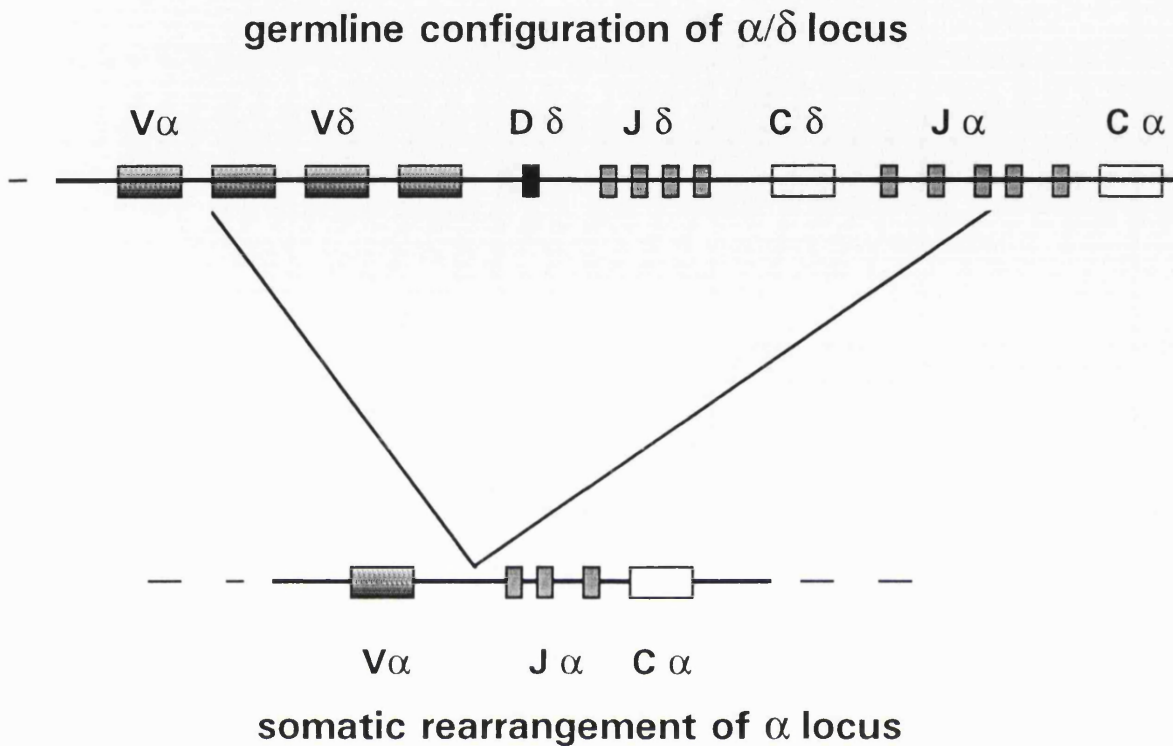
germline configuration of α/δ locus

somatic rearrangement of α locus

rearrangements (and hence *a*/ß TCR expression) can occur. It was initially proposed that one cell bore one specific receptor type and that functional expression of the TCR on the cell surface prohibited further allelic rearrangements. Whilst this is true for the ß chain, it is not so for the *a* chain, and T cells bearing two functional and independent *a*ß receptors have been found in the mouse (Padovan *et al* 1993). A subset of human gamma-delta cells expressing two functionally discrete receptors, encoded by different gamma alleles, also exists (Davodeau *et al* 1993).

b) T CELL SIGNALLING.

The way in which a T cell responds to presented antigen depends not only on the trimolecular TCR-antigen-MHC interaction but also on the cytokine microenvironment, the avidity of adhesive interactions with the APC and the binding of a number of co-stimulatory molecules. These interplays shape the ultimate outcome of antigen presentation: for example, apoptosis or positive selection in immature thymocytes, or, in mature T cells, differentiation, anergy or death. Over 70 molecules are specifically regulated during T cell activation between two hours and seven days after stimulation (Crabtree 1989). In order to divide and diversify, IL-2 (and IL2-R) transcription is necessary, and it is the exquisite control of this cytokine, through a variety of kinases and phosphatases, that has been the central focus of T cell signalling studies (e.g. Weiss 1993,).

That the TCR/CD3 complex mediates signalling in peripheral T cells was first suggested by increases in the levels of cytoplasmic free Ca^{2+} after CD3 dependent activation (Weiss *et al* 1984). This increase in cellular Ca^{2+} and the accompanying activation of protein kinase C (PKC) is brought about by the second messengers 1,4,5, inositol triphosphate (IP3) and 1,2-sn-diacylglycerol (DAG), respectively. IP3 and DAG are formed from

phosphatidyl inositol 4,5-bisphosphate when phospholipase C gamma 1 is tyrosine phosphorylated after TCR engagement. Though precise mechanisms are unclear, there is a causal relationship with IL-2 production (Imboden and Weiss 1987), since calcium ionophores can elicit IL-2 transcription in T cell tumour lines. However, IL-2 is still generated in the absence of this pathway (Sussman *et al* 1988), indicating that other signals are involved. The influence of Ca^{2+} on signalling has been clarified by studying the mechanism of action of the immunosuppressive drugs CsA and FK506 (Thomson and Starzl 1994, O'Keefe *et al* 1992). These drugs have different structures but both bind to immunophilins, which have cis-trans peptidyl-prolyl isomerase activity. Their mode of action, when incorporated as a drug/immunophilin complex, involves interacting with (and inhibiting) calcineurin, a Ca^{2+}/calmodulin dependent serine phoshphatase (Liu *et al* 1991b, Clipstone and Crabtree 1992). This discovery focussed interest on the role of calcineurin in the regulation of IL-2. For example, overexpression of calcineurin results in hypersensitivity to T cell signalling in Jurkat T cell lines (Schreiber 1992).

Normally, in the absence of immunosuppression, elevated Ca^{2+} levels activate the latter of two nuclear protein transcription factors, NF-IL2IA and NF-AT (e.g. Jain *et al* 1993), that bind to two discrete sites within the IL-2 enhancer and whose induction is both Ca^{2+} dependent and CsA/FK506 sensitive (Flanagan *et al* 1991). NF-AT has two subunits: the first is a preformed, CsA/FK506 sensitive 110-140kD cytoplasmic phosphoprotein that translocates to the nucleus upon dephosphorylation by calcineurin (McCaffrey *et al* 1993). The second is a smaller, nuclear component, probably a Fos/Jun heterodimer, to which its partner binds. This protein is activated by ras, a 21kD GTP binding protein and GTPase that is itself regulated by both nucleotide exchange proteins (e.g. sos and vav) and GTPase activating proteins (GAPs). Fos and Jun are controlled by

both post-translational modifications and their rates of transcription. Fos induction is controlled by phosphorylation of its own transcription factor, TCF, by two MAP kinases, ERK 1 and ERK 2 (Gille *et al* 1992). Jun induction is also mediated by a MAP kinase, called JNK (Derijard *et al* 1994), which was the first protein whose effects were shown to integrate several discrete signalling pathways (Su *et al* 1994). Fos and Jun can also bind as an AP-1 complex to a separate IL-2 enhancer site. The intricate details of these kinase cascades are also being unravelled in non-lymphoid cells (Crews and Erikson 1993) and appear to be conserved in a wide range of cell types.

Events at the plasma membrane also heavily depend on tyrosine kinases and serine/threonine kinases after TCR triggering. Two discrete families of tyrosine kinases are involved:

1) the src-related tyrosine kinases lck, fyn and yes; these are plasma membrane associated, myristylated on their N-terminal glycine residues and contain both an SH3 domain and a negative regulatory site for tyrosine phosphatase (e.g. Rudd *et al* 1994).

2) the ZAP 70 protein tyrosine kinase family (Chan *et al* 1992), whose members posses two SH3 domains and a terminal catalytic domain (e.g Rudd *et al* 1994). They are not thought to be membrane associated.

The relevance of lck and fyn has been demonstrated in both mice and in T cell lines that are either deficient in or over express these proteins (e.g. Abraham *et al* 1991). In the former case, events associated with signal transduction are impaired, whereas in the latter, increased sensitivity to TCR stimulation is observed (Karnitz *et al* 1992, Veillette *et al* 1991a, Davidson *et al* 1992). ZAP 70, which is exclusive to T and NK cells, associates with tyrosine-phosphorylated forms of the CD3 zeta chain via its SH2 domains (Wange *et al* 1993). Humans lacking ZAP 70 are severely immunodeficient, lack $CD8^+$ T lymphocytes and have peripheral $CD4^+$ T

pm

invariant
chain
protein

tyr

tyr

p

p

p

p

p

p

src type
PTK

syk/ZAP 70
type PTK

cytoplasm

p

src type
PTK
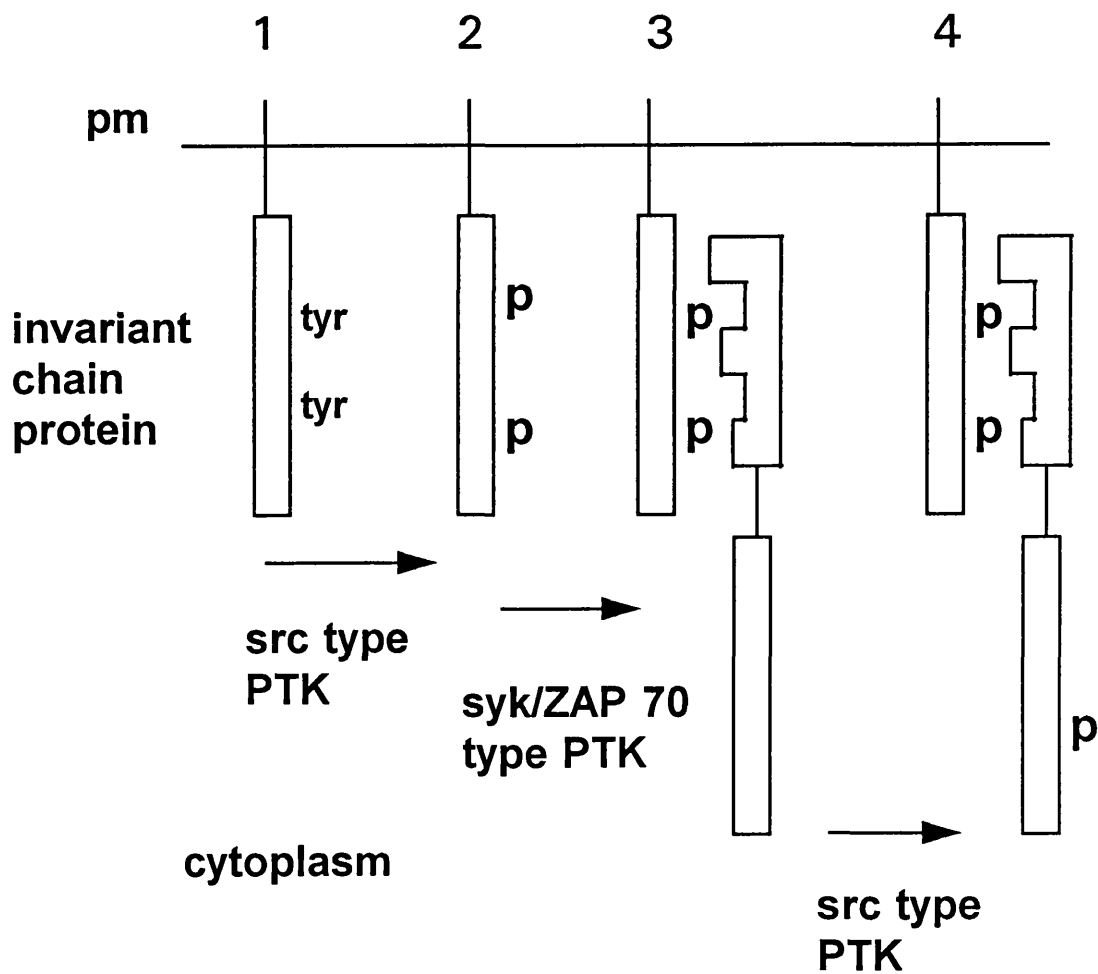
FIGURE 6:

Hypothetical illustration of signalling events following TCR engagement and concomitant co-receptor binding: 1-2) the invariant chain of the TCR/CD3 complex is tyrosine phosphorylated by src. 2-3) ZAP70 is recruited to the phosphorylated invariant chain. 3-4) src phosphorylates the C-terminal region of ZAP70. Signalling events further downstream are unclear. pm= plasma membrane, tyr= tyrosine residue, P= phosphorylated tyr residue. The diagram is based upon that of Weiss and Littman 1994.

cells that don't proliferate or produce IL-2 (Arpia *et al* 1994, Elder *et al* 1994, Chan *et al* 1994). The suggestion is that the TCR recruits first src then ZAP 70 PTK's sequentially (Iwashima *et al* 1994, Weiss and Littman 1994), although mechanisms further downstream are unclear (see Figure 6). Serine/threonine kinases also play a part: raf, for example, may work in an analagous way to ZAP 70, but by influencing transduction of the gamma and δ chains rather than the zeta chain (Loh *et al* 1994).

c) CO-RECEPTORS IN T CELL SIGNALLING.

Mere engagement of the T cell receptor is insufficient to produce an immune response: at least one co-stimulatory signal is also required (Bretscher 1992). Signalling is also far more efficient if the co-stimulation is delivered by the same cell that is presenting the antigenic ligand, as shown by experiments that utilised transfected fibroblasts and Mab-induced receptor blocking (Liu and Janeway 1992).

CD4 or CD8 co-receptor signalling, either through CD4 binding to the ß2 subunit of class II molecules (Doyle and Strominger 1987, Cammarota *et al* 1992) or through CD8 contacting the α subunit of a class I molecule (Norment *et al* 1988, Konig *et al* 1992) augments TCR signalling by up to 100 fold. It also results in the intracellular binding of lck to common cys-containing domains within CD4 or CD8 (Veillette 1991b) which may serve to position lck closer to the TCR. CD4 seems to rely more heavily on tyrosine kinase activation than CD8, which is itself more heavily phosphorylated by PKC (Ravichandran and Burakoff, 1994).

The plasma membrane protein tyrosine phosphatase CD45, expressed as a number of alternatively spliced 180-220kD products on most haematopoietic cells, is another important co-receptor (Trowbridge 1991, Trowbridge and Thomas 1994). In addition, the different CD45 isoforms have been used to distinguish T cell subsets: for example, mature

T cells express high levels of CD45RO whereas naive lymphocytes express CD45RA, -RB, and -RC. CD45 deficient T cells fail to signal properly through the TCR and thymocyte development is impaired at the CD4$^+$ CD8$^+$ stage (Kishihara *et al* 1993). CD45 may negatively regulate lck and/or fyn tyrosine phosphorylation; CD45/lck association is independent of CD8, CD4 and TCR stimulation (Ross *et al* 1994). Truncated CD45 molecules (Volarevic' *et al* 1993) and CD45 knockout mice (Thomas 1994) have been created in order to examine the effects of this molecule in thymocyte maturation.

Another important co-receptor is CD28, a 44kD disulphide linked homodimeric glycoprotein present on 80% of human peripheral T cells (June *et al* 1990c). Biochemical analysis of this molecule and the discovery of its ligands and homologues has shed light on the complex nature of co-stimulatory pathways. For example, anti-CD28 monoclonal antibodies augment PKC dependent T cell signalling and alter cellular cytokine production patterns (June *et al* 1990a). Studying CD28 has helped to unravel the mechanisms of clonal anergy, a state brought about by TCR engagement in the absence of co-stimulation.

CD28 stimulation in the absence of TCR engagement has little effect, whereas blocking CD28 with anti-CD28 Mabs prevents the cell from subsequently responding to antigen (Harding *et al* 1992). In CD4$^+$ T cells, CD28 may work by stabilising IL-2 mRNA transcripts that contain AU-rich instability sequences in their 3' UTRs (June *et al* 1990b) as well as acting post-transcriptionally to increase the levels of IL-2 and other cytokines (June *et al* 1994). The cytosolic domain of CD28 also has an IP3 kinase binding consensus sequence, indicating that it may be a kinase receptor.

CD28 interacts with B7-1 (renamed CD80), a type I membrane bound 30kD glycoprotein with two extracellular Ig-like domains, that is expressed on active (but not resting) B cells and is constitutively expressed

on dendritic cells (Linsley and Ledbetter 1993); its expression can be induced by a number of cytokines. A second ligand for B7, CTLA-4 (Brunet *et al* 1987), appears at low levels following T cell activation and is twenty times more avid than CD28 (Linsley *et al* 1991), though its exact function is uncertain. A soluble immunoglobulin-CTLA-4 fusion protein, termed CTLA-4Ig, has been valuable both to transplantation and tolerance studies (Linsley *et al* 1992). CTLA4-Ig also binds to B7 (Chen *et al* 1992, Hathcock *et al* 1993) and inhibits a number of different T cell responses *in vitro* and *in vivo* (Linsley *et al* 1992). Stimulation of T cells in the presence of CTLA4-Ig leads to antigen specific anergy (Linsley *et al* 1992) and permits the long term survival of xenografted pancreatic islets in the mouse (Lenschow *et al* 1992). Similar treatment can also extend the survival time of rat cardiac allografts (Turka *et al* 1992), though this effect is not indefinite. Mice that overexpress CTLA-4-Ig have more $CD4^+$ cells after antigen stimulation and have impaired antibody responses to T-dependent but not T-independent antigens (Ronchese *et al* 1994), suggesting that this co-stimulatory network may influence T-B interactions.

A second ligand, B7-2 (simultaneously discovered as B70) has been found (Freeman *et al* 1993a and b, Azuma *et al* 1993) and more ligands are likely to be identified (Boussiotis *et al* 1993). The expression patterns of these molecules suggests that B7-1 is the principal ligand for CTLA-4, whereas B7-2 is primarily involved in CD28 binding.

The nature of the signals induced by CD28/CTLA-4 engagement is unclear, especially since many studies have been performed using immortalised cell lines. However, a model is emerging in which CD28 couples two distinct signal transduction pathways. One may operate in mature T cells, in which primed CD28 cross-linked by PTK activation raises Ca^{2+} levels in a CsA sensitive fashion; the second pathway, in naive thymocytes, with low levels of cross-linked CD28, may involve a CsA

insensitive message mediated by serine/threonine kinases or phosphatases (June *et al* 1994).

Our understanding of co-stimulation is thus far from complete. For example, the discovery that CD28 deficient mice were not completely immunodeficient (Shahinian *et al* 1993) led to the detection of heat stable antigen (HSA), a 45-55 kD GPI-linked glycoprotein that is also involved in co-stimulating naive CD4 T cells (Liu *et al* 1992a). HSA is expressed at high levels on immature thymocytes. Transgenic mice over expressing HSA are grossly deficient in single and double positive thymocytes, but have normal levels of double negative precursors (Hough *et al* 1994).

d) T CELL SUBSETS.

Helper T cells can also be subdivided into distinct T_H1 and T_H2 subsets according to their cytokine profiles, as was first shown in the mouse (Mossmann and Coffman 1989). T_H1 cells secrete IL-2, IFN-gamma and TNFß and encourage cytolitic responses and DTH reactions whilst inhibiting IgE production. T_H2 cells manufacture IL-4, IL-5, IL-6 and IL-10 and mediate mainly humoral responses. In culture, both murine T_H1 and T_H2 cells arise from $CD45^{lo}$ precursors, but whereas T_H1 clones remain $CD45^{lo}$, T_H2 cells become $CD45^{hi}$ (Lee and Vitetta 1992).

Controlling whether an antigen stimulates a predominantly T_H1 (cytolitic) or T_H2 (humoral) pathway is important in infectious disease and could have implications in transplantation. *In vitro* work using naive OVA- and $I-A^d$-specific TCR transgenic mouse T cells suggested that stimulation of macrophages by *Listeria* caused the production of IL-12 by these APCs and drove the T cells to differentiate into T_H1 rather than T_H2 clones (Hsieh *et al* 1993). IL-4 on the other hand drives the equilibrium towards T_H2 formation (e.g. Kopf *et al* 1993). The outcome is crucially dependent on the APC involved. This work raised the possibility of using IL-12 as an adjuvant

in vaccinations against infectious diseases. This procedure has now been done with some success in mice that subsequently developed protective cell-mediated immunity against *Leishmania major* (Afonso *et al* 1994).

The situation is not always that simple, however. For example, in a rodent model of malaria, both T_H1 and T_H2 CD4$^+$ cells provide protective immunity, the former doing so by a nitric oxide-dependent process, the latter by facilitating IgG1 antibody production (Taylor-Robinson *et al* 1993). The existence of T_H1 and T_H2 subsets in the rat had previously been shown using CD4$^+$ T cells from LEW rats that had received (LEWxBN)F1 cardiac allografts (Papp *et al* 1992).

1.5 TOLERANCE.

a) THYMIC T CELL TOLERANCE.

The main centre for tolerance induction for T cells is the thymus (Ritter and Crispe 1992). It is here that the processes of positive and negative selection provide the means by which thymocytes are gated. Those with the potential to recognise foreign antigen in association with self-MHC are allowed to mature and leave the thymus, whereas those that recognise self MHC in association with self peptide are eliminated by apoptosis.

Initially, stem cells enter the thymus either from the fetal liver or adult bone marrow. At this stage their lineage commitment is unknown. From a CD4^{lo} state (Wu *et al* 1991), they then develop into CD4$^-$ CD8$^-$ double negative lymphocytes, begin to express CD8 and then become CD4$^+$ CD8$^+$ double positive cells (see also section 1.5c). A variety of other cell surface markers are also expressed at varying levels as the cells mature (e.g. Ritter and Crispe 1992). Selection acts on these developing thymocytes when they co-express CD4, CD8 and low levels of TCR (Fowlkes and Pardoll 1989).

Acquisition of self tolerance has been known for some time to occur during the development of an individuals immune system (Owen 1945, Billingham *et al* 1953), though it continues to be induced throughout an individuals lifetime. Tolerance was shown to be due to a host 'radiation-resistant' entity by using fully and semi-allogeneic bone marrow chimaeras (Singer *et al* 1981). Allogeneic thymic chimeras (Singer *et al* 1982) soon demonstrated that this element was the thymus and supplemented previous findings that the thymus controls the specificity of both cytotoxic and helper T cell clones (Sprent 1978a and b). Grafting experiments further demonstrated that T cell depleted mice given thymuses carrying foreign MHC molecules produced T cells that were tolerant of both the host and graft MHC (Kindred 1978). Actual deletion of precursor cytotoxic lymphocytes was observed *in vitro* by fusing developing H-2 fetal thymuses with fetal liver cells (Good *et al* 1983): CTLs were found towards third party alloantigens, but not against the MHC molecules of the thymus or liver cells. Clonal deletion (i.e. negative selection) in the thymus was formally proven with a Mab towards the TCR element $V_{\beta}17a$ (reactive against the murine class II molecule I-E). This enabled the fate of developing lymphocytes to be followed (Kappler *et al* 1987 and 1988). Those cells expressing $V_{\beta}17a$ were selectively eliminated before reaching the periphery of I-E mice.

It was not until the advent of TCR transgenic mice that positive selection could be directly demonstrated (Kisielow *et al* 1988b, Von Boehmer 1994). Mice with MHC molecules unable to recognise an introduced transgenic T cell receptor do not develop single positive T cells (Scott *et al* 1989), whereas class I specific TCR transgenics have increased numbers of $CD8^{+}$ cells (Sha *et al* 1988).

The need for direct TCR/MHC contact during positive selection was

also suggested by experiments that used transgenic mice expressing a peptide-specific TCR (for a pigeon cytochrome c peptide) that was I-E^k restricted (Berg *et al* 1989). H-2^k TCR transgenics had an increased number of T cells, both overall and reactive to the cytochrome c peptide when compared to those cells from an H-2^b mouse. These latter animals had arrested T cell development at the double positive stage, a phenomenon that was reversed if I-E was expressed on the thymic epithelium.

b) THE SITE OF TOLERANCE INDUCTION.

For T cell selection to proceed, interaction of developing lymphocytes with MHC class I or class II bearing cells within the thymus is necessary (e.g. Boyd and Hugo 1991). The thymus essentially consists of two types of cells:

i) haematopoeitic cells such as dendritic cells, macrophages and lymphocytes themselves. These originate from the bone marrow and are sensitive to both radiation and deoxyguanosine treatment.

ii) epithelial cells of the cortex and medulla. These non-marrow cells are radiation resistant (e.g Robey and Fowlkes 1994).

The cell types upon which selection occurs has been open to debate. Utilising the differential susceptibility of these cells to radiation and deoxyguanosine, reconstituted or chimeric animals have been used to suggest that epithelial cells mediate positive selection (e.g. Lo and Sprent 1986, Bevan 1977, Sprent 1978b). These early studies have been supported by work in which positive selection occurs only when the restricting MHC element is expressed on the thymic epithelium of TCR transgenic mice (Kisielow *et al* 1988a and b) and by recent *in vitro* reconstitution studies (e.g. Anderson *et al* 1994). Cortical, rather than medullary, epithelium appears to drive positive selection (e.g. Vukmanovic' *et al* 1992, Hugo *et al* 1992).

Similarly, reconstitution experiments also suggested that bone marrow derived cells are involved in the selection process (e.g Longo and Schwartz 1980) and that they are necessary for the negative selection of self reactive clones (e.g. Sprent *et al* 1975, Zinkernagel 1978, Mazda *et al* 1991, Inaba *et al* 1991). However, the situation is not that simple. Thymic epithelium can also mediate negative selection, as shown by experiments that used chimeras and transgenic mice (e.g. Salaün *et al* 1990, Houssaint and Flajnik 1990). Distinguishing between deletion and anergy was not always possible in these experiments, though deletion by thymic epithelium does seem to happen in the absence of haematopoietic cells (Speiser *et al* 1992). Such epithelial-driven negative selection is relatively inefficient compared with that of BMDDC (e.g. Robey and Fowlkes 1994).

There is also some evidence that class I molecules on haematopoeitic cells can be involved in positive selection of CD8$^+$ cells (Bix and Raulet 1992), though again this form of selection is relatively inefficient. In addition, immature CD4$^+$CD8$^+$ TCR transgenic thymocytes will differentiate into CD4$^+$ cells *in vitro* in the presence of non-thymic APCs and antigen (Kaye and Ellenberger 1992). Whether these double positive cells reflected a legitimate thymocyte population, however, was uncertain. The injection of mouse fibroblasts expressing H-2K^b class I molecules into the thymuses of H-2^k β_2m-negative recipients can restrict emerging T cells to H2K^b-bearing targets (Pawlowski *et al* 1993). This shows that the restricting element need not necessarily be derived from either a haematopoietical or thymic-epithelial origin.

Since MHC molecules are expressed on both medullary and cortical cells, the question of how differential positive and negative selection occur are achieved is an intruiging one. The discovery of a Mab, Y-Ae, that specifically targeted thymic medullary APCs expressing I-A^b in association

with a self I-E^b-derived peptide (Murphy *et al* 1989, Rudensky *et al* 1991a) led to the proposal that differential selection was due to different ligands being expressed by different cells of the thymus. This opposes the view that selection is achieved by different affinities of the same ligand (e.g. Sebzda *et al* 1994). More recently, however, the idea that related peptides can switch a thymocyte towards either cell death or survival has taken shape (Nikolic' Zugic' and Bevan 1990, Allen 1994). Elegant studies using ß$_2$m$^{-/-}$ and TAP$^{-/-}$ TCR transgenic mice allowed the influence of peptides on selection to be studied (Hogquist *et al* 1994, Ashton-Rickardt *et al* 1993 and 1994). Class I expression can be reconstituted by adding back ß$_2$m and/or specific peptide in fetal thymic organ cultures (Jenkinson and Owen 1990). Using a CD8$^+$ TCR specific, antigenic OVA peptide, Hogquist *et al* got negative selection - but slight sequence alterations of the peptide enabled positive selection of the developing T cells to occur.

Whether a peptide mediates positive or negative selection also depends on the level of class I expression, since the same peptide caused positive selection in ß$_2$m$^{-/-}$ mice but negative selection in ß$_2$m$^+$ animals. Ashton-Rickardt *et al* used variants of a LMCV peptide to rescue their TAP-deficient transgenics and induce positive selection. They also found that low doses of the antigenic peptide resulted in positive selection and performed titrations in which the antigenic peptide caused a change from positive to negative selection over a range of 3μM to 300μM. It appears that normally, self peptides resembling an antigenic peptide will positively select, but that strong recognition will result in elimination. However, an increase in class I density or peptide concentration may cause a shift such that peptides previously capable of inducing positive selection are efficacious enough to trigger cell death. Furthermore, dissimilar peptides can stimulate the same T cell receptor, implying that there may be a number of promiscuous peptides in the thymus that are used to select a

number of different clones. However, earlier comparisons of class II bound peptides eluted from spleen and thymic cortex and /or medullary cells showed that there was no difference in the peptide profiles (Marrack *et al* 1993). Evidently, there is still much to resolve in the area of thymic selection.

Avidity of co-receptor interactions may also play a part in the selection process. TCR contact with CD8 is necessary (Ingold *et al* 1991), but over expression of CD8 can alter the fate of lymphocytes from positive to negative selection (Lee *et al* 1992, Robey *et al* 1992). Lowering the affinity for CD8 also enables TCRs with higher affinity for self-MHC to survive negative selection (Sherman *et al* 1992).

c) T CELL LINEAGE AND CELL DEATH.

The control of lineage commitment during positive selection is uncertain. CD8⁻ CD4⁻ TCR⁻ T cell precursors differentiate first into CD4⁺ CD8⁺ TCR^{lo} 'double positive' cells. Maturation from CD4⁺ CD8⁺ double positive cells to the single positive stage requires MHC/TCR αß binding (Von Boehmer and Kisielow 1993). Contact of the TCR with MHC class I antigens leads to the development of CD8⁺ cells (Teh *et al* 1988), whereas contact with MHC class II molecules leads to CD4⁺ cell maturation (Kaye *et al* 1989); for CD8⁺ cells, the presence of the CD8 ß chain is also required (Nakayama *et al* 1994), but selection is independent of CD8α-lck interaction (Chan *et al* 1993a). Similarly, helper T cell development does not require an association between CD4 and lck kinase (Killeen and Littman 1993).

Commitment to a lineage may either be 'instructive', (whereby TCR binding to class I results in CD8⁺ T cell development and that to class II results in CD4⁺ T cell development) or 'stochastic', (whereby CD4⁺ and CD8⁺ commitment occurs regardless of receptor specificity). Somewhat

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counter-intuitively, evidence for the latter model was obtained from both class II and class I deficient mice which had apparently maturing subsets of $CD4^{hi}CD8^{lo}$ and $CD4^{lo}CD8^{hi}$ cells, respectively (Chan *et al* 1993b). Moreover, class I deficient mice over expressing a CD4 transgene had functional $CD8^+$ cells that did not express CD4 (Davis *et al* 1993). Further support of the stochastic model comes from $ß_2$m-negative mice that have activated T cells with intermediate CD4 levels, high CD8 levels and intermediate TCR expression (Van Meerwijk and Germain 1993). These cells do not develop into $CD8^+$ single positive cells, suggesting that commitment but not maturation is independent of MHC class I expression. These experiments invoke a mechanism in which either CD4 or CD8 expression is blocked in developing thymocytes, with the relevant cells being rescued from cell death.

Over 90% of thymocytes die during development, and the cellular mechanisms controlling this process are only beginning to be understood. Regulated cell death or apoptosis is partly controlled by bcl-2, an inner mitochondrial membrane protein found in medullary but not cortical T cells (Pezzella *et al* 1990, Hockenbery *et al* 1991), that can inhibit apoptosis in $CD4^+CD8^+$ thymocytes (Stentman *et al* 1991, Strasser *et al* 1991). Transfection of a bcl-2 transgene into T cells enabled them to survive lymphotoxic treatments that killed control lymphocytes *in vitro*. Furthermore, such transfectants were able to respond to immunisation in an enhanced manner (Strasser *et al* 1994). Thy-1, an 18kD, N-linked, GPI-anchored protein that is found on peripheral and thymic lymphocytes of mice (Williams and Gagnon 1982) and in other species, may also be involved in programmed cell death. In the mouse, thy-1 can trigger bcl-2 resistant thymocyte cell death (Hueber *et al* 1994). This was visualised using a Mab to thy-1 that initiated cell death in thymocytes but not in peripheral T cells in culture.

The 35kD cell surface antigen fas-1 (e.g. Hanabuchi *et al* 1994) also contributes to the negative selection of autoreactive T cells in the thymus (Watanabe-Fukunaga *et al* 1992). Mice with fas-1 mutations develop autoimmune disease and produce a variety of self reactive T cells that have escaped clonal elimination. There is still much to be discovered concerning programmed cell death in the thymus.

d) PERIPHERAL T CELL TOLERANCE.

Tolerance is not just a thymic phenomenon. How, for example, is tolerance achieved towards tissue-specific antigens that are not found in the thymus? Some T cells with the potential to cause autoimmunity escape the thymus and are subsequently dealt with in the periphery. For example, a T cell with a receptor that has been selected in the thymus could somatically mutate and become autoreactive, as has been shown *in vitro* towards MHC antigens (Augustin and Sim 1984). Some peripheral T cells with the potential to react with presented antigen remain quiescent: this state is called anergy.

Anergy was originally demonstrated *in vitro* (Jenkins and Schwartz 1987, Lamb *et al* 1983) and its signature is the failure of anergic T cells to produce IL-2 or functional AP-1 (Kang *et al* 1992), even though such T cells have the same number of T cell receptors as normal cells (Schwartz 1990). *In vivo*, transplantation studies that produced tolerance without the loss of self reactive T cells suggested that peripheral mechanisms could be involved (Talmage *et al* 1986). However, the first apparent *in vivo* demonstration of anergy came in 1988, when transgenic mice were created expressing the H-2K^b class I gene coupled to the rat insulin promoter (to localise expression to the pancreas and avoid thymic education). These animals develop diabetes, but lack an immune response against the

pancreatic ß cells expressing the transgene (Allison *et al* 1988, Morahan *et al* 1989). Their spleen cells fail to kill H-K^b-expressing targets but their thymic cells do, an effect that is reversed by the provision of IL-2. This indicated that abnormal IL-2 provision to anergic T cells (perhaps by a localised infection) might result in their activation and a subsequent autoimmune response (Bottazzo *et al* 1983, Sarvetnick *et al* 1988). However, the possibility that antigen was recirculated to the thymus or that low levels of transgene were undetectably expressed in the thymus could not be conclusively ruled out in this and other similar experiments (Lo *et al* 1988, Lo *et al* 1989, Burkly *et al* 1990). Indeed, it was later shown that low levels of the H-2K^b transgene were expressed in the thymus. The current explanation for these experiments is that the majority of H-2K^b reactive cells are deleted in the thymus. Those that remain in the periphery are able to reject H-2K^b skin grafts but not H-2K^b on pancreatic islets unless high levels of IL-2 are present in the microenvironment (Heath *et al* 1992).

Anergy *in vivo* was also demonstrated using the mouse Mls superantigen experimental model (Rammensee *et al* 1989). T cells specific for Mls-1^a, bearing the Vß6$^+$ TCR gene segment, were tracked in Mls-1^b mice. Mls-1^b mice are unresponsive to the Mls-1^a cell surface superantigen. Using Mabs to Vß6, T helper cells were detected that were not clonally deleted, but anergic, with a characteristic failure to produce IL-2.

Anergy can also occur if the TCR delivers an inappropriate signal to the T cell interior, even if co-stimulation is provided (Sloan-Lancaster *et al* 1993); this depends on the nature of the peptide ligand. The TCR also responds differently to superantigens as opposed to peptide antigens (Webb *et al* 1990, Liu *et al* 1991a) and the cytokine microenvironment (Scott 1993), suggesting that anergy may be the end result of a number of different factors.

Tolerance has also been examined in the periphery to the normally antigenic protein hen egg-white lysozyme (HEL) that was ubiquitously expressed in transgenic mice (Cibotti *et al* 1992). The nature of T cell unresponsiveness depended on the serum levels of the protein. Furthermore, tolerance seemed to be limited towards a few dominant peptides with subdominant epitopes playing a role in autoimmunity. The concentration of antigen or tolerogen almost certainly determines the level of T cell tolerance. Experiments using tissue specific expression of K^b in transgenic mice led to T cell tolerance when extremely low levels of the transgene were expressed on hepatocytes. K^b gene expression was controlled by an inducible promoter from the human complement reactive protein. Giving the animals LPS specifically enhanced transcription off the promoter and allowed levels of K^b to be elegantly controlled (Ferber *et al* 1994). Tolerance at low levels of K^b expression was achieved by partial downregulation of the $CD8^+$ cell's TCR. Higher levels of expression resulted in complete TCR downregulation, suggesting that peripheral tolerance is a multistep, sequential process (Hämmerling *et al* 1993).

Circulating antigens can also induce tolerance if the dose is particularly high (e.g. Mitchison 1968). This phenomenon, called 'high zone tolerance', was first observed by Mitchison in 1964. Work using non-cytopathic LCMV has provided a possible explanation for this observation (Moskophidis *et al* 1993). Administration of low doses of virus (10^2 plaque forming units - PFU) to C57BL/6 mice results in a coventional cytotoxic T cell response and characteristic immunopathology. Higher doses (10^7 PFU) activate the $CD8^+$ T cell population so effectively that they become exhausted before they can proliferate. The animal, therefore, becomes tolerant and the virus persists. This outcome was also shown to be thymus, cytokine and APC-independent. Such a form of tolerance could

also be possible in transplantation. Challenging male mice with CD8$^+$ T cells that had a transgenic TCR for the male H-Y transplantation antigen led to the vigorous expansion and then disappearance of 70% of these introduced T cells with no apparant injury to the host (Rocha and Von Boehmer 1991). The remaining T cells appeared to be anergic. A mechanism for high zone tolerance has been suggested (using a different model) in which activated T cells judge the intensity of the immune response through the cell cycle (Critchfield *et al* 1994). Re-engagement of the T cell receptor at high antigen doses may then lead to apoptotic cell death and protect the host from injury caused by an overzealous immune response.

It has been postulated (Fuchs and Matzinger 1992) that the state of the T cell as well as that of the APC influence the outcome of antigen presentation and this may also have interesting implications for transplantation. As mentioned earlier, there is evidence both for (Rock *et al* 1984, Lanzavecchia 1985) and against (Lasila *et al* 1988, Inaba and Steinman 1984) antigen presentation by B cells. Fuchs and Matzinger investigated this further by exploiting the response of naive and memory female T cells to the H-Y antigen. There were two important findings from this work:

i) injection of naive T cells and resting B cells into female mice, followed by male spleen cell challenge, resulted in tolerance i.e. no CTL response and acceptance of male tissue grafts.

ii) activated B cells induced tolerance more efficiently than their resting counterparts in naive females, but were able to stimulate activated memory T cells.

They used these findings to explain high and low zone tolerance and the acceptance of grafts under certain circumstances. In the former, antigen at low levels will be presented mainly by antigen-specific B cells

due to the efficiency of their Ig receptors - this will tolerise responding T cells. High antigen load should allow all B cells to take up antigen non-specifically, and, since they outnumber 'professional' APCs, tolerance should again occur. Antigen concentrations between these two extremes have an immunising effect, since professional APCs will outnumber antigen specific B cells in the competition for antigen.

In the latter case, the 'blood transfusion effect' (prolonged graft acceptance following a pre-transplant blood transfusion) may be caused by the large number of introduced B cells inducing tolerance in naive alloreactive T cells in the recipient. The effect may not last because recipient memory T cells would not be tolerant and blood donor professional APCs may also have an immunising effect.

e) SUPPRESSION.

One way that peripheral tolerance could be maintained is by lymphocytes specifically regulating the activity of autoimmune cells. This was first suggested by adoptive transfer experiments in which the T cells from tolerant animals were able to transfer tolerance to naive recipients provided that specific antigen was given simultaneously (e.g. Kilshaw *et al* 1975, Tilney *et al* 1978). Though suppressor T cell clones have been isolated in mouse (Fresno *et al* 1981) and man (Lamb and Feldmann 1982), no cell phenotype has ever been shown to be associated with suppression and the phenomenon has therefore been hard to investigate. However, recent *in vitro* experiments with alloreactive human cells indicate that anergic T cells can suppress potentially reactive cells of the same antigen specificity (Lombardi *et al* 1994), presumably by competition for antigen, IL-2 and costimulatory molecules on the APC. This provides a possible explanation of how peripheral tolerance is maintained.

f) ORAL TOLERANCE.

Oral tolerance is a dose dependent phenomenon that is usually associated with specific unresponsiveness to dietary antigens that pass through the intestinal mucosa (Mowat *et al* 1987). However, oral tolerance may have some relevance to both transplantation and autoimmunity, since specific peripheral unresponsiveness can occur after feeding with particular antigens (Weiner *et al* 1994). Clinical trials have taken place involving the oral administration of bovine myelin to multiple sclerosis patients (Weiner *et al* 1993). This double blind study noted a significant improvement in the health of the treatment group, but the findings were confounded by the larger numbers of females and HLA-DR haplotypes in the placebo group (Weiner *et al* 1993).

The cellular mechanisms involved in oral tolerance are unclear, but a recent study of T cells tolerised by oral ovalbumin claimed that systemic tolerance was probably mediated by anergy, since specific and bystander suppression could be ruled out (Melamed and Friedman 1993). Different mechanisms may play a part in other systems. For example, rats that develop experimental allergic encephalomyelitis (EAE) after MBP immunisation have their symptoms alleviated by prior feeding with MBP. CD8$^+$ T cells from such animals mediate bystander suppression when adoptively transferred to naive animals subsequently immunised with MBP (Lider *et al* 1989). This effect is independent of cell contact but requires antigen-specific triggering of the suppressor cell (Miller *et al* 1991). TGFß is involved in this process, since anti-TGF Mabs prevent suppression, and administration of anti-TGFß immune sera to non-tolerised animals increases the disease severity (Miller *et al* 1992).

In transplantation, oral tolerance has apparently been achieved by feeding LEW RT1^l rats with BN RT1^n splenocytes, sensitising them with

(LEWxBN)F1 skin grafts and then challenging them with cardiac allografts (Watschinger *et al* 1994). Extended survival times of seven days were observed, the same as for non-sensitised animals. Further studies by the same group involved feeding B^u and D^u class II allopeptides to LEW rats prior to conventional immunisation (Sayegh *et al* 1992). An antigen specific reduction of cell mediated immunity and DTH responses was observed. Other ways in which tolerance has been achieved in transplantation are discussed in section 1.6c.

1.6 TRANSPLANT IMMUNOLOGY.

a) ALLORECOGNITION.

There are far more cytotoxic precursors with the potential to interact with MHC alloantigens than there are towards other allodeterminants. It has been estimated that between 1-10% of peripheral lymphocytes respond to allogeneic MHC antigens in the absence of antigen priming (Fischer-Lindahl and Wilson 1977). This compares with the 0.001% of T cells that react with processed recipient alloantigens (Liu *et al* 1993a). One explanation for this was provided by Matzinger and Bevan (Matzinger and Bevan 1977). They discovered that CTLs could be generated between MHC identical strains of mice that differed only in their minor histocompatibility proteins. They proposed that intact, polymorphic cell surface antigens or foreign protein formed a complex with the restricting MHC element. The large number of potentially polymorphic proteins in an allogeneic situation, including MHC molecules themselves, then provides an explanation for the high precursor T cell frequency. Seven years later, Bevan proposed an alternative hypothesis (Bevan 1984). He argued that all allogeneic donor MHC molecules would contain foreign sequences that could potentially be recognised by recipient T cells (i.e. about 10^5 determinants per cell). This high 'concentration' or density of antigen might thus activate T cells of

relatively low affinity. Conventional antigens, present on the cell surface at a lower density, would require higher affinity interactions to activate T cells.

These two hypotheses are not mutually exclusive, but the elucidation of the crystal structures of MHC molecules (Bjorkman *et al* 1987a and b, Brown *et al* 1993) indicate that a modified form of the Matzinger and Bevan hypothesis may be more accurate. Other theories to explain allorecognition have also been proposed. For example, Lechler and colleagues, using results from HLA-DR primed T cell clones, have considered allogeneic MHC molecules as essentially bifunctional proteins. One region, corresponding to the external faces of the *a* helices, contacts the TCR whereas the other site, corresponding to the inner aspect of the *a* helices and the ß sheet 'floor', contacts bound peptide (Lechler *et al* 1990, Lechler *et al* 1992). T cell reactivity is therefore mediated by differences at these sites. Peptide need not play a role in this model of allorecognition, providing that the TCR can recognise the allo-MHC through molecular mimicry.

The requirement (or otherwise) for MHC-bound peptide during allorecognition has received much attention (Sayegh *et al* 1994). Most studies have used *in vitro* CTL assays to investigate this; far fewer experiments have addressed these issues in an *in vivo* transplantation context. That peptide recognition may be an important factor in class I allorecognition was demonstrated using human cells transfected with murine K^b molecules. K^b specific CTLs were analysed for their reactivity towards K^b (bearing human peptides): some of the few clones that failed to respond subsequently did so if murine peptides were provided (Heath *et al* 1989).

The class I processing mutants RMA-S and T2 (see also section 1.3c) have also been invaluable in understanding allorecognition.

Transfection of such cells with K^b results in relatively normal K^b cell surface expression, although with little, if any, associated peptide. Most K^b specific CTLs fail to respond to K^b in this context (Marrack and Kappler 1988), indicating the requirement for peptides. Those clones that do respond to K^b show enhanced lysis if cyanogen bromide-cleaved cytoplasmic peptides are added, demonstrating that recognition of both peptide and MHC together can optimise alloresponsiveness.

Experiments in which numerous CTLs were generated against T2 cells transfected with HLA-A2, $H-2D^d$ and $H-2L^d$ MHC molecules suggests that about 85-90% of MHC specific clones need peptide to be present (e.g. Crumpacker *et al* 1992). Transfection of murine cell lines with human HLA class I antigens e.g. A2 and B7, has also demonstrated that CTL specificity towards human alloantigens is MHC dependent (e.g. Bernhard *et al* 1987, Koller *et al* 1987), although one slightly flawed similar study suggested that species restricted allorecognition might be due to differences in accessory molecules between mice and men (Mentzer *et al* 1986). One interesting observation (though not in a transplantation context) is that MHC conformation can depend on peptide sequence (Catipovic' *et al* 1992). RMA-S cells expressing empty $H-2K^b$ molecules that are stably expressed at 20°C were used. These empty class I molecules are disrupted at higher temperatures unless K^b binding peptides are added. Two different $H-2K^b$ conformations were stabilised by different peptides and could be detected by discrete Mabs. Amino acid substitution in one of these peptides (derived from VSV-NP) enabled it to stabilise a different K^b molecule. Another study, using HLA-A2 variants that differ by known amino acid substitutions in the $a2$ helix or ß sheet floor of the peptide binding groove, showed that allorecognition could be abolished by just two amino acid substitutions (Mattson *et al* 1989).

Whether peptide dependence means peptide specificity has been

questioned by data obtained from clones that recognise K^b in the presence of synthetic K^b-binding proteins that are non-cellular in origin (e.g. Guimezanes *et al* 1992). Perhaps the conformation of the MHC molecule (induced by peptide binding), rather than the peptides amino acid sequence, is important in this, and other, examples of allorecognition (Bluestone *et al* 1993). When considering these results, it is also worth bearing in mind the concept of affinity, as discussed by Bevan (Bevan 1984), since many of the experiments involve saturating cell surface MHC molecules with exogenous peptides. This may be enough to trigger low affinity T cell clones and does not necessarily reflect what would happen if the same peptide(s) were generated by conventional processing *in vivo*.

MHC class II restricted allorecognition has also been studied by similar approaches. B cell lines defective in class II antigen processing, but still able to express DR3 (Mellins *et al* 1990), were not recognised by three of four anti-DR alloreactive T cell clones (Cotner *et al* 1991). Again, this highlighted the potential importance of peptide in the alloresponse. Class II alloreactivity was also examined in DR1 expressing human and murine cells (Lombardi *et al* 1989a and b). As before, some, but not all, anti-DR1 clones were specific for cellularly derived peptides in the context of allo-MHC.

Some investigators have attempted to identify the peptides that are bound 'naturally' during allorecognition. Elution of a broad range of material from murine APCs has led to the suggestion that many peptides can be recognised by a single T cell clone (e.g. Sherman and Chattopadhyay 1994). A number of different peptide species are likely to be involved in allorecognition (Rötzschke *et al* 1991) including MHC class I and class II-derived peptides (e.g. Chicz *et al* 1993, Chicz and Urban 1994, Chen *et al* 1990). The proteins from which allopeptides are derived need not be particularly polymorphic either (e.g serum albumin, Germain 1986).

The potential importance of the T cell receptor in allorecognition

should not be overlooked (e.g. Casanova *et al* 1993). Lymphocytes from mouse strains with assigned mutations in their K^b molecules have been exploited to investigate CTL specificity towards mutant or wild type K^b MHC molecules (Sherman *et al* 1982). A large number of different T cell receptor specificities were found, even when the difference between donor and recipient K^b was just a few amino acids. T cell receptor cloning also revealed that TCR specificities were extremely diverse, even towards the same alloantigen (Bill *et al* 1989). The same region of the TCR has been shown to be involved in both alloantigen recognition and conventional antigen/MHC recognition (Matis *et al* 1987). Other studies suggest a more limited TCR Vß gene usage (Liu *et al* 1993b).

These and numerous other studies are sobering because they demonstrate the vast potential diversity of the alloresponse. This, together with the fact that tissues expressing the same MHC molecules may present a different array of peptides (Heath and Sherman 1991), may make donor-specific immunosuppression at the level of the peptide a difficult goal.

b) MECHANISMS OF ALLOGRAFT REJECTION.

It has been extensively demonstrated *in vitro* that allorecognition can occur and that it is usually peptide dependent to some extent. However, it is important to discover what role allorecognition plays *in vivo* in causing allograft rejection.

Dendritic cells have been mentioned earlier as important antigen presenting cells, so unsurprisingly they are also important in the rejection response. Retransplanting (ASxAUG)F1 kidney transplants to AS rat recipients normally prolongs the survival time of the graft (Batchelor *et al* 1979). Injecting purified, donor strain dendritic cells at the time of regrafting, however, restores the rapid rejection response (Lechler and

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Batchelor 1982). Dendritic cells are therefore likely to play a crucial role in chronic rejection responses, presumably by 'direct T cell allorecognition' in which their class II (and class I) molecules can be immediately and directly recognised, without antigen processing, by recipient effector T cells. This might be independent of the presence of peptide and may perhaps compare to superantigen binding. Later acute rejection responses are more likely to involve 'indirect T cell allorecognition', in which recipient APCs conventionally process donor graft antigens and present them to donor CD4$^+$ effector T cells. The antigens most likely to be recognised as foreign are class I and class II molecules and other polymorphic minor transplantation antigens.

The notion that the direct pathway may be more important in acute rejection has received some experimental support. Kidney grafted rats receiving CD4$^+$ T cells 'primed' to direct recognition reject their grafts acutely. However, the provision of the same cells by adoptive transfer to passenger cell-depleted kidney allografted recipients has no influence on the rejection response (Braun et al 1993a). Rats receiving kidneys depleted of passenger cells do not get acute rejection, even in the absence of immunosuppression (see Chapter 5). This experiment suggested what one would guess intuitively i.e. that different T cell populations mediate chronic and acute rejection responses and that the indirect pathway is probably more important in chronic rejection. In this regard, it is interesting that in the clinic, whilst CsA and other therapies have dramatically improved short term allograft survival (i.e acute rejection), long term prognosis (chronic rejection) remains unchanged (e.g. Calne 1987).

For indirect recognition to occur in vivo, potentially antigenic material must be available for uptake by antigen presenting cells. The discovery of intact MHC class I molecules in normal human circulation (e.g. Van Rood et al 1970) and in kidney transplant recipients (Suciu-Foca et al 1991)

suggested that processing of these polymorphic molecules was feasible in a transplant setting. Adoptive transfer experiments showed that donor splenocytes could sensitise recipient mice for accelerated skin allograft rejection, presumably by the indirect pathway (Sherwood *et al* 1986). Somewhat more rigorous studies, in which LEW rats were immunised with soluble, allogeneic DA class I or class II molecules, also resulted in accelerated rejection when the animals were subsequently challenged with (DAxLEW)F1 skin grafts. Concomitant donor class I antibody production was also observed (Dalchau *et al* 1992).

Indirect T cell allorecognition has been shown to occur and to be physiologically relevant in non-vascularised rat skin allograft rejection (Fangmann *et al* 1992 a and b). This work is discussed further in the Results section, since it forms the basis for the experiments contained within this thesis. In the mouse, challenge with allogeneic skin grafts or splenocytes resulted in the activation of recipient T cells that could proliferate *in vitro* towards MHC class II derived allopeptides (Benichou *et al* 1992), though the *in vivo* influence of indirect recognition was not demonstrated. Other workers have used synthetic class II allopeptides to indicate that indirect recognition can potentially occur in rat vascularised cardiac allograft rejection (Watschinger *et al* 1994). In this study, LEW RTI^l rats primed with RTI^u cardiac allografts had T cells that specifically responded to synthetic, RTI D^{u}ß-derived 25mer peptides *in vitro*. However, this did not demonstrate that indirect recognition was actually responsible for rejection *in vivo*. Interestingly, in contrast to the studies in our lab (Fangman *et al* 1992a and b), the most immunogenic peptides came from the ß sheet (floor) of the peptide binding groove rather than the α helices of the donor MHC molecule.

In man, the potential of allogeneic T helper cells to process denatured class II antigens and proliferate towards class II-derived peptides

in vitro has also been demonstrated (Saskia de Koster *et al* 1989). Here, peptides derived from the hypervariable region of the HLA-DR3 ß chain were used to prime T cells that could then proliferate in the presence of cells bearing HLA-DR3 and DP3. Presentation was probably on DP molecules, since anti-DP Mabs blocked the alloresponse. In another study, T cells responding to synthetic peptides from the DRß1*0101 molecule were generated. These T cells could recognise DRß1*0101 in the context of DR11 (Liu *et al* 1992b). One study with interesting implications for transplantation involved isolating cytotoxic T cells that were specific for an HLA-B7 (class I) peptide presented in the context of DR-11.1 (class II). These CTL were CD4$^+$ and were able to kill cells expressing both B7 and DR11.1 without the addition of peptide (Chen *et al* 1990). Class II presentation in this system occurred by the endogenous route. As yet, there is no *in vivo* evidence for indirect T cell allorecognition in human transplantation.

Though both direct and indirect T cell allorecognition are important in mediating rejection, the effector mechanisms by which they do so remain to be established (e.g Bradley and Bolton 1992). CD8$^+$ effector cells and/or antibody driven by T cell help (Lee *et al* 1994), or DTH-like reactions could all feasibly be involved. In non-vascularised, MHC class I disparate skin grafts, adoptively transferred CD8$^+$ T cells can cause rejection (Sprent *et al* 1986a and b) and anti-CD8 antibody therapy can prolong graft survival (Ichikawa *et al* 1987). With class II skin disparities, CD4$^+$ T cell s seem to initiate rejection (Rosenberg *et al* 1987, Rosenberg *et al* 1988).

The situation may be different in vascularised allografts. For example, the nature of the kidney rejection response was compared in PVG RT1^u (high responder) and RT1^c (low responder) rats bearing RT1^a class I disparate allografts (Gracie *et al* 1990). The former recipients reject their allografts vigorously, whilst the latter go on to indefinite survival. Both sets

of animals have comparable cellular infiltrates and in the RT1^u rats, anti-CD8 antibody therapy has no effect on rejection despite depleting the CD8$^+$ T cell population. However, these rats mount a strong alloantibody response to graft antigens and passive transfer of immune sera from these animals is able to provoke the allospecific rejection of RT1^a kidneys in CsA treated recipients. A role for antibody mediated cellular cytotoxicity or for activation of complement was proposed.

Another study looked at prolonged survival (induced by CsA therapy) of PVG class I and class II mismatched cardiac allografts in DA rats (Hall *et al* 1990). In this system, a role has been invoked for host CD4$^+$ suppressor T cells that can prevent CD8$^+$ T cells from eliciting the rejection response and can nullify normal CD4$^+$ T cells.

It seems likely that alloantibodies, cytokines, CD8$^+$ T cells and CD4$^+$ T cells are all important in rejection, but the extent to which each is dominant may vary with a number of conditions. The nature of T$_H$1 versus T$_H$2-driven responses also needs to be thoroughly investigated in the context of transplantation. For example, might a predominantly T$_H$2 response dampen down cytotoxic destruction in certain situations? The aim should be to pinpoint the 'rate determining step' for a particular rejection response, since this would be the ideal target for therapy. This may well be the CD4$^+$ cell (e.g Guerder and Matzinger 1992).

c) GRAFT FUNCTION AND TRANSPLANTATION TOLERANCE.

Tolerance is of particular interest in transplantation, since 'donor specific tolerance' is necessary for long term graft stability (e.g. Charlton *et al* 1994). Since the pioneering experiments of Medawar and colleagues, in which tolerance to allografts was achieved in neonatal mice (Billingham *et al* 1953), transplantation has strived to achieve specific tolerance in the adult (e.g. Qin *et al* 1989). There has been some limited success in

experimental animals (depending on the extent of the mismatch) using allogeneic bone marrow transplantation, in conjunction with a number of therapies such as total lymphoid irradiation (Slavin *et al* 1977, Ilstad and Sachs 1984, Slavin 1987), anti lymphocyte antisera (Monaco *et al* 1971, Monaco and Wood 1970), blood transfusion (Fabre and Morris 1972, Opelz *et al* 1973), and monoclonal antibody therapies (e.g Isobe *et al* 1992). However, none of these procedures is alloantigen specific and all have side effects.

The most significant advance in transplantation tolerance came with the discovery of immunosuppressive drugs. In kidney transplantation, for example, the 1960's and early 1970's heralded the use of azathioprine, a purine antiproliferative agent (Elion 1967) in conjunction with prednisone as a general immunosuppressive regimen (Naimark and Cole 1994). Anti-lymphocyte immunoglobulin was introduced in the late 1970's, but it was not until the introduction of CsA (e.g. Borel *et al* 1977, Borel 1981) in the early 1980's that 1 year graft survival began to improve dramatically (e.g. Calne 1987). Subsequently, alternative immunosuppressive drugs have become available such as the macrolides FK506 (Kino *et al* 1987, Todo *et al* 1988, Starzl *et al* 1989) and rapamycin (Morris 1992). These are being introduced into the clinic with some success in controlling acute rejection.

Though current immunosuppressive protocols can control acute rejection, they rarely (if ever) result in donor specific tolerance and as a result have had little bearing on the long term survival of allografts, which are usually lost through chronic rejection (e.g. Paul and Benediktsson 1993, Häyry *et al* 1993). The biology of chronic rejection is poorly understood (Azuma and Tilney 1994). In chronic kidney allograft rejection, a number of immune processes have been implicated ranging from macrophage activation (Hancock *et al* 1993) to overzealous cytokine production

(Fellström and Larsson 1993). These may combine with other factors such as ischemia and reperfusion injury during transplantation (e.g. Munger *et al* 1993, Yilmaz and Häyry 1993). Once sufficient functional kidney mass has been lost, hyperfiltration followed by glomerulosclerosis and systemic hyperfiltration can lead to graft loss.

Numerous retrospective studies have examined the risk factors for long term allograft survival, especially amongst renal patients. As one would expect, the level of preformed antibodies to donor leukocytes appears to be important (e.g. Almond *et al* 1993), whereas pretransplant blood transfusion is probably beneficial (Norman *et al* 1986, Feduska *et al* 1984, Brand and Lagaaij *et al* 1989). The benefits of HLA matching, however, remain controversial. Some suggests that this improves long term prognosis (e.g. Takemoto *et al* 1992, Thorogood *et al* 1990) whilst others find it does not (Lundgren *et al* 1986, Mendez *et al* 1991). Early rejection episodes do have an impact on long term graft survival (e.g. Gulanikar *et al* 1992, Lindholm *et al* 1993). In one study, no chronic kidney loss was observed unless at least one prior rejection episode had occurred (Basadonna *et al* 1993). Unfortunately, multiple variables such as age, sex, donor type, sex, race, drug regime, transplant centre and minor transplantation antigenic differences can all confound such retrospective analyses.

Adhesion molecules are also considered to be important mediators of rejection responses, since they are crucially involved in the interactions of lymphocytes with endothelium (e.g. Kirby and Wilson 1994). The cell surface expression of such molecules is upregulated as a consequence of inflammation during rejection (e.g. Pober and Cotran 1990). Mabs raised against the adhesion molecule ICAM-1 have shown promise in blocking rejection in cynomolgous monkeys (Cosimi *et al* 1990) and in phase 1 clinical trials of human renal allograft recipients (Haug *et al* 1993).

Combining anti-ICAM-1 and anti LFA-1 Mab therapy has also been successful in the mouse (Isobe *et al* 1992). Previous experience with Mabs in transplantation, such as OKT3 (e.g. Alloway *et al* 1994) shows that care must be taken to avoid unpleasant side effects and immune responses against the Mab itself.

Other therapies that were hoped to result in donor-specific immunosuppression have been less successful. For example, administration of classical, soluble MHC class I preparations in the rat had no effects on rat kidney allograft viability (Priestly *et al* 1989). Hope that this type of approach would be successful was provided by murine *in vitro* studies. $H\text{-}2K^b$ reactive T cell hybridomas were prevented from proliferating in response to cells transfected with $H\text{-}2K^{bm10}$ (an $H\text{-}2K^b$ mutant) by purified soluble $H\text{-}2K^b$ and by a K^b derived peptide (Schneck *et al* 1989).

The induction of tolerance in experimental animals and the deciphering of the mechanisms underlying such phenomena have been of great interest in the transplantation community. Though tolerance can often be achieved in animals, the conditions required are usually complex and often unsuitable for clinical application. One explanation for the lifelong tolerance seen in mice exposed to anti CD4 and CD8 Mabs is that tolerance is infectious (Qin *et al* 1993). This fits in with the findings of Lombardi *et al* (section 1.5e). In Qins' experiments, spleen cells from tolerant animals that have received Mab therapy can suppress naive lymphocytes by adoptive transfer, whilst having no effect on the rejection of third party grafts. Using transgenic mice expressing human CD2 as skin graft recipients (to enable isolation of host and donor lymphocytes), it was shown that naive donor lymphocytes became tolerant after residing in the recipient with its own tolerant cells for between 7-14 days. Host cells can be eliminated by monoclonal antibody to CD2, in which case the donor lymphocytes still maintain tolerance to subsequent grafts in the absence of Mab therapy.

An explanation for tolerance even more controversial than suppressor cells is the concept of microchimaerism. Microchimaerism is the term given to the co-existence of donor and recipient T cells within a (usually liver) allograft over long periods of time (Starzl *et al* 1992). The donor cells are maintained and are tolerogenic, and patients with established microchimaerism appear to require only minimal immunosuppression. The donor nature of these cells has been confirmed using Mabs to donor MHC molecules or fluorescence *in situ* hybridisation (FISH) after female into male organ transplantation (Starzl *et al* 1992 and 1993). However, the phenotype of these cells has not been established.

Tolerance has also been achieved by intra-thymic transplantation of whole donor cells such as islets (Posselt *et al* 1990 and 1993) or glomeruli (Remuzzi *et al* 1991), as measured by subsequent donor-specific unresponsiveness and prolonged allograft survival. In these experiments, thymic processing of alloantigen may lead to it being seen as self during negative selection. Injection of RT1.B^u and RT1.D^u MHC class II peptides into RT1^l LEW rats can lead to indefinite survival of RT1^u kidney allografts without resorting to immunosuppression. This is despite the MHC class I disparity in these animals (Sayegh *et al* 1993). The means by which such apparently effective tolerance is achieved is uncertain (e.g. Coutinho *et al* 1993), but may involve the recirculation of activated T cells to the thymus (Agus *et al* 1991).

Many experimental transplant models that achieve tolerance have dysregulated cytokine profiles, usually including downregulation of IL-2 (e.g. Dallman 1992, Dallman *et al* 1993). It would be a surprise if cytokines were not crucial to the outcome of rejection since they are critical to the activation of effector cells and also control the outcome of the T_H1/T_H2 equilibrium (at least in the mouse). One possibility was demonstrated *in*

vitro with the switching of cytotoxic CD8$^+$ T cells by IL-4 to a CD8$^-$ CD4$^-$ phenotype that secreted IL-4, IL-5 and IL-10 and behaved like T$_H$2 cells (Erard *et al* 1993). The plasticity of the immune system urges caution, however. Interleukin 2 deficient mice, for example, have relatively normal *in vivo* helper, cytotoxic and B cell responses, although secondary cytotoxicity *in vitro* is heavily dependent on exogenous IL-2 (Kündig *et al* 1993). The IL-2 receptor gamma chain is also part of the IL-7 and IL-4 receptors (Kondo *et al* 1994), so cytokine production and sensitivity are likely to be complexly regulated.

A full understanding of the mechanisms of tolerance is still some way off, but the last few years have revealed some important clues, both in the clinic and in experimental systems. Meanwhile, the unambiguous identification of risk factors and the optimisation of immunosuppressive drug doses is needed to help improve the long term survival of vascularised allografts.

CHAPTER 2:   MATERIALS AND METHODS.

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CHAPTER 2:

MATERIALS AND METHODS.

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

2.1 Animals:

Rats:

Inbred adult male LEW (RT1^l), WAG (RT1^u), PVG (RT1^c), DA (RT1.A^{av1}) and (DAxLEW)F1 rats were purchased from Harlan Olac, UK.

Mice:

Inbred male and female BALB/c mice were also purchased from Harlan Olac.

2.2 Buffers:

TBS: 0.15M NaCl, 0.025M Tris, 0.02% NaN$_3$ pH 7.5 at 25°C.

PBS: 0.01M phosphate buffer, 0.0027M KCl, 0.137M NaCl, pH 7.4 at 25°C.

X-GAL: 2mM MgCl$_2$, 0.1% sodium deoxycholate, 0.02% Nonidet P-40, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 0.1% X-Gal in PBS.

2.3 Monoclonal antibodies:

The following mouse monoclonal antibodies were used during the course of this work:

BMAC-5- IgG1 subclass. Detects rat macrophages (Spencer and Fabre 1990).

F16-4-4- IgG1 subclass. Detects partially purified DA MHC antigens (Hart and Fabre 1981a).

MN4-91-6- IgG1 subclass. Detects rat RTl.A class I MHC antigen

polymorphic determinant. (Milton and Fabre 1985).

MRC OX6- IgG1 subclass. Detects a monomorphic determinant on rat RTI.B MHC class II MHC antigens (Fukumoto *et al* 1982).

MRC OX8- IgG1 subclass. Detects rat CD8 molecules and labels all T cells left unlabelled by W3/25 (Brideau *et al* 1980).

MRC OX12- IgG2a subclass. An rat kappa chain antibody (Hunt and Fowler 1981).

MRC OX27- IgG2a subclass. Detects a polymorphic rat RT1.A determinant (Jefferies *et al* 1985).

MRC OX35- IgG2A subclass. A rat CD4 antibody that labels T cells and macrophages (Jefferies *et al* 1985).

NDS 60- Detects a polymorphic LEW RTI.A determinant. A gift from Dr. M. Dallman, Nuffield Department of Surgery, Oxford (Tellides 1988).

W3/25- IgG1 subclass. An rat CD4 antibody that labels thymocytes, macrophages and T helper cells (Williams *et al* 1977).

2.4 Peptides:

Peptide 1 (P1) is a 24mer corresponding to amino acids 57 to 80 of the α helical region of the α1 domain of the DA RT1.A class 1 molecule (Rada *et al* 1990) and the 15mers are derived from it. Peptide 3 (P3) is a 22 mer from the α2 domain of the same molecule (amino acids 143 to 164). A control peptide P2 was used in the proliferation assays. This peptide is derived from the ß sheet of the same class I molecule (amino acids 94-117). A second irrelevant control peptide (Px) was also used; this was derived from the mouse H2Ab[b] class II molecule (Larhammar *et al* 1983). The sequence of the peptides in the one letter code is given below:

```
P1   PEYWEQQTRIAKEWEQIYRVDLRT
A1   PEYWEQQTRIQKEWE
A2    EYWEQQTRIAKEWEQ
A3     YWEQQTRIAKEWEQI
A4      WEQQTRIAKEWEQI
A5       EQQTRIAKEWEQIYR
A6        QQTRIAKEWEQIYRV
A7         QTRIAKEWEQIYRVD
A8          TRIAKEWEQIYRVDL
A9           RIAKEWEQIYRVDLR
A10           IAKEWEQIYRVDLRT

P3   TRNKWERARYAERLRAYLEGTC
P2   TIQEMYGLDVGSDGSLLRGYRQDA
Px   YWMNSQPEILERTRAEL
```

All peptides except Px were synthesised by Cambridge Research Biochemicals (Cambridge, GB). Peptides 1,2 and 3 were provided lyophilised in powder form. They were dissolved in water using sonication to assist solution and further purified by desalting on a Sephadex G-10 column. They were then freeze-dried and reconstituted in 0.15M NaCl at 1mg ml^{-1}. The 15mers were provided in solution at 5mg ml^{-1}. These were sonicated, millipore filtered, and made up to 1mg ml^{-1} in 0.15M NaCl. Px was synthesised by Dr. V. Fowler at the Blond McIndoe Centre, East Grinstead, on a PepSynthesiser (CRB, Cambridge) using the F-moc polyamide method of solid phase synthesis. Px was then extracted with ether and dissolved in 0.1M NH_4 HCO_3.

2.5 Immunisations:

Rats were immunised subcutaneously with free (unconjugated) peptide emulsified in an equal volume of Freunds complete adjuvant. Fifty μg of peptide in 0.2ml of emulsion was given to each hind footpad. Boosts were given 4 weeks after the initial immunisation, using the same dose of peptide emulsified in Freunds incomplete adjuvant. Control animals were immunised with Freunds Adjuvant emulsified with saline.

2.6 Iodination of rabbit anti rat and mouse antibodies.

Purified, immunoadsorbent rabbit F(ab')$_2$ anti-rat F(ab')$_2$ (RAR) was prepared as previously described (Dalchau and Fabre 1979) and iodinated using the chloramine T method. Briefly, 10μl of 2mg ml^{-1} chloramine T in Phosphate buffer were added to 1mCi carrier free ^{125}Iodine (Amersham, England) along with 25μl of either Rabbit anti-rat (RAR) or Rabbit anti-mouse (RAM) antibodies at 1mg ml^{-1} in 0.5% BSA/TBS. After 2 min, 50μl of 0.5mg ml^{-1} tyrosine solution in phosphate buffer were added to quench the reaction. After a further 2 min, 100μl 10% BSA/TBS were added and the mixture applied to a Pharmacia PD10 column, previously equilibrated at room temperature with 10ml 0.1% BSA in TBS. Aliquots of 1ml were collected by eluting with 0.1% BSA/TBS. The iodinated material is pooled and diluted to a specific activity of approx. 2MBq ml^{-1}.

2.7 Indirect radioactive binding assays for alloantibodies to peptides:

Ninety six well PVC microtiter plates (Dynatech Ltd., UK) were coated with peptide by adding 25μl peptide at 100μg ml^{-1} in 0.15M NaCl overnight. Control wells were incubated with 0.15M NaCl only. Plates were washed thrice with 0.1% bovine serum albumin (BSA) in PBS. One hundred microlitres of 5% BSA/PBS was added to each well for 1 hour to block protein binding sites, and the plates were washed again as above. Tripling dilutions of the sera to be analysed were made in 0.5% BSA/PBS, and 25μl were added in duplicate to the peptide coated plate. After a 1 hour incubation, the sera were removed by aspiration, the plates were washed as before, and 50μl of ^{125}I-labelled RAR was added (approximately 300,000 cpm per well in 0.5% BSA/PBS). The plates were again incubated for 1 hour before aspirating and washing as above. Individual wells were then cut out and bound radioactivity was measured on a LKB Minigamma spline gamma counter. All incubations were at 4°C.

2.8 Indirect antibody binding assay to detect antibodies to whole class I molecules:

Red blood cells (RBC) from a DA (RT1.A^{av1}) rat were used as target cells (since they express MHC class I molecules) at a concentration of 10^9 ml^{-1} in 0.5% BSA/PBS, having initially been washed thrice in PBS to remove lymphocytes. Tripling dilutions of sera were made up in 0.5% BSA/PBS and 25μl of each dilution were added to 25μl of target RBC. After 1hr incubation, tubes were washed twice in 0.5% BSA/PBS before 100μl of ^{125}I-labelled RAR was added (approximately 300,000 cpm per well in 0.5% BSA/PBS). One hour later, the samples were washed again as before, the pellets reconstituted in PBS and counted on a gamma counter. All spins were at 2000rpm and all incubations at 4°C.

2.9 Typing assays to determine rat MHC haplotypes:

Rats to be typed had 1ml blood removed from the tail vein into 0.5ml saline / 2% heparin solution. The red blood cells were washed twice in PBS and the buffy coat removed. The cells were resuspended in 0.5ml 0.5% BSA/PBS and 25μl duplicates were incubated for 1hr with an equal volume of each of the following monoclonal antibodies individually: MN4 (1/100 dilution), F16 (1/100 dilution), OX27 (1/10 dilution), NDS60 (1/10 dilution) and saline only. The cells were then washed as before and approx. 300,000 cpm of ^{125}I-RAM in 25μl 0.5% BSA/PBS was added for 1hr. The cells were washed as before, reconstituted in 0.5ml PBS and counted on a gamma counter. All spins were at 2000rpm and all incubations at 4°C.

2.10 Cell preparations:

Cervical, popliteal and para-aortic lymph nodes from experimental rats were made into a single cell suspension in Hepes buffered RPM1 1640 medium (Gibco BRL, UK) containing 1% normal rat serum (NRS), 2mM L-glutamine, 5 x 10^{-5}M 2-mercaptoethanol, 100μg ml^{-1} penicillin and 100μg ml^{-1} streptomycin at 4°C. The cells were washed twice, harvested by centrifugation and resuspended at 2 x 10^6 cells ml^{-1} in RPM1 1640 medium without Hepes, supplemented as above, but with 5% NRS (the "culture medium").

2.11 T cell subset analysis:

Cell suspensions at 2x10^6 cells ml^{-1} were depleted of adherent cells by incubating them for 30min on Sephadex G-10 columns that had been equilibrated with Hepes-buffered RPMI 1640 medium at 37°C. The cells were then eluted with one column volume of fresh buffer and kept at 4°C for the remainder of the procedure. The CD8$^+$ T cell fraction at 5x10^7 cells ml^{-1} was incubated with the following monoclonal antibodies at saturating concentrations: MRC OX12, MRC OX35, W3/25, MRC OX6 and BMAC-5 (to deplete B cells, CD4$^+$ cells and macrophages). For the CD4$^+$ enriched T cell population, the same procedure was performed, except that MRC OX35 and W3/25 were replaced with MRC OX8 (to remove CD8$^+$ T cells). After a 30min incubation, the cells were washed twice in medium and resuspended at 10^8 cells ml^{-1}. Sheep anti-mouse IgG-coupled Dynabeads (Dynal Ltd., Wirral, Merseyside, U.K.) were added to the suspensions at a ratio of 10 beads per cell and incubated for 1hr on ice, with frequent mixing. The beads were removed using a magnetic particle concentrator (Dynal) and the remaining purified cell populations were washed once and resuspended in culture medium at 2x10^6 cells ml^{-1}.

2.12 Proliferation assays:

Purified T cell subsets, or lymph node cell preparations of 2×10^5 lymph node cells, in a total volume of $200\mu l$ of culture medium were incubated in individual wells of 96 well plates (Sterilin, UK) at 37°C in 5% CO_2 in air. The cells were continuously exposed to peptides at various concentrations (see figure legends). Concanavalin A at $10\mu g$ ml^{-1} was used as a positive control. Each well was pulsed with $1\mu Ci$ ^3H-thymidine 24hr before harvesting. Cells were harvested at 3,4 or 5 days after initiation of the culture. Individual filters were immersed in Optiscint Hisafe III scintillation fluid (LKB/Pharmacia, Stockholm) and cell bound radioactivity measured on a scintillation counter.

2.13 Transformation of lymphocytes with retroviral vectors:

Lymph node cells from the cervical and mesenteric lymph nodes of PVG RT1^c rats were dispersed into RPMI medium supplemented with 5% FCS, 2mM L-glutamine, $5 \times 10^{-5}M$ 2-mercaptoethanol, $100\mu g$ ml^{-1} penicillin and $100\mu g$ ml^{-1} streptomycin buffered with 10mM Hepes pH 7.4. Viable lymphocytes were obtained from these suspensions by isopycnic density gradient sedimentation over 1/3 volume lymphopaque (Nyegaard, Norway) at 2000rpm for 20min at 20°C. The interface cells were removed, resuspended in medium and counted on a haemocytometer, checking viability with 0.2% w/v trypan blue in PBS. The cells were spun down at 400g 10min 20°C and resuspended at 10^6 ml^{-1} in RPMI supplemented with 2mM L-glutamine, $5 \times 10^{-5}M$ 2-mercaptoethanol, $100\mu g$ ml^{-1} penicillin, $100\mu g$ ml^{-1} streptomycin, 10% FCS, 13% CAS and $5\mu g$ ml^{-1} Con A. X-irradiated (2500 rad) syngeneic spleen cells at $2-5\times10^5$ ml^{-1} were used as APCs.

Lymphocytes were periodically removed from this bulk culture and

co-cultured with confluent, x-irradiated (2500 rad) AM12 fibroblast packaging cells that had previously been transfected with a retroviral vector containing the *E. coli lac z* gene. Hexadimethrine Bromide at $2\mu g$ ml^{-1} in PBS was added to the co-cultures and the lymphocytes were fed and cleaned over lymphopaque every two days.

To analyse the lymphocytes for *lac z* expression, 10^6 viable cells were removed, washed twice in PBS and cytospun onto microscope slides. The cells were fixed in 0.5% glutaraldehyde/PBS for 15min at 27°C, washed again in PBS and then incubated at 37°C for 2-4hr in X-Gal solution. The slides were then washed in PBS and counter-stained using 0.1% w/v Eosin (Gurr) in PBS. Cells expressing *lac z* stain blue.

2.14 Kidney transplantation:

All initial transplants and interstitial dendritic cell-free retransplants were left orthotopic kidney grafts employing end to end anastamosis of the renal artery, vein and ureter. Total ischemia times all ranged between 25 and 35 minutes and biopsies of the transplanted kidney were taken at the time of grafting and 7 days after transplantation for histological analysis. The procedure has been described in detail (Fabre *et al* 1971). CsA (a gift from Sandoz, Switzerland) was administered orally by gavage to the first transplant recipients at a concentration of 10mg kg^{-1} (dissolved in olive oil at 60°C) for 10 days or 15mg kg^{-1} for 14 days. When performing retransplants, grafting was performed 10 days after the recipients had been boosted with antigen:- these animals did not receive CsA. Control, untreated single transplant, LEW (RT1^l) rats receiving a (DAxLEW)F1 kidney were not immunised and did not receive CsA.

2.15 Histology:

Biopsies and sections of intact kidneys taken from terminated animals were stored in 10% v/v formalin prior to alcohol dehydration, wax embedding and staining with haematoxylin and eosin to observe the gross structure of the kidney.

2.16 Urea tests:

These were performed using a urea testing kit (Boehringer-Mannheim, Germany) in accordance with the manufacturers instructions. Test were repeated once and rats were terminated if blood urea levels exceeded 300mg/100ml sera.

2.17 Flow cytometry:

a) Single colour analysis using directly labelled FITC-antibodies:

Viable lymphocytes at 10^8 ml^{-1} in 0.5% BSA/PBS were prepared from rat lymph nodes after mechanical dispersion by density gradient centrifugation over 1/3 volume lymphopaque (Nyegaard, Sweden) at 2000rpm for 20min at 25°C. Twenty-five microlitres of cells were mixed with 25μl of the appropriate FITC-labelled monoclonal antibody and incubated on iced water for 30min. The cells were washed twice with 1.6ml 0.1% BSA/PBS (400g 10min 4°C) and resuspended in 2% formalin/PBS to await analysis.

b) Single colour analysis using PE-labelled antibodies:

Between $2x10^5$ and 10^6 viable lymphocytes were incubated with 10μl PE labelled W3/25 and/or MRC OX 8 antibodies (Serotec, Oxford) in a total volume of 40μl for 30min on ice. Cells were washed in 0.5% BSA/PBS and resuspended in 2% formalin/PBS prior to analysis.

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c) Double colour analysis using PE-labelled antibodies and FDG:

To analyse the nature of rat T cells transfected with a retrovirus containing the *E. Coli lac z* gene, between 2×10^5 and 10^6 viable lymphocytes were incubated with 10μl PE labelled W3/25 and/or MRC OX 8 in a total volume of 40μl for 30min on ice. The cells were pelleted at 400g 10min 4°C and resuspended in 50μl 'staining buffer' (15mM NaPO$_4$ pH 7.3, 150mM NaCl, 10mM Hepes pH 7.3, 4% v/v FCS). The cells were incubated at 37°C for 10min before rapidly mixing with 50μl 2mM FDG. After a 1 min incubation at 37°C, the reaction was quenched by adding 2ml ice cold 'staining buffer' and the tubes placed on ice for immediate analysis.

All FACS analyses were performed on a Becton/Dickson flow cytometer.

CHAPTER 3:   **THE IMMUNE RESPONSE TO DA MHC**

               CLASS I-DERIVED PEPTIDES IN DIFFERENT

               RAT STRAINS.

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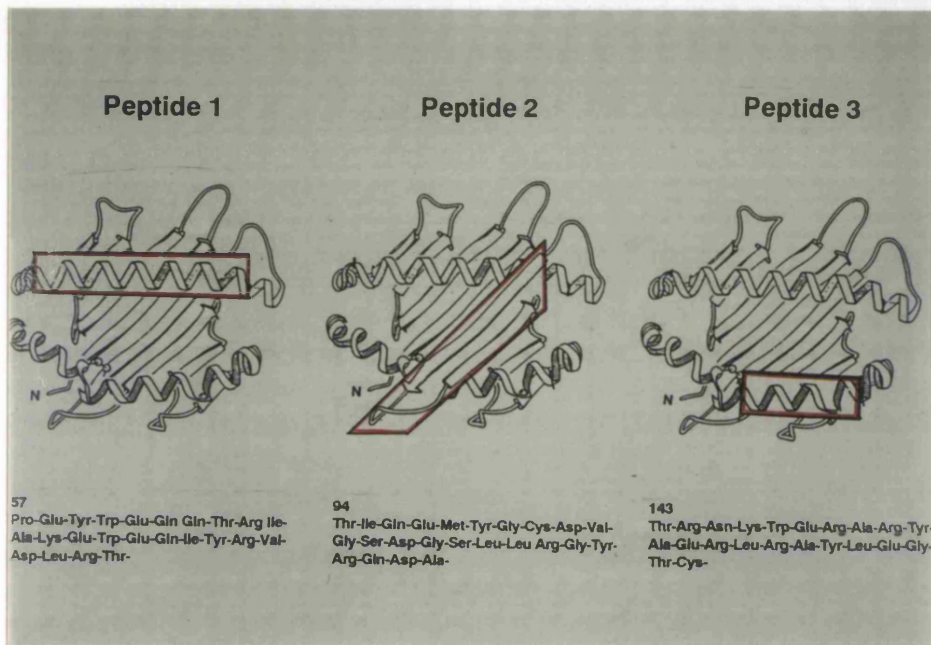
CHAPTER 3:

THE IMMUNE RESPONSE TO DA MHC CLASS I-DERIVED PEPTIDES IN DIFFERENT RAT STRAINS.

3.1 Allorecognition Of DA RTI.A^{av1} MHC Class I Derived Peptides:

Three peptides were synthesised by Cambridge Research Biochemicals (UK) that were central to most of the work in this thesis. They were derived from the hypervariable a helical and ß sheet regions of the classical DA MHC class I molecule and termed Peptide 1 (P1), Peptide 2 (P2) and Peptide 3 (P3). The regions from which they were derived, along with their lengths and sequences are given in FIGURE 1. The regions of the DA class I molecule corresponding to these peptides were chosen because the equivalent regions in man and mouse are highly polymorphic (Parham *et al* 1988).

Previous work in our laboratory showed that two of these peptides, P1 and P3, could elicit a strong and specific antibody response when injected into allogeneic LEW RTI^{l} rats, whereas P2 had only a weak effect on antibody production (Fangmann *et al* 1992a). This was observed without conjugating the immunising peptides to carrier proteins. The way in which carrier-free peptides are presented by B cells is illustrated in FIGURE 2. The immune response to these peptides was probably T helper cell dependent, since peptide specific CD4^{+} T cells could be found in lymph node tissue.

Immunisation of LEW rats with P1 and P3 resulted in the statistically significant acceleration of subsequent DA skin allograft rejection (Fangmann *et al* 1992a). This proved that priming to indirect recognition, in the absence of priming to direct recognition (i.e. peptides rather than intact allo-MHC class I molecules) influenced rejection responses *in vivo* and was a relevant physiological mechanism.

Peptide 1

Peptide 2

Peptide 3

57
Pro-Glu-Tyr-Trp-Glu-Gln Gln-Thr-Arg Ile-
Ala-Lys-Glu-Trp-Glu-Gln-Ile-Tyr-Arg-Val-
Asp-Leu-Arg-Thr-

94
Thr-Ile-Gln-Glu-Met-Tyr-Gly-Cys-Asp-Val-
Gly-Ser-Asp-Gly-Ser-Leu-Leu Arg-Gly-Tyr-
Arg-Gln-Asp-Ala-

143
Thr-Arg-Asn-Lys-Trp-Glu-Arg-Ala-Arg-Tyr-
Ala-Glu-Arg-Leu-Arg-Ala-Tyr-Leu-Glu-Gly-
Thr-Cys-

FIGURE 1:

Representation of the MHC class 1 regions from which Peptides 1, 2 and 3 were derived along with their amino acid sequences (see also Chapter 2). This diagram was created by Josef Fangmann.

86
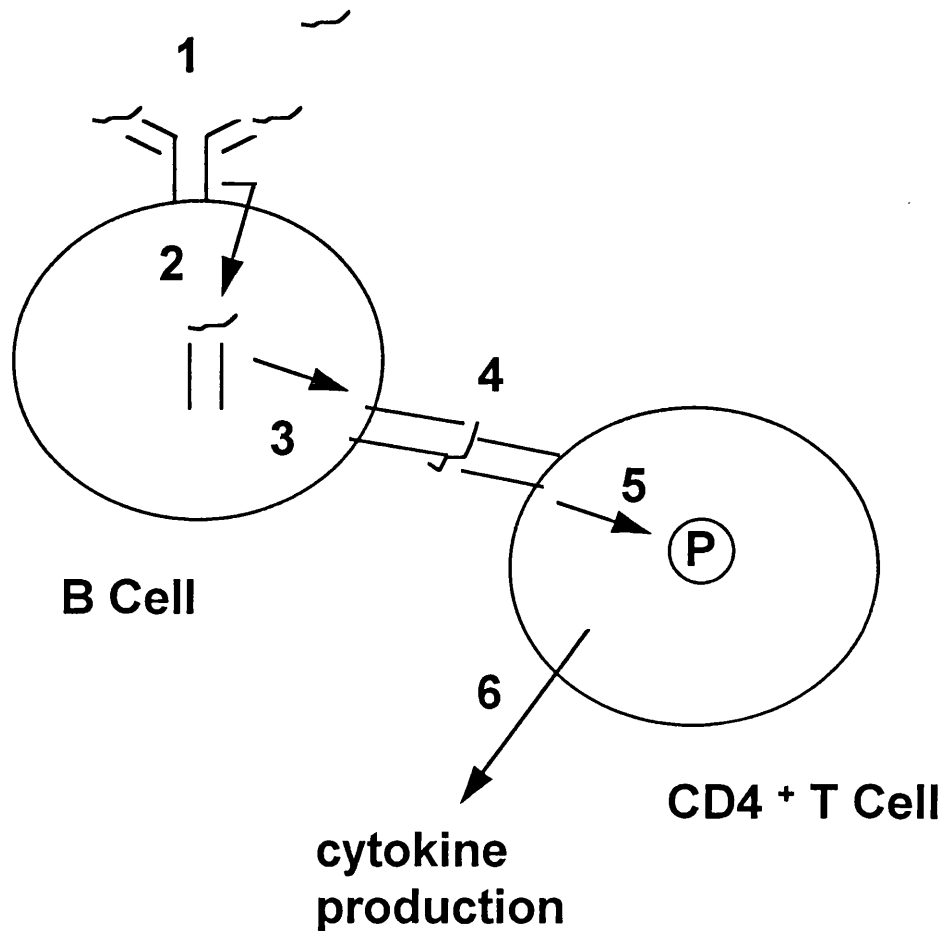
FIGURE 2:

Schematic representation of B cell antigen presentation.

1 Free peptide specific for the B cell Ig receptor binds at the cell surface.

2 Ig receptor-peptide antigen complex is internalised and degraded.

3 Processed (or possibly intact) peptide associates with MHC class II molecules and routes to the cell surface.

4 Class II-peptide complex interacts with the TCR on CD4$^+$ T cell. Various co-receptors and accessory molecules are also involved.

5 The T cell is triggered and various kinase pathways are activated.

6 Upregulation of cytokine production, primarily IL-2, enables T cell to initiate helper immune responses.

LEW rats receiving DA skin and kidney allografts also had splenic CD4$^+$ T cells that proliferated *in vitro* when stimulated with P1, but not with P3 or P2. This demonstrated that at least for P1, a physiologically relevant epitope was being studied. However, this effect was not apparent in T cells derived from lymph nodes (Fangmann *et al* 1992b). This raised interesting questions about antigen processing *in vivo*, the influence of different antigen presenting cells and the 'form' of the antigen in relation to the allorecognition response.

With this background, I initially investigated the immunity to these peptides in a wider range of class I disparate rat strains with a view to further studying their role in allorecognition. I concentrated on P1 and P3, as these were the most immunogenic according to the studies mentioned above.

3.2 RESULTS:

3.2a Antibody Responses In Peptide Immunised Rats:

The alloantibody response to the RTI.A^{av1} peptides P1 and P3 was investigated by immunising LEW RTI^l, WAG RTI^u and PVG RTI^c rats in the hind footpad with 50μg of either unconjugated P1 or P3. In the latter set of experiments, DA RT1.A^{av1} rats were also immunised with P3. The animals were boosted after four weeks. Their sera were taken for analysis using the immunising peptide as a target in an indirect radioactive binding assay.

FIGURE 3 shows a typical primary antibody response to P1 obtained 28 days after immunisation. Both LEW and WAG strain rats mount a strong peptide-specific response even before secondary immunisation. The PVG animal has a negligible antibody response, as might be expected in a strain that finds RTI.A^{av1} antigens poorly immunogenic (Butcher and Howard 1982). The secondary responses of the LEW and WAG rats are strong but
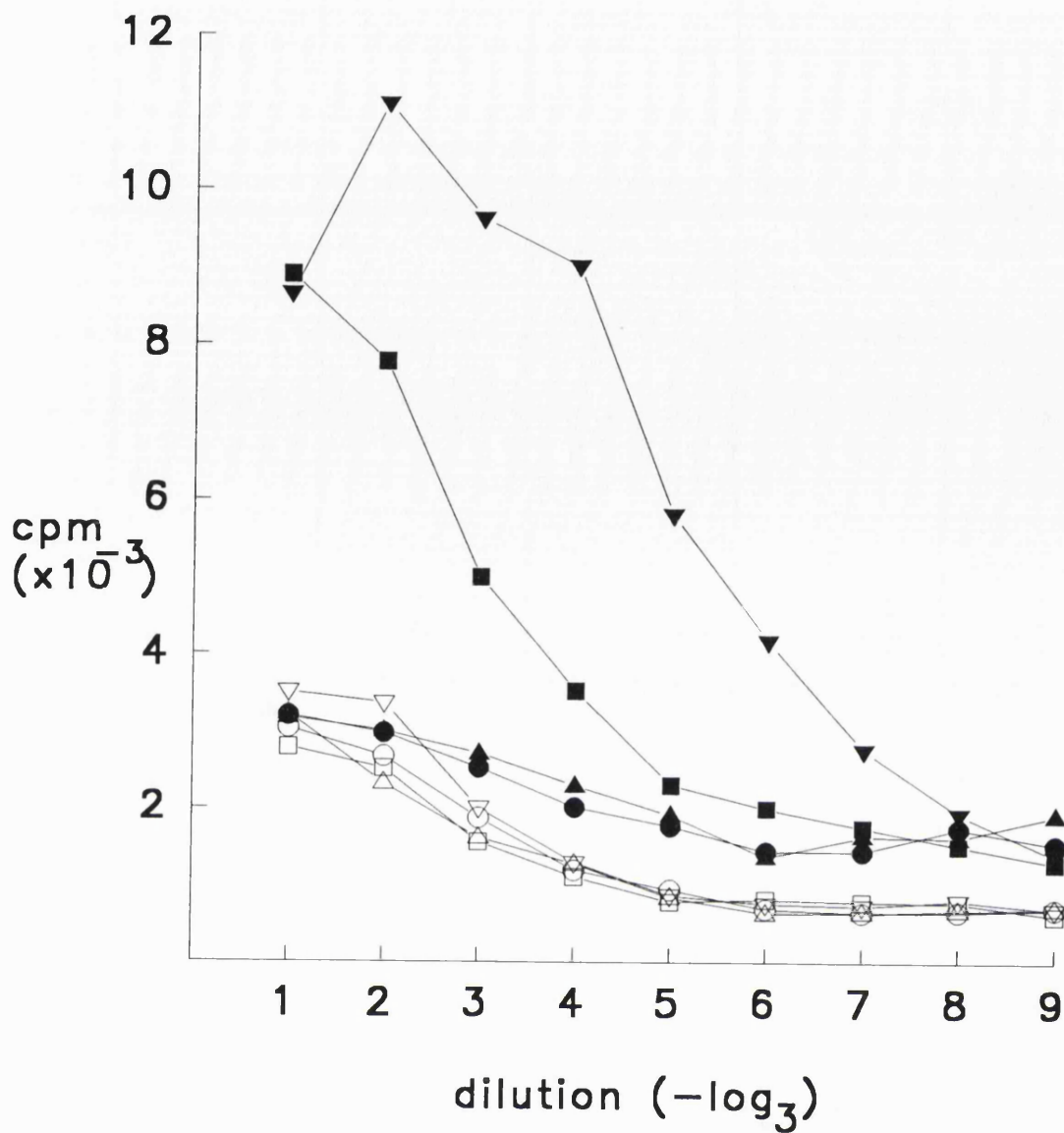
FIGURE 3:

Primary Antibody Response to DA Class I Peptide 1.

Sera were taken from WAG (▼), LEW (■) and PVG (●) rats 28 days after immunisation with P1 in FCA. A LEW control animal was immunised with saline in FCA (▲). Titrations were performed in tripling dilutions on P1 coated plates (filled symbols) or control saline plates (open symbols). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 0.5% and 9% of the mean values obtained.
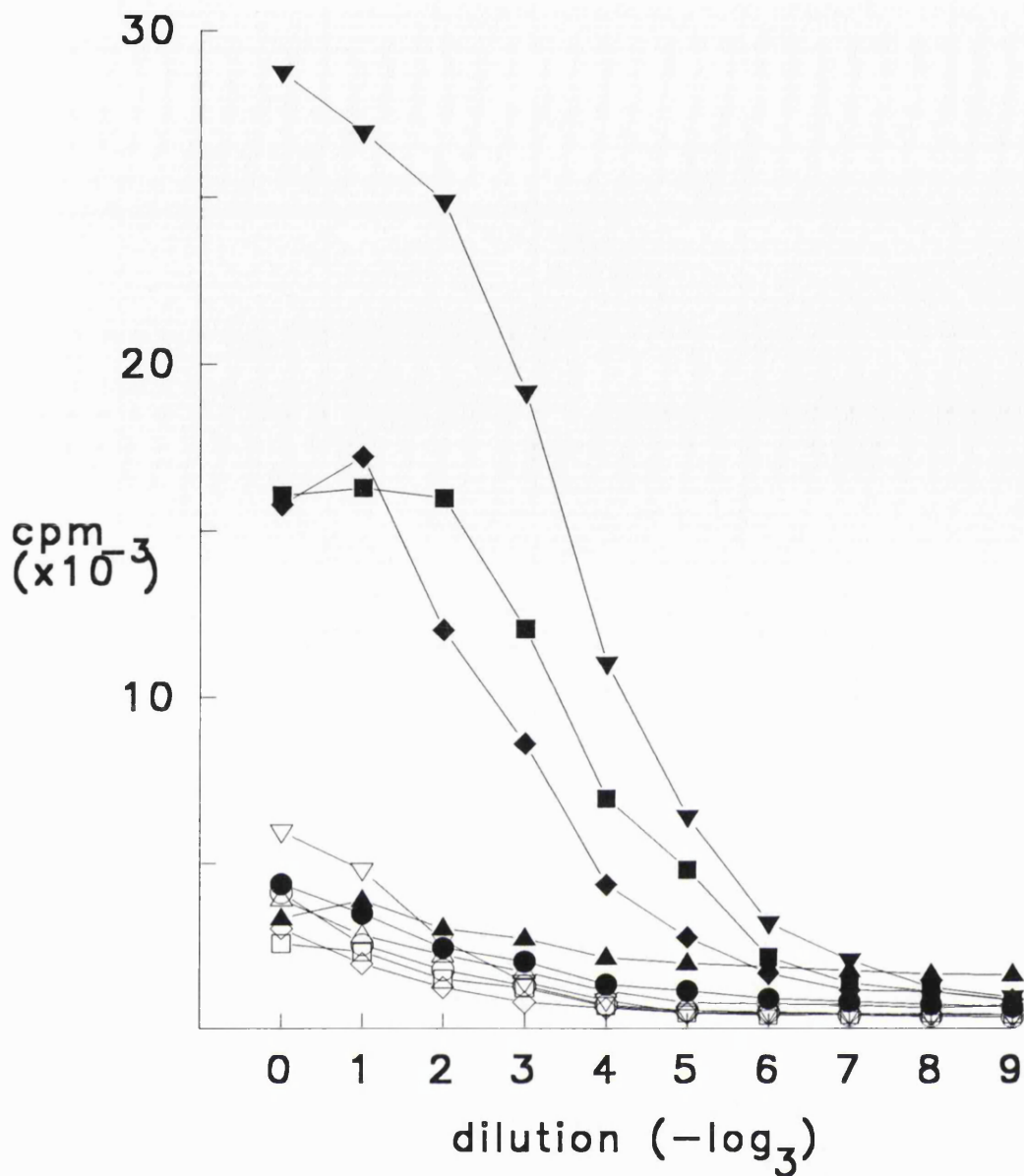
FIGURE 4:

Primary Antibody Response to DA Class I Peptide 3.

Sera were taken from WAG (▼), LEW (■) and PVG (●) and DA (◆) rats 28 days after immunisation with P3 in FCA. A control LEW animal was immunised with saline in FCA (▲). Titrations were performed in tripling dilutions on P3 coated plates (filled symbols) or control saline plates (open symbols). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 0.6% and 10% of the mean values obtained.

comparable to the primary response; that of the PVG rat shows a slight increase above background in most cases (data not shown).

FIGURE 4 similarly shows the primary antibody response to P3. Again, both LEW and WAG rats have good titers to the alloantigen, whereas PVG produces no RT1.A^{av1} alloantibodies. This pattern of reactivity is also reflected in the secondary responses of these animals (data not shown).

Surprisingly, however, the DA animal immunised with its own RT1.A^{av1}-derivative peptide exhibited primary and secondary antibody responses that were comparable with the LEW rat. This response was noted in repeat assays and with each of the three experimental animals used in the series.

3.2b T Cell Responses In Peptide Immunised Rats:

The B cell compartment of WAG and LEW rats immunised with RTI.A^{av1} peptides is capable of alloantibody production, whereas that of the PVG rat is not. To investigate whether the same was true of the T cell compartment, the animals used for the experiments shown in FIGURES 3 and 4 were terminated 10 days after boosting and their lymph nodes removed from the cervical, popliteal, mesenteric and para-aortic regions as detailed in Chapter 2. Single cell suspensions of these cells were set up *in vitro* in three or four day culture with the immunising peptide at different dilutions as the stimulus. P2 was used as a peptidic control.

FIGURE 5 shows that both LEW and WAG LN cells (T cells) proliferate very strongly and specifically to P1 *in vitro* at both 25 and 2.5μg ml^{-1}. PVG LN cells lack any T cell response to the antigen. The control LEW animal, which has been immunised with saline and Freunds adjuvant, has a relatively high but nonspecific proliferative response that acts as a

background for the other animals. In the LEW rat, this response is restricted to the CD4$^+$ T cell subset and is APC dependent (data not shown, but see Chapter 7).

FIGURE 6 shows the same experiment performed with LN cells taken from P3 immunised rats. The findings are similar to the P1 experiment in that both WAG and LEW rat LN cells respond strongly and specifically to P3 at 25μg ml^{-1} and 2.5μg ml^{-1}. However, the WAG rat invariably mounts a stronger response to this allopeptide than does its LEW counterpart. This can be compared with the stronger LEW response to P1 in FIGURE 5. Typically, proliferation rises from day 3 to day 4 in the WAG strain, whereas with the LEW rat, peak counts are usually obtained on day 3. PVG rats show no response to P3 - indeed, their T cell responses are usually lower than those of the LEW control. Counts obtained from control LEW animals vary somewhat from experiment to experiment, but the response obtained to peptide and to medium only is always similar. Interestingly, the DA rat showed no evidence of significant T cell responsiveness to P3 despite its apparent antibody production. However, these *in vitro* experiments are insufficiently sensitive to completely rule out T cell responsiveness *in vivo*.

3.2c Immunisation Using Allogeneic DA Spleen Cells:

The experiments thus far have examined the immune response to synthetic peptides by immunising with the peptide and then measuring an *in vitro* reaction to the same peptide. This does not prove that these allopeptides are physiologically relevant, since the possibility exists that a small organic or minor peptide contaminant is the cause of the immune response. To eliminate this possibility, LEW, WAG and DA rats were immunised with fresh, red blood cell free, DA spleen cells emulsified in adjuvant. This represents a biological source of DA class I molecules
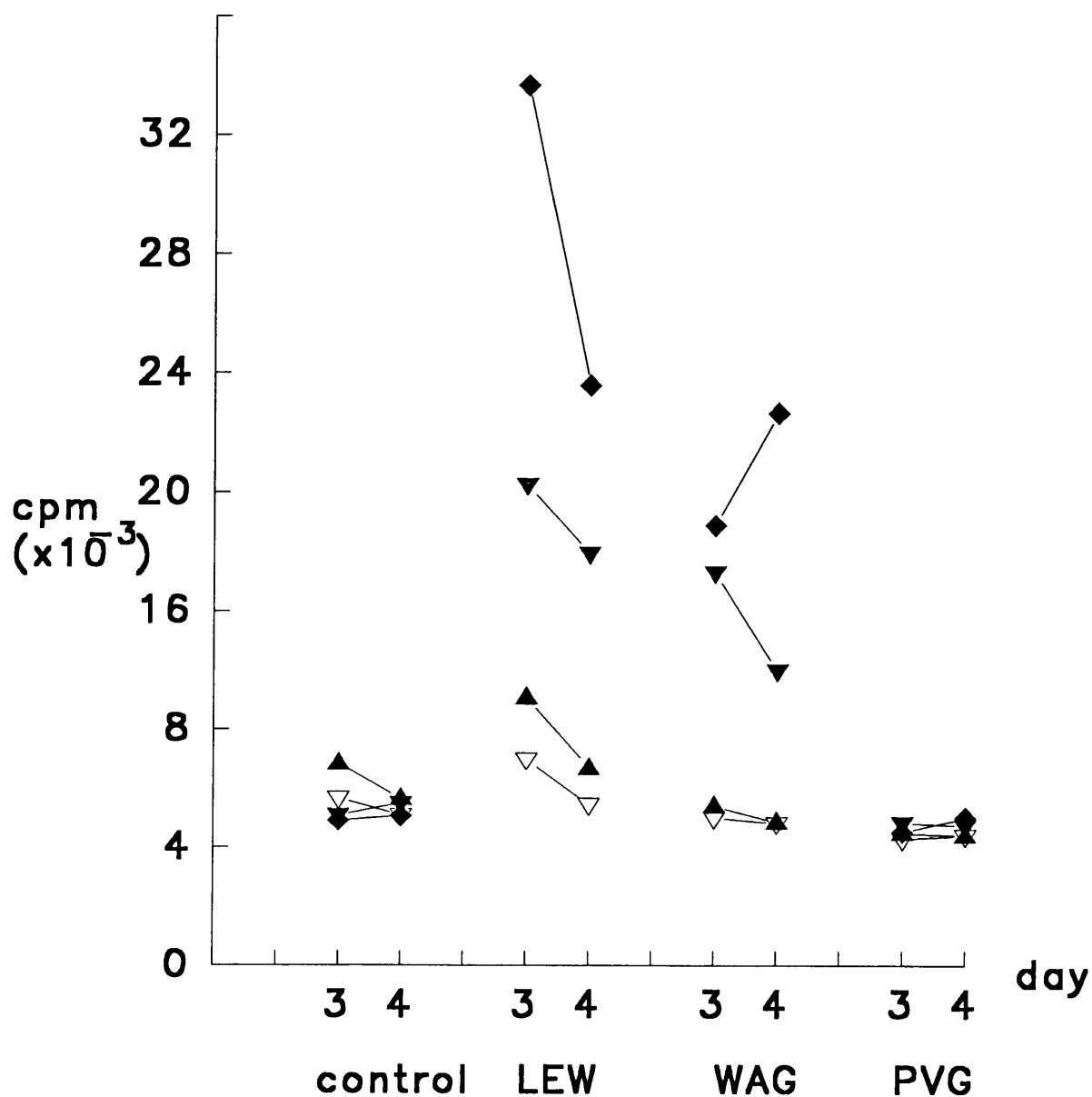
FIGURE 5:

Proliferative Responses to DA Derived Peptide 1.

LEW, WAG and PVG animals were immunised with P1 in FCA and boosted. A control LEW rat was immunised with saline emulsified in adjuvant. Their LN cells were stimulated *in vitro* with either 25μg ml⁻¹ P1 (♦), 2.5μg ml⁻¹ P1 (▼), 25μg ml⁻¹ irrelevant peptide Px (▲) and medium only (▽). Cells were harvested 24hr after pulsing with ³H-thymidine and 3 or 4 days after stimulation. cpm refers to counts per minute of incorporated tritium. Standard errors were between 1% and 11% of the mean values obtained.

cpm
$(\times 10^{-3})$

18
16
14
12
10
8
6
4
2
0

3 4   3 4   3 4   3 4   3 4   day
Control LEW WAG PVG DA
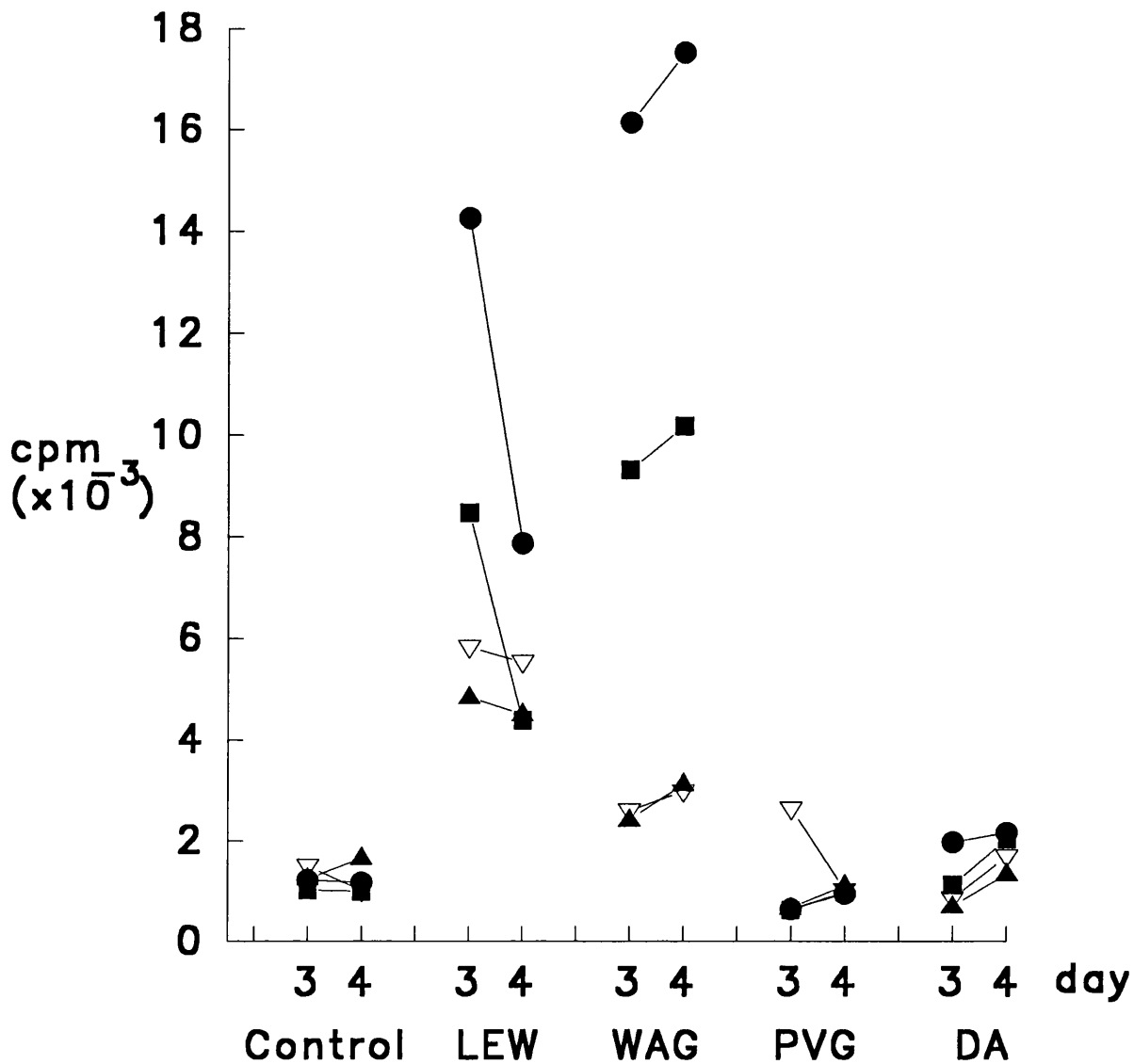
FIGURE 6:

Proliferative Responses to DA Derived Peptide 3.

LEW, WAG, PVG and DA animals were immunised with P3 in FCA and boosted. A control LEW rat was immunised with saline emulsified in adjuvant. Their LN cells were stimulated *in vitro* with either 25μg ml⁻¹ P3 (●), 2.5μg ml⁻¹ P3 (■), 25μg ml⁻¹ irrelevant peptide P2 (▲) and medium only (▽). Cells were harvested 24hr after pulsing with ³H-thymidine and 3 or 4 days after stimulation. cpm refers to counts per minute of incorporated tritium. Standard errors were between 0.8% and 10% of the mean values obtained.

(amongst other things) which can be processed 'naturally'. These animals were investigated *in vitro* for antibody production and T cell reactivity towards the synthetic peptides P1, P2 and P3. Any T or B cell response can only be due to natural processing of the DA RTI.A^{av1} alloantigens present on the surface of splenic cells. This then provides us with evidence that these peptides are valid tools with which to investigate alloimmunity.

In previous work (Fangmann *et al* 1992b), LEW rats transplanted with DA skin and kidney allografts had CD4$^+$ T cells that proliferated *in vitro* on exposure to P1. This demonstrated that P1 was of physiological relevance.

3.2d Antibody Responses To Peptides In DA Spleen Cell Immunised Rats:

LEW, WAG and DA rats were immunised with approximately 10^7 DA spleen cells emulsified in a total volume of 0.5ml in adjuvant. The animals were boosted after 28 days with spleen cells from a different naive DA littermate. The primary antibody response to the P1, P2 and P3 DA class I-derived peptides was then investigated *in vitro* in an indirect radioactive binding assay (FIGURE 7). Quite high backgrounds were obtained in these experiments and even at a 1 in 6561 dilution, levels of antibody binding could not be reduced to those of saline control wells in both the control (FIGURE 7d) and experimental animals (FIGURE 7a-c). Taking this into account, it is still evident that in both the LEW (FIGURE 7b) and WAG (FIGURE 7c) rat, weak primary antibody responses are found to P3 in animals immunised with DA spleen cells. Their P1 responses, however, remain at background levels.

Interestingly, the DA rat, immunised with spleen cells derived from a littermate, possibly shows a weak response to P3. This is very unlikely to be due to contaminants and raises questions about the nature of peripheral tolerance to class I antigens *in vivo*.

FIGURE 7:

Primary Antibody Responses to DA MHC Derived Peptides.

DA (a), LEW (b) and WAG (c) rats were immunised with 10^7 DA spleen cells emulsified in FCA. A control LEW rat was immunised with saline emulsified in FCA only (d). Their primary antibody responses were titrated in tripling dilutions against the DA MHC class I derived peptides P1 (●), P3 (■) and saline (▽) bound to PVC plates. cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 1% and 12% of the mean values obtained.

96
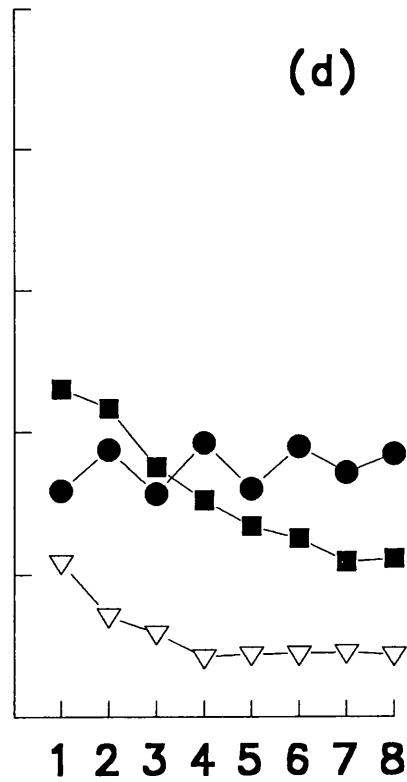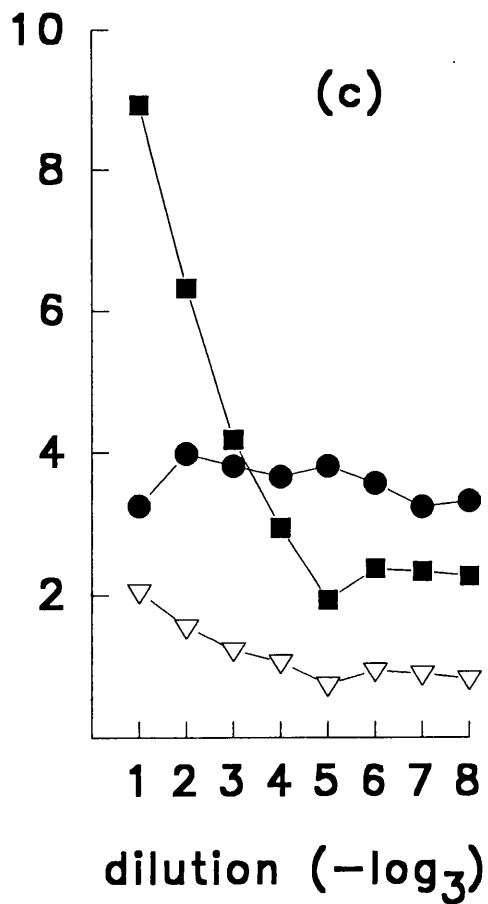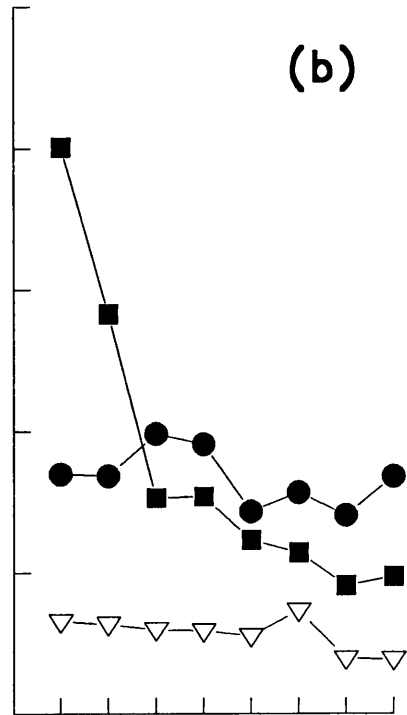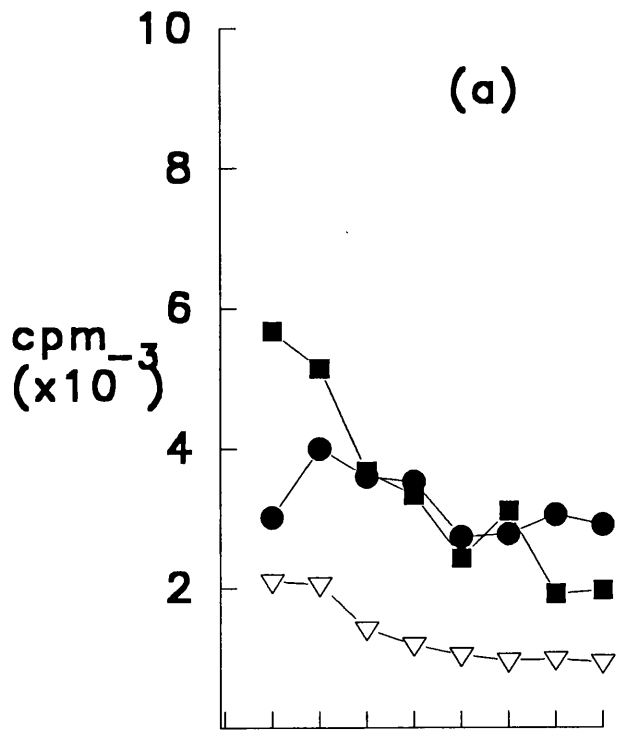
Secondary antibody responses in these experiments were comparable to primary responses (data not shown). It thus appears that class I molecules present on spleen cells can be processed and presented to B cells into a form related to P3. Whether or not P1 is naturally processed could still be open to question from these assays alone.

3.2e T Cell Responses In Spleen DA Cell Immunised Rats:

To investigate the T cell response to our synthetic peptides in animals immunised with biological material, LN cells were removed from the same experimental subjects as used in FIGURE 8. Single cell suspensions from these LEW, WAG and DA rats, as well as the saline immunised control, were cultured with either P1, P2 or P3 for 3 or 4 days *in vitro*. Both the LEW and WAG rats show proliferative responses to P1 that rise from day 3 to day 4. In this experiment, the LEW animal also appears to have T cells that respond to P3 and P2, whereas the WAG rat has levels of responsiveness that correspond to background with these peptides. The DA rat exhibits no evidence of T cell activity to these synthetic autologous peptides in comparison with the low apparent antibody response observed above. The control LEW animal has high background responses to these peptides that are comparable to the responses obtained in the spleen cell immunised LEW rat. This therefore casts some doubt over the peptide specificity of the response in this particular LEW animal.

To eliminate the possibility that proliferation was non-peptide specific, an irrelevant peptide control (derived from the mouse H-2A^{b}b class II molecule and termed here Px), was included in two subsequent assays, one of which is shown in FIGURE 9. In this figure, LEW and WAG rats respond specifically to P1 and also towards P2, but there is no response to P3. The DA rat has no significant response to these peptides. Unfortunately, a control LEW animal was not available for analysis in this

experiment.

The counts obtained in these two experiments were quite low, although stimulation of these cultures with concanavalin A mitogen as a positive control always resulted in counts between 40,000 and 150,000 cpm. The 'concentration' of class I molecules in these spleen cell preparations and the efficiency of processing them into the P1, P2 or P3 determinants above all the others may explain the relatively low (but detectable) peptide specific response *in vitro*. Nevertheless, the WAG response to P3 in particular is notable after spleen cell immunisation.
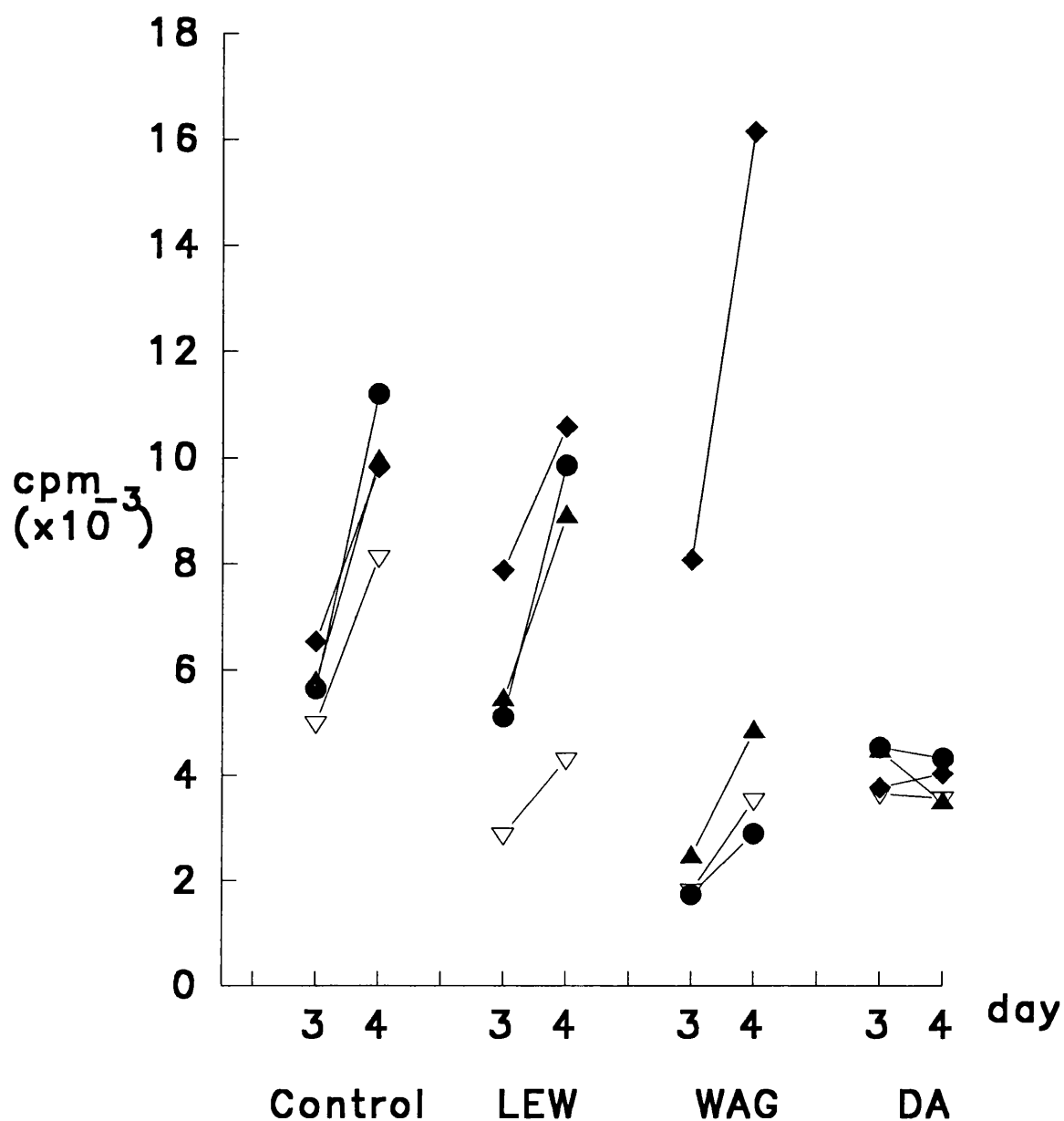
FIGURE 8:

Proliferative Responses to DA MHC Derived Peptides.

LEW, WAG and DA rats were immunised with 10^7 DA spleen cells emulsified in FCA. A control LEW rat was immunised with saline in FCA only. After boosting, their LN cells were stimulated *in vitro* with P1 (◆), P2 (▲), and P3 (●) at 25μg ml⁻¹ or medium (▽). Cells were harvested 24hr after pulsing with ^{3}H-thymidine and 3 or 4 days after stimulation. cpm refers to counts per minute of incorporated tritium. Standard errors were between 1.5% and 12% of the mean values obtained.
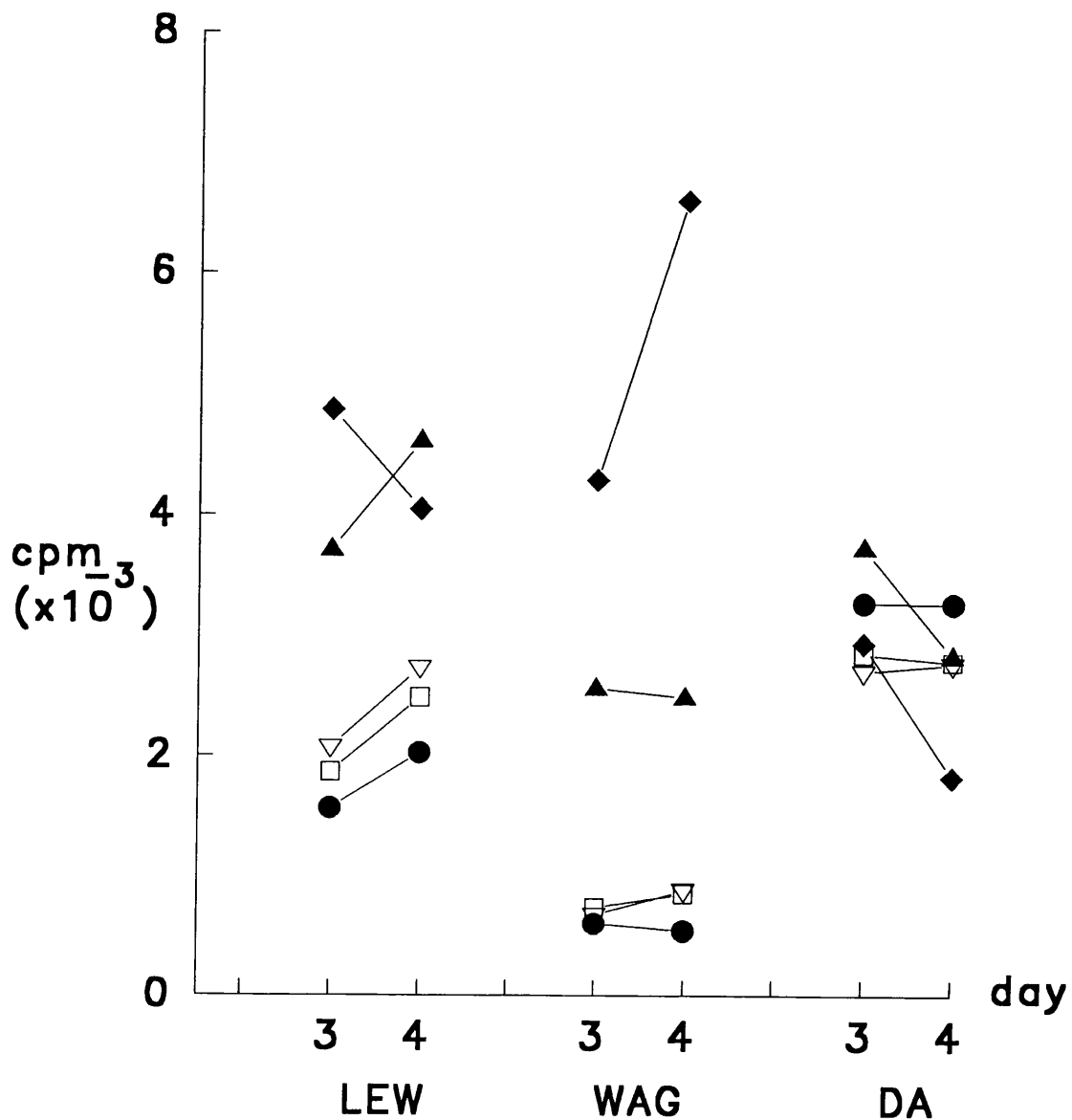
FIGURE 9:

Proliferative Responses to DA MHC Derived Peptides.

LEW, WAG and DA rats were immunised with 10^7 DA spleen cells emulsified in FCA. After boosting, their LN cells were stimulated *in vitro* with P1 (◆), P2 (▲), P3 (●), irrelevant peptide Px (□) all at $25\mu g$ ml^{-1} and medium (▽). Cells were harvested 24hr after pulsing with ^{3}H-thymidine and 3 or 4 days after stimulation. cpm refers to counts per minute of incorporated tritium. Standard errors were between 1.5% and 11% of the mean values obtained.

3.3 DISCUSSION:

These data illustrate several points concerning alloimmunity in the rat. It is easy to immunise WAG and LEW rats with unconjugated peptides P1 and P3 derived from the classical DA class I disparate MHC class I molecule. A strong primary antibody response is invariably obtained. Both LEW and WAG rats have peptide specific T cell responses when immunised with these peptides down to a concentration of $2.5 \mu g$ ml^{-1}. However, PVG rats show some discrepancy in their immune responses to peptide in that they can exhibit a weak secondary antibody response but never any T cell proliferation; this poses the question of how T-dependent B cell help is initially generated if T cell activity is undetectable *in vitro*. One explanation might be that T cells require activation by antigen presenting cells that are unlikely to be present in our lymph node preparations. Alternatively, since antibody production is an amplification of the original helper response, it may be that the level of antigen specific T cell activity *in vitro* is too small to detect in a relatively crude proliferation assay.

DA rats immunised with their own peptides appear to mount a specific alloantibody response, but their T cells are unresponsive and they show no signs of autoimmunity. DA rats immunised with DA spleen cells show a similar but weaker pattern of B cell reactivity. This response has not been investigated further, but if substantiated may be interesting with regard to peripheral tolerance. The presence of contaminating peptide species in the P3 samples would also have to be definitively ruled out.

LEW and WAG rats immunised with DA spleen cells mount weak antibody responses to P3, but variable T cell responses to the allopeptides. They both recognise P1, LEW can respond to P3 (FIGURE 8) and both can occasionally proliferate weakly to P2 (FIGURE 9). The T cell responses of these rats appear to be the inverse of the antibody studies. Nevertheless,

these results, coupled with other work (Fangmann *et al* 1992b), demonstrate that these peptides are worthy tools with which to study allorecognition.

CHAPTER 4: FINE SPECIFICITY OF THE DETERMINANTS OF PEPTIDE 1.

CHAPTER 4:

FINE SPECIFICITY OF THE DETERMINANTS OF PEPTIDE 1.

4.1 Introduction:

For many years the rejection of allografts was thought to occur primarily by the 'direct' pathway of T cell allorecognition. In this direct pathway, intact polymorphic MHC antigens on the surface of foreign donor cells stimulate recipient T cells directly, without the need for processing and presentation by syngeneic (recipient) APCs (e.g. Lechler *et al* 1990).

It is now apparent that peptide fragments of polymorphic graft proteins can be treated as conventional antigens i.e. processed and presented by recipient APCs. This 'indirect' pathway of T cell allorecognition was initially demonstrated by analysis of the specificity of T cell clones *in vitro* (Saskia de Koster *et al* 1989, Chen *et al* 1990, Essaket *et al* 1990) and *in vivo* by immunisation with MHC peptides (Parker *et al* 1992). Any polymorphic protein of the graft (whether internal or cell surface) may be recognised in this way, but one would expect that donor MHC proteins should provide most of the polymorphic peptides. Indirect T cell recognition of donor MHC peptides has now been demonstrated during *in vivo* rejection responses (Benichou *et al* 1992, Fangmann *et al* 1992b) and more importantly, it has been proven to play a significant role in the effector mechanisms of rejection (Dalchau *et al* 1992, Fangmann *et al* 1992a). Mechanistically, indirect recognition may ultimately lead to graft destruction by either an intragraft Delayed Type Hypersensitivity (DTH) reaction or by the provision of T cell help for the generation of cytotoxic T cells and for the production of antibodies (e.g Fangmann *et al* 1992a).

Priming LEW(RT1^l) rats with the two unconjugated 22-24 amino acid peptides P1 and P3, derived from the polymorphic regions of the RT1.A class I α helices of the DA(RT1.A^{avl}) strain, results in the accelerated rejection of DA skin grafts (Fangmann *et al* 1992a). Moreover, LEW rats

rejecting DA skin and kidney grafts have CD4$^+$ T cells that proliferate *in vitro* when stimulated with one of these peptides, P1 (Fangmann *et al* 1992b). This indicates that P1 (or related derivatives), is/are produced by the normal physiological processing of the DA class I molecule during rejection. Since the most common size of peptide presented to T cells in the MHC class II groove is approximately 15 amino acids (Chicz *et al* 1993), it is possible that our peptides undergo further processing when they are used for *in vivo* immunisations.

In this chapter, I have investigated whether the 24 amino acid P1 (from the α helix of the α1 domain) contains a single T cell epitope as would be expected from previous studies with conventional antigens (e.g. Westhof *et al* 1984), or multiple T cell epitopes. This is potentially important not only for understanding the molecular mechanisms of rejection in our model, but also for future studies on immunosuppression. A nested series of 15 amino acid peptides, spanning the N to the C terminus of P1, was therefore synthesised and used to elucidate the antigenically important regions of P1. The sequences of these peptides are given below and the details of their preparation given in Chapter 2.

|	
P1	PEYWEQQTRIAKEWEQIYRVDLRT
A1	PEYWEQQTRIAKEWE
A2	EYWEQQTRIAKEWEQ
A3	YWEQQTRIAKEWEQI
A4	WEQQTRIAKEWEQI
A5	EQQTRIAKEWEQIYR
A6	QQTRIAKEWEQIYRV
A7	QTRIAKEWEQIYRVD
A8	TRIAKEWEQIYRVDL
A9	RIAKEWEQIYRVDLR
A10	IAKEWEQIYRVDLRT

4.2 RESULTS:

4.2a T Cell Proliferation Studies Following Immunisation With P1:

PVG, LEW and WAG rats were immunised with unconjugated P1, to allow *in vivo* development of responses to naturally processed fragments of the peptide. T cell activity was then tested *in vitro* to the nested set of 15mers.

The proliferation response of LN cells was determined on days 3 and 4 after initiation of the cultures, with day 3 usually giving the stronger response. FIGURE 1 shows the day 3 response to P1 and all ten 15mers in a LEW, WAG, PVG and control LEW rat.

In FIGURE 1, both LEW and WAG strains responded strongly to P1 at 25μg ml^{-1}, whereas the PVG strain responded poorly. Similar responses were obtained at peptide doses of 2.5μg ml^{-1}. While the weak response of the PVG strain was expected, in view of its low responsiveness to the whole class I molecule (Butcher and Howard 1982 and chapter 3), it is interesting that the WAG and LEW strains both respond to the same peptide. Since the class II genes of these strains are dissimilar, a difference in the peptide specificity of the response was expected. Indeed, the 15mer studies do suggest a difference in fine specificity towards this alloantigen. The LEW rat responds strongly to all the 15mers, with no single peptide giving a significantly greater response than the others (FIGURE 1). In the WAG rat, the situation is different: the N-terminal 15mers stimulate strongly, but the response to the C terminal 15mers is weak. This is seen more clearly when lower doses of peptide were used for the *in vitro* stimulations (FIGURE 2). This suggests that, in the WAG strain, the region of importance in T cell recognition is confined to the N-terminal region of P1, while this is not the case for the LEW strain.
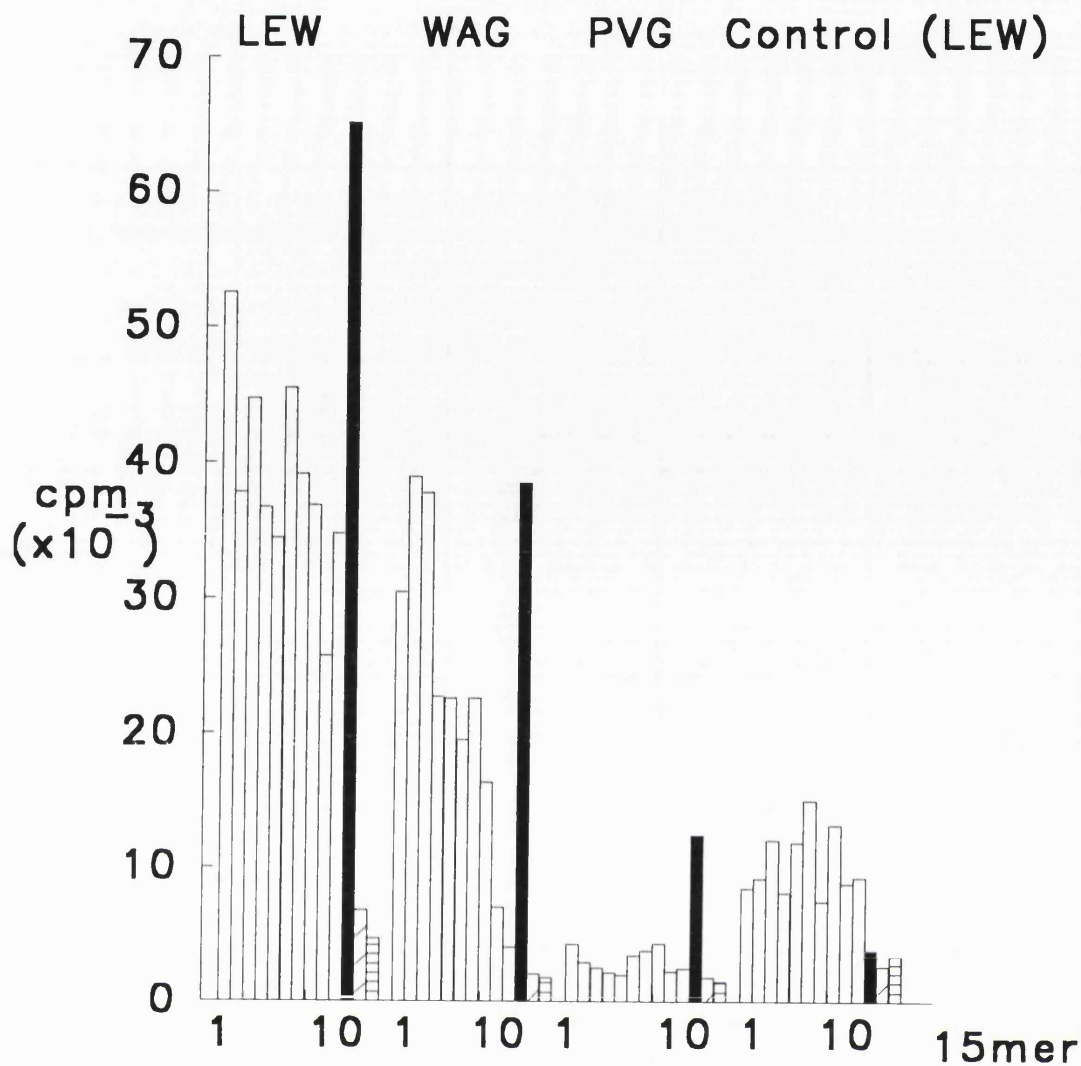
FIGURE 1:

   Proliferative Response of P1 Immunised Rats to P1 and the Derivative 15mers *In Vitro.*

LEW, WAG and PVG rats were boosted with P1 and a control LEW rat was boosted with Freunds adjuvant. Their LN cells were stimulated *in vitro* against P1 at 25μg ml^{-1} (■), individual 15mers A1 to A10 at 25μg ml^{-1} (□), control peptide P2 at 25μg ml^{-1} (◪) or medium only (▤). The cultures were maintained for three days and harvested after pulsing with ^{3}H-thymidine for 24hrs. Concanavalin A was used as a positive control and gave counts of between 100-150,000 cpm. cpm refers to counts per minute of incorporated tritium. Standard errors were between 1.5% and 12% of the mean values obtained.
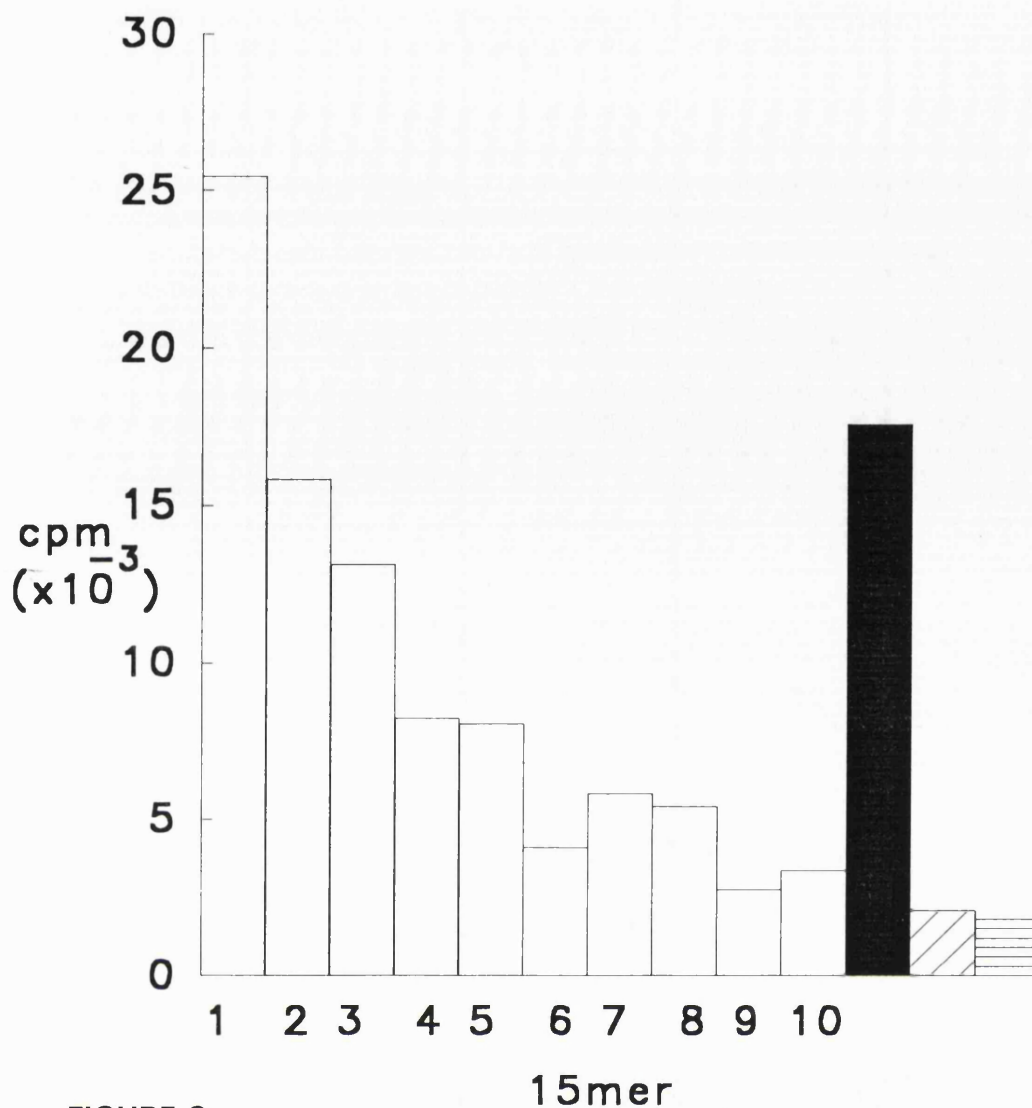
FIGURE 2:

Proliferative Response of P1 Immunised WAG Rats to P1 and the Derivative 15mers *in vitro.*

A WAG rat was boosted with P1 and its LN cells were stimulated *in vitro* against P1 at 2.5μg ml^{-1} (■), individual 15mers A1 to A10 at 2.5μg ml^{-1} (□), control peptide P2 at 2.5μg ml^{-1} (◪) or medium only (⊟). The cultures were maintained for three days and harvested after pulsing with ^{3}H-thymidine for 24hrs. Concanavalin A was used as a positive control and gave counts of between 130-180,000 cpm. cpm refers to counts per minute of incorporated tritium. Standard errors were between 1.2% and 11% of the mean values obtained.
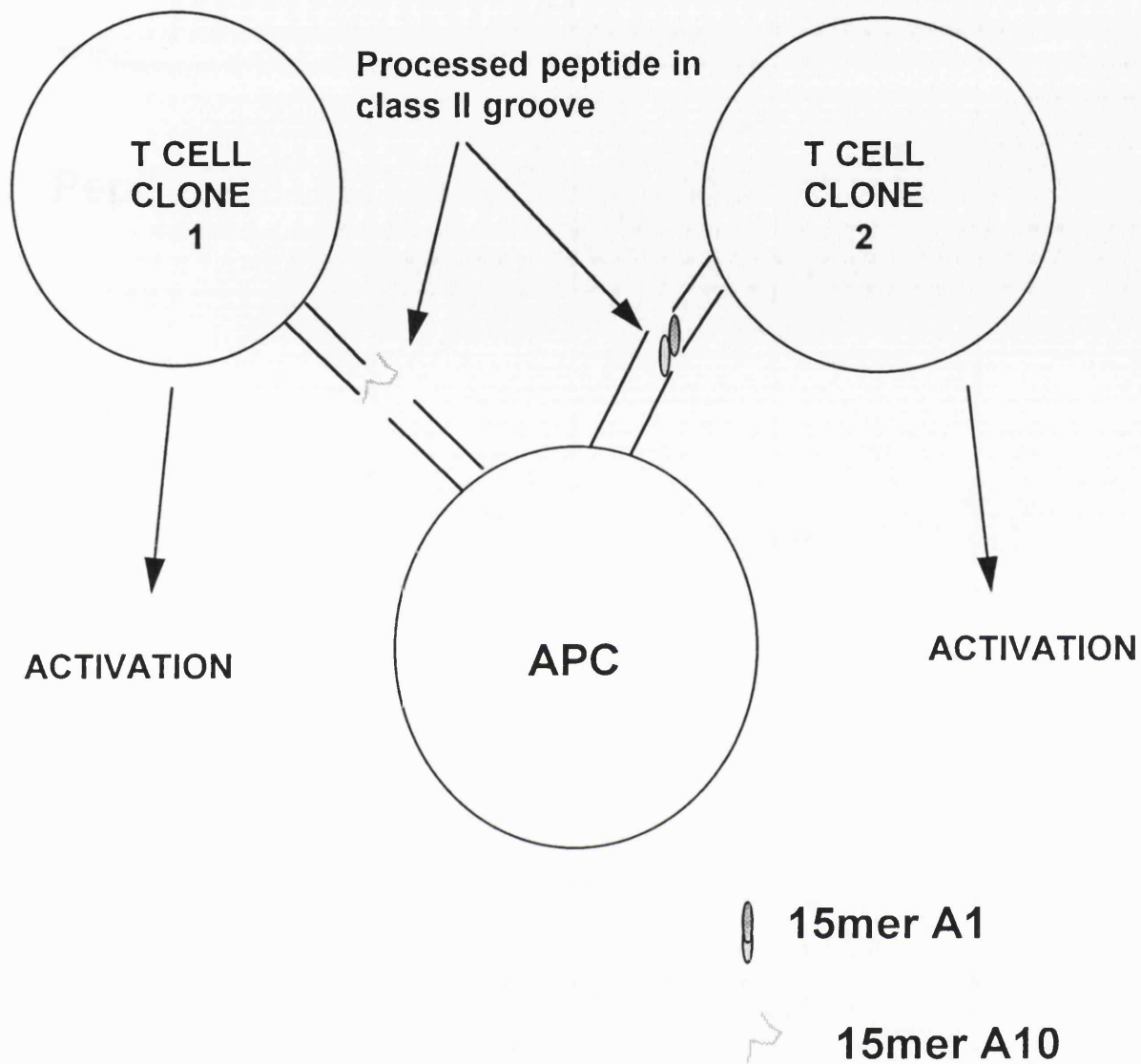
4.2b T Cell Proliferation Studies Following Immunisation With Individual 15 mers:-

I) The LEW Strain:

The response of P1 immunised LEW rats to all the 15mers has 2 possible explanations:

a) A polyspecific T cell response. The number of T cell epitopes within a protein antigen is variable, but is often about 1 per 50 amino acid residues (Westhof *et al* 1984). However, it may be the case that more than one epitope exists within our 24mer. The original peptide may be processed in different ways by either the same, or different, APCs and the various peptide fragments presented to T cells with different peptide specificity. The class II molecules on which the different peptides are presented may also be different, but need not be so. This is illustrated in FIGURE 3.

b) Monospecific T cell response and degenerative presentation of peptides. The structure of a human MHC class II molecule has been determined (Brown *et al* 1993) and is probably almost identical in the rat. The peptide binding groove of the MHC class II molecule is open ended, so there is no strict restriction on the size of peptide in the cleft. All the 15mers share a common hexamer sequence IAKEWE located at the centre of P1. This hexamer might contribute to MHC binding of the peptides. All of the 15mers might occupy the same MHC groove, but with varying degrees of overhang from one or other end of the class II molecule. If these IAKEWE residues are important for T cell recognition, then all of the 15mers might be able to stimulate the same group of T cells. This is illustrated in FIGURE 4.

Class II α helix

Peptide A1

T Cell Receptor View
Of Class II Groove
+Peptide

Peptide A5

Peptide A10

TCR Recognises And Responds
To Central Residues IAKEWE
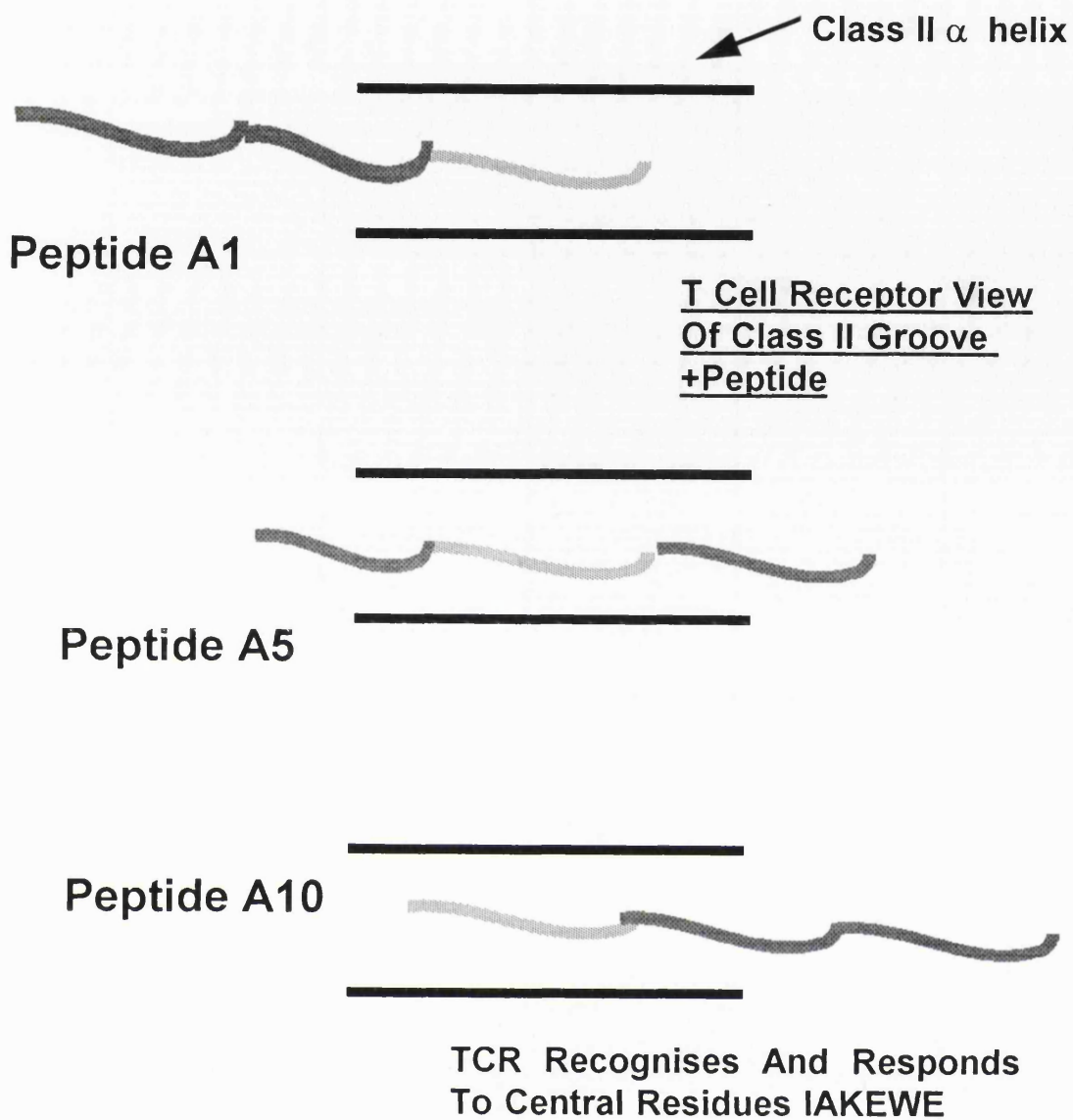
FIGURE 4:

Degenerative Processing Of 15mers.

All the 15mers are able to occupy the same MHC class II cleft by virtue of its open endedness. If the five common residues IAKEWE are important in T cell recognition, then all the 15mers might be able to stimulate the same T cell clone.

Immunising LEW rats with individual 15mers allowed these ideas to be tested. If the first hypothesis (polyspecific T cell responses) is correct, then a rat immunised with the A1 15mer (for example) will have T cells which are not stimulated *in vitro* by the other 15mers, since these other 15mers will be recognised by different T cells which will not have been primed by peptide A1. If the second hypothesis (monospecific T cell responses) is true, then an animal immunised with A1 should have T cells that can respond *in vitro* to the common hexamer of any of the 15mers.

LEW rats were immunised subcutaneously in the hind footpad with individual 15mers, either A1, A4, A7 or A10, and the proliferation response of their lymph node cells was examined *in vitro* for stimulation with each of the 15mers as well as P1. The results are given in FIGURE 5 and show that each LEW rat responds most strongly to the 15mer with which it has been immunised. This indicates that each 15mer stimulates a distinct set of T cells, and favours the hypothesis that the T cell response to peptide 1 is polyspecific, i.e. involves multiple T cell determinants. The fact that neighbouring 15mers are also capable of inducing proliferation in each case suggests that there is some degeneracy in the way in which 15mers are bound to the class II MHC molecules. This is to be expected from what is known of the structure of the class II molecule (Brown *et al* 1993).

In FIGURE 5, lymph node cells from rats primed *in vivo* with 15mers responded less strongly *in vitro* to P1 than to the immunising 15mer. However, this is not as marked as it seems at first sight, since the control rat has a higher background with the 15mer preparations than with P1 and Px. The response to P1 was somewhat variable, being equal to that of the immunising 15mer in one of the repeat experiments. One possible explanation for this is that P1 requires further processing for optimal presentation of the different T cell epitopes within it, and that while this
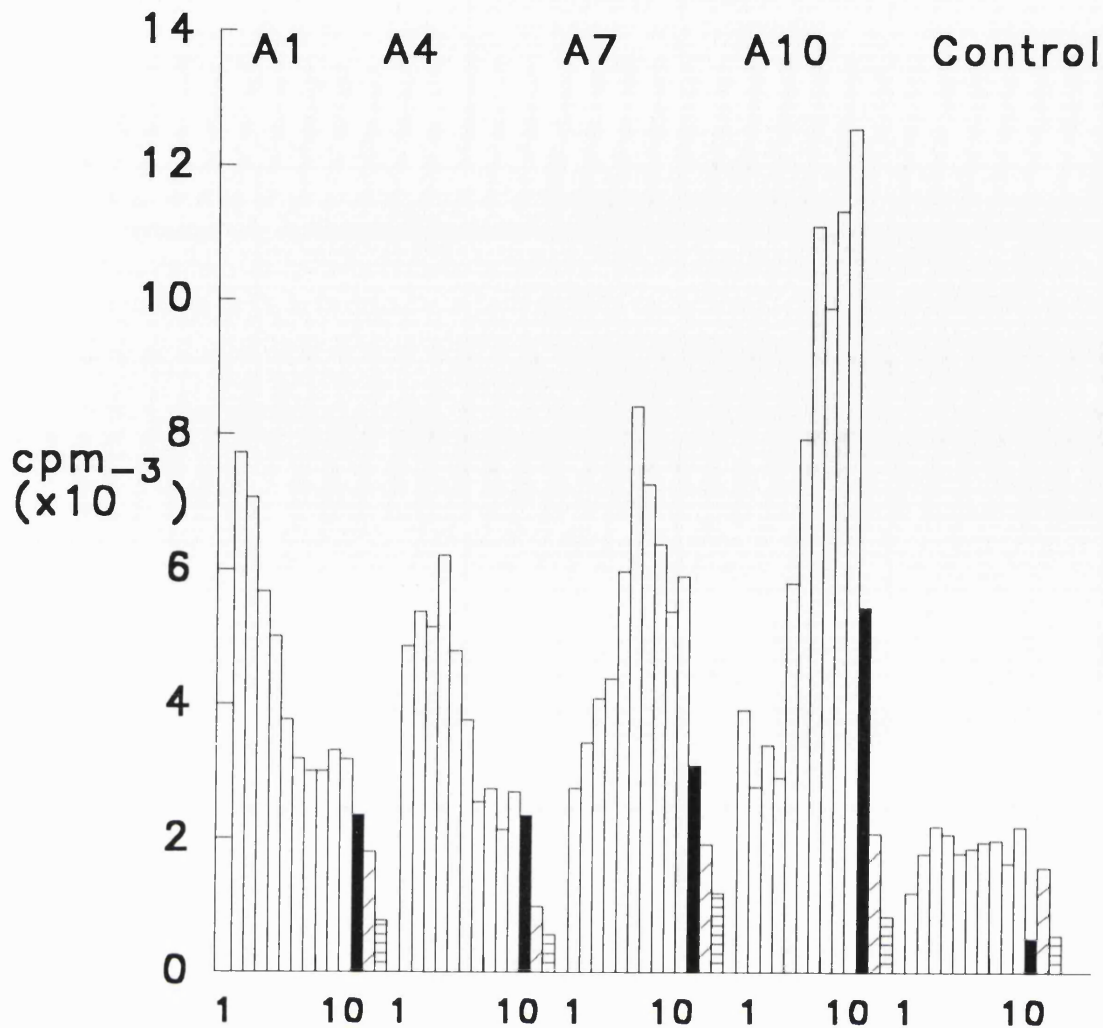
15 mer

Proliferative Response of 15mer Immunised Rats to P1 and the Derivative 15mers *In Vitro.*

LEW rats were boosted with A1, A4, A7 or A10 and a control LEW rat was boosted with Freunds adjuvant. Their LN cells were stimulated *in vitro* against P1 at 25µg ml⁻¹ (■), individual 15mers A1 to A10 at 25µg ml⁻¹ (□), control peptide Px at 25µg ml⁻¹ (▨) or medium only (▤). The cultures were maintained for three days and harvested after pulsing with ³H-thymidine for 24hrs. Concanavalin A was used as a positive control and gave counts of between 50-80,000 cpm. cpm refers to counts per minute of incorporated tritium. Standard errors were between 1% and 13% of the mean values obtained.

processing occurs readily *in vivo* (FIGURE 1), this might be less efficient *in vitro*.

The level of stimulation induced by the 15mers in the 15mer immunised rats is also consistently lower than that found in rats immunised with P1. This might be explained if the optimal size of the peptide presented in the class II cleft is not 15 amino acid residues, but one or a few residues longer or shorter. In this case, immunisation with a 15mer might give rise to less than optimal binding to the class II molecules, with consequent reduced stimulation of T cells *in vivo*.

II) The WAG Strain:

Although the WAG T cell response to 15mers after immunisation with P1 was confined more to the N-terminal based derivatives, its response to individual 15mers was still worth analysing. This might reveal differences in peptide specificity between the LEW and WAG strains of rat. Thus individual WAG animals were immunised with A2, A5, A8 or adjuvant only and their LN cells taken ten days after boosting. As with the LEW animals, these cells were stimulated *in vitro* for 3 or 4 days with all the 15mers as well as P1. FIGURE 6 shows that the A2 immunised animal responds well to A2 *in vitro* and towards its two neighbours in a manner comparable to the LEW response (FIGURE 5). For the 15mers that provoked little response after immunisation with P1 (i.e. A5 and A8) the T cell response is both weaker and broader. Like the individually immunised LEW animals, proliferation is lower than in P1 immunised rats. These data further support the notion that in the WAG rat, the N terminal region is the important one in allorecognition. The low counts and the result in the A2 animal suggest that 15 is probably not the optimal size of peptide in the groove. Perhaps a 17mer spanning A1 to A3 is a likely candidate. It can

FIGURE 6:

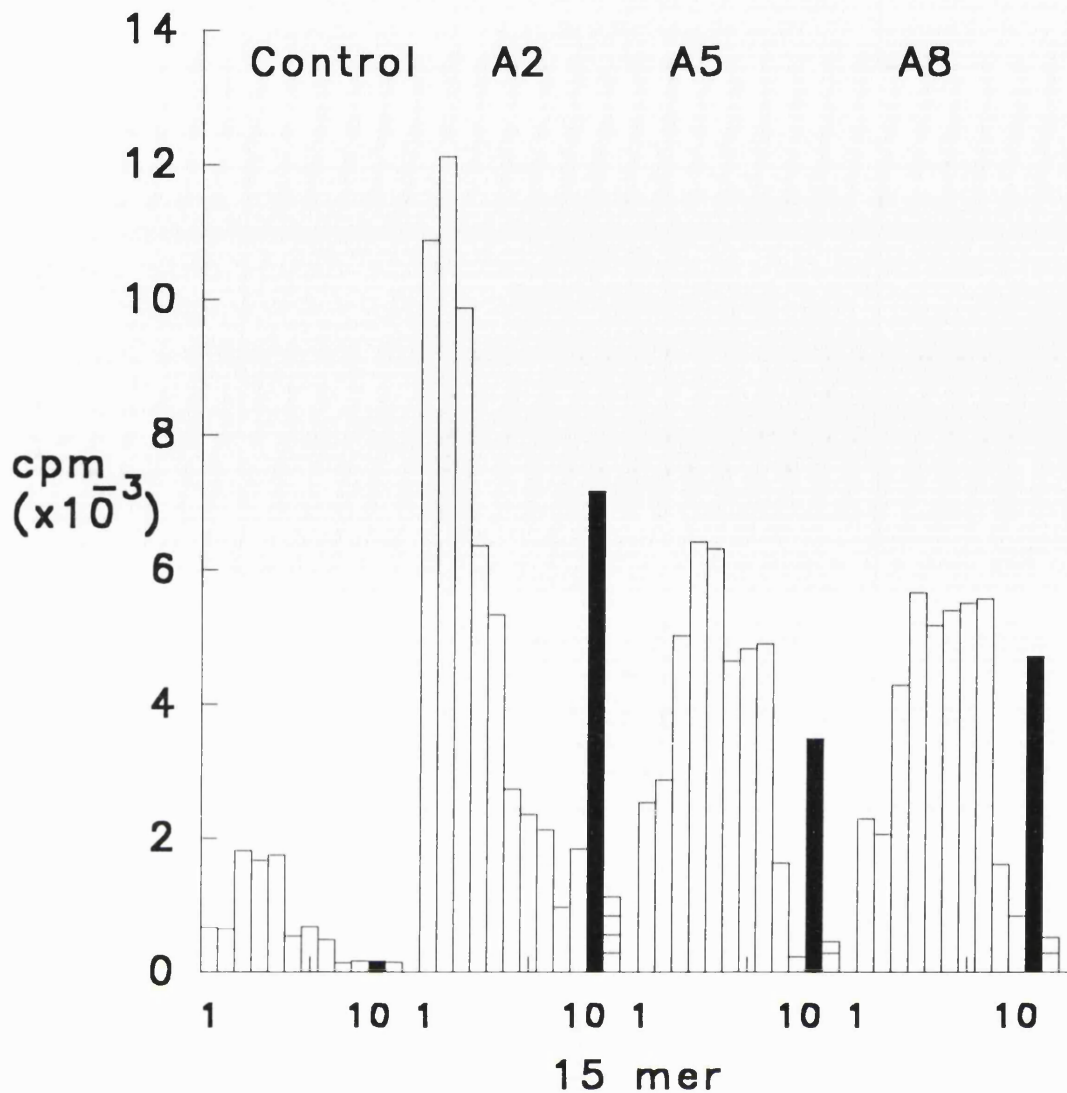Proliferative Response of 15mer Immunised WAG Rats to P1 and the Derivative 15mers *in vitro*.

WAG rats were boosted with A2, A5 or A8 and a control rat was boosted with Freunds adjuvant. Their LN cells were stimulated *in vitro* against P1 at $25\mu g$ ml^{-1} (■), individual 15mers A1 to A10 at $25\mu g$ ml^{-1} (□) or medium only (▤). The cultures were maintained for three days and harvested after pulsing with ^{3}H-thymidine for 24hrs. Concanavalin A was used as a positive control and gave counts of between 150-170,000 cpm. cpm refers to counts per minute of incorporated tritium. Standard errors were between 2% and 15% of the mean values obtained.

also not be discounted that part of the epitope lies outside the sequence defined by the original P1.


4.2c The Antibody Response Following Immunisation With Peptide 1:

Having investigated the T cell responses of rats immunised with P1 and 15mer derivatives, it was decided to utilise the sera obtained from these animals to investigate their antibody responses to the same peptides. FIGURE 7 shows data obtained from the secondary antibody responses in P1 immunised rats. The antibody response to peptide 1 was strong in the LEW and WAG rats, but very weak in PVG rats. Previously, it has been demonstrated that the *in vitro* proliferation response of LEW rats to P1 is entirely confined to the CD4$^+$ T cell subset (Fangmann *et al* 1992a). The ability of these strains to respond *in vitro* in proliferation assays to P1 (FIGURE 1) presumably correlates with the capacity for T-B collaboration to P1 in these strains.

If P1 can provide T helper determinants for antibody production, the fine specificity of the antibodies produced should be constrained only by the B cell repertoire and the capacity of P1 to interact with the surface immunoglobulin on B cells. When the antisera to P1 were analysed using the 15mers as targets, the response was very strong to the C terminal peptide A10, strong to the N terminal peptide A1, but virtually absent on all other peptides (FIGURE 7). This pattern was seen not only with the LEW and WAG strains, but also with the weak antibody response of the PVG strain. The only difference between peptide A10 (which provided an excellent target for the antisera) and peptide A9 (which provided a very poor target) is the C terminal amino acid threonine. Similarly, the only difference between peptides A1 and A2 is the N terminal amino acid proline. The implication is that the amino acids at either end of P1 play a

FIGURE 7:

Secondary Antibody Response to P1 and the Derivative 15mers. Antisera were obtained from LEW, WAG and PVG rats 10 days after boosting with P1 and from a control LEW animal boosted with Freunds adjuvant only. The sera were tested in indirect plate binding assays using the following targets: P1 (●-●), 15mer A1 (●··●), 15mer A10 (●---●), 15mers A2 to A9 (o), control peptide Px (■) or saline only (▼). Titrations were performed in tripling dilutions. cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors obtained were between 1% and 9% of the means.
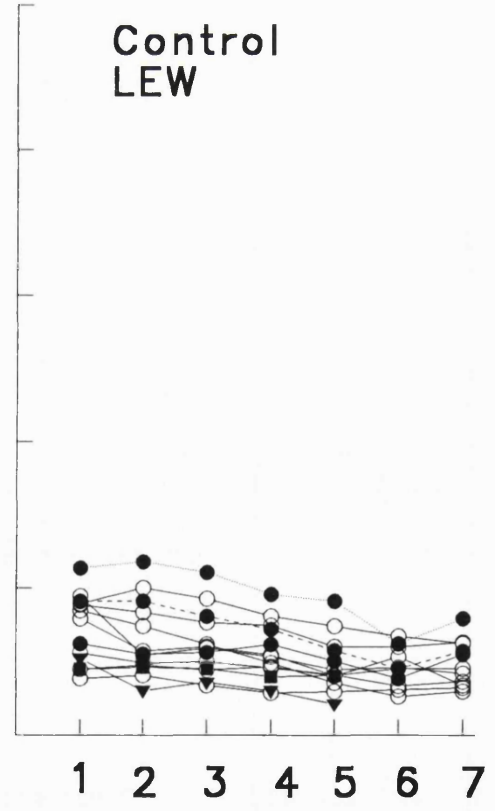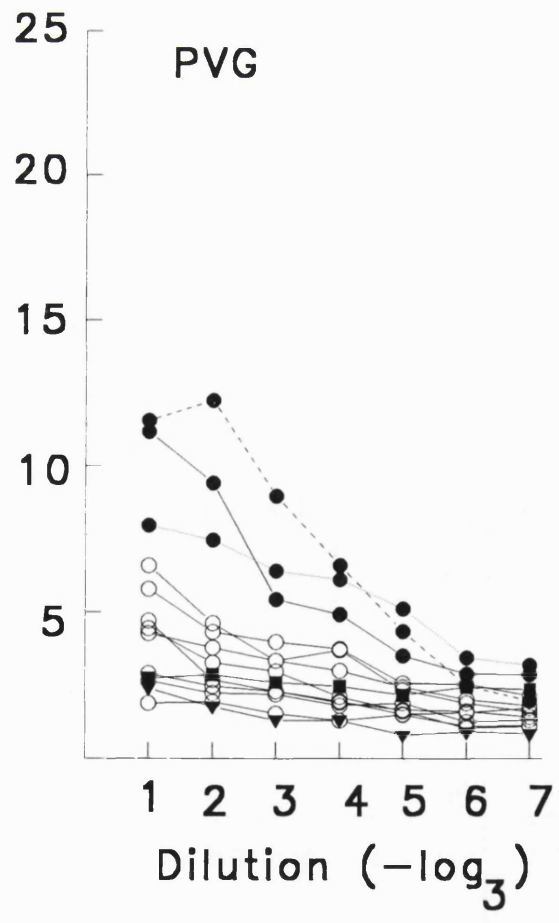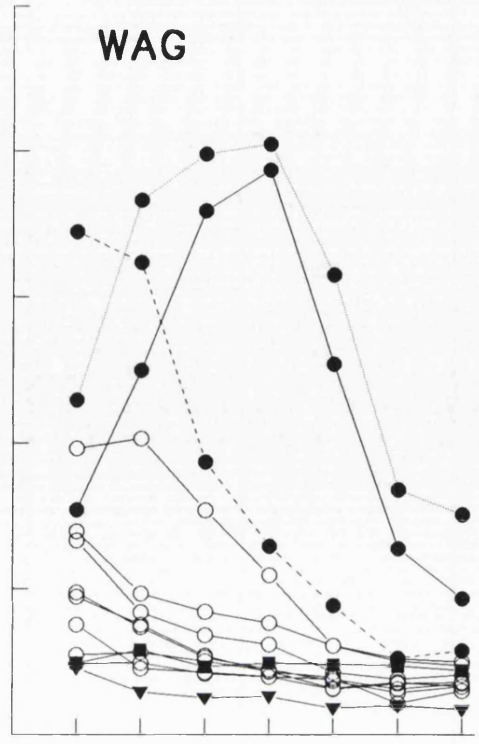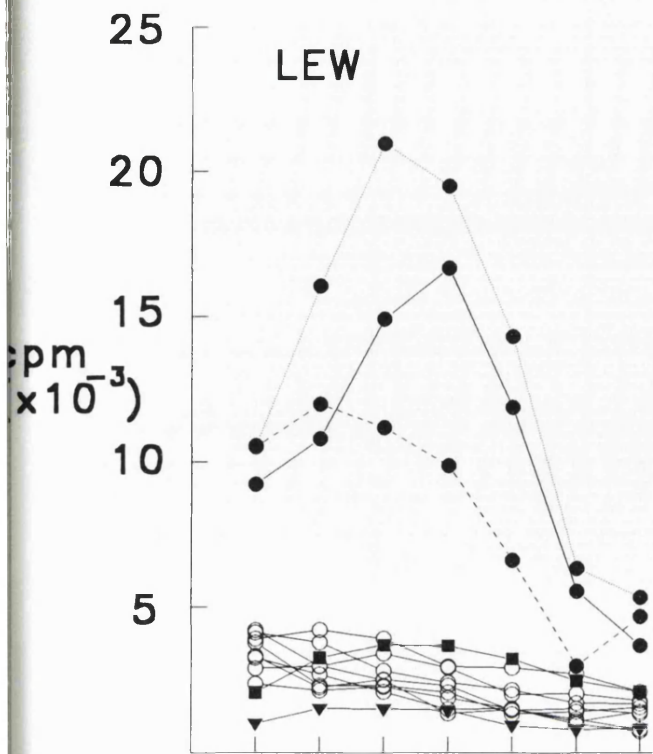
crucial role in binding to the surface immunoglobulin of B cells. The problem is not one of the B cell repertoire, since LEW rats immunised with the A1, A4, A7 and A10 peptides all mounted good antibody responses to these individual 15 mers (FIGURE 8 and TABLE 1).

4.2d The Antibody Response Following Immunisation With Individual 15mers:

FIGURE 8 represents the antisera from animals boosted with individual 15mers and set up in an indirect binding assay using all the 15mers as targets. The titrations for A1, A4, A7 and A10 have been included on each graph together with the 15mers neighbouring the immunogen in each case. Control LEW sera, boosted with saline in FIA, was also set up against the 15mers and showed background binding (not shown). As the graph is somewhat cluttered, the data are also presented in tabulated form (TABLE 1) as titres, taking the half maximal response obtained from the immunising peptide as the standard titer for each individual animal. Good responses are obtained to the immunising peptide in each case, though the strongest curves are obtained with the A1 and A10 sera. Interestingly, the antisera to these 15 mer peptides were not dependent on the N and C terminal amino acids for specificity.

Similarly, sera was taken from the WAG rats boosted with individual 15mers and assayed for antibodies towards the immunising 15mer as well as all the other derivative peptides. As with the LEW rats this is presented graphically in FIGURE 9 and in table form (TABLE 2). Only the antibody curves against A2, A5, A8 and the 15mers neighbouring the immunising peptide are shown in FIGURE 9, along with that of P1 and the saline control. Px was used as a control peptide in these assays and gave background levels comparable to the saline wells. Taking into account the relatively high background in the control animal, A2 is the only 15mer to

which a notable antibody response is obtained in the A2 and A5 immunised animals. This 15mer is, of course, one of the N-terminal peptides that provoked a high T cell response *in vitro* (FIGURE 1). Table 2 provides the complete data at a glance in the form of titres. In the A2 immunised animal, antibodies can also recognise the neighbouring 15mers A1, A3 and A4 as well as the parent peptide P1. Immunisation with A5 produces virtually no antibody response, so the titres all appear as 0, despite the fact that A2 is detected by antibodies in this sera. A8 is also poor at inducing antibody secretion though slightly better than A5. This provides further evidence that the N-terminus of P1 (and possibly beyond) is important in allorecognition in the WAG strain. It also suggests that 15mers are relatively poor antigens when administered without carriers *in vivo*.

FIGURE 8:

Secondary Antibody Response to P1 and the Derivative 15mers. Antisera were obtained from LEW rats 10 days after boosting with either A1, A4, A7 or A10 and from a control LEW animal boosted with Freunds adjuvant only (not shown). The sera were tested in indirect plate binding assays using the following targets: P1 (●), 15mers A1 to A10 (O) and saline only (▼). Titrations were performed in tripling dilutions. cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors obtained were between 0.9% and 10% of the mean values.

A1 Sera

A4 Sera

A7 Sera

A10 Sera

cpm (x10^{-3})

Dilution (−log$_3$)
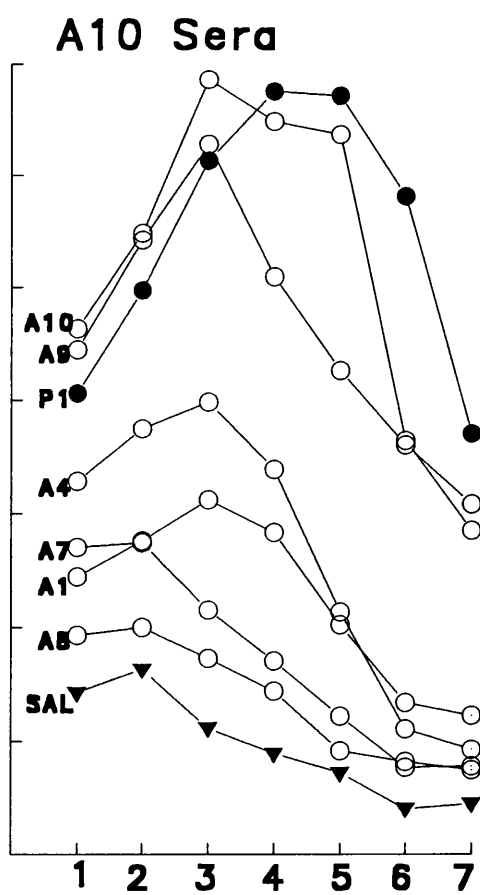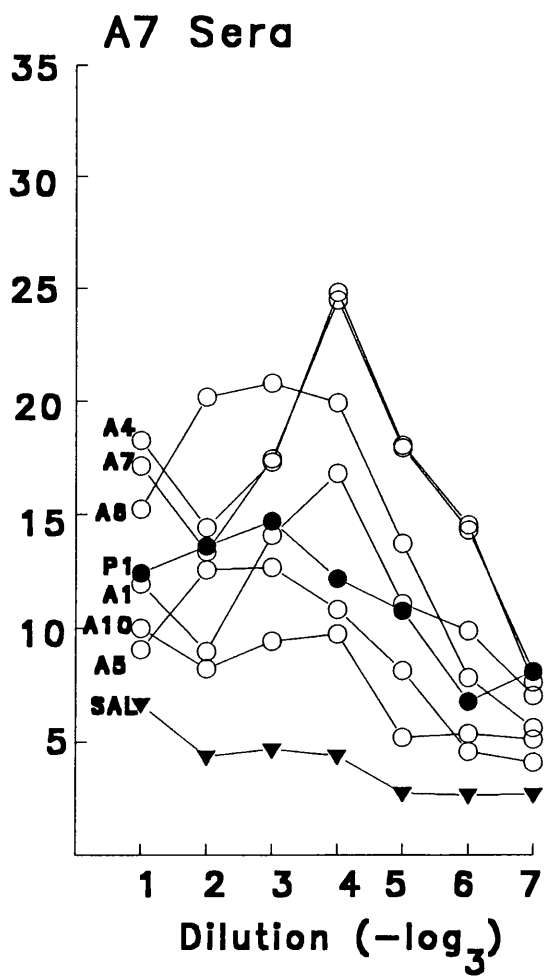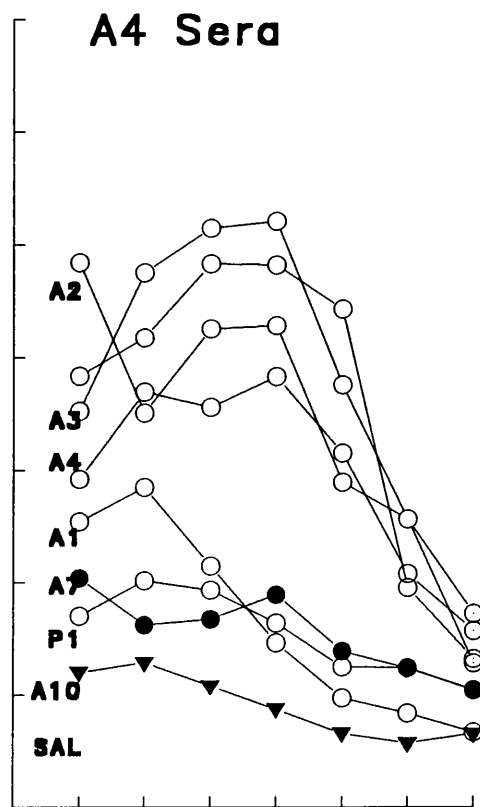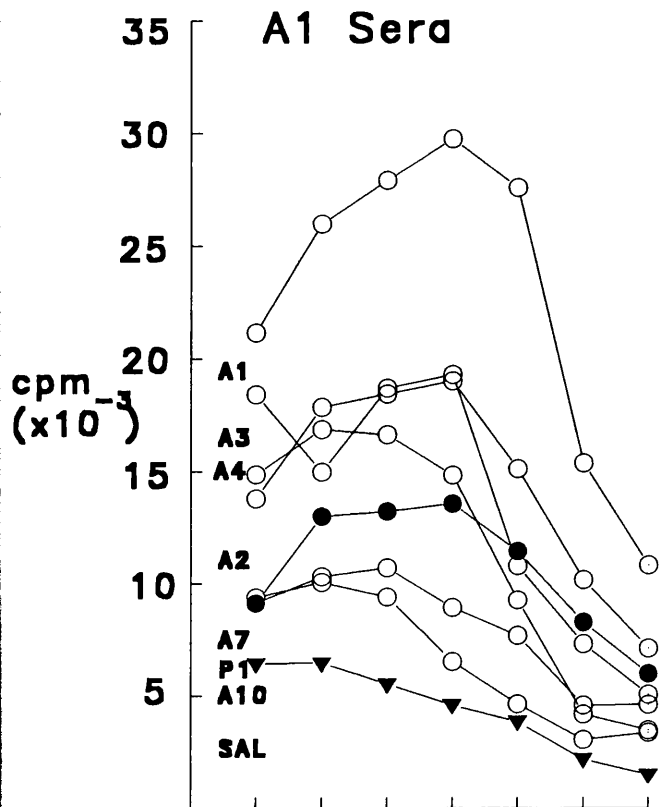
Analysis of LEW Antisera to Individual 15mers of Peptide 1 of the DA RT1.A^{av1} Class I Molecule.

LEW rats were immunised with individual 15mers A1, A4, A7 and A10. The sera were tested in plate binding assays using P1 and the individual 15mers A1 to A10 as targets. Maximum cpm of ^{125}-RAR obtained for sera assayed on immunising peptides were approximately 20,000 for A1, 26,000 for A4, 24,000 for A7 and 32,000 for A10. The titres represent the reciprocal of the last dilution at which greater than half maximum binding was obtained in comparison to the immunising peptide. 'Saline only' wells gave constant background counts of between 5-6,000 cpm. LEW rats immunised with Freunds adjuvant only did not respond to any of the peptides.

Peptide target	Titres of antisera against peptide			
	A1	A4	A7	A10
A1	729	243	81	0
A2	243	243	243	0
A3	243	243	729	3
A4	81	243	729	81
A5	0	3	27	0
A6	3	27	243	0
A7	0	9	729	0
A8	0	3	81	0
A9	0	0	81	729
A10	0	0	0	729
P1	243	0	27	2187

FIGURE 9:

Secondary Antibody Response to P1 and the Derivative 15mers.
Antisera were obtained from WAG rats 10 days after boosting with either A2, A5, or A8 and from a control WAG animal boosted with Freunds adjuvant only. The sera were tested in indirect plate binding assays using the following targets: P1 (●), 15mers A1 to A10 (○) and saline only (▼). Titrations were performed in tripling dilutions. cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors obtained were between 0.8% and 14% of the mean values.

TABLE 2:

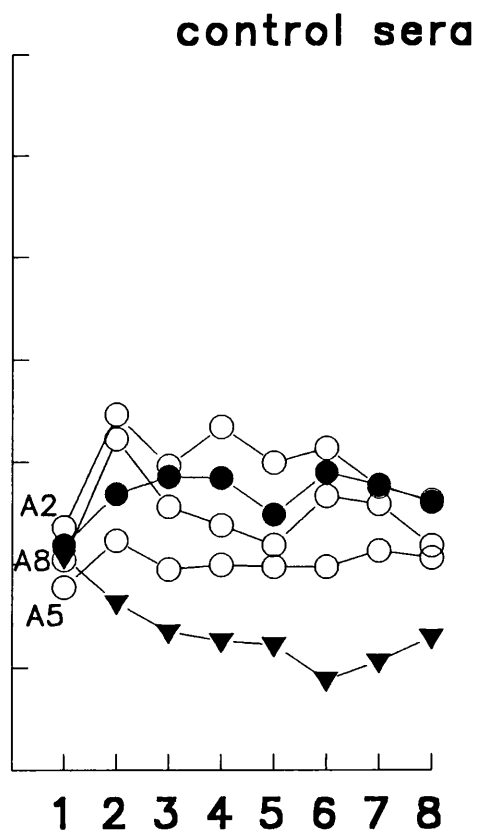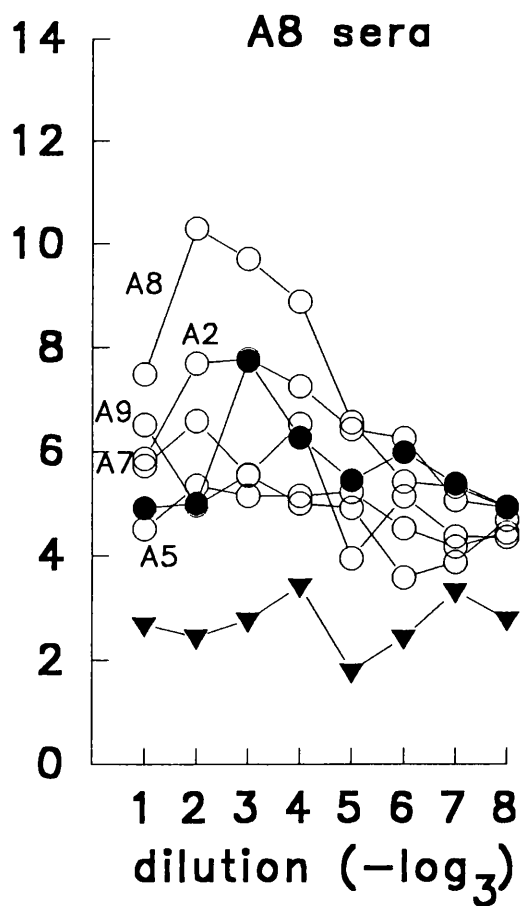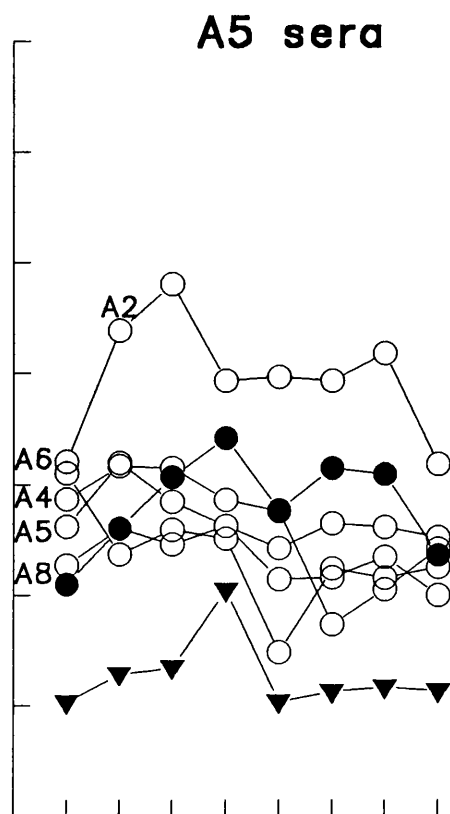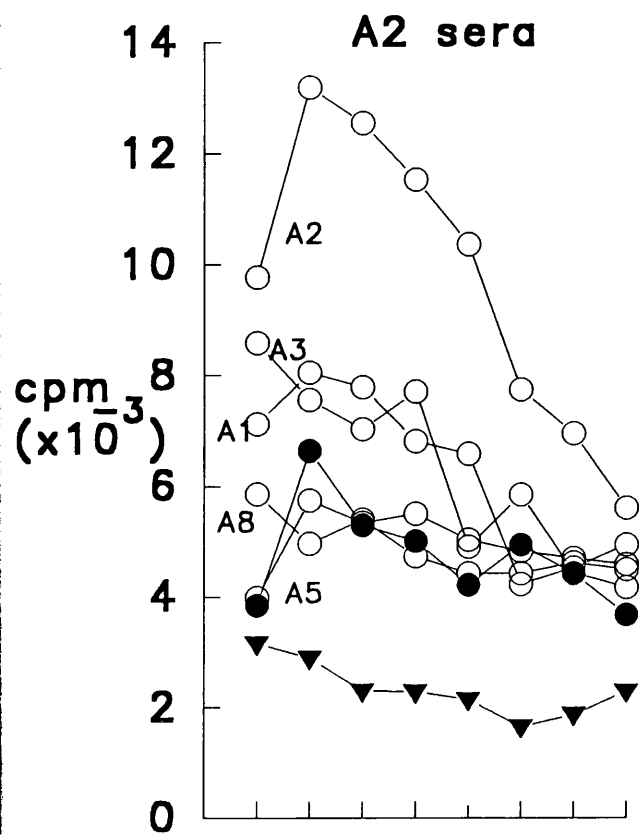Analysis of WAG Antisera to Individual 15mers of Peptide 1 of the DA RT1.A^{av1} Class I Molecule.

WAG rats were immunised with individual 15mers A2, A5, and A8. The sera were tested in plate binding assays using P1 and the individual 15mers A1 to A10 as targets. Maximum cpm of ^{125}-RAR obtained for sera assayed on immunising peptides were approximately 14,000 for A2, 6,000 for A5, and 10,000 for A8. The titres represent the reciprocal of the last dilution at which greater than half maximum binding was obtained in comparison to the immunising peptide. 'Saline only' wells gave constant background counts of between 5-6,000 cpm. WAG rats immunised with Freunds adjuvant only did not respond to any of the peptides.

Peptide target	Titres of antisera against peptide		
	A2	A5	A8
A1	81	0	243
A2	2187	0	729
A3	81	0	2187
A4	81	0	243
A5	0	0	243
A6	0	0	3
A7	0	0	27
A8	0	0	2187
A9	0	0	81
A10	0	0	243
P1	9	0	3

4.3 DISCUSSION:

These studies demonstrate that the indirect T cell allo-recognition response in transplantation is likely to be very complex, and variable from recipient to recipient, even to the same donor antigen. A single 24 amino acid stretch from the α1 domain of the DA RT1.A class I MHC molecule contains multiple T cell determinants as viewed by the LEW rat strain. The α helix of the α2 domain of this molecule is known to provide at least one additional determinant for LEW T cells in the form of P3 (Fangmann et al 1992a) and other determinants might be present in other regions of the molecule. If one adds to this the large number of potential T cell epitopes from all class I and class II MHC molecules, as well as other polymorphic proteins, the potential complexity of the indirect T cell response to donor tissue becomes apparent. This is of particular importance if one is considering specific immunosuppression strategies directed at the indirect response: either the treatment must ensure exposure to all potential determinants for indirect T cell recognition (e.g. by treatment with actual donor tissue) or reliance must be placed on suppressor responses to some epitopes having an overall suppressive effect by way of bystander or linked suppression.

The crucial importance of the N and C terminal amino acids in defining the B cell epitopes for the 24mer peptide 1 was unexpected. Theoretical problems with the B cell repertoire and technical problems with peptide binding to plates were excluded by showing that immunisation with internal 15mers produced good antibody responses in the LEW rat. Another possibility is that the B cell determinants within P1 are conformationally disrupted in the internal 15mers. However, small peptides tend not to assume any constant secondary structure in solution (Tainer et al 1984). Perhaps an individual B cell (with a particular specificity) needs to recognise a frequently adopted peptide conformation to interact with and

internalise sufficient peptide before T cell help can proceed. If this is so, the more constant conformations might involve the terminal regions in our longer peptide.

CHAPTER 5: INDIRECT T CELL ALLORECOGNITION AND THE REJECTION OF VASCULARISED KIDNEY ALLOGRAFTS.

INDIRECT T CELL ALLORECOGNITION AND THE REJECTION OF VASCULARISED KIDNEY ALLOGRAFTS.

5.1a Introduction:

It is now well established that indirect recognition can contribute to the effector mechanisms of rejection of non-vascularised skin allografts in experimental animals (Fangmann *et al* 1992a, Dalchau *et al* 1992, Auchincloss *et al* 1993). The occurrence of indirect recognition in rodents receiving skin and kidney grafts has also been demonstrated (Fangmann *et al* 1992b). In man, indirect recognition has also been shown to occur *in vitro*, using allogeneic cells in a mixed lymphocyte reaction that artificially creates a cellular donor:recipient mismatch (Liu *et al* 1993a). However, it remains to be established that this pathway is a relevant effector mechanism in the rejection of vascularised allografts or in human transplants. Vascularised transplants are of more clinical relevance than those of skin, since skin allografting is only rarely performed on very badly burnt patients.

The work in this chapter uses an experimental retransplant model to demonstrate that indirect T cell allorecognition is a physiologically relevant component of vascularised allograft rejection in the rat. Kidney retransplantation experiments were first performed in the rat in the early 1970's (Stuart *et al* 1970, Marquet *et al* 1971); in these experiments, delayed allograft rejection was observed in a (LEWxBN)F1 to LEW model and in one example of a BN to WAG retransplant.

5.1b The Experimental Model:

To establish indirect recognition as an *in vivo* component of vascularised allograft rejection, a retransplantation protocol was chosen,

using (DAxLEW)F1 kidney donors and LEW recipients, which offered two important criteria:

i) a reduced susceptibility to the direct pathway of T cell allorecognition. This is likely to be mediated early on in the rejection response by donor dendritic cells resident in the kidney. Harbouring a kidney in the original recipient for 100 days prior to retransplantation depletes the graft of donor interstitial dendritic cells, since they are mobile. It is these APCs that are likely to be responsible for direct recognition responses (e.g Hart *et al* 1980, Lechler and Batchelor 1982).

ii) the rate of rejection is much slower than normal first transplant kidneys (Fabre and Morris 1972). This represents an ideal model for testing indirect T cell allorecognition responses, since accelerated rejection caused by priming to indirect recognition can more clearly be observed.

The model involved transplanting LEW (RT1^l) rats with a (DAxLEW)F1 kidney under CsA cover (10mg kg^{-1} for 10 days) and allowing these animals to become long survivors with functional grafts (FIGURE 1). At >100 days, such grafts have been demonstrated to be devoid of donor interstitial dendritic cells (Hart and Fabre 1981) but otherwise to have a normal pattern of MHC expression (Hart *et al* 1980). The dendritic cell-free kidneys were then retransplanted into LEW (RT1^l) rats that had previously been primed either with a mixture of P1 and P3 in adjuvant or with saline in adjuvant (controls). The two peptides have been used previously and are derived from the α helices of the DA (RT1.A^{av1}) class I molecule as previously described; they prime for indirect recognition without influencing the direct T cell allorecognition pathway (Fangmann *et al* 1992a).

The animals were bled regularly for antibody studies, their blood urea levels monitored and the survival times noted. Biopsies at time of transplantation, d7 post transplantation and day of termination were also examined.

FIGURE 1: Retransplantation Protocol Used To Observe Delayed (DAxLEW)F1 Kidney Allograft Rejection In The LEW Rat.

(DA x LEW) F1 kidney transplanted to naive LEW rat under CsA cover.

(DAxLEW) F1 rat

LEW rat left to become long survivor.

Interstitial dendritic cell-free kidney retransplanted to immunised LEW rat after >100 days.

d0

Wk4

Wk 6

LEW rat challenged with peptides derived from DA class I or with saline in adjuvant (control).

Animals boosted with peptides or saline in FIA

Observe :
a) urea Levels
b) survival time
c) antibodies
d) biopsy

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5.2 RESULTS:

5.2a Transplantation Studies:

The blood urea levels of long surviving animals prior to retransplantation were monitored and were all between 50 and 100mg per 100ml serum. One animal had a blood urea level of 150mg per 100ml serum; its kidney was given to a control animal that subsequently had extended graft survival. Biopsies of these interstitial dendritic cell-free kidneys were also taken before retransplantation, and H and E staining showed them all to be in good condition.

The blood urea levels after retransplantation are given in TABLE 1 for both peptide immunised and control rats. Four of the seven peptide treated rats primed for indirect recognition had very high urea levels 10 days after receiving an interstitial dendritic cell-free kidney and these four showed accelerated graft rejection. All seven control animals had low urea levels at day 10 and they all survived for >3 weeks. All peptide challenged rats had higher urea levels than the controls 10 days after retransplantation. The survival figures (p = 0.03) and the urea levels (p = 0.0001) are significantly different between the two groups on a Fischers Exact Test. These data demonstrate that priming for indirect recognition does contribute to the effector mechanisms of vascularised allograft rejection.

Five control, single transplant LEW (RT1^l) rats were also studied as a comparison. All these control LEW (RT1^l) rats received a (DAxLEW)F1 kidney in the absence of CsA cover and immediately rejected their grafts by d10 with blood ureas between 322 and 486mg per 100ml sera. With these animals, the donor kidneys are not depleted of mobile, dendritic cells, enabling direct allorecognition to mediate the rapid rejection response.

H and E stained sections from the dendritic cell-free kidneys were examined prior to retransplantation and seven days after retransplantation.

<u>TABLE 1:</u>

Results Of Retransplanting Long Surviving (DAxLEW)F1 Kidneys from the original LEW Recipients to Fresh LEW Recipients Immunised With DA Derived MHC Class I Peptides.

Treatment group	Blood Urea (mg /100 ml) at		Survival Time (days)
	day 10	day 21	
Peptide θ Immunised	617	-	10
	542	-	10
	492	-	10
	452	-	10
	206	222	49
	169	200	49
	124	174	>100
Saline φ Immunised	109	94	>100
	96	121	>100
	93	201	56
	92	299	28
	92	444	21
	68	448	21
	63	367	21

Θ Peptide immunised rats received 50μg P1 and 50μg P3 subcutaneously in Freunds complete adjuvant and were boosted with the same dose of peptide in Freunds incomplete adjuvant 4 weeks later. Kidney grafting was performed 10 days after boosting.

φ Control rats were treated with Freunds incomplete adjuvant and saline using the above protocol.

FIGURE 2 shows a typical series of biopsies from a rejecting, peptide immunised rat (FIGURE 2a), and a non-rejecting, control rat (FIGURE 2b). A normal LEW rat kidney is also shown for comparison (FIGURE 2c).

Rejecting kidneys from peptide immunised rats show evidence of increased leucocytic infiltration and glomerular destruction when compared with the controls. Interestingly, one of the peptide treated rats that survived for 49 days also showed evidence of a deterioration in kidney structure at 7 days post-transplantation.
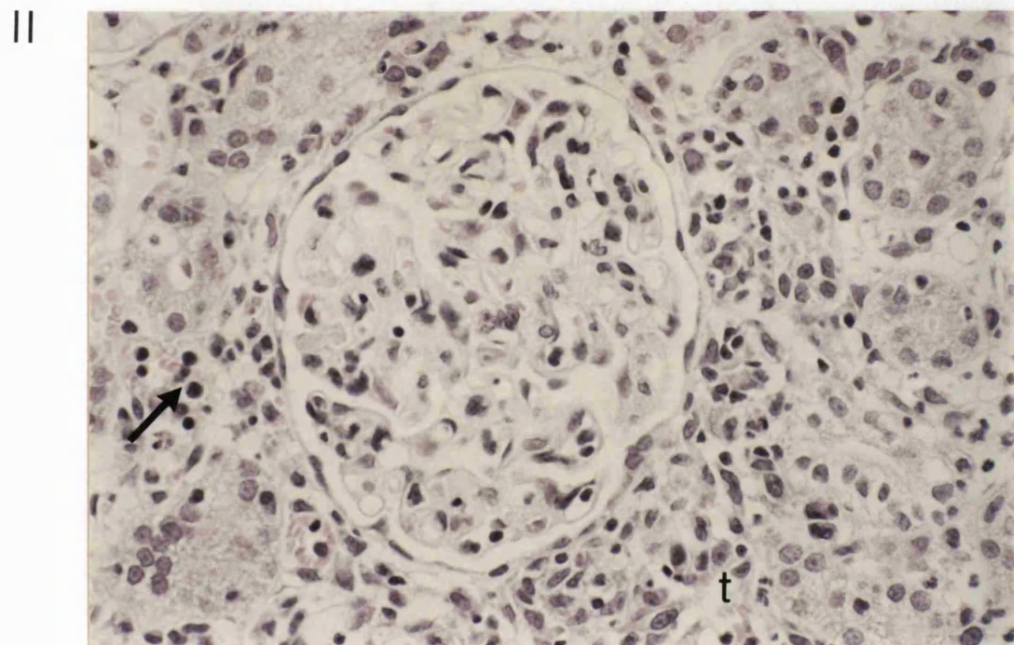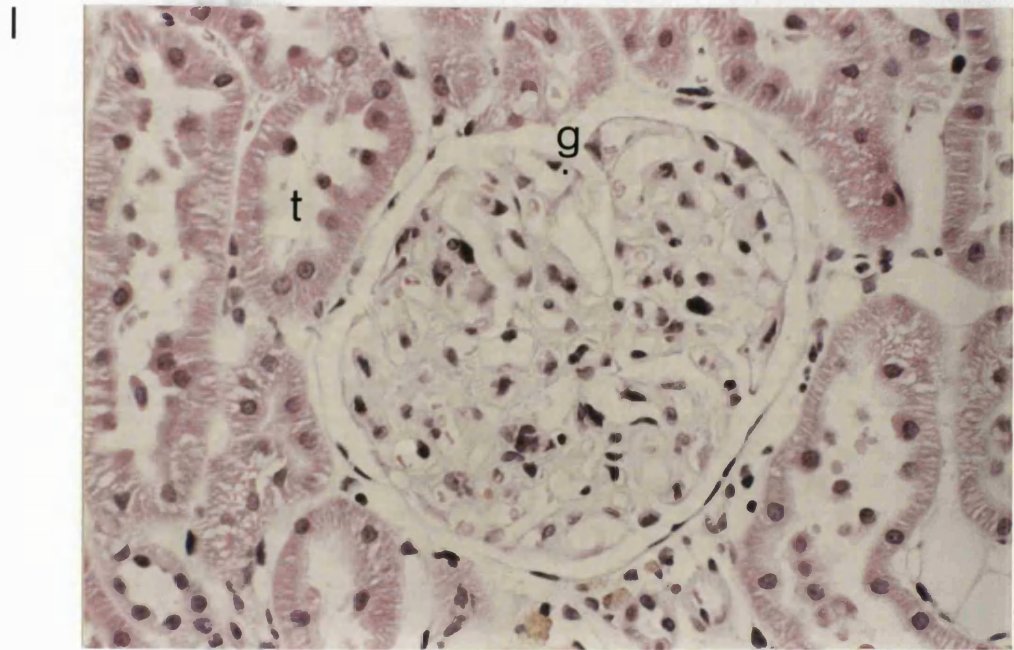
FIGURE 2a:

Biopsies From the Kidney of a Rejecting, Peptide Treated Rat.

I Prior to retransplantation, the glomerulus (G) and tubules (T) of the kidney are in reasonable condition.

II Seven days post-retransplantation, the kidney shows increased signs of leukocytic infiltration (arrowed) and deterioration of the tubules (T).

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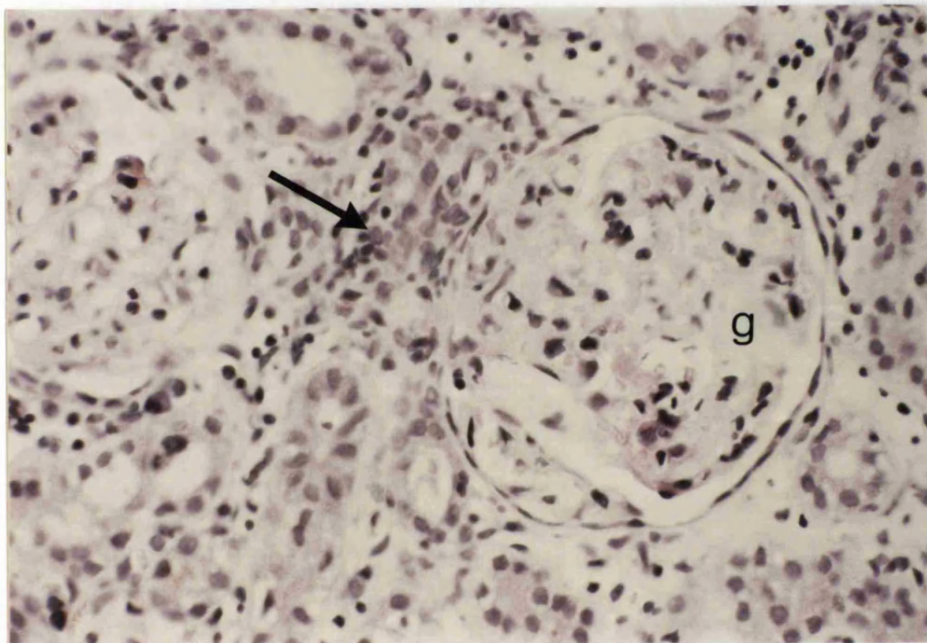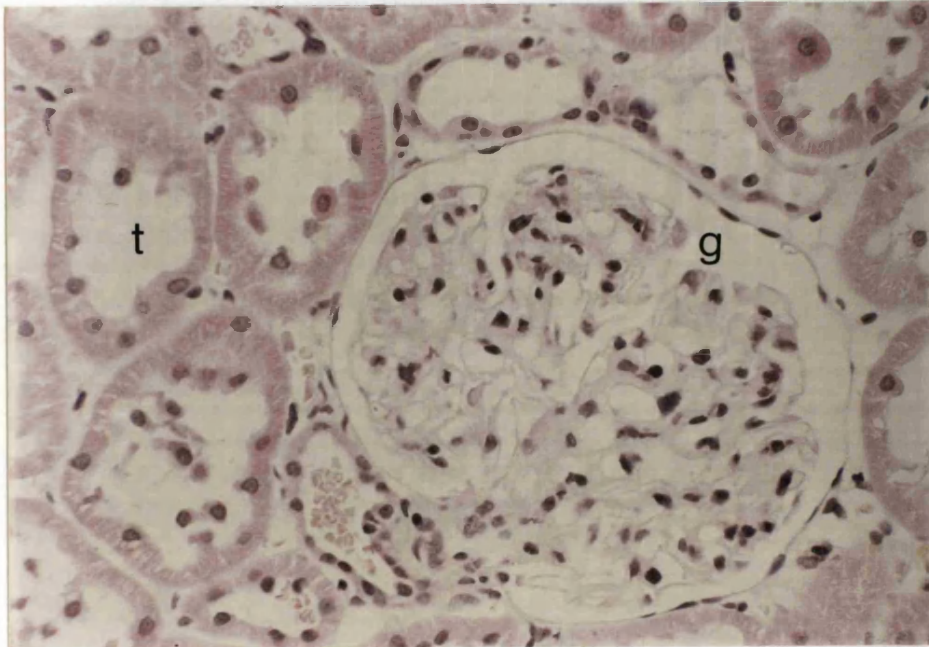
FIGURE 2b:

Biopsies From the Kidney of a non-Rejecting, Control Treated Rat.

I Prior to retransplantation, the tubules (T) and the glomeruli (G) of the kidney are in reasonable condition.

II Seven days post-retransplantation, the kidney shows increased levels of leukocytic infiltration (arrowed). The glomeruli (G) are in good condition compared to the peptide immunised animal (2a).
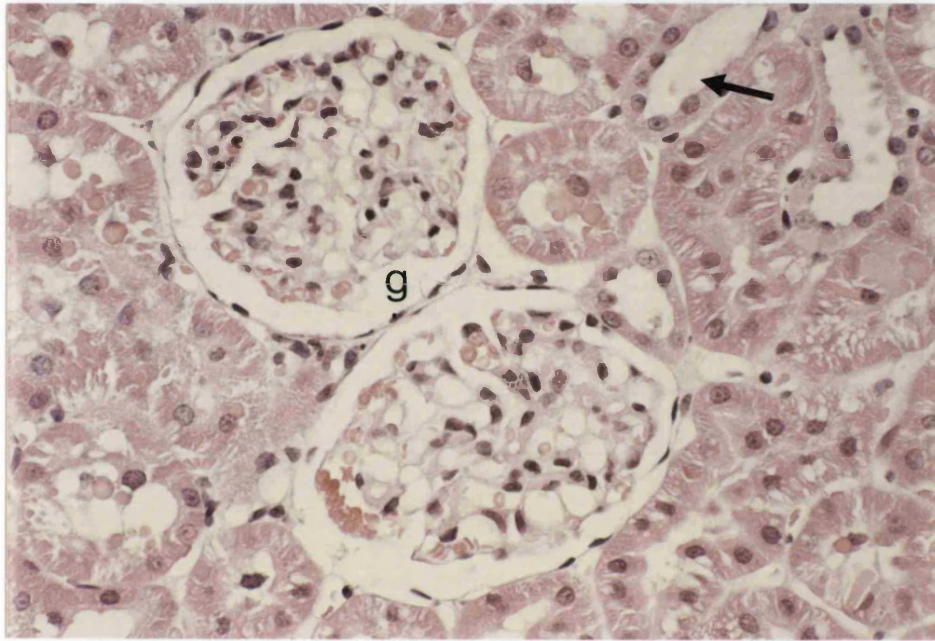
FIGURE 2c:

Biopsy From the Kidney of a Control Rat That Has Received no Treatment.

The fresh kidney is in good condition, with intact tubules (arrowed) and healthy glomeruli (G).

5.2b Alloantibody Studies:

5.2b i) Peptide specific antibodies: All peptide immunised and saline immunised LEW (RT1^l) rats were examined for the appearance of antibodies to P1 (FIGURE 3a and b) and P3 (FIGURE 3c and d), both before and after grafting with interstitial dendritic cell-free kidneys. All peptide immunised rats developed a strong antibody response to both unconjugated peptides, which is not enhanced by the additional stimulus of the graft (FIGURE 3a and c). Conversely, all saline immunised animals have no antibody to peptide (FIGURE 3b and d). After grafting, the (DAxLEW)F1 kidney potentially provides a source of P1 and P3 in the form of DA RTI.A^{av1} class I molecules. The absence of antibody after grafting in these controls implies that B cells are not efficiently exposed to these particular determinants, even though peptide specific CD4$^+$ T cells are almost certainly present (Fangmann et al 1992b).

5.2b ii) Donor MHC Class I Specific Antibodies: Alloantibodies to intact DA class I molecules were measured using DA RBC as targets in an indirect binding assay for both control and peptide immunised animals. Each peptide immunised animal was compared with a saline immunised animal in an individual binding assay using the same positive control sera throughout (FIGURE 4) . The titration curves obtained were similar in each case and demonstrate two important points:

a) In FIGURE 4a, peptide immunised rats show an accelerated rate of donor class I antibody production compared with saline controls, regardless of whether or not they reject their kidneys. On the day of transplantation, there was no antibody response to intact donor class I in either group of animals, showing that immunisation with peptides does not give rise to antibodies that cross react with the entire molecule. By day 7

post-transplantation, the response of saline immunised animals is still very weak and about 100 times less than that of the peptide immunised group.

b) FIGURE 4b demonstrates that saline immunised control rats do develop an anti-class I response with time that reaches the same level as that of the peptide immunised rats by 2-4 weeks after grafting, but these creatures do not reject their grafts. The essential difference between the peptide immunised and control immunised LEW recipients of interstitial dendritic cell-free (DAxLEW)F1 kidneys was in the far more rapid kinetics of the antibody response in the peptide immunised rats. Whilst the rate of antibody production often correlates with rejection, the presence of class I antibodies is not in itself sufficient to effect rejection.
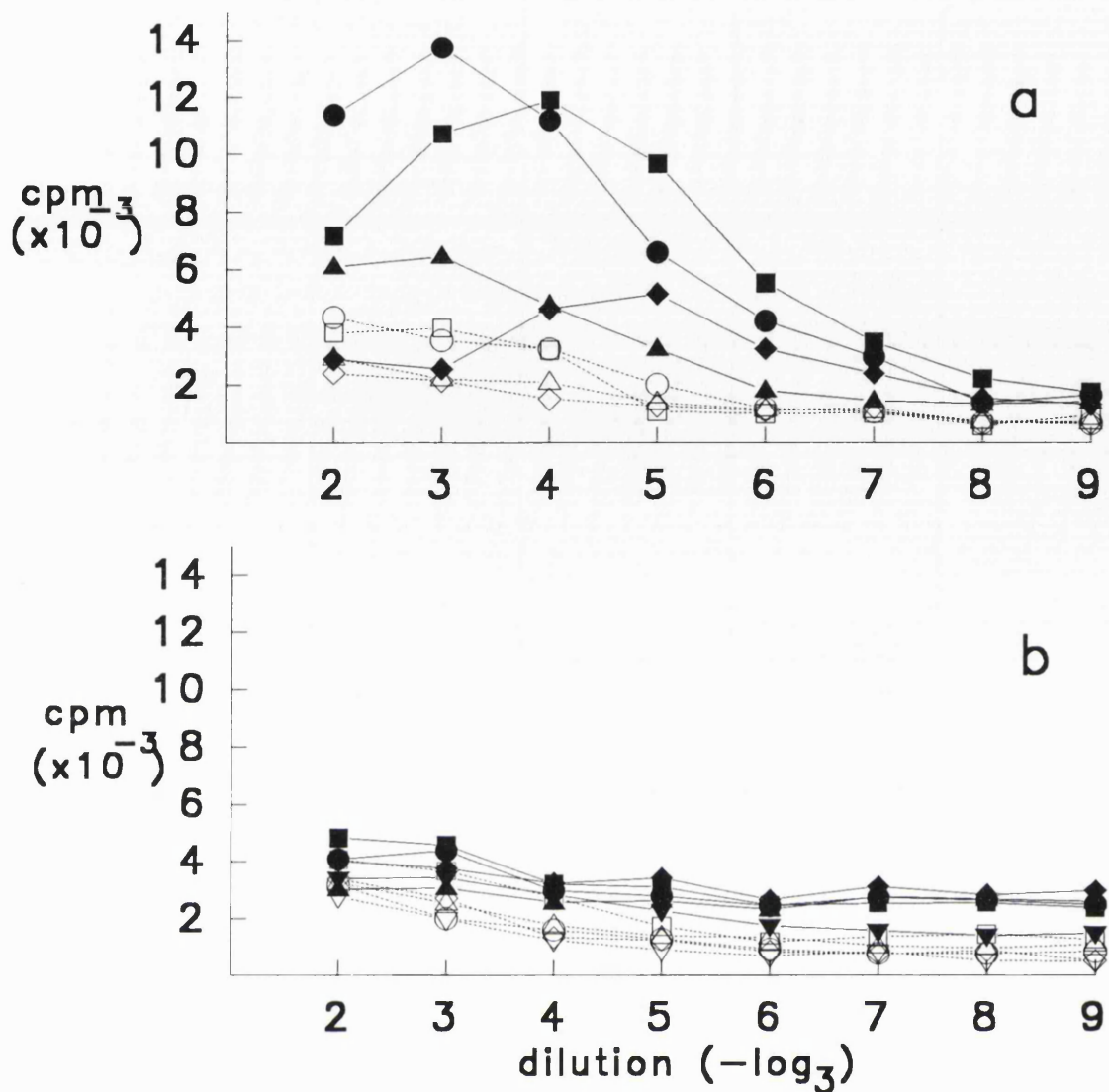
FIGURE 3a and b:

Antibody Response to Donor Class I Peptide 1.

LEW rats were immunised with P1 (a) or saline in adjuvant (b) and given a dendritic cell-free (DAxLEW)F1 kidney allograft 10 days after boosting. Sera from these animals were tested against P1 (solid lines, filled symbols) or saline only (dashed lines, open symbols) on PVC plates 14 days post-immunisation (♦), 28 days post-immunisation (▲), 10 days post-boost, which is also the day of transplantation (■), and days 7 (●) and 28 (▼,b only) post-grafting. cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 1% and 11% of the mean values obtained.
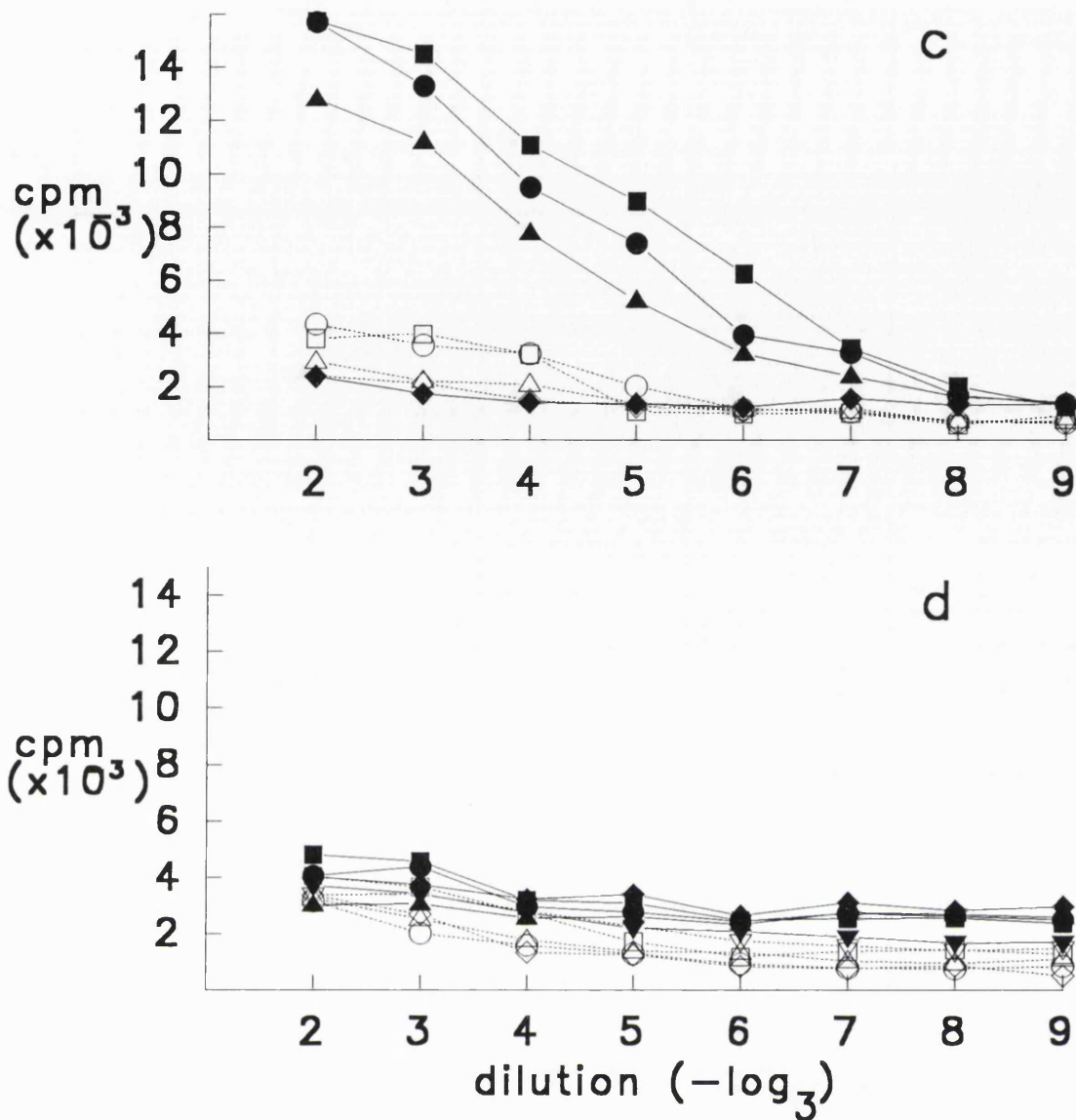
FIGURE 3c and d:

Antibody Response to Donor Class I Peptide 3.

LEW rats were immunised with P3 (c) or saline in adjuvant (d) and given a dendritic cell-free (DAxLEW)F1 kidney allograft 10 days after boosting. Sera from these animals were tested against P3 (solid lines, filled symbols) or saline only (dashed lines, open symbols) on PVC plates 14 days post-immunisation (♦), 28 days post-immunisation (▲), 10 days post-boost, which is also the day of transplantation (■), and days 7 (●) and 28 (▼,d only) post-grafting. cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 0.5% and 13% of the mean values obtained.

FIGURE 4a and b:

Antibody Responses to Intact Donor MHC Class I Molecules.

Peptide immunised (O) and saline immunised (●) LEW rats that had received interstitial dendritic cell-free kidney grafts were tested for antibodies to intact donor class I molecules using 10^7 DA RBC as targets. In (a), sera taken on the day of grafting (●···●) and at day 7 post transplantation (●-●) are compared. In (b), sera from a saline immunised rat on the day of grafting (●···●) and at day 7 (●-●), day 14 (●-·-●) and day 28 (●---●) post-transplantation are shown. These are compared with a peptide immunised rat at day 7 (O-O). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 1.5% and 13% of the mean values obtained. These assays were performed on five sets of animals, with similar results.

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5.2b iii) Alloantibody Response To Intact MHC class I In a Rejecting And Non-rejecting Peptide Immunised Rat:

It was of interest to compare the antibody response to intact donor MHC class I molecules in two peptide immunised rats, one of which rejected its interstitial dendritic cell-free kidney by day 10 and one of which retained a functional graft until day 100 (FIGURE 5). The assay conditions are the same as in FIGURE 4. Both animals have negligible titres at the time of grafting as expected. The non-rejecting animal develops an antibody response of similar magnitude and at the same rate as the rejecting one. Unfortunately, day 7 post-transplantation sera were not available for the rejecting animal in this assay.

Thus the appearance of alloantibodies does not necessarily correlate with rejection. This applies both to the peptide immunised group (all of which had a strong antibody response by day 7, but only 4 of which rejected their grafts acutely), and the saline immunised group (all of which developed a strong antibody response by 4 weeks after grafting, with only 3 achieving long term survival).

5.2b iv) Alloantibody Response To Intact DA MHC Class I Molecules In Control Single Transplant Rats.

Five control, single transplant LEW (RT1^l) rats with (DAxLEW)F1 kidneys in the absence of CsA cover also had their antibody production to whole DA (RT1.A^{av1}) class I molecules monitored (FIGURE 6). Antisera from all these animals have such antibodies by d7 post-transplantation at a level that approaches or exceeds that of peptide challenged, dendritic cell free-kidney allografted rats. Thus rapid kinetics of antibody production occurs either in rats primed to indirect recognition or in rats also exposed to the direct arm of the rejection response.

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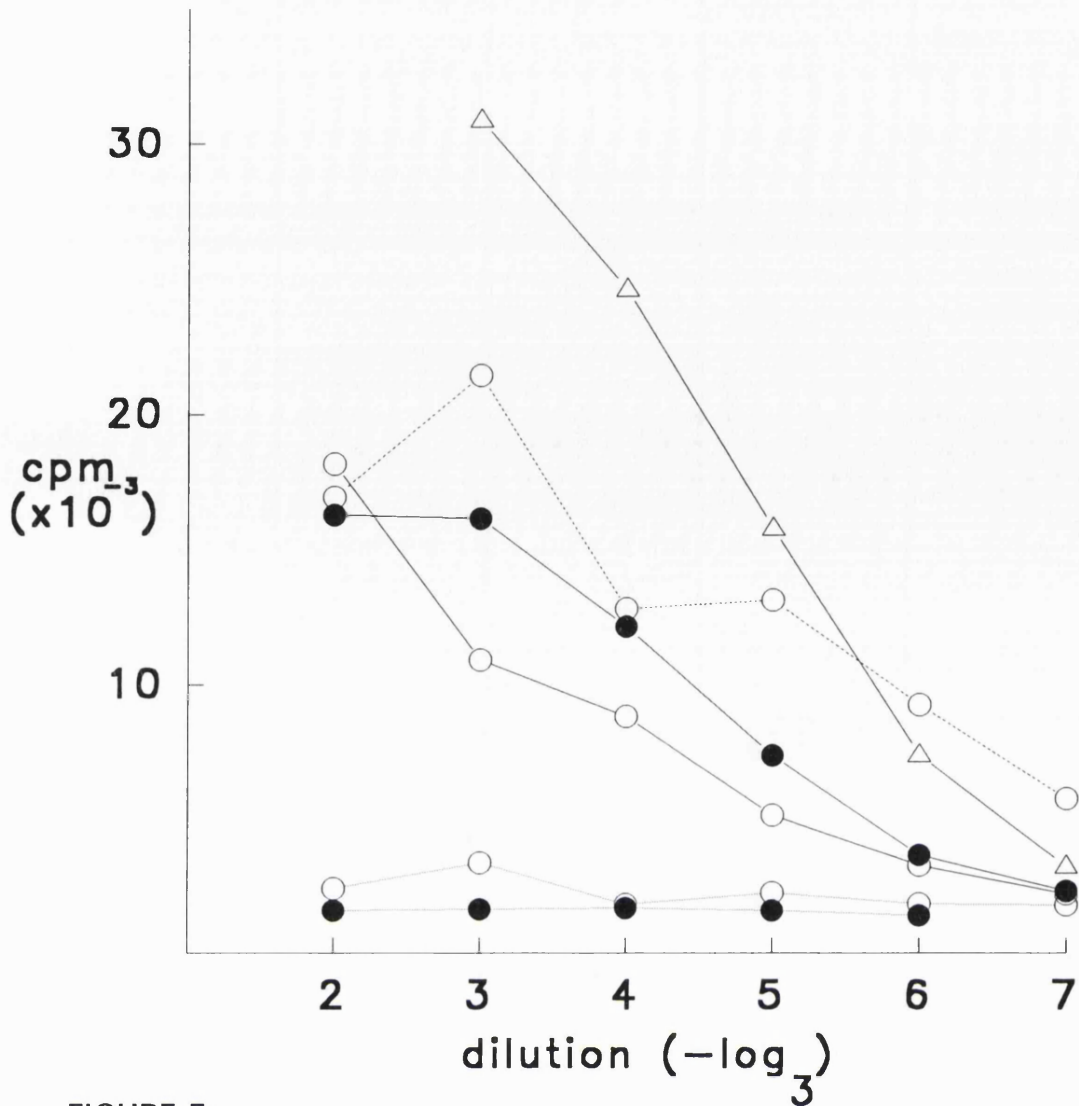
FIGURE 5:

Antibody Responses to Intact Donor MHC Class I Molecules in Rejecting and Non-rejecting P1 Immunised Recipients.

Sera from a rejecting rat (●) and non-rejecting rat (○) with interstitial dendritic cell-free kidney grafts were tested for antibodies to intact DA class I molecules using 10^7 DA RBC as targets. Sera were taken on the day of grafting for both animals (···), day 10 after grafting for the rejecting animal (●-●) and days 7 (○-○) and 14 (○--○) after grafting for the non-rejecting animal. Sera from a LEW animal immunised with soluble DA MHC class I molecules was used as a positive control (△). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 0.5% and 13% of the mean values obtained.
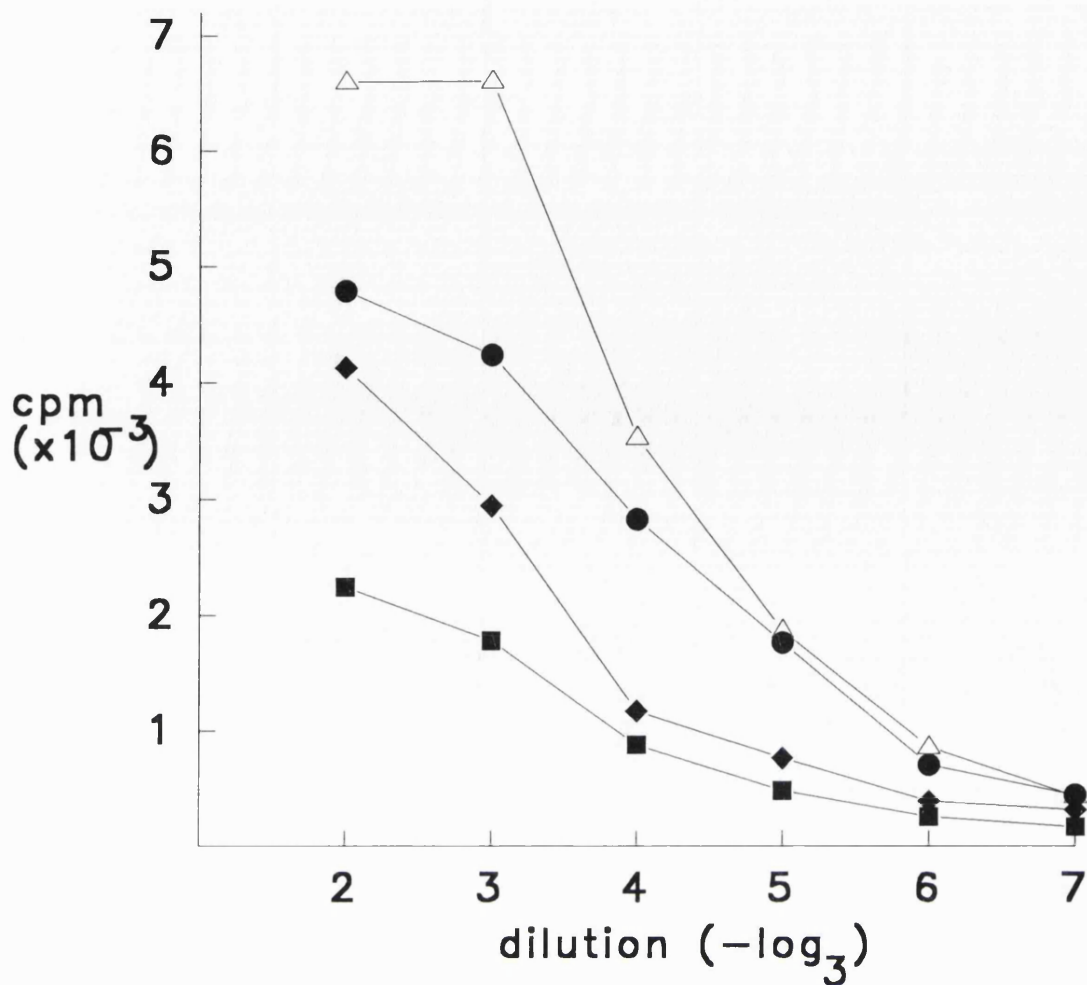
FIGURE 6:

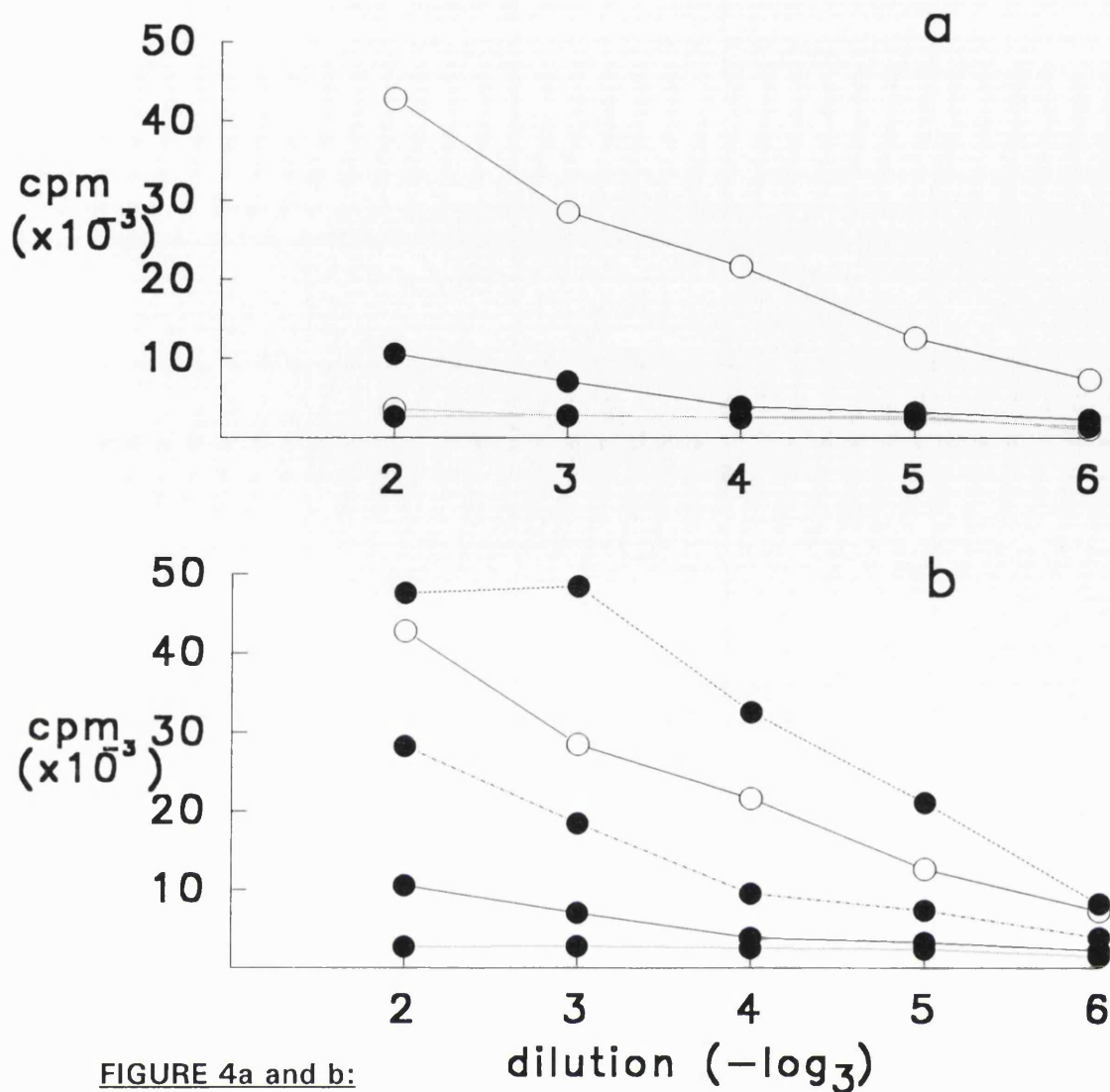**Antibody Responses to Intact Donor MHC Class I Molecules.**

Day 10 sera from a LEW rat that had received a (DAxLEW)F1 kidney without CsA immunosuppression (●) was compared with day 7 sera from a peptide immunised LEW rat with a dendritic cell-free kidney (◆) and day 7 sera from a control LEW rat with an interstitial dendritic cell-free kidney (■). The sera were assayed for antibodies to intact DA class I molecules using 10^8 RBC as targets. Positive control sera from a LEW rat immunised with soluble DA class I molecules was also used (△). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors obtained were between 2% and 10% of the mean values. All 5 single transplant LEW animals examined showed similar results.

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5.3 DISCUSSION:

These data demonstrate that indirect T cell allorecognition of donor MHC antigens can contribute to the effector mechanisms of organ allograft rejection, and that this mode of T cell recognition is of potential clinical significance. The presence of antibodies to donor antigens did not correlate with rejection in this rat model. It is therefore probable that the provision of T cell help for antibody production is not functionally important in this situation. Of the three effector mechanisms proposed to be associated with the indirect pathway, the provision of help for $CD8^+$ effector cells or alternatively $CD4^+$ T cell-mediated intragraft DTH responses are likely to be of primary importance. However, in clinical transplantation (Jeannet *et al* 1970) and in other models (e.g Gracie *et al* 1990), antibodies to donor antigens are likely to be of far greater import.

The diminished early antibody response to donor MHC class I antigens on interstitial dendritic cell-free kidney allografts confirms previous studies done by our group using lymphocytotoxicity (Fabre and Morris 1972, Hart *et al* 1980). The data suggest that interstitial dendritic cells are crucial to the early antibody response to alloantigens. The fact that delayed kinetics of antibody production can be restored to 'normal' levels by priming with peptides for indirect recognition strongly suggests that the T helper response is rate-limiting in the antibody response to allografts.

It was hoped that this model could be further used to study the effector arm of the indirect pathway in more depth. However, the model is not consistent enough in terms of rejection times to allow this to be done. If a model could be developed in which priming for indirect recognition (in the absence of the direct pathway) consistently resulted in accelerated rejection, then depleting monoclonal antibodies towards $CD8^+$ and $CD4^+$ T cells, combined with the use of antisera from rejecting rats, could be used

to prove which effector mechanism is crucial to the rejection response. Attempts to develop such a system are partially addressed in the next chapter.

CHAPTER 6:   THE ALLORESPONSE IN (PVGxLEW)F1

RECIPIENTS OF PVG RTI^{r1} KIDNEY GRAFTS.

THE ALLORESPONSE IN (PVGxLEW)F1 RECIPIENTS OF PVG RTI^{r1} KIDNEY GRAFTS.

6.1a The Experimental Model:

The (PVGxLEW)F1 rat carries the MHC genes of both the PVG RT1^c and LEW RT1^l parental strains, whereas the RTI^{r1} rat carries the DA RT.A^{av1} class I locus on a PVG background. It was initially hypothesised that F1 rats receiving RTI^{r1} kidneys would reject them in the absence of immunosuppression, since there is a pure MHC class I difference between these rat strains. The original intention was to use the RTI^{r1} kidney into (PVGxLEW)F1 model in studies on tolerance. By administering our peptides specific for indirect T cell allorecognition, it was hoped that the 'indirect' rejection response against the class I molecules from which these peptides were derived (i.e donor MHC class I) could be reduced. However, there remained a possibility that DA class I molecules on the graft could be recognised 'directly' by recipient CD8$^+$ T cells and still initiate an acute rejection response. FIGURE 1 outlines how the allo-MHC molecules on the graft and in the recipient might result in rejection by either the indirect or direct pathways.

6.1b Induction Of Tolerance:

Various protocols for tolerance induction exist. For example, oral tolerance to MHC antigens has been achieved through feeding with MHC class I and class II allopeptides (Sayegh *et al* 1992). In the mouse, autoimmune diseases such as EAE can be alleviated by specific intraperitoneal MBP-derived peptide administration in Freunds incomplete adjuvant (Gaur *et al* 1992). It is well established that at least three factors are crucial in the induction of tolerance as opposed to sensitisation. These are:

i) Type of antigen presenting cell and its activation state.

ii) Site of antigen administration.

iii) Dose of antigen/tolerogen.

Other factors influencing tolerance include the physical nature of the antigen and the age of the patient or animal. These issues are further discussed in Chapter 1, sections 1.5 and 1.6c.

Having referred to the work of Gaur *et al*, it was decided to attempt to induce tolerance to DA peptides in male (PVGxLEW)F1 animals by immunising them with both P1 and P3 in the hind footpad in the conventional way, but using incomplete rather than complete adjuvant. Since RTI^{r1} kidney transplantation was to be subsequently performed, it was felt that intraperitoneal peptide injections would irritate the gut and hinder transplantation. This procedure was therefore avoided.
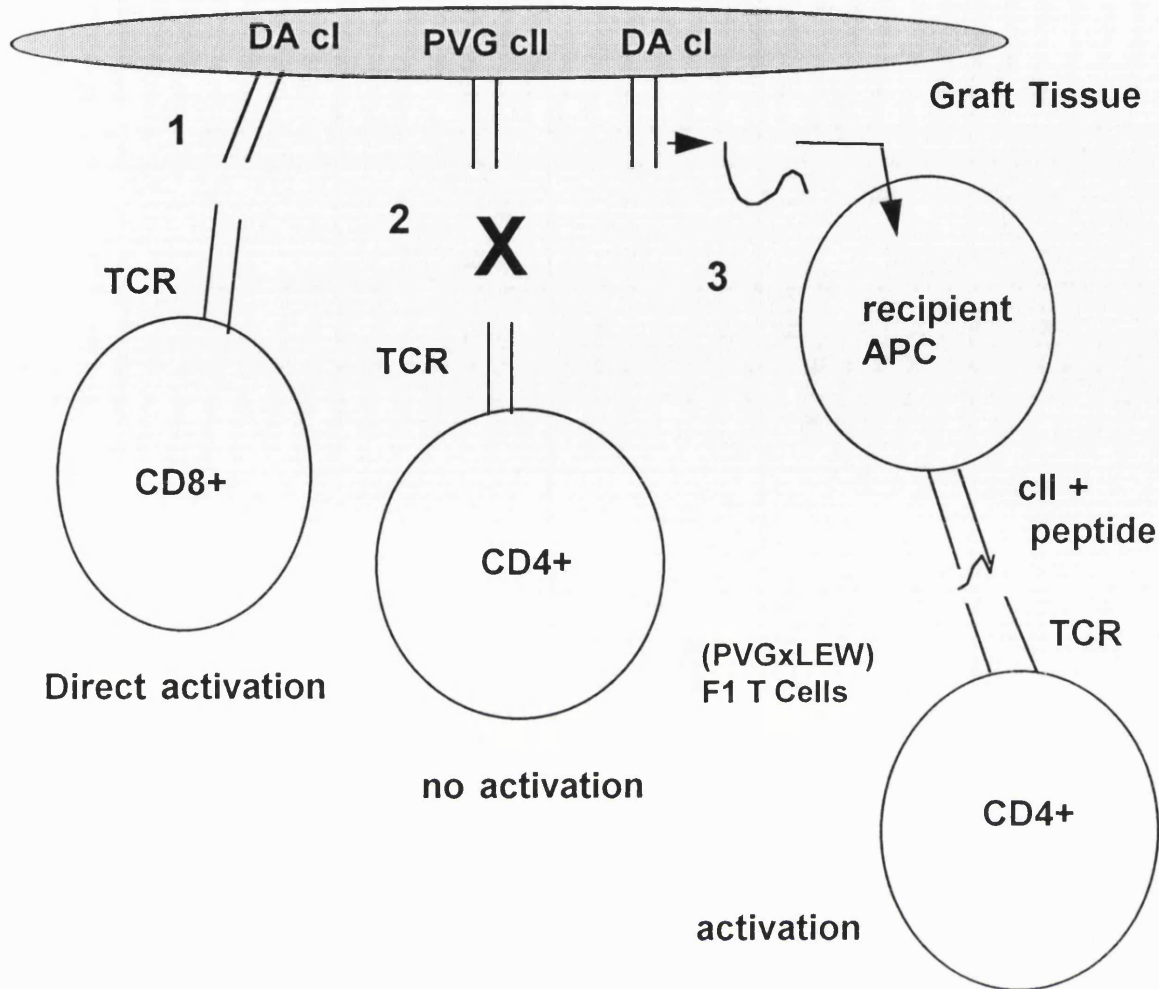
FIGURE 1:

Potential T Cell Responses in the (PVGxLEW)F1 Recipient of an RT1^{r1} Allograft.

1 Direct activation of recipient CD8^{+} T cells by DA class I molecules of allograft. This may be inefficient due to lack of CD4^{+} T cell help.

2 No direct activation of recipient CD4^{+} T cells by PVG class II molecules, since these are also present in the host.

3 Indirect recognition of DA class I molecules through conventional antigen processing and presentation to recipient CD4^{+} T cells.

cI = MHC class I, cII = MHC class II.

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6.2 RESULTS:

6.2a MHC Class I Typing Of Rat Strains:

Since PVG, (PVGxLEW)F1 and PVG RTI^{r1} rat strains are visually indistinguishable, the animals used in these experiments were typed using rat MHC class I specific monoclonal antibodies to ensure that the correct animals were being used as graft donors and acceptors. Naive animals were bled into heparin coated tubes. Approximately 10^8 red blood cells, which express class I molecules in the rat, were incubated with an equal volume of each of the following monoclonal antibodies: MN4 (anti DA class I), F16 (anti rat class I), OX27 (anti PVG class I) and NDS60 (anti LEW class I). A sample of the results obtained using ^{125}I labelled RAM to detect the first antibody is given in FIGURE 2. All the animals tested expressed the MHC class I molecules expected of them. The MN4 antibody is a particularly good one and masks the results obtained with the other antibodies in this graph. The epitope recognised by F16 does not always come up on analysis (as for the PVG rat in FIGURE 2).

6.2b Immunisation Using Freunds Incomplete Adjuvant Prior To Kidney Transplantation:

Two groups of three age matched (PVGxLEW)F1 rats were initially used in a trial experiment. Male rats were immunised in individual hind footpads with 20μg each of peptides P1 and P3 emulsified in FIA and transplanted 28 days later with age-matched male PVG RTI^{r1} kidneys. No immunosuppressive therapy was administered. Control animals were sham immunised with saline emulsified in FIA. The animals were bled regularly before and after transplantation and their blood urea levels were monitored from day 10 post-grafting, following removal of the recipients remaining kidney, to assess graft function. Kidney biopsies were taken seven days
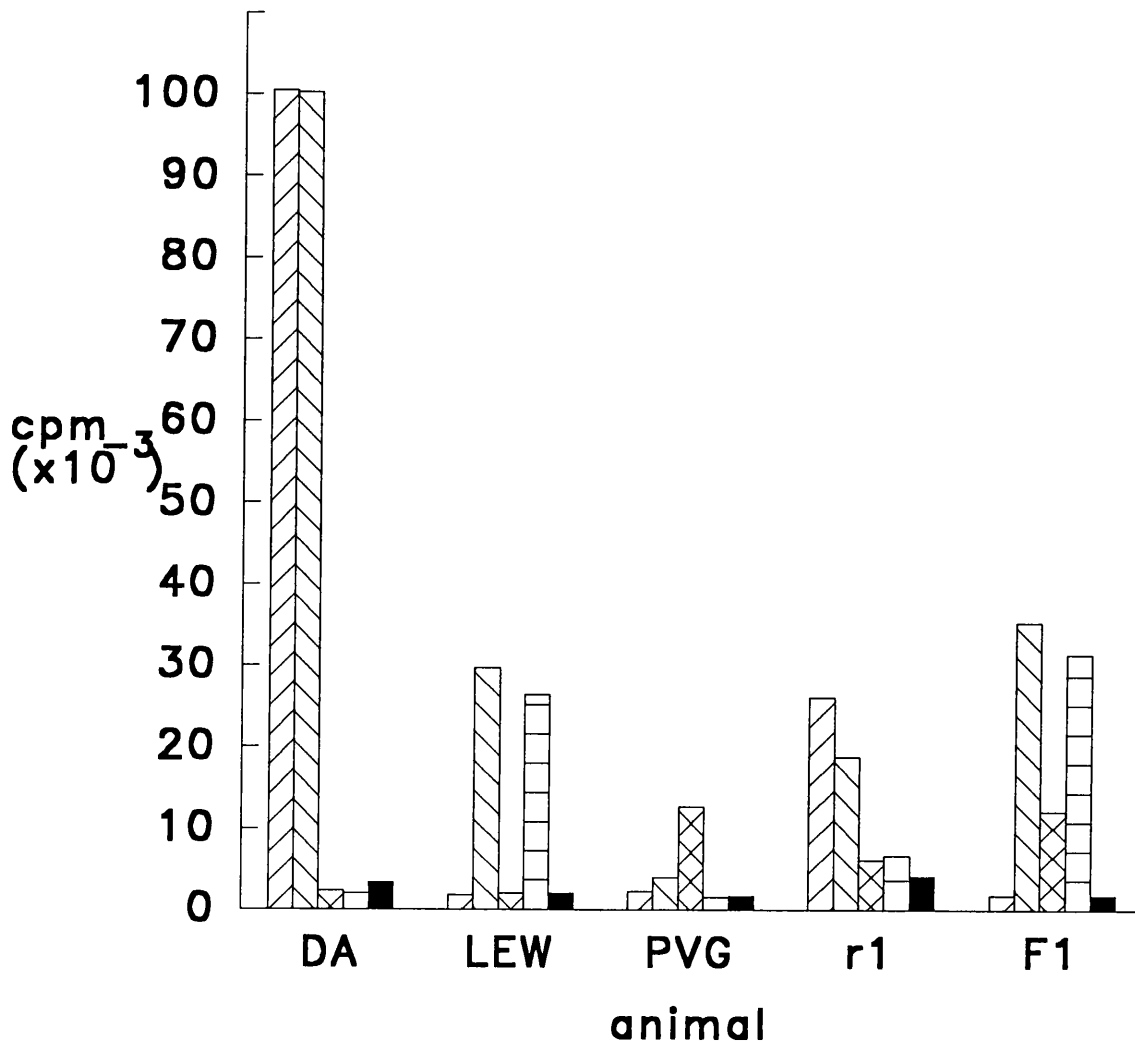
FIGURE 2:

MHC Class I Typing of Rat Strains.

Heparinised blood samples were taken from the rat strains shown above and 25μl containing about 10^8 RBC were incubated with equal volumes of the following monoclonal antibodies: MN4 (anti-DA class I ▨), F16 (anti-class I ▨), both at 1 in 100 dilution, OX27 (anti-PVG class I ▨) and NDS60 (anti-LEW class I ▤), both at 1 in 10 dilution. Medium was used as a negative control (■). Antibody binding was detected by using ^{125}I-RAM as a second antibody. cpm refers to counts per minute of bound radioactivity. Standard errors were between 1% and 15% of the mean values obtained.

after transplantation to study the morphology of the organ by H and E staining. Rats were terminated when their blood urea levels exceeded 200mg per 100ml blood and their kidneys removed for histological analysis.

6.2b i) Kidney Function And Morphology:

The blood urea levels at day 10 post transplantation for these six rats is given in TABLE 1 alongside their survival times. All three peptide treated animals had high blood urea levels at day 10 and were therefore sacrificed. By contrast, the saline control group showed a mixed rejection profile: one animal went on to become a long survivor, maintaining a functional graft and low urea levels for over 100 days. One rat had an elevated urea level at day 10 but did not lose function until day 17. The third rat rejected its kidney acutely with an elevated blood urea level at day 10. The histology of H and E sections through these kidneys is compared on the day of termination in FIGURE 3.

Surprisingly, in the saline immunised group in which consistent allograft rejection of class I mismatched kidneys was expected, a diverse pattern of rejection responses is seen. The reason for this is unknown, but perhaps the provision of adjuvant, coupled with the non-specific effects of surgery, is sufficient to push some of these animals towards rejection. By contrast, the peptide treated group, in which it was hoped to achieve tolerance to indirect recognition, uniformly acutely rejected their grafts. Our protocol may have primed these animals for indirect recognition, rather than tolerised them, and thus provided the necessary impetus to consistently stimulate rejection. This further emphasises the importance of the site of antigen administration in achieving tolerance (e.g. Gaur *et al* 1992).

To verify the immune status of these animals, I examined their antibody responses to P1, P3 and intact donor RTI.A^{av1} MHC class I molecules.

Urea Levels and Survival Times of RTI[r1] Kidney Grafted (PVGxLEW)F1 Male Rats.

Peptide immunised rats received 20μg ml^{-1} of both P1 and P3 in FIA into both hind footpads 28 days before transplantation. Saline immunised rats received saline emulsified with FIA and were treated analogously.

| Treatment Group | Urea Level (mg/100ml) at | | Survival Time (days) |
	d10	d14	
P1 and P3 In FIA	353	-	10
	379	-	10
	345	-	10
Saline In FIA	56	58	>100
	171	170	17
	462	-	10
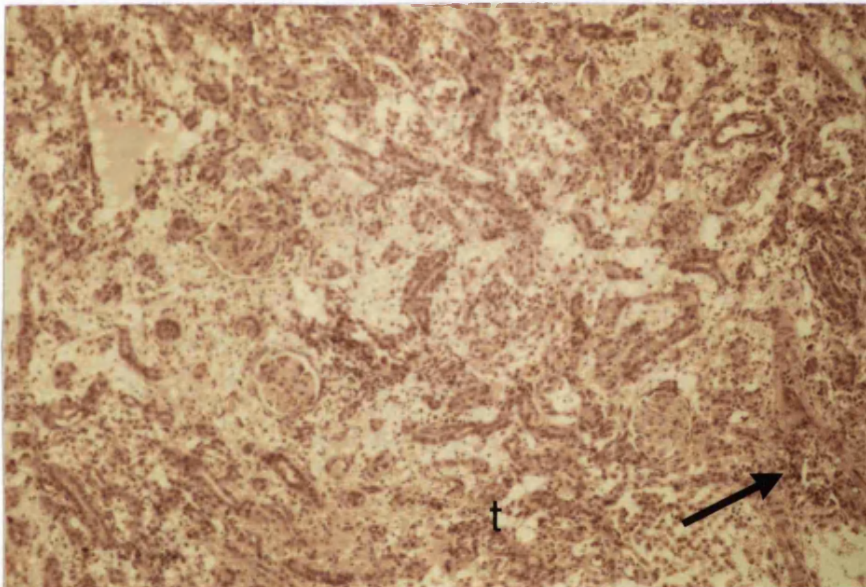
FIGURE 3a:

Histology of Peptide Treated Male Rat d7 post-transplantation.

There is marked tubular destruction (T) and heavy leukocytic infiltration (arrowed) indicating acute rejection is in progress.

I
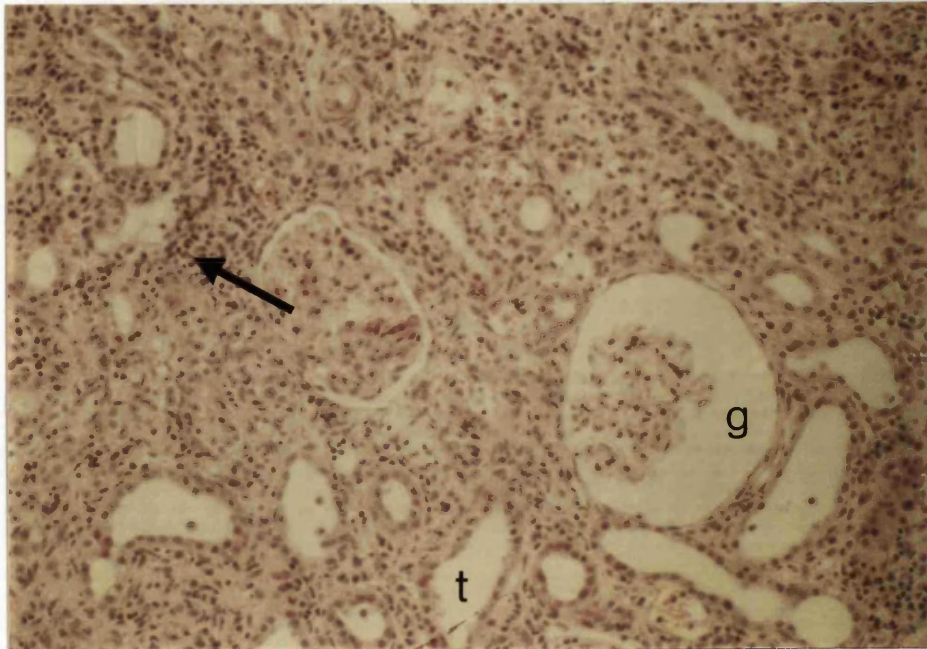
II
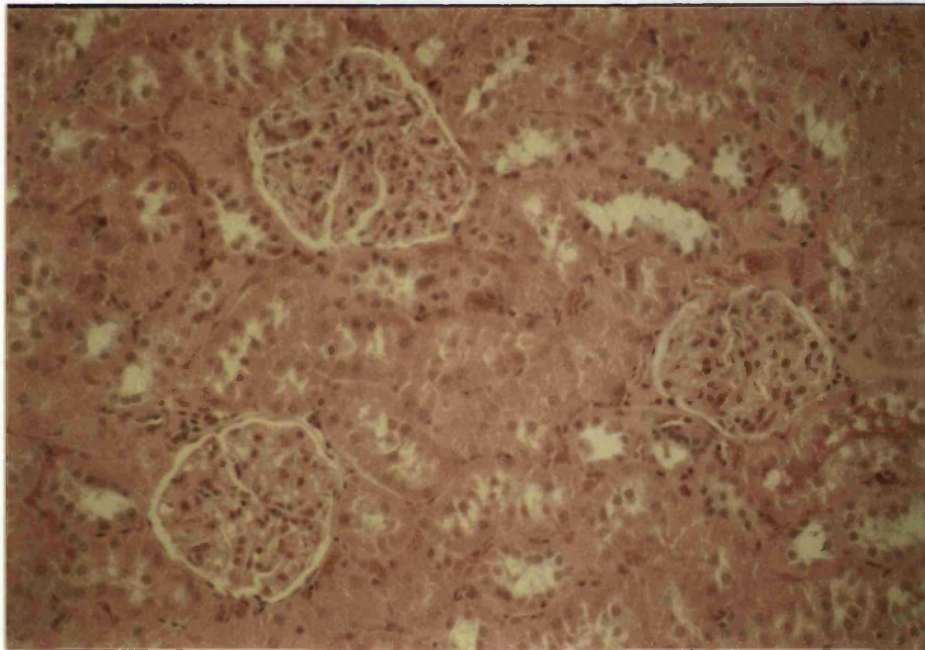
FIGURE 3b:

Histology of Two Saline Treated Male Rats d10 (I) and d100 (II) post-transplantation.

The kidney of the rat which rejected at d10 (I) is in far worse condition than that of the animal which was functional up to d100 (II). II exhibits intense leukocytic infiltrate (arrow) and glomerular (G) plus tubular (T) damage.

6.2b ii) The Antibody Response To P1 and P3:

Each animal was assayed for the presence of peptide specific antibodies towards P1 and P3 both before and after transplantation using an indirect radioactive binding assay with immobilised antigen as the target. FIGURE 4 reveals a typical antibody response to P1 before and after grafting, comparing an animal that has been immunised with peptides in FIA with one that received saline in FIA. In the peptide-treated rat, a strong primary antibody response is obtained both before and after grafting. Thus this protocol results in peptide sensitisation, as measured by antibody production, despite the fact that incomplete Freunds adjuvant was used. The presence of an RTI^{r1} graft bearing $RT1.A^{av1}$ class I molecules does not markedly boost this antibody titer. The saline treated animal has few, if any, peptide specific antibodies either before or after grafting.

FIGURE 5 represents data from the same animals but with P3 as the target antigen rather than P1. Here, the results are essentially the same in that there is a strong antibody response to P3 in the peptide immunised rat. In this animal, the titer was slightly greater before rather than after grafting and the strength of the anti-P3 response is greater than anti-P1. This varies between animals, but the overall trend is the same.

Thus peptide priming in FIA results in sensitisation rather than tolerance.

6.2b iii) The Antibody Response To Intact $RT1.A^{av1}$ Class I Molecules:

Sera from each of the six experimental animals was also used in an indirect binding assay to detect antibodies against intact $RT1.A^{av1}$ class I molecules. DA red blood cells expressing class I were used as targets and are relevant since the RTI^{r1} kidney also expresses these molecules. Sera from a control and peptide immunised rat were compared in each assay
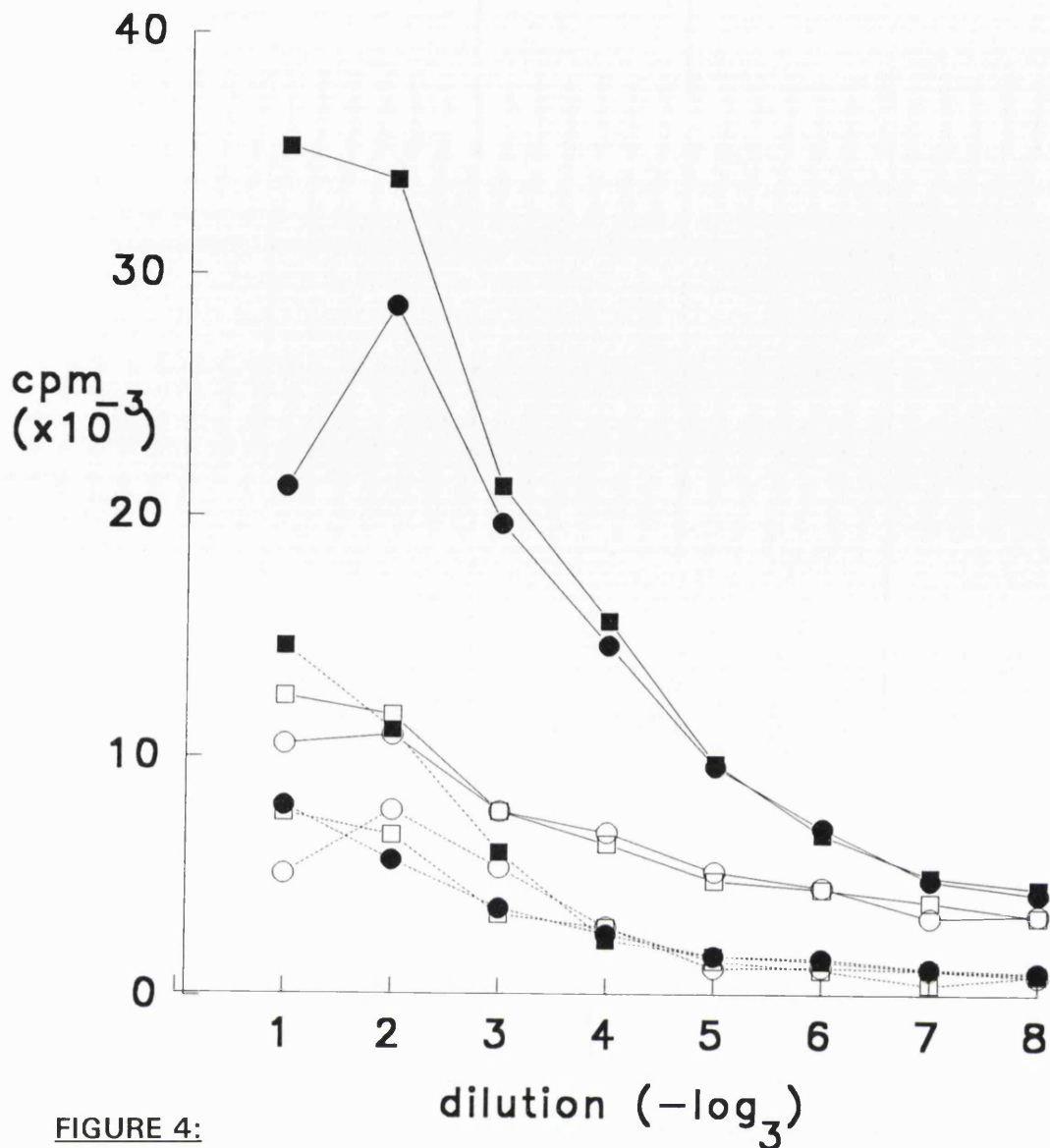
FIGURE 4:

Antibody Responses to P1 in (PVGxLEW)F1 Rats.

Sera were taken from a (PVGxLEW)F1 rat that was immunised with P1 and P3 in FIA (filled symbols) 28 days after immunisation (●) and 10 days after transplantation with a PVG r1 kidney (■). Sera were also taken from an F1 rat immunised with saline in FIA (open symbols) 28 days after immunisation (O) and 14 days after r1 kidney transplantation (□). Titrations were performed in tripling dilutions on P1 coated PVC plates (solid lines) or control saline coated plates (dashed lines). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 1% and 14% of the mean values obtained.

Antibody Responses to P3 in (PVGxLEW)F1 Rats.

Sera were taken from a (PVGxLEW)F1 rat that was immunised with P1 and P3 in FIA (filled symbols) 28 days after immunisation (●) and 10 days after transplantation with a PVG r1 kidney (■). Sera were also taken from an F1 rat immunised with saline in FIA (open symbols) 28 days after immunisation (O) and 14 days after r1 kidney transplantation (□). Titrations were performed in tripling dilutions on P3 coated PVC plates (solid lines) or control saline coated plates (dashed lines). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 0.5% and 15% of the mean values obtained.

before and after transplantation. The results obtained from the saline immunised animals in each of the three assays were different, so each one is shown here in FIGURE 6a,b and c. A positive control sera, derived from a LEW animal immunised with solubilised DA class I molecules, was used and is included to allow comparisons to be made between the different assays. FIGURE 6a represents data from the saline treated animal that did not reject its kidney, whereas 6b illustrates the response of the saline immunised animal that suffered acute graft loss. FIGURE 6c shows data from the saline control that rejected its kidney at day 17. The information from these three graphs can be summarised as follows:

i) Prior to transplantation, none of the six animals have antibody responses against intact class I molecules. In the peptide immunised rats, there is therefore no cross-reactivity between peptide B cell epitopes and MHC class I epitopes, as we have found previously in other models (Chapter 3, Chapter 5, Chapter 7).

ii) All peptide treated animals secrete class I antibodies after challenge with an RTI[r1] graft in a response that is both strong and rapid. By 7 days post-transplantation, the antibody titer is almost maximal.

iii) The three saline immunised animals have different kinetics of class I antibody production that correlates with the rate at which they reject their allografts. The rat that reached long-survivor status (FIGURE 6a) had a very weak antibody response at day 10 that is about one hundred fold lower than that of the rejecting peptide-treated animal. The saline treated rat that rejected acutely (FIGURE 6b) had a strong class I antibody response at day 10 that was comparable to the peptide animal and was also about one hundred times as great as its counterpart in FIGURE 6a. The saline treated rat that rejected at day 17 showed slower kinetics of class I antibody production that reached the same level as the peptide treated animal by day
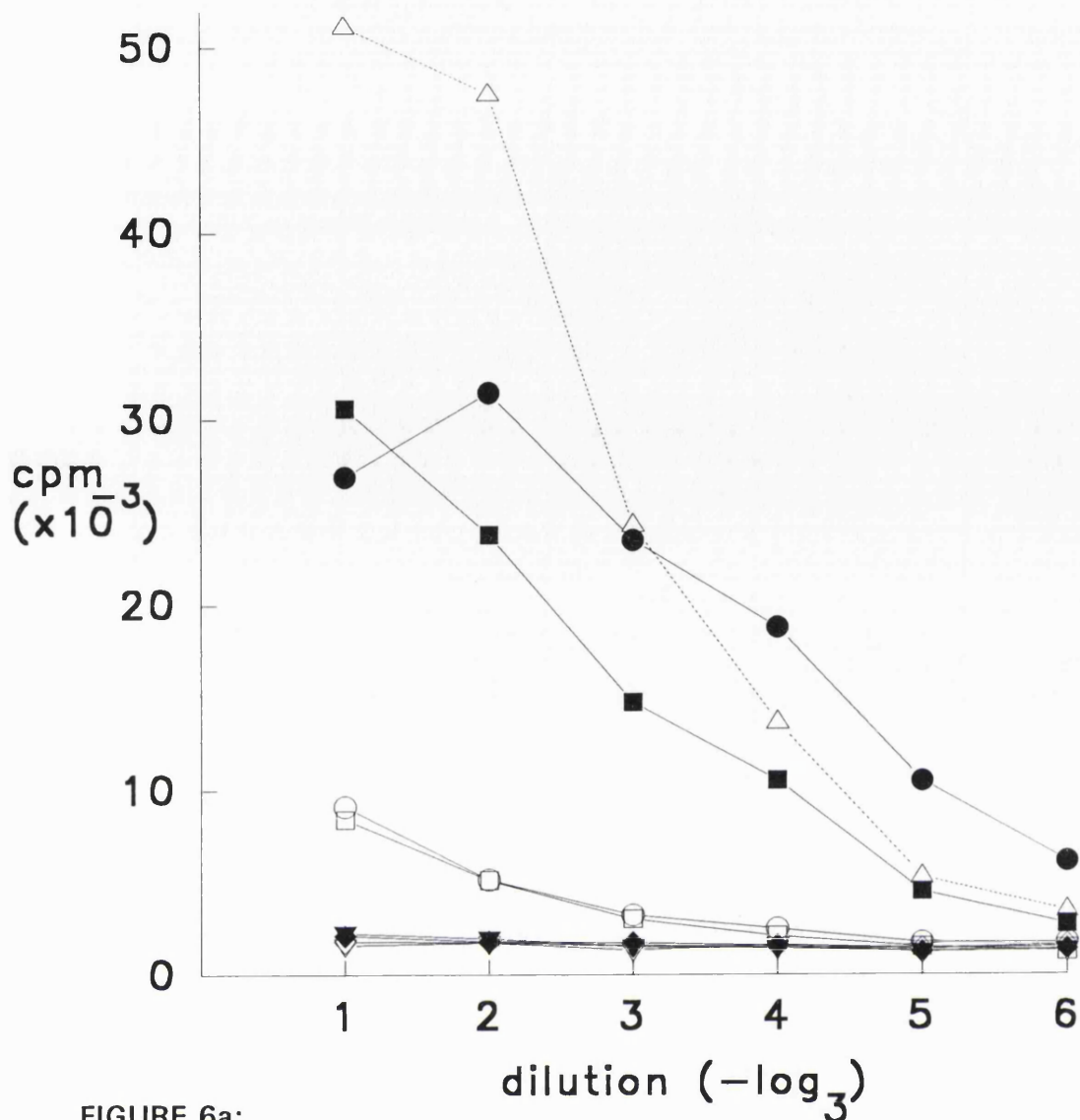
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Antibody Response to DA Class I Molecules in (PVGxLEW)F1 Rats.

Sera were taken from a (PVGxLEW)F1 animal immunised with P1 and P3 in FIA (filled symbols) or immunised with saline in FIA only (open symbols). The animals were grafted with r1 kidneys 28 days after immunisation. The antibody response was measured to intact DA class I molecules 14 days after immunisation (▼), on the day of transplantation (◆), 7 days post-transplantation (■) and 10 days post-transplantation (●). Sera from a DA class I immunised LEW rat was used as a positive control (△). cpm refers to counts per minute of ^{125}I-RAR. Standard errors were between 2% and 12% of the mean values obtained.

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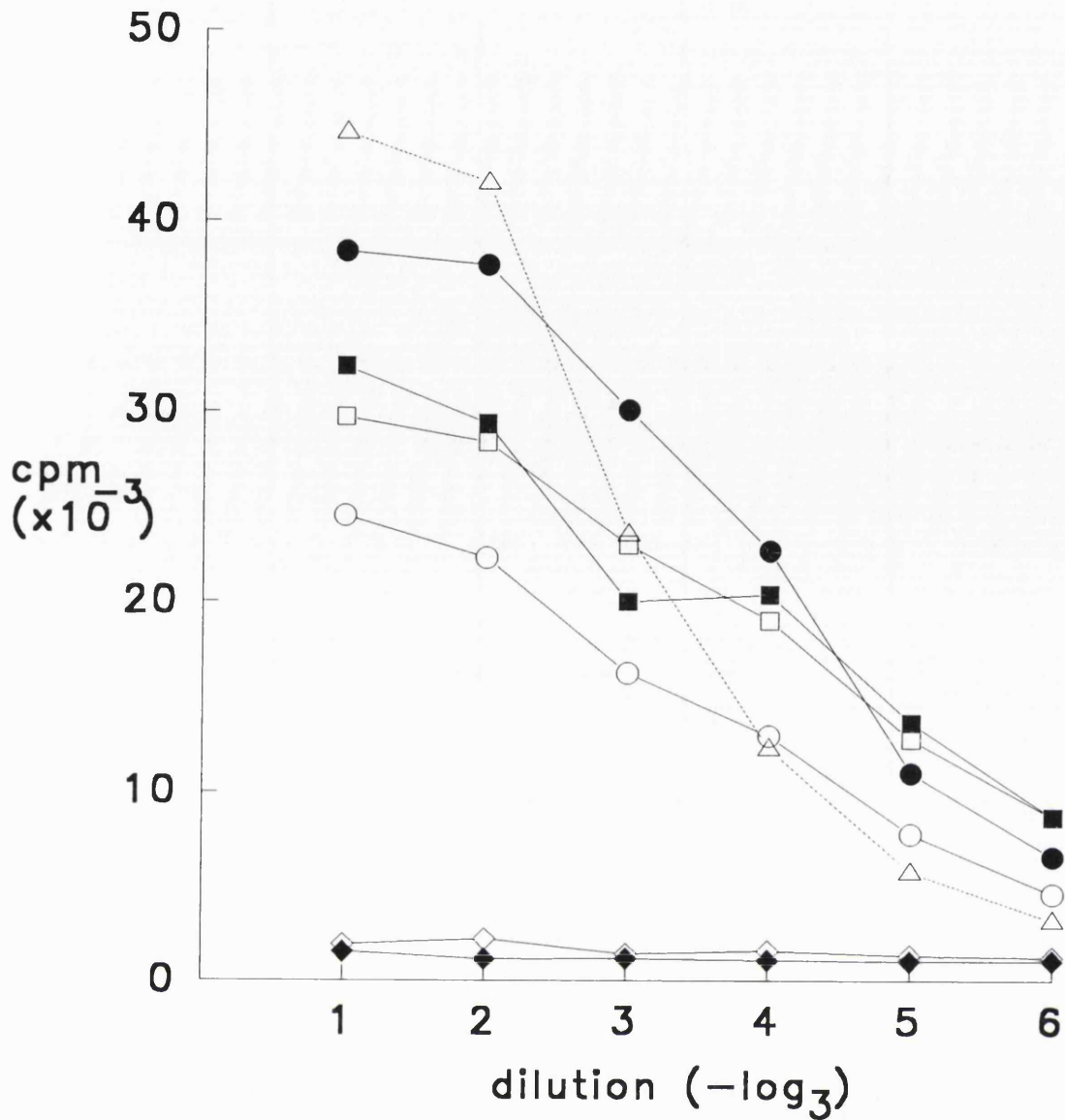
FIGURE 6b:

Antibody Response to DA Class I Molecules in (PVGxLEW)F1 Rats.
Sera were taken from a (PVGxLEW)F1 animal immunised with P1 and P3 in
FIA (filled symbols) or immunised with saline in FIA only (open symbols).
The animals were grafted with r1 kidneys 28 days after immunisation. The
antibody response was measured to intact DA class I molecules on the day
of transplantation (◆), 7 days post-transplantation (■) and 10 days
post-transplantation (●) for both animals. Sera from a DA class I immunised
LEW rat was used as a positive control (▲). cpm refers to counts per minute
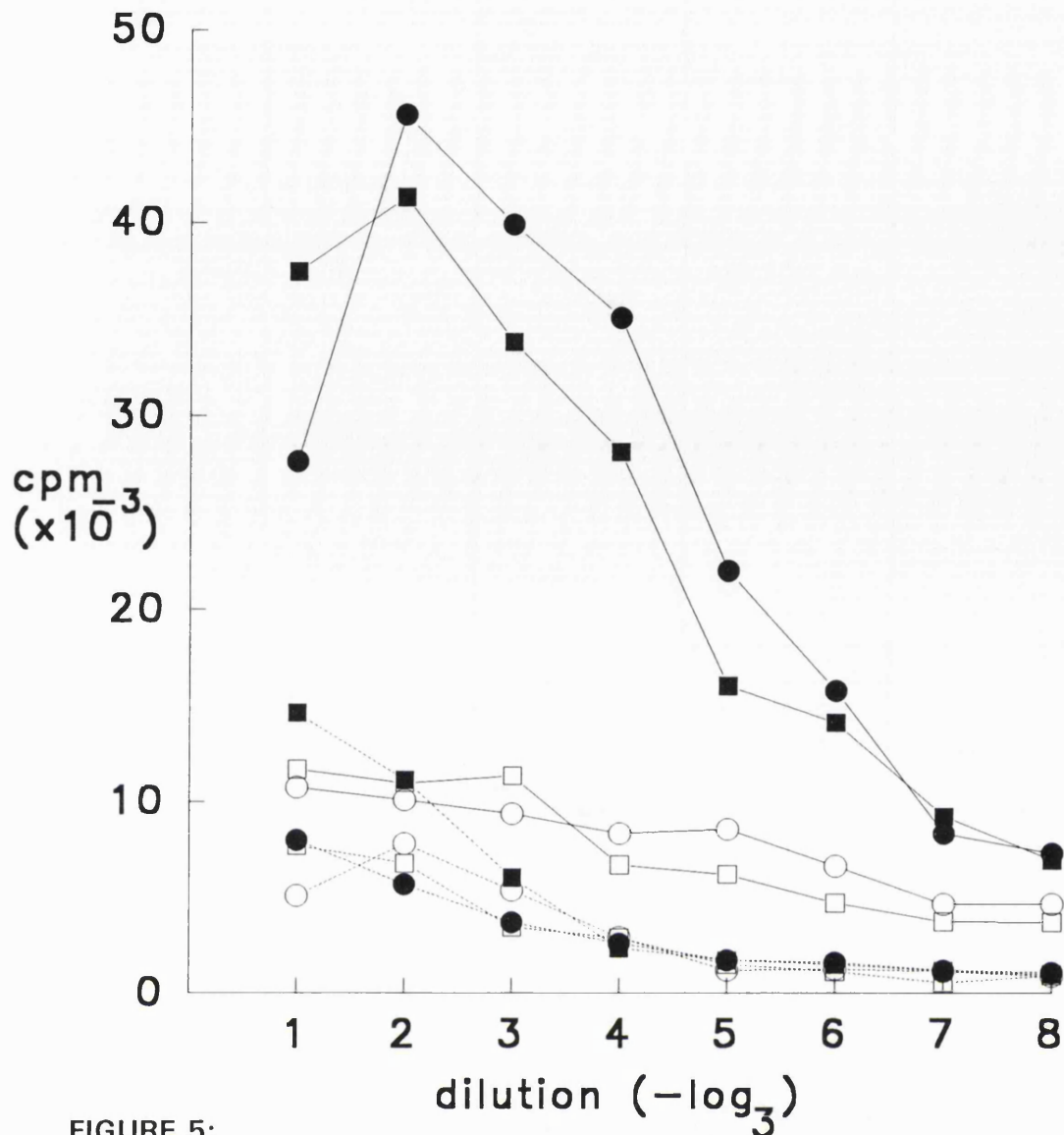of ^{125}I-RAR. Standard errors were between 1.5% and 15% of the mean
values obtained.

164
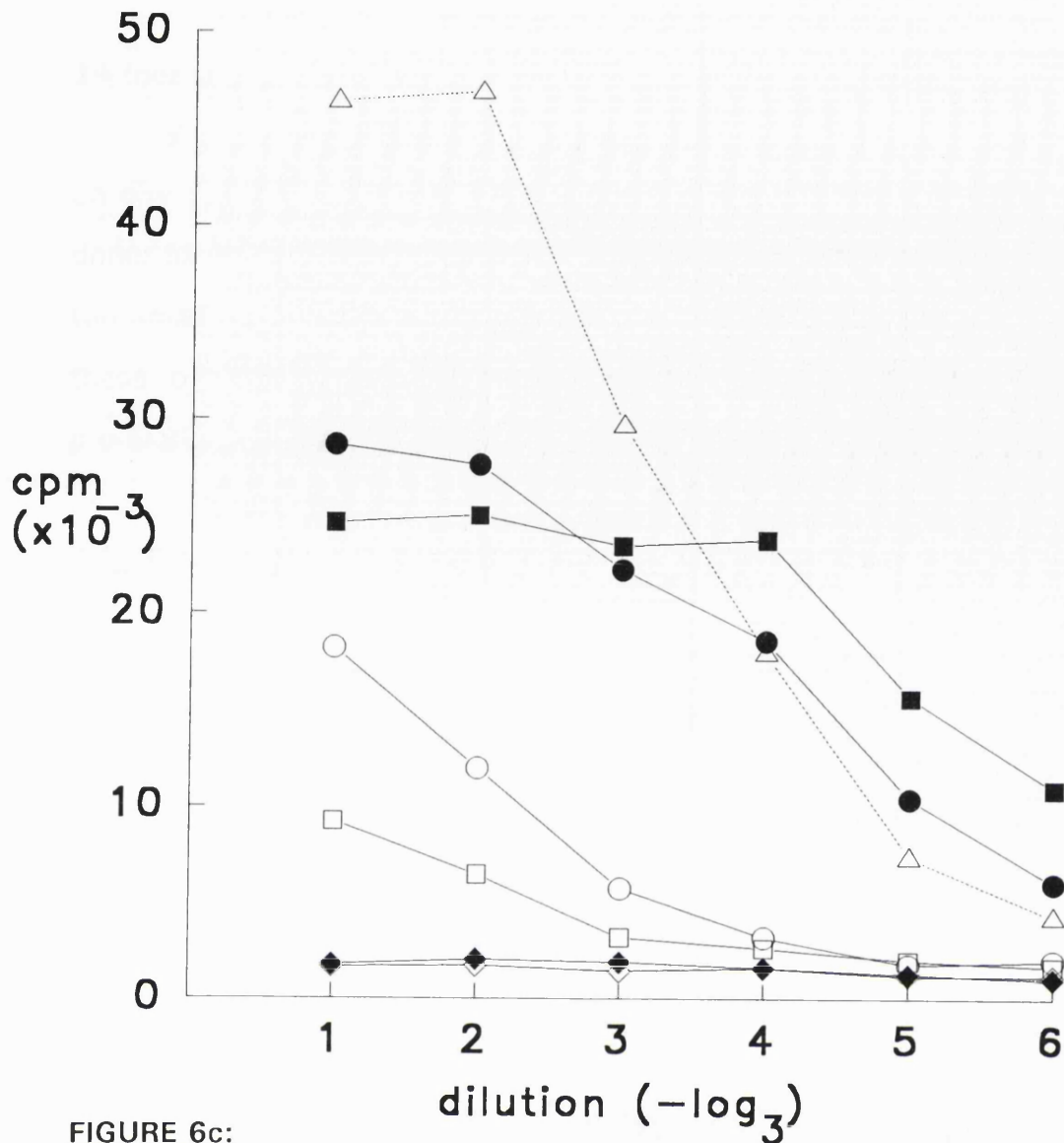
FIGURE 6c:

Antibody Response to DA Class I Molecules in (PVGxLEW)F1 Rats.

Sera were taken from a (PVGxLEW)F1 animal immunised with P1 and P3 in FIA (filled symbols) or immunised with saline in FIA only (open symbols). The animals were grafted with r1 kidneys 28 days after immunisation. The antibody response was measured to intact DA class I molecules on the day of transplantation (◆), 7 days post-transplantation (■) and 10 days post-transplantation (●) for both animals. Sera from a DA class I immunised LEW rat was used as a positive control (△). cpm refers to counts per minute of ^{125}I-RAR. Standard errors were between 0.9% and 11% of the mean values obtained.

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14 (not shown).

Whilst it is impossible to examine the mechanisms involved, there is an obvious correlation between the swiftness of rejection and the rate of donor MHC class I antibody production. However, the numbers involved are too small to establish statistical significance. These findings are similar to those of Chapter 5, though it is impossible to say whether antibody production is the cause of graft destruction or a result of it.

6.2c Further Observations Of The (PVGxLEW)F1 Model:

The results given above made it apparent that the protocol was unsuitable for investigating tolerance. However, it was felt that there was still some potential in the model as a tool for investigating indirect T cell allorecognition. If a reproducible system could be devised in which 100% of peptide treated, allografted animals rejected but 100% of control allografted animals did not, then the mechanisms involved in indirect T cell allorecognition could be established by depleting with anti-CD8 and anti-CD4 monoclonal antibodies or by using immune alloanti-sera from rejecting rats.

6.2c i) The Role Of Adjuvant In RTI^{r1} Kidney Rejection By F1 Rats:

To establish whether immunisation with FIA was instrumental in provoking allograft rejection in this model, three control grafts were performed. Male age-matched RTI^{r1} kidneys were transplanted into F1 recipients without any immunisation. These animals were biopsied and their urea levels monitored exactly as before. All three had acceptable urea levels at d100 post-transplantation and went on to become long survivors (TABLE 2). An example of a biopsy obtained from one of these rats at day 10 is given in FIGURE 7. Thus immunisation with incomplete adjuvant can be enough to elicit rejection.

Sera were also taken from these animals before and after grafting. They all lacked antibodies to P1 (data not shown) but did develop some RT1.A^{av1} class I antibodies which appeared with relatively slow kinetics as the animals aged (FIGURE 8). The same positive control sera is also included to allow comparison with the other assays in this chapter. Each of the three animals had an indistinguishable donor class I antibody profile.

As with the (DAxLEW)F1 kidney into LEW recipient model discussed in the previous chapter, it appears that animals can still develop graft specific antibodies without necessarily suffering from graft loss.

Urea Levels and Survival Times of Unimmunised RTI[r1] Kidney Grafted (PVGxLEW)F1 Male Rats.

The urea levels were monitored fortnightly from day 28. The third animal, with a high urea at day 10, had a value of 83 at day 14, indicating that there was a mild rejection episode.

| Treatment Group | Urea Level (mg/100ml) at | | Survival Time (days) |
	d10	d28	
unimmunised males	55	72	>100
	65	72	>100
	199	73	>100
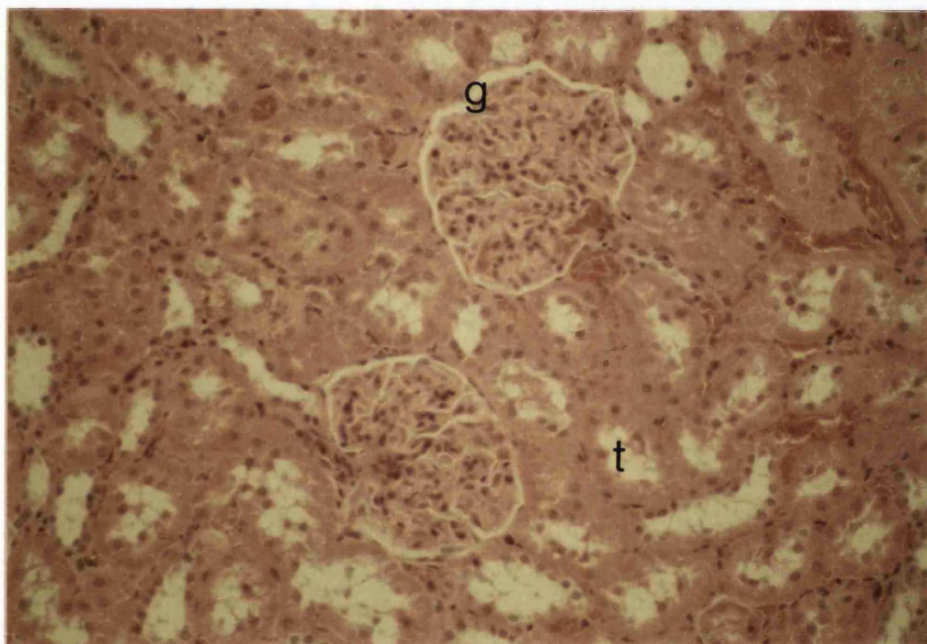
FIGURE 7:

Histology of Unimmunised Male Rat Kidney d100 post transplantation.

There is little glomerular destruction (G) or tubular damage (T) indicating that the kidney is in good condition.
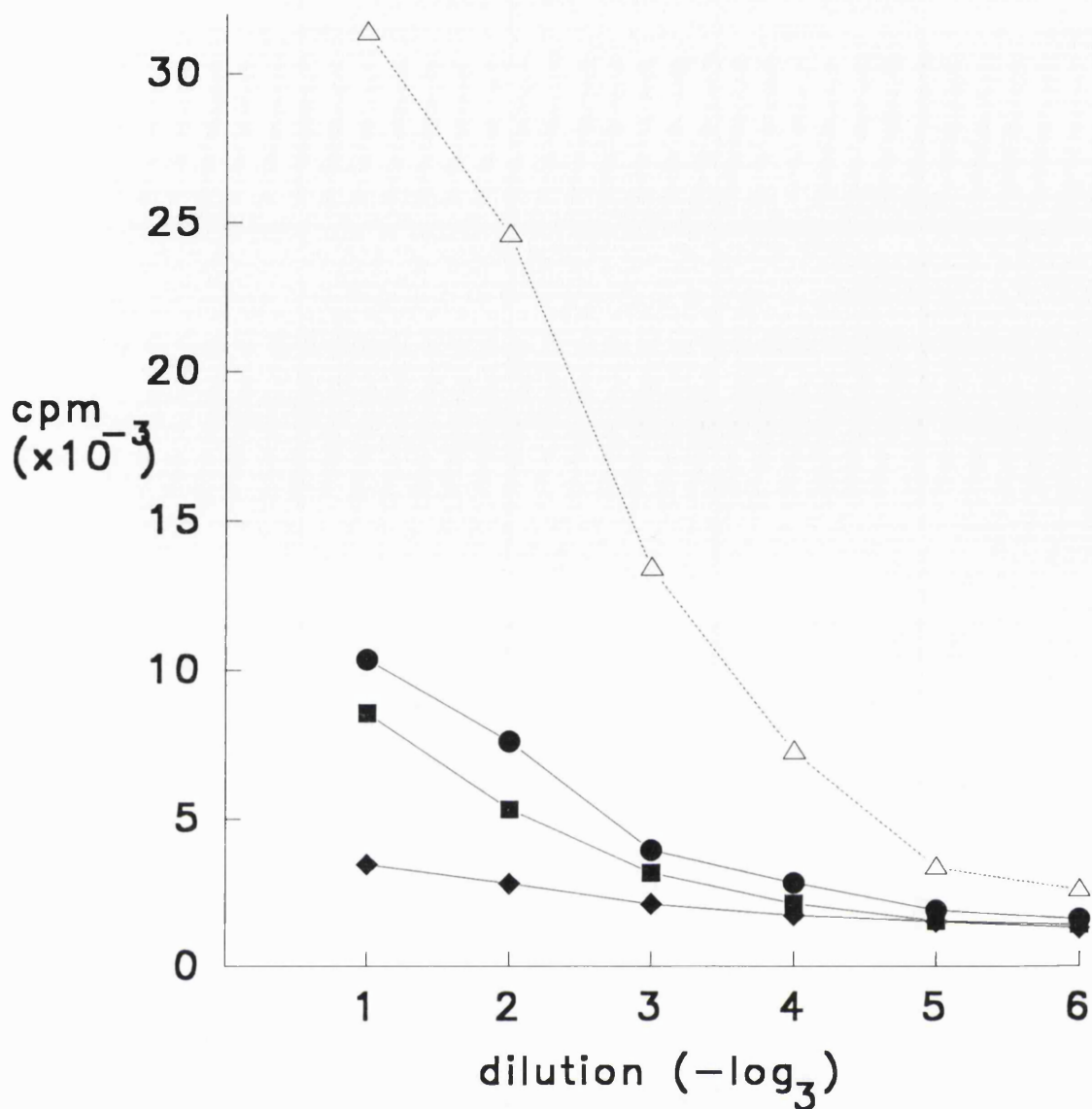
FIGURE 8:

Antibody Response to DA Class I Molecules in Control (PVGxLEW)F1 Rats.

Sera were taken from male (PVGxLEW)F1 rats that received r1 kidneys with no prior immunisation. The antibody response was measured to intact DA class I molecules 7 days (◆), 21 days (■) and 28 days (●) after transplantation. Sera from a DA class I immunised LEW rat was used as a positive control (△). cpm refers to counts per minute of ^{125}I-RAR. Standard errors were between 2% and 9% of the mean values obtained.

<u>6.2d Intradermal Peptide Immunisations:</u>

In the light of these control experiments, an immunisation protocol was required that omitted adjuvant in order to ensure that none of the control grafts underwent rejection. In a small-scale pilot experiment, two groups of two male and female F1 rats were therefore immunised intradermally with P1 at various sites on the back to maximise drainage to the local lymph nodes. These animals were boosted with P1 after two weeks and subsequently given a sex-matched and age-matched RTI^{r1} kidney graft.

<u>6.2d i) Allograft Rejection And Morphology:</u>

As with the other animals, the recipients were biopsied day 7 post-transplantation and on the day of termination. They were regularly bled and their blood urea levels monitored. TABLE 3 shows that both females had blood urea levels >200mg per 100ml blood and were therefore terminated at day 10. The single surviving male, by contrast, had a low urea level at day 10 and on subsequent bleeds and went on to establish a long surviving graft. Autopsy of the male that died of technical failure (a blocked ureter) at day 7 found that the kidney was in good condition.

Histological analysis of a male (FIGURE 9.I) and female (FIGURE 9.II) confirms at a gross level that rejection is occurring in the latter but not the former animal.

<u>6.2d ii) Antibody Responses:</u>

No antibody to P1 could be detected in any of the intradermally immunised male or female rats either before or after grafting (data not shown). However, assays for RTI.A^{av1} class I alloantibodies showed an interesting discrepancy (FIGURE 10). Prior to grafting, neither male nor

TABLE 3:

Urea Levels and Survival Times of Intradermally Immunised RTl[r1]

Kidney Grafted (PVGxLEW)F1 Male and Female Rats.

Both male and female animals were immunised subcutaneously with 20μg

ml[-1] of P1 and boosted 14 days before transplantation.

* This animal died of technical failure and did not appear to be rejecting its

graft.

Treatment Group	Urea Level (mg/100ml) at		Survival Time (days)
	d10	d28	
P1 intradermal females	253	-	10
	214	-	10
P1 intradermal males	36	98	>100
	-	-	7 *
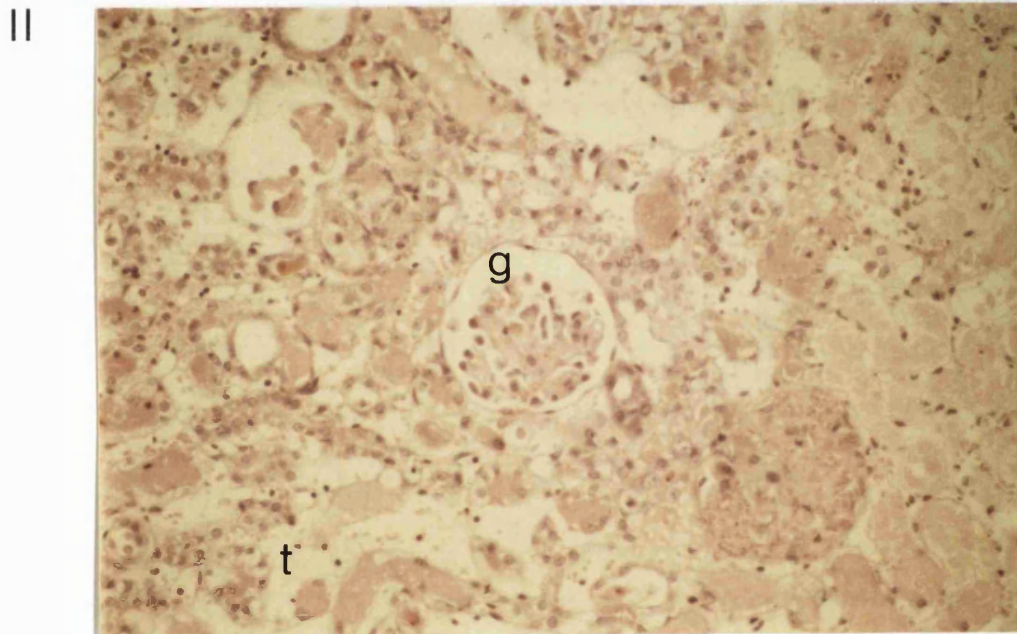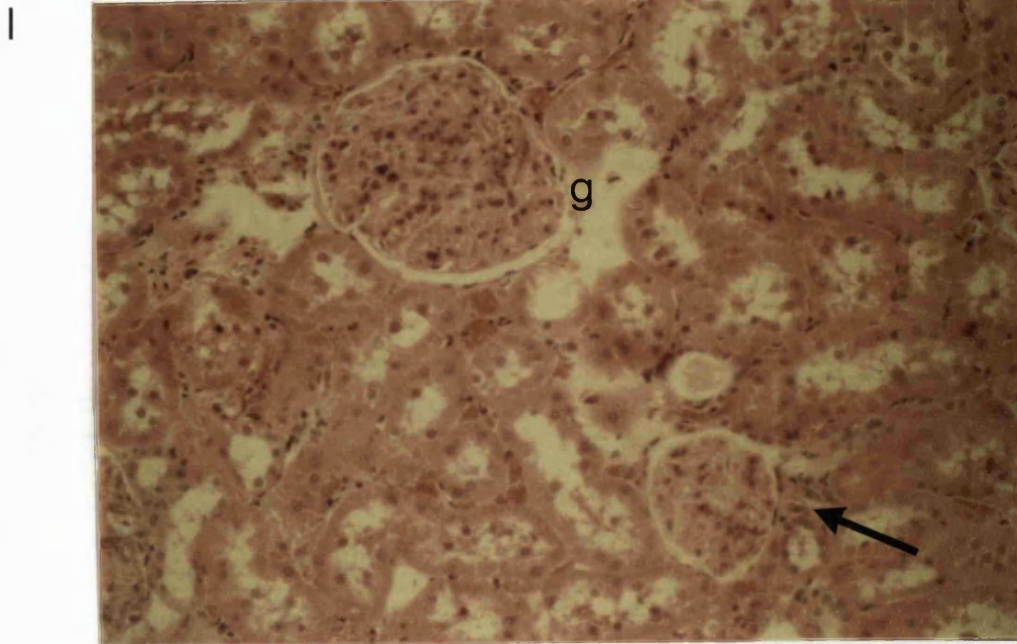
FIGURE 9:

Histology of An Intradermally Immunised Male Rat (I) and Female Rat (II) Kidney d100 and d10 post-transplantation.

In the male, there is little glomerular destruction (G) or infiltration (arrowed) indicating that the kidney is in good condition. In the female rat, increased tubular damage (T) and glomerular destruction (G) are indicative of rejection.

female rats have any antibodies to intact class I, as expected. But just 7 days after transplantation there is a rapid induction of alloantibody in the female rats that go on to reject their grafts. This appears in the absence of an antibody response to peptide. By contrast, the male rat has an extremely weak antibody response.

Again, the same positive control sera has been used in this assay for comparative purposes.

6.2e The Female (PVGxLEW)F1 Model:

From the above observations, it seemed that the female (PVGxLEW)F1 rat might satisfy the criteria for studying the mechanisms of allograft rejection, even though peptide priming did not result in a peptide specific antibody response. Two further groups of female control grafts were therefore performed. In the first group, three female (PVGxLEW)F1 animals received female RTl^{r1} grafts with no immunisation. In the second group, two female (PVGxLEW)F1 rats were intradermally immunised and boosted with saline only prior to transplantation. Three more females were also intradermally immunised with P1 and transplanted with $RT1^{r1}$ kidneys to extend the number in this group to 5.

The urea levels at day 10 post-transplantation and their survival times are given in TABLE 4. The histological analysis of biopsy material taken on the day of termination is given in FIGURE 11 for one unimmunised (non-rejecting) rat (11.I) and one saline intradermal (rejecting) animal (11.II). Though all three unimmunised females went on to become long survivors, one of them did have quite a high urea level at day 10. Of the two saline immunised females, one died of a technical failure and the other showed an elevated urea level at day 14 and was therefore terminated. One of the three peptide treated animals also failed to reject its kidney and maintained a functional graft for 100 days.

<u>TABLE 4:</u>

Urea Levels and Survival Times of Female RTI^{r1} Kidney Grafted Rats.
Urea levels of the long surviving rats were monitored every three to four weeks and remained low.

* Animal died of technical failure unrelated to rejection status.

Treatment Group	Urea Level (mg/100ml) at		Survival Time (days)
	d10	d14	
unimmunised females	70	80	>100
	197	95	>100
	72	71	>100
saline intradermal females	300	-	14
	-	-	7*
peptide intradermal females	388	-	10
	346	-	10
	130	170	>100

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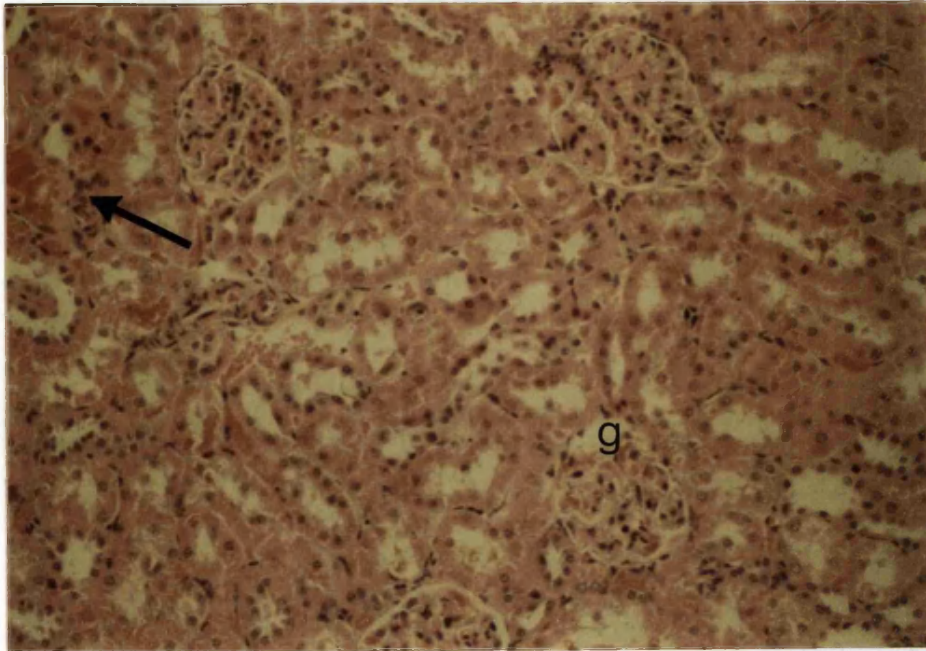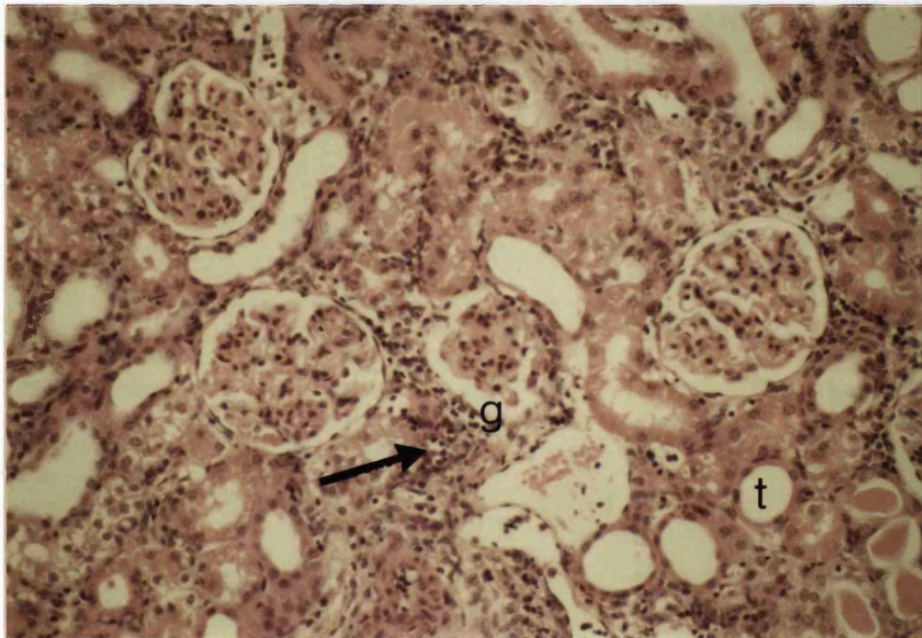
FIGURE 11:

Histology of An Unimmunised Female Rat (I) and a Saline Intradermally Immunised Female Rat (II) Kidney d7 post-transplantation.

In (I), there is little glomerular destruction (G) and some leukocytic infiltration (arrowed) indicating that the kidney is in good condition. In (II), increased leukocytic infiltration (arrowed) and tubular (T) plus glomerular destruction (G) are indicative of a rejection response.

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It was thus decided that the female intradermally immunised (PVGxLEW)F1 model would not be a reliable model in which to study the mechanisms involved in indirect T cell allorecognition and the experiments were therefore discontinued.

6.3 DISCUSSION:

According to these preliminary investigations, the PVG RTI^{r1} kidney into the (PVGxLEW)F1 recipient is, for us, a poor model for studying the mechanisms of indirect T cell allorecognition and for investigating tolerance to peptides involved in this rejection response. TABLE 5 summarises the findings from the small number of rats used in this work. Of particular note was that peptide sensitisation was easy to achieve using FIA only and without boosting. This further demonstrates the high immunogenicity of these MHC class I derived peptides. It was also interesting that, contrary to expectations, rejection did not occur in control male, unimmunised RTI^{r1} recipients. This is despite the presence of DA class I molecules on the allograft that should be able to provide either a 'direct' or 'indirect' stimulus to the LEW MHC class I and class II components of the (PVGxLEW)F1 recipient (FIGURE 1). This fundamental finding underlines how little is known about the mechanisms of allograft rejection.

The results indicate that kidney rejection is easy to initiate, given that stimulation with saline emulsified with incomplete adjuvant can be sufficient to cause graft loss. Perhaps peripheral tolerance is precariously maintained until an immunological insult upregulates nonspecific immune responses that are sufficient enough to tip the balance in favour of rejection. There may be some parallells here with autoimmune responses and with the switches that operate to determine the activation of T_H1 versus T_H2 pathways.

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Summary of Immune Response to RTI[r1] Kidneys in Male and Female (PVGxLEW)F1 Rats.

(yes) indicates a weak antibody response.

* this was true for four of the five animals tested.

abs = antibodies.

	Treatment	rejection by d10	class I abs	peptide abs
male	FIA + Peptides FIA + saline intradermal P1 no treatment	yes yes/no no no	yes yes/no no (yes)	yes no no no
female	intradermal P1 intradermal sal no treatment	yes* yes no	yes - -	no - -

Another interesting feature of this model was the different susceptibilities of males and females to rejection. This has been known for some time in a number of experimental and clinical situations (e.g Sarris *et al* 1994). In this chapter, intradermal immunisation of P1 resulted in allograft rejection in four of the five females tested but not in the males. However, since the single surviving saline intradermal female also suffered early rejection, it is uncertain whether or not priming for rejection is peptide specific and therefore due to indirect recognition. The males and females intradermally immunised with P1 also failed to mount a B cell response to the immunogen, and in the absence of T cell studies, it is uncertain whether peptide specific priming was achieved.

Were the work to be carried further, interesting experiments could be done to investigate the T cell response in these animals. One might ask whether intradermally immunised animals have peptide specific $CD8^+$ or $CD4^+$ T cells in the absence of a B cell response. Furthermore, in the male rats immunised with P1 and P3 in FIA, one could also establish whether direct recognition ($CD8^+$ response to whole donor class I) or indirect recognition ($CD4^+$ response to P1 or P3) is involved in the observed accelerated rejection of kidney grafts.

As in the interstitial dendritic cell-free model of Chapter 5, the donor class I antibody studies in the (PVGxLEW)F1 recipient are also noteworthy. Peptide primed rats invariably reject their RTI^{r1} kidneys and develop a strong donor class I antibody titer. The 'saline-plus-FIA' immunised rats develop antibodies to donor class I molecules at a rate which mirrors that of rejection. The same is true for intradermally immunised females. Thus in the (PVGxLEW)F1 model, the rate of donor class I antibody production is a good indicator of rejection. Whether the mere presence of alloantibody to class I is enough to initiate a rejection response could be addressed by immunising (PVGxLEW)F1 recipients of RTI^{r1} kidneys with allosera from an

F1 rat that had been previously immunised with purified donor MHC class I molecules.

CHAPTER 7: <u>**T AND B CELL RESPONSIVENESS TO**</u>

<u>**DONOR MHC MOLECULES AND PEPTIDES**</u>

<u>**IN LONG SURVIVORS OF RAT KIDNEY**</u>

<u>**ALLOGRAFTS.**</u>

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CHAPTER 7:

T AND B CELL RESPONSIVENESS TO DONOR MHC MOLECULES AND PEPTIDES IN LONG SURVIVORS OF RAT KIDNEY ALLOGRAFTS.

7.1 Introduction:

During the course of my studies on indirect T cell allorecognition, a number of LEW rats with long surviving (DAxLEW)F1 kidney allografts were generated. These animals were used to investigate the nature of tolerance in both the T cell and B cell compartments in the presence of an MHC class I disparate graft. The rat provides a potentially valuable model system in which to study tolerance because rats receiving kidney allografts only require a short (approx. 10 day) course of Cyclosporin A immunotherapy in order to develop permanent tolerance to their grafts (e.g. Homan *et al* 1980), though the reasons for this are unclear. In the clinic, with the possible exception of some liver grafts (Starzl *et al* 1992), continuous immunosuppression is required to prevent a rejection response from occurring. Discovering why a rat but not a person becomes permanently tolerant could shed light on potential therapeutic approaches in the clinic.

Having shown that indirect T cell allorecognition is an important component of vascularised allograft rejection, many interesting questions remain to be addressed in the rat model. In chapter 6, preliminary attempts were made to induce tolerance to 'indirect recognition' using our RTI.A^{av1} derived peptides. In this chapter, another question is asked, namely what is the nature of tolerance in stably accepted (DAxLEW)F1 allografts and can peptides involved in indirect T cell allorecognition be used to break this tolerance. One hypothesis is that the CD4$^+$ T cell is crucial in directing the course of a rejection response, particularly for the indirect pathway. This could have implications for the course of chronic rejection if T helper responses can be switched from one pathway to another. At this stage, all

the migratory donor APCs responsible for direct recognition have probably disappeared from the graft, leaving conventional processing to cause rejection.

7.2 RESULTS:

7.2a Antibodies To Donor MHC Class I Molecules In Long Surviving Rats:

It was decided to examine stably grafted LEW rats for antibodies to donor class I molecules to see whether alloantibody production occurred in the face of long term graft acceptance. Nineteen long surviving LEW RT1^l rats with (DAxLEW)F1 kidney allografts were examined for the presence of alloantibodies to DA RT1.A^{av1} MHC class I molecules after they had become established long survivors (>100 days). They were therefore tolerant of their grafts. Fourteen of these animals had received a short 10 day course of 10mg kg^{-1} CsA therapy to prevent rejection immediately following transplantation. The remaining 5 animals received a 15 day course of 15mg kg^{-1} of CsA. There was no difference in terms of graft survival or antibody production between the two groups of animals. Serum urea levels remained acceptably low throughout the course of the experiments and H and E stained biopsies showed that the kidneys were in reasonable condition (data not shown).

Approximately 10^8 DA RBC were used as targets in indirect radioactive binding assays to check for antibodies to donor MHC class I molecules. Positive control sera obtained from a LEW rat that had been immunised with soluble DA class I molecules was used in each indirect binding assay so that the results from different assays could be compared if necessary. A sample of the binding curves obtained is given in FIGURE 1a. Of the nineteen animals tested, seventeen had a specific antibody response to intact donor class I molecules. Only two animals had no antibody response whatsoever. The seventeen responders fell into three categories
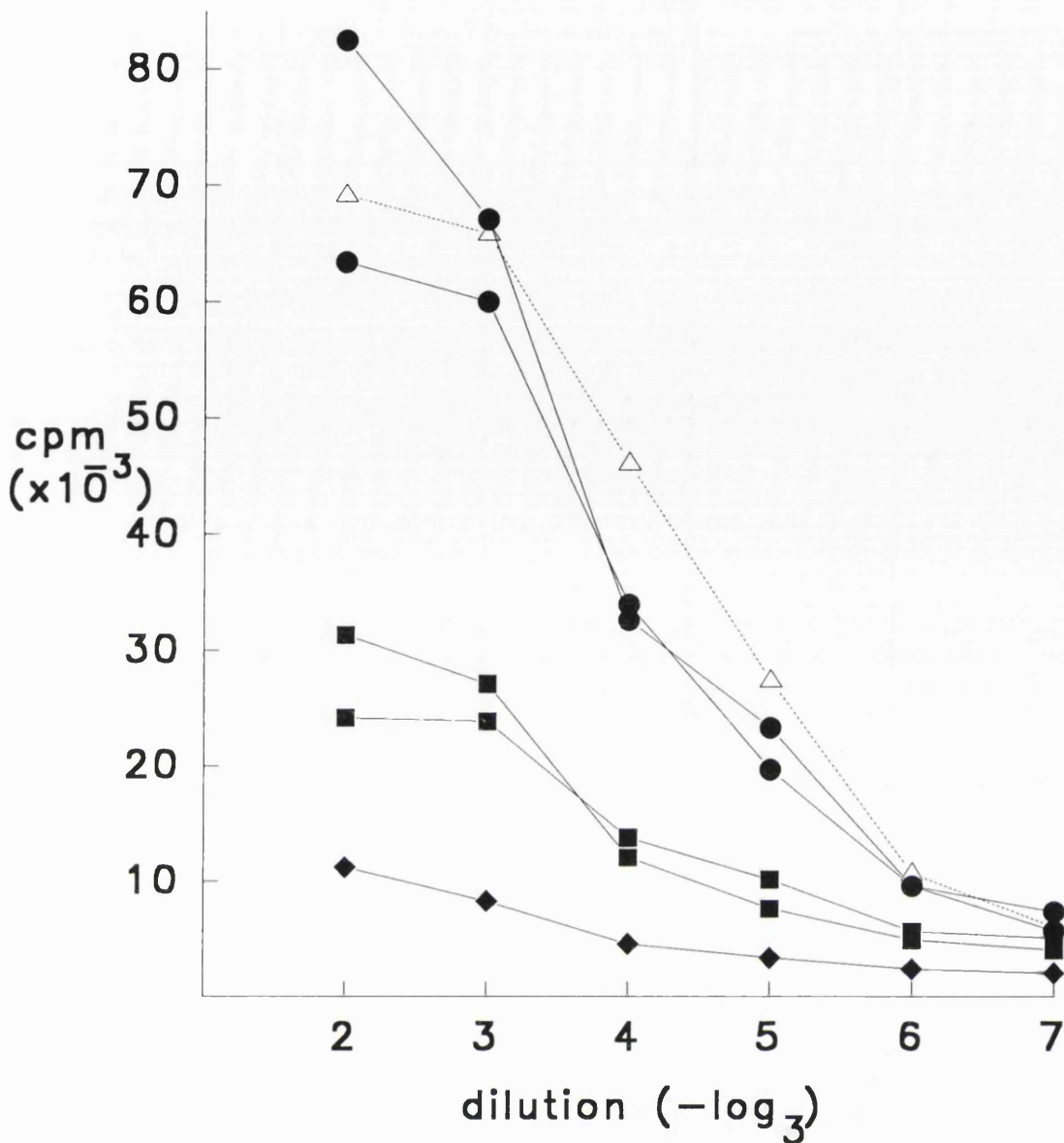
<u>FIGURE 1a:</u>

Titration Curves For DA Class I Antibodies in LEW Rats With (DAxLEW)F1 Kidneys 100 Days After Transplantation.

There are three categories of antibody producer: low (◆), medium (■) and high (●). The positive control sera is from a LEW rat immunised with soluble DA class I molecules (△). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors are between 1% and 13% of the mean values obtained.

when they were assayed in comparison to the positive control: high, medium and low. Examples of each of these are given in FIGURE 1a. It is important to note that the strength of the antibody response was variable between animals and that these three categories serve as a guide rather than a strict subdivision.

Eight of the antibody-secreting animals were then examined in retrospect, using sera from past regular bleeds. This was to determine when the antibody response appeared. The 2 non-antibody producers were also examined to discover whether or not episodes of antibody production had occurred in the past (e.g. FIGURE 1c). FIGURE 1b shows a typical profile from an antibody producing animal. At 24 days post-transplantation, there is no antibody to donor MHC class I but by 6 weeks there is a strong response. All the eight 'antibody producers' developed class I specific antibodies between four and six weeks post grafting. However, no week 5 bleeds were available for examination due to the retrospective nature of the study. So although these animals are tolerant of graft tissue, they secrete specific alloantibodies to a major, polymorphic allograft protein. Antibody production towards a molecule known to be a target of indirect T cell allorecognition in our long survivors is interesting, since it is known that CsA suppresses T dependent antibody responses in animals after grafting (Borel *et al* 1977). One would expect that the 2 week presence of a constant antigenic source (i.e. a vascularised allograft) during CsA therapy would give time for all potentially graft-reactive T helper cells to become tolerant. But in 90% of our rats, B cells can remain functionally active.
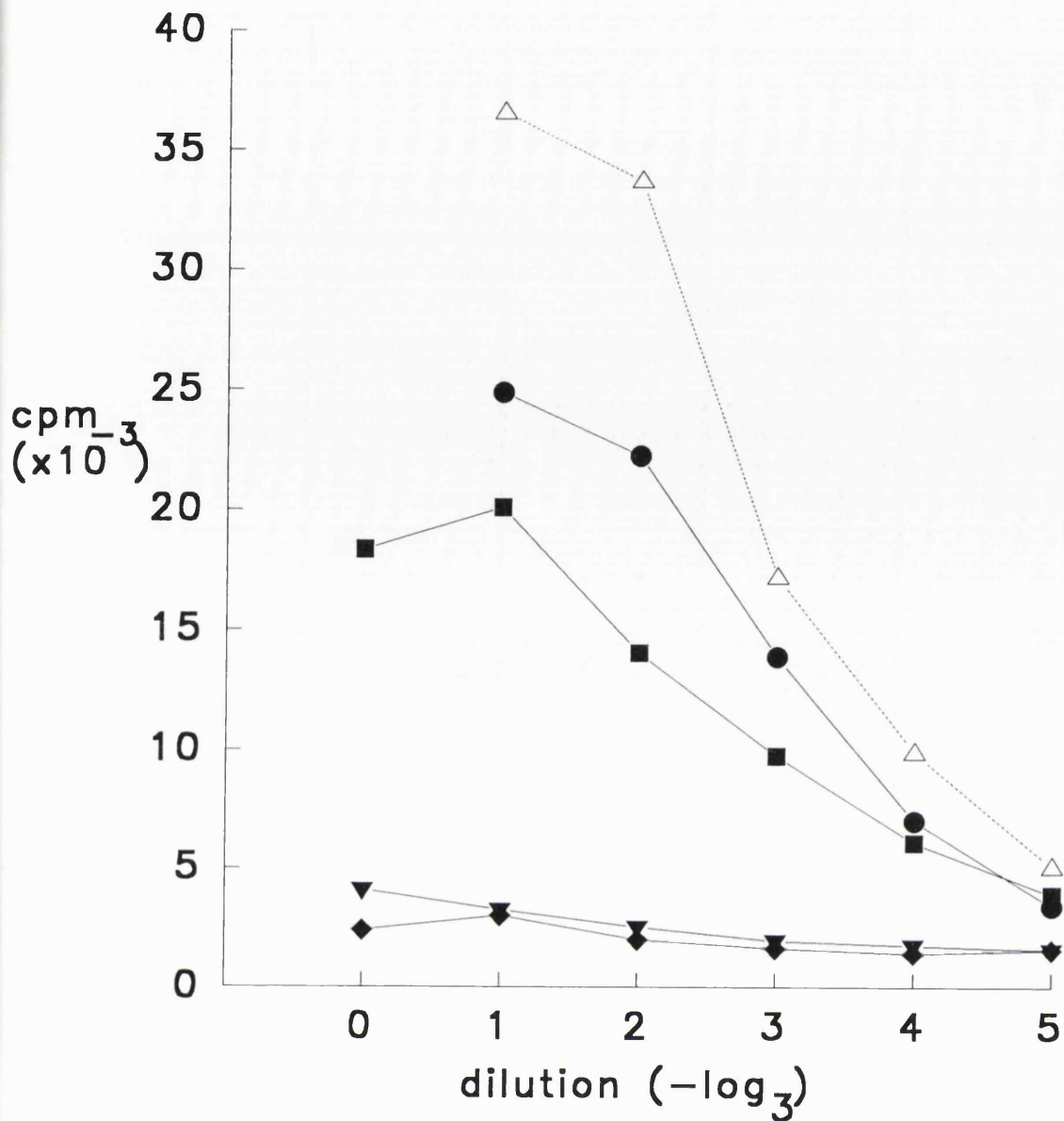
FIGURE 1b:

Development of Donor Class I Antibodies in a LEW Rat With a (DAxLEW)F1 Kidney Allograft.

An animal with a long surviving graft was titered retrospectively for donor class I antibodies using 10^8 DA RBC as targets. The response was measured 14 days post-transplantation (▼), 24 days post-transplantation (♦), 6 weeks post-transplantation (■) and 100 days post-transplantation (●). The positive control sera was used as in 1a (△). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 1% and 11% of the mean values obtained.

7.2b Specific Analysis Of Three Individual Animals

The donor class I antibody profiles of these 19 animals were very interesting in that there was a surprisingly high number of secretors and only two whose B cells were quiescent towards this alloantigen. The two non-responders and a high responder were therefore used for more detailed further analysis. The two non-responders are referred to as rat 1 and rat 2 and the antibody secretor as rat 3 throughout this chapter. Rats 1 and 2 were investigated because although they are rare, they represent an important immunological situation in which B cells are quiescent or tolerant towards a potentially immunogenic donor molecule (i.e. MHC class I).

These three rats all received CsA at 10mg kg^{-1} for 10 days at the time of allografting.

7.2b i) Time course Of Antibody Production In Rats 1, 2 and 3

The history of antibody production was examined in each of these three animals. The antibody profile of Rat 1 is shown in FIGURE 1c, though only four time points are given for clarity. The animal does not develop an anti-MHC class I response, but one might argue that there are two potential 'blips' after 7 days (during the course of immunosuppression) and 9 weeks post-transplantation. Rat 2 had a similar profile (data not shown) but with a potential low-level 'blip' after 10 days. Other than that, the titrations were flat and remained virtually at background until 100 days post-transplantation. Rat 3 developed its antibody response between 4 and 6 weeks post-transplantation (FIGURE 1b), and this remained high up until transplantation.
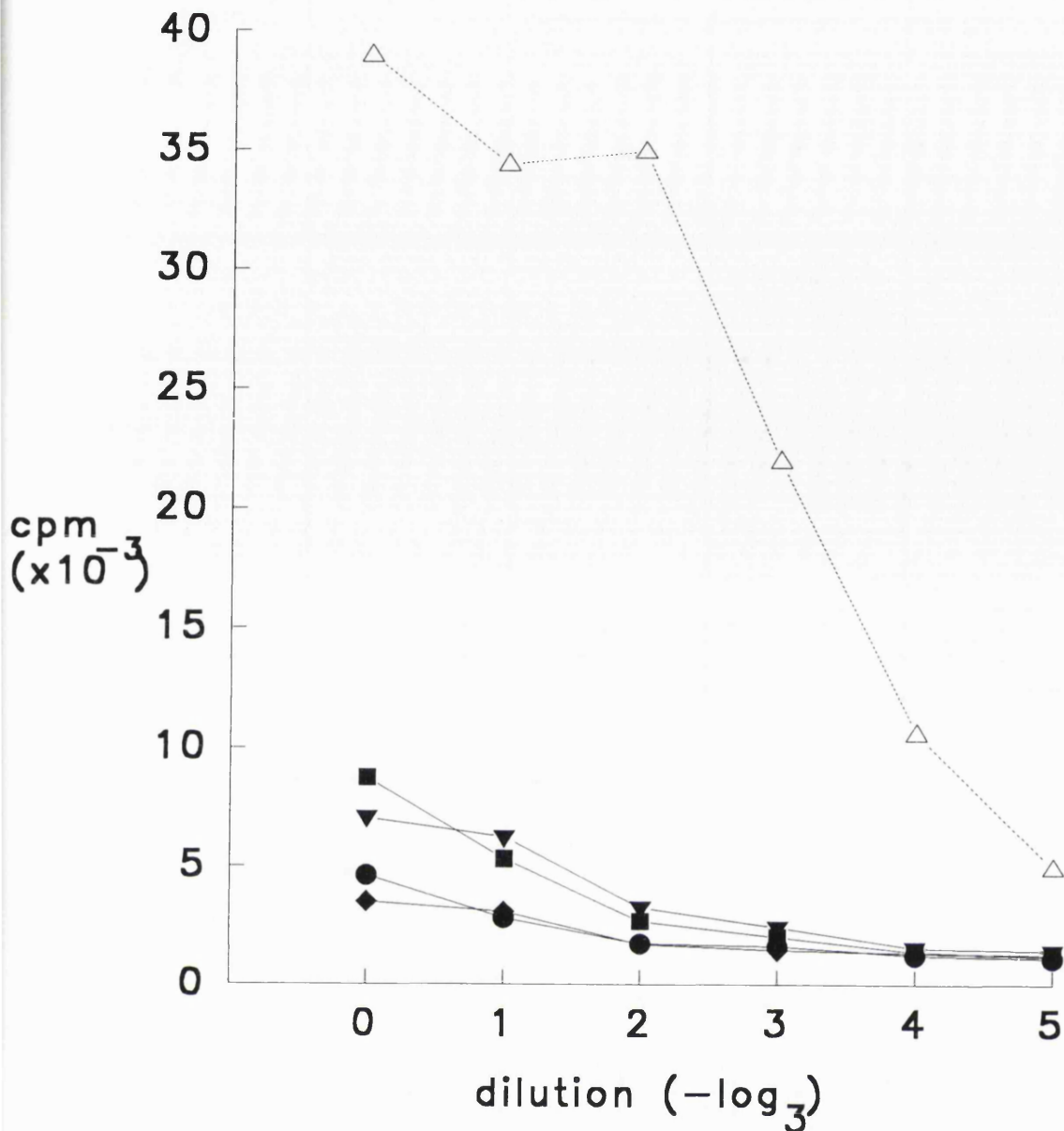
<u>FIGURE 1c:</u>

Development of Donor Class I Antibodies in a LEW Rat 1.

The sera from rat 1 was titered retrospectively for donor class I antibodies using 10^8 DA RBC as targets. The response was measured 7 days post-transplantation (▼), 20 days post-transplantation (◆), 7 weeks post-transplantation (■) and 9 weeks post-transplantation (●). The positive control sera was used as in 1a (△). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 0.5% and 10% of the mean values obtained.

7.2c Immunisation Of Rats 1, 2 and 3:

The three rats were immunised with unconjugated P1 (derived from the DA RT1.A^{av1} MHC class I a helix) in FCA in an attempt to break tolerance to the allografts and to discover if rats 1 and 2 were capable of T cell responses to peptides, despite the long-term acceptance of graft derived class I peptides. This might also help establish whether unresponsiveness lies in the B or T cell compartments. The *in vitro* T cell responses and antibody profiles (to both peptide and donor MHC class I molecules) of these animals were then examined. This was done to see whether B and T cell tolerance could be influenced by administering a peptide specific for indirect T cell allorecognition.

Rat 1:

7.2c i) B Cell Responses

Data obtained from investigations into the immune status of rat 1 are presented in FIGURE 2a-d. After immunisation with P1, FIGURE 2a shows that there is a good primary antibody response to the immunising peptide which is of a comparable magnitude to the naive LEW rats similarly immunised in Chapter 3. FIGURE 2b shows that, in an indirect binding assay to detect donor MHC class I alloantibodies, strong antibody production is induced by primary peptide immunisation. This animal has potentially reactive B cells that are not able to secrete class I alloantibodies until peptide priming has occurred. The lack of provision of T cell help before peptide immunisation could be holding back antibody production in this case.

7.2c ii) T Cell Responses

Ten days after rat 1 was boosted with P1, its lymph node cells were removed and stimulated with P1 *in vitro*, with the cells being harvested

after either 4 or 5 days in culture. A control rat that had been immunised with P1 but that had not received a kidney graft was also analysed. LEW rats receiving primary (DAxLEW)F1 kidneys do not develop P1 specific CD4$^+$ LN cells (Fangmann *et al* 1992b). FIGURE 2c shows that both rat 1 and the control had P1 specific T cells that in the case of rat 1 responded most strongly on day 4, down to a concentration of 5μg ml^{-1} P1. The peptide-specific T cell response of rat 1 is almost half that of the control in this experiment. It is tempting to speculate that some peptide-specific T cell clones have been switched off by the tolerising presence of the allograft and therefore result in reduced peptide specific proliferation. However, further repeat experiments are necessary before any such conclusions can be made.

T cell subset studies were also performed on 4 day cultures that had been depleted of either CD4$^+$ or CD8$^+$ T cells and stimulated with P1. FIGURE 2d shows that the proliferative response is both peptide specific, since there is no cell division when a control mouse MHC class II-derived peptide is added, and CD4$^+$ T cell restricted, since the CD8$^+$ T cell response is almost at background levels. Proliferation is also dependent on antigen presentation, since the removal of syngeneic APCs from cultures obliterates T cell activation, either by P1 or by concanavalin A mitogen.

Thus in rat 1, peptide priming results in the activation of peptide specific CD4$^+$ T cells and in the activation of B cells secreting alloantibodies to MHC class I. This suggests that priming helper T cells for indirect T cell allorecognition can result in help being provided to donor class I antibody producing B cells in this animal. However, this animal still maintained a functional graft with steady blood urea levels, despite the activation of donor MHC class I-specific B cells and peptide specific CD4$^+$ T cells.
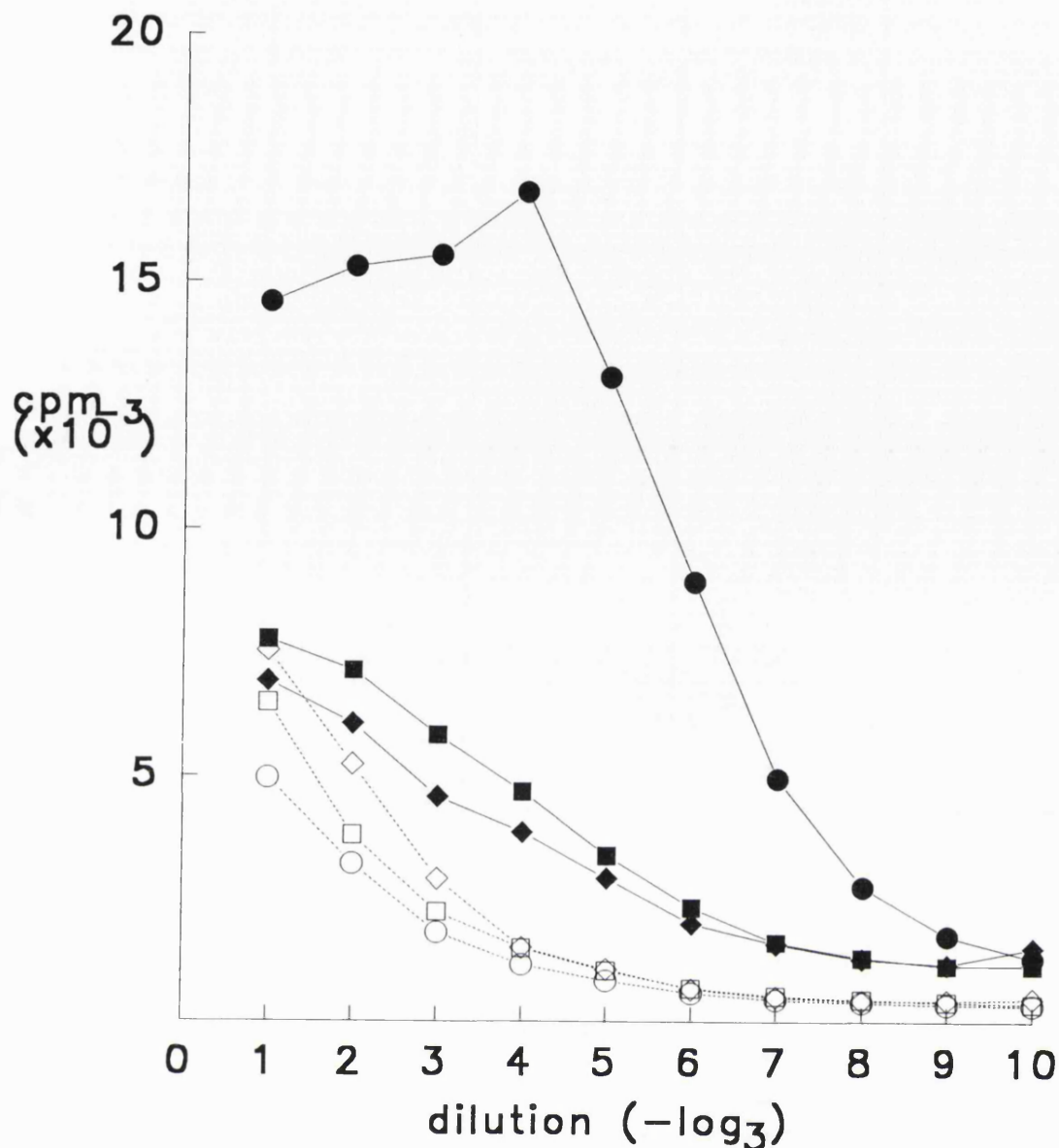
FIGURE 2a:

Primary Antibody Response to P1 in LEW Rat 1.

Rat 1 was immunised and boosted with 20μg P1 in the hind footpad after accepting a (DAxLEW)F1 kidney for 100 days. Titrations were performed on P1 coated PVC plates (filled symbols, solid lines) or on saline control plates (open symbols, dashed lines). The antibody response is shown on the day of immunisation (◆), 14 days after immunisation (■) and 28 days after immunisation (●). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 1% and 9% of the mean values obtained.
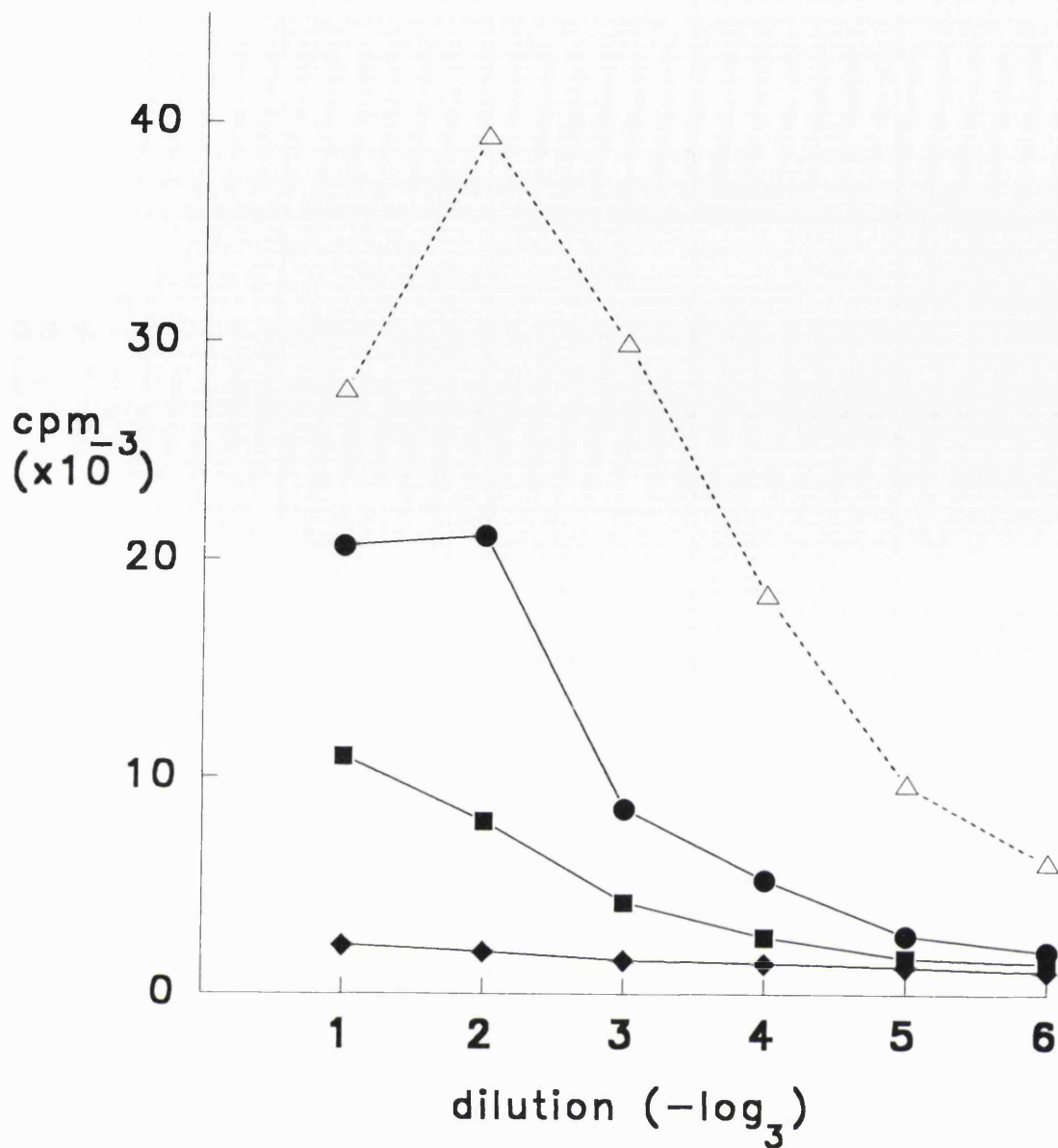
FIGURE 2b:

Antibody Response to DA MHC Class I in LEW Rat 1.

The response to intact donor MHC class I molecules was measured after 100 day acceptance of a (DAxLEW)F1 kidney allograft (◆), 14 days after immunisation with P1 (■) and 28 days after immunisation with P1 (●). The positive sera is the same as that for 1a (△). Approx. 10^8 RBC were used as targets. cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 0.7% and 11% of the mean values obtained.

60

50

40

cpm
$(\times 10^{-3})$

30

20

10

0

4   5   4   5   day

control   rat 1
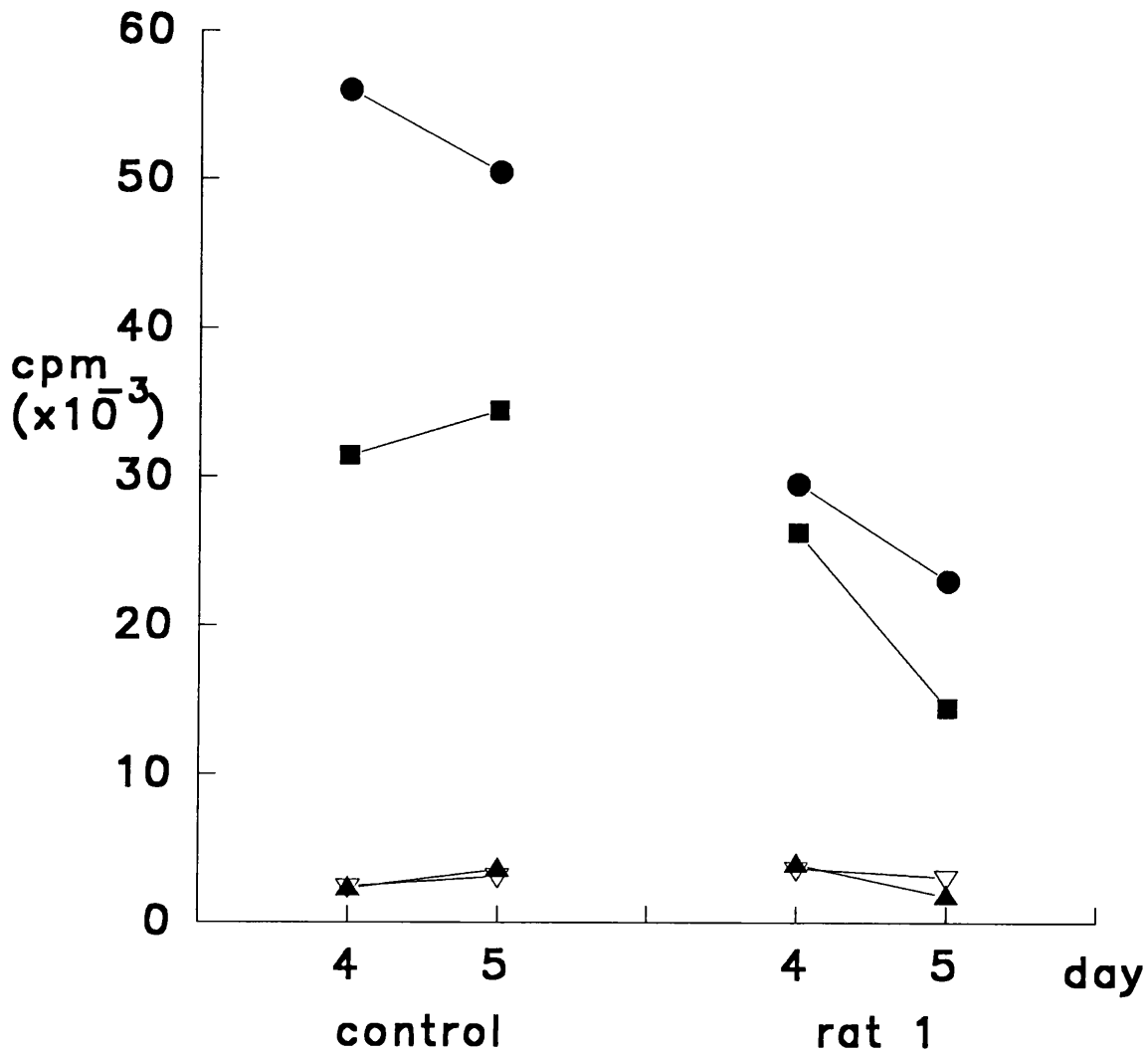
FIGURE 2c:

Proliferative Response of Rat 1 LN Cells to P1.

Whole LN cell preparations were dispersed at 10^5 ml^{-1} 10 days after the animal was boosted with 20μg P1. Single cell suspensions were stimulated with either 15μg ml^{-1} P1 (●), 5μg ml^{-1} P1 (■), 15μg ml^{-1} Px as a control peptide (▲) and medium only (▽) for four or five days. The cells were pulsed with 20μCi ^{3}H-thymidine and harvested 24hr later. cpm refers to counts per minute of incorporated tritium. The control rat was immunised but had not received an allograft. Standard errors were between 1% and 14% of the mean values obtained.
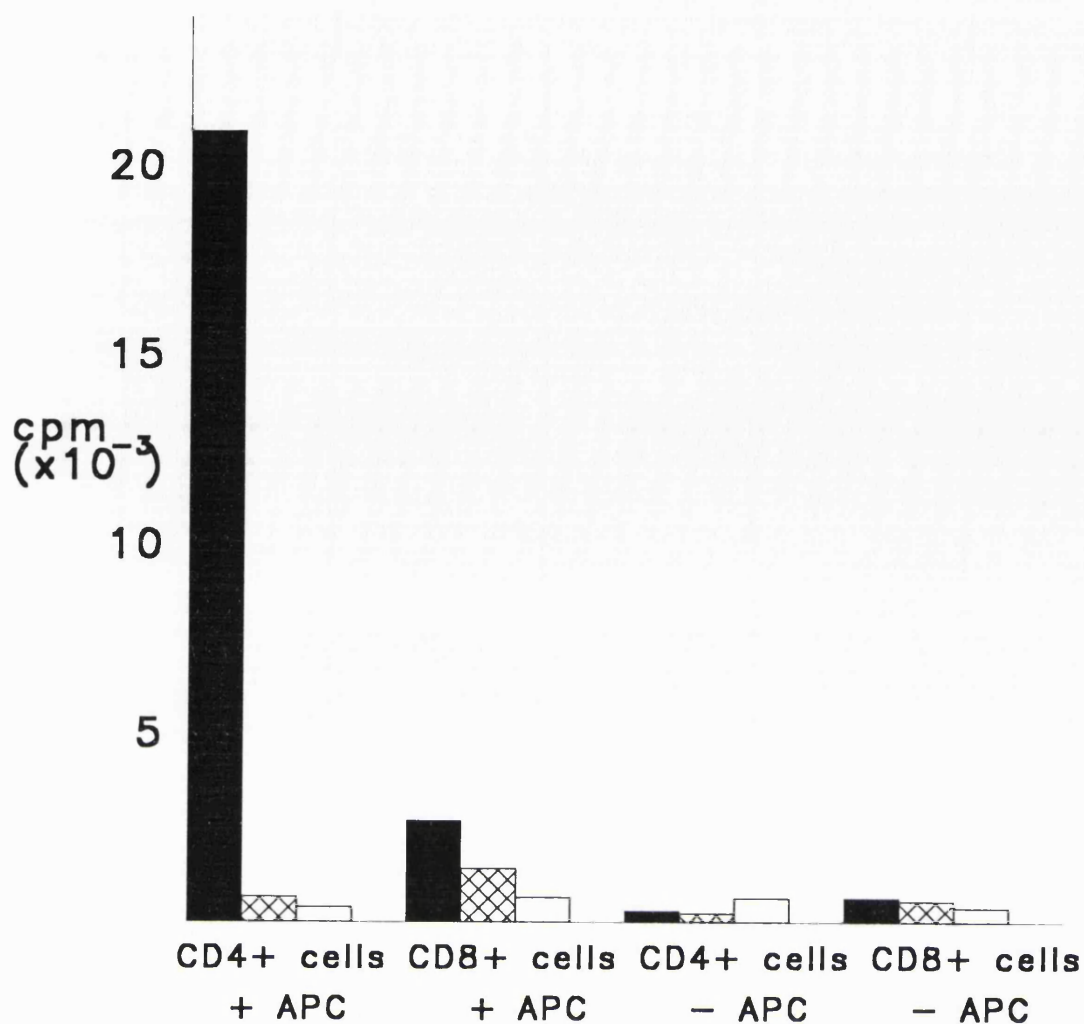
FIGURE 2d:

T Cell Subset Studies on Rat 1 LN Cells.

Rat 1 was boosted with P1 and its LN cells removed. Single cell suspensions were made and cultures were depleted of either CD8$^+$ or CD4$^+$ T cell subsets. APCs were also removed from half of the CD4$^+$ and CD8$^+$ T cell depleted cultures. Cells at 10^5 ml^{-1} were stimulated for 3 days in culture with either 25μg ml^{-1} P1 (■), 25 μg ml^{-1} irrelevant peptide Px (▨) or medium only (□). The cultures were pulsed with 20μCi ^{3}H-thymidine and harvested 24hr later. cpm refers to counts per minute of incorporated radioactivity. Standard errors were between 0.5% and 8% of the mean values obtained.

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<u>Rat 2:</u>

<u>7.2d i) B Cell Responses</u>

FIGURE 3a-d shows data obtained on the immune status of rat 2 after immunisation with unconjugated P1. The responses of this rat are different from rat 1, even though it is also a non donor class I allo-antibody secretor prior to peptide immunisation. Like its counterpart, rat 2 also develops a primary and secondary peptide specific antibody response that is comparable to that of normal LEW rats immunised with P1 (FIGURE 3a). FIGURE 3b shows that, unlike rat 1, priming this animal with P1 does not result in subsequent donor MHC class I alloantibody production. Rat 2 demonstrates that the nature of the tolerant state may differ, even under the same treatment regime. This is not entirely surprising, as different animals will have different specificities in their TCR and Ig receptor repertoires. Peptide immunisation alone is not sufficient to provide help for class I antibody production in this animal. Perhaps exposure to graft antigens at the time of immunosuppression has resulted in B cell anergy towards intact donor class I molecules.

<u>7.2d ii) T Cell Responses</u>

FIGURE 3c shows that LN cells taken from rat 2 ten days after boosting with P1 exhibited peptide specific activity after 3 and 4 days of stimulation in culture. The conditions of this assay are different to those used in the analogous experiment with rat 1. P2 rather than Px was used as a control peptide and the concentrations of peptide were changed to 25 and 2.5μg ml^{-1} rather than 15 and 5μg ml^{-1}. Cultures were also stimulated for 3 and 4 days rather than 4 and 5 days.

As for rat 1, rat 2 also has antigen specific T cell activity. The proliferative response rises between days 3 and 4 and is detectable down to a peptide concentration of 2.5μg ml^{-1}. The amount of tritium

incorporated is comparable with a control LEW animal that had been boosted with P1 but that had not received an allograft. FIGURE 3d offers a more detailed examination of the T cell response to P1. In this depletion assay, analogous to that shown in 2d, the response is again peptide specific and confined exclusively to the CD4$^+$ T cell compartment. It is also dependent on allogeneic APCs. This data strongly suggests that P1 immunisation activates peptide specific helper T cells as for rat 1. However, in this case, activation of a B cell specific response to intact donor MHC molecules does not occur. The generation of peptide specific CD4$^+$ T cells is insufficient to break tolerance towards the intact allograft, as this rat also showed no signs of rejection after peptide priming.
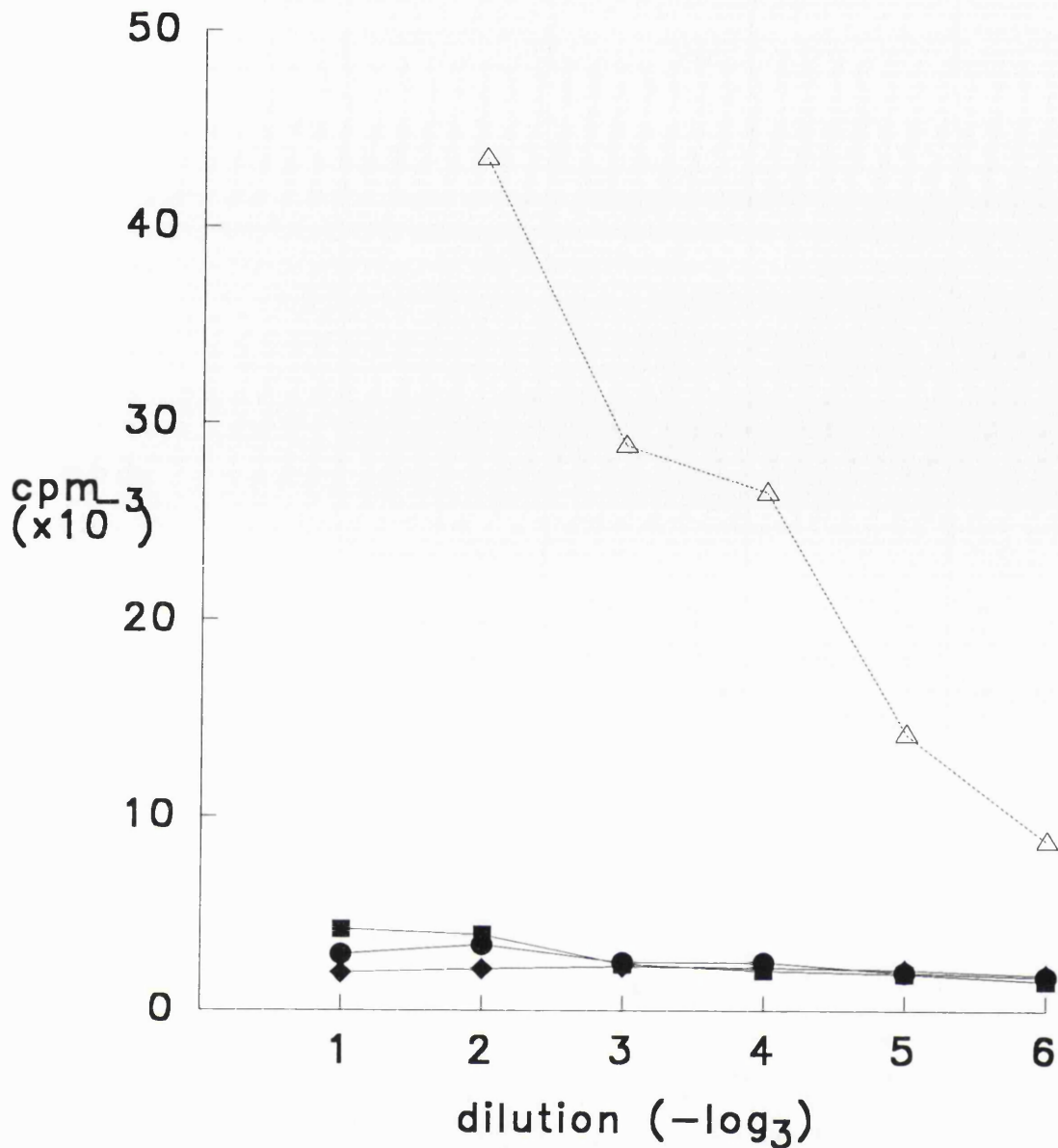
FIGURE 3b:

Antibody Response to DA MHC Class I in LEW Rat 2.

The response to intact donor MHC class I molecules was measured after 100 day acceptance of a (DAxLEW)F1 kidney allograft (♦), 14 days after immunisation with P1 (■) and 2 weeks after boosting with P1 (●). The positive sera is the same as that for 1a (△). Approx. 10^8 DA RBC were used as targets. cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 0.5% and 15% of the mean values obtained.
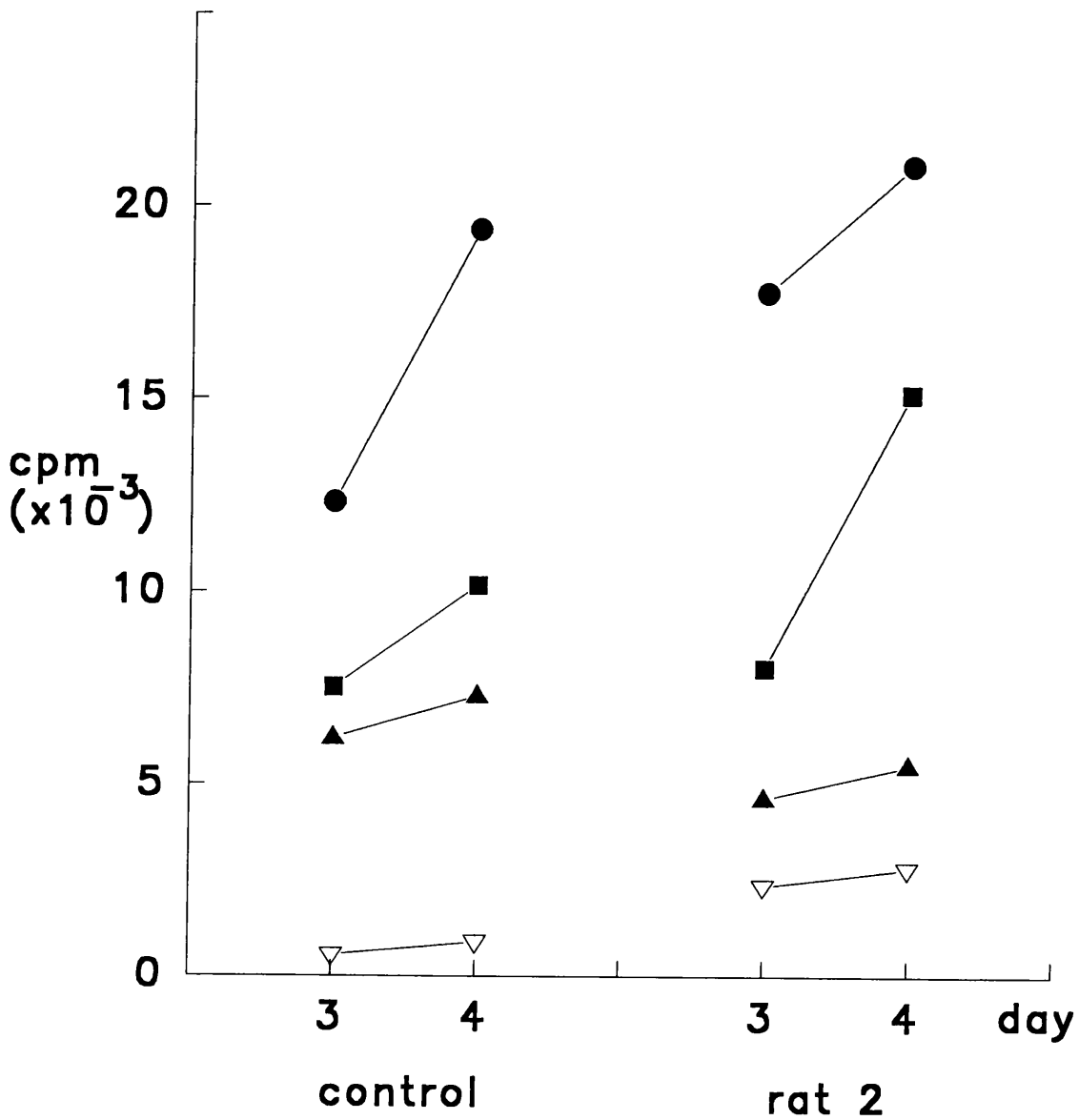
FIGURE 3c:

Proliferative Response of Rat 2 LN Cells to P1.

Whole LN cell preparations were dispersed at 10^5 ml^{-1} 10 days after the animal was boosted with 20μg P1. Single cell suspensions were stimulated with either 25μg ml^{-1} P1 (●), 2.5μg ml^{-1} P1 (■), 25μg ml^{-1} P2 as a control peptide (▲) and medium only (▽) for three or four days. The cells were pulsed with 20μCi ^{3}H-thymidine and harvested 24hr later. cpm refers to counts per minute of incorporated tritium. The control rat was immunised with P1 but had not received an allograft. Standard errors were between 1% and 7% of the mean values obtained.

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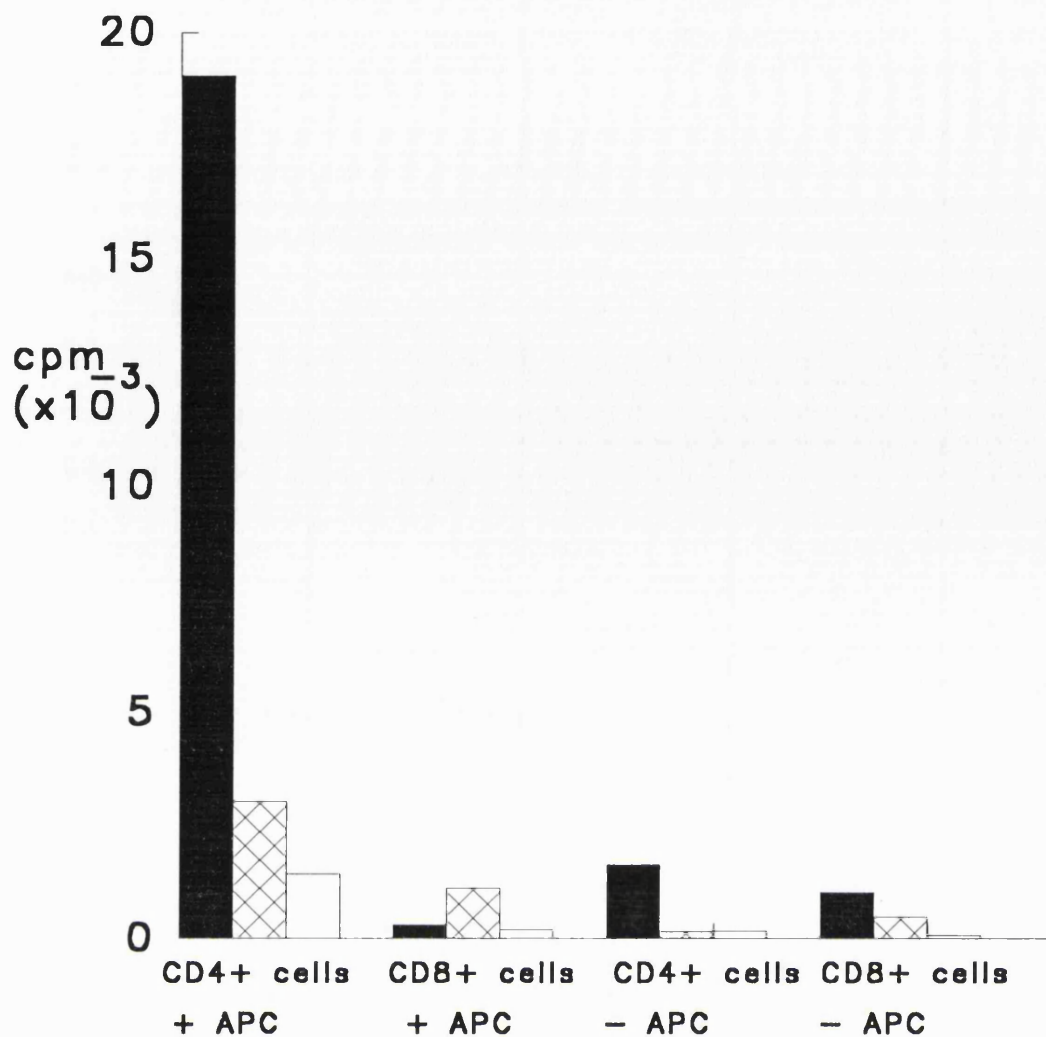
FIGURE 3d:

T Cell Subset Studies on Rat 2 LN Cells.

Rat 2 was boosted with P1 and its LN cells removed. Single cell suspensions were made and cultures were depleted of either CD8^{+} or CD4^{+} T cell subsets. APCs were also removed from half of the CD4^{+} and CD8^{+} T cell depleted cultures. Cells at 10^5 ml^{-1} were stimulated for 3 days in culture with either 25μg ml^{-1} P1 (■), 25 μg ml^{-1} irrelevant peptide Px (▨) or medium only (□). The cultures were pulsed with 20μCi ^{3}H-thymidine and harvested 24hr later. cpm refers to counts per minute of incorporated radioactivity. Standard errors were between 0.5% and 9% of the mean values obtained.

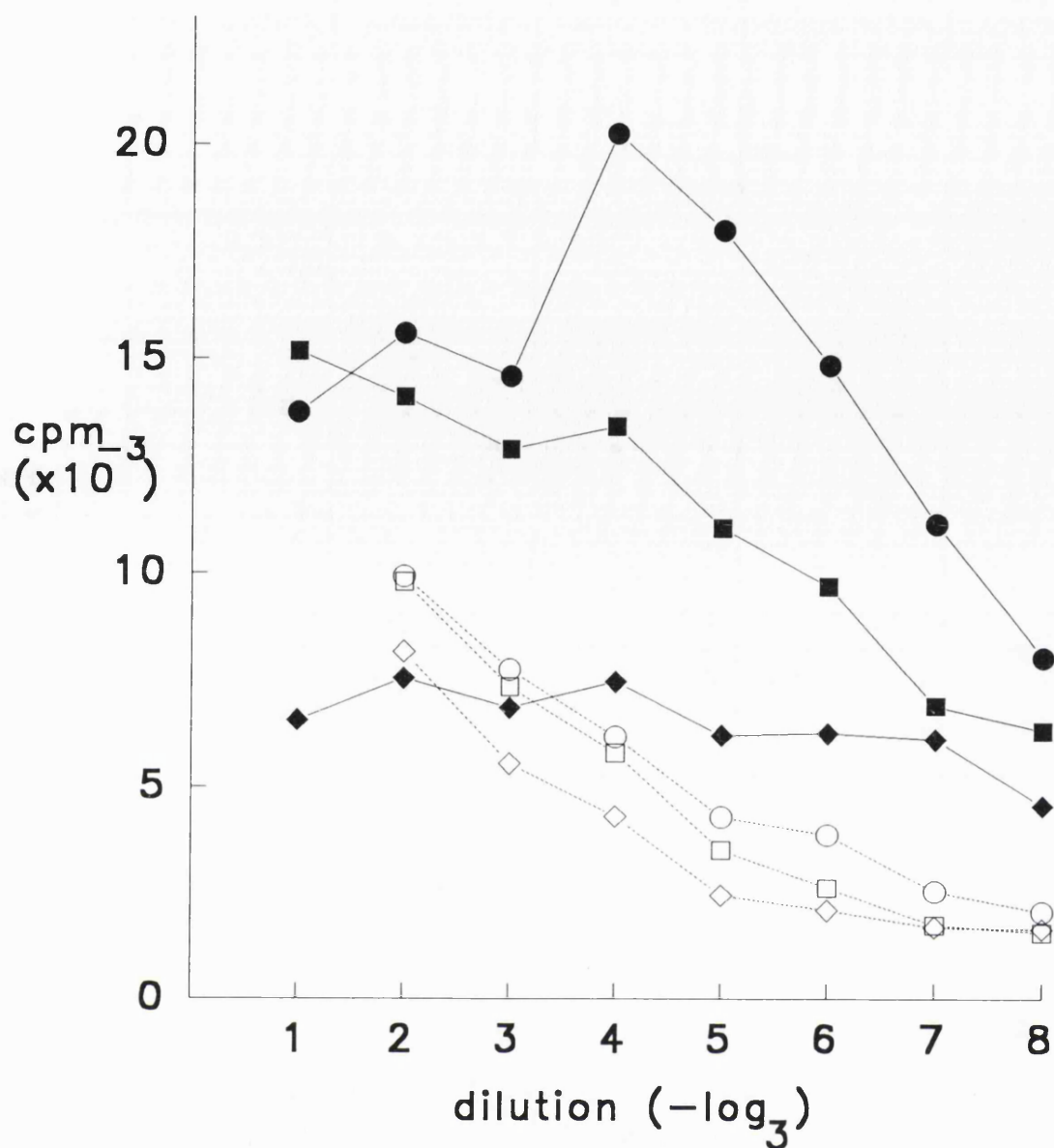<u>Rat 3:</u>

<u>7.2e i) B Cell Responses</u>

Unlike rats 1 and 2, rat 3 was already secreting high titers of donor MHC class I alloantibodies prior to P1 immunisation. FIGURE 4a shows that as for rats 1 and 2, rat 3 generated a good primary and secondary (not shown) antibody response to the unconjugated peptide. The saline background is high because the sera were not spun to remove debris beforehand. FIGURE 4b is an analysis of the response to intact donor MHC alloantigens after peptide priming. The strong response that is seen prior to immunisation remains unaffected by P1 administration.

<u>7.2 e ii) T Cell Responses</u>

FIGURE 4c shows data from a proliferation assay using LN cells from rat 3 taken ten days after boosting with P1. The conditions used were the same as those for rat 2. A similar P1 immunised control rat that had not received an allograft was also assayed. Rat 3 responds strongly and specifically to P1 at a concentration of $25 \mu g$ ml^{-1}. At a concentration of $2.5 \mu g$ ml^{-1}, proliferation is at the level of the control peptide on day 3, although there is a difference at day 4. The control rat is slightly less responsive to P1 and also shows little difference in activity between P1 at $2.5 \mu g$ ml^{-1} and the control peptide P2. Thus the T cell response seems to require higher peptide concentrations than the non-antibody secreting animals. FIGURE 4d represents the subset analysis of the T cell response and is a little harder to interpret than those in FIGURE 2d and 3d. Positive control counts using concanavalin A as a non-specific mitogen gave values of around 30,000-35,000 for the $CD8^+$ and $CD4^+$ subsets with APCs, which is on the low side of what is normally obtained. The cells used in this assay were split from those used for FIGURE 4c, showing that they are

capable of peptide specific proliferation. However, the CD4$^+$ subset incorporates only a low number of counts. In the context of the experiment at least, the response is restricted to APC dependent CD4$^+$ T cells. Peptide priming and peptide specific T cell activation have no effect on donor MHC alloantigen production or on allograft survival in this animal.
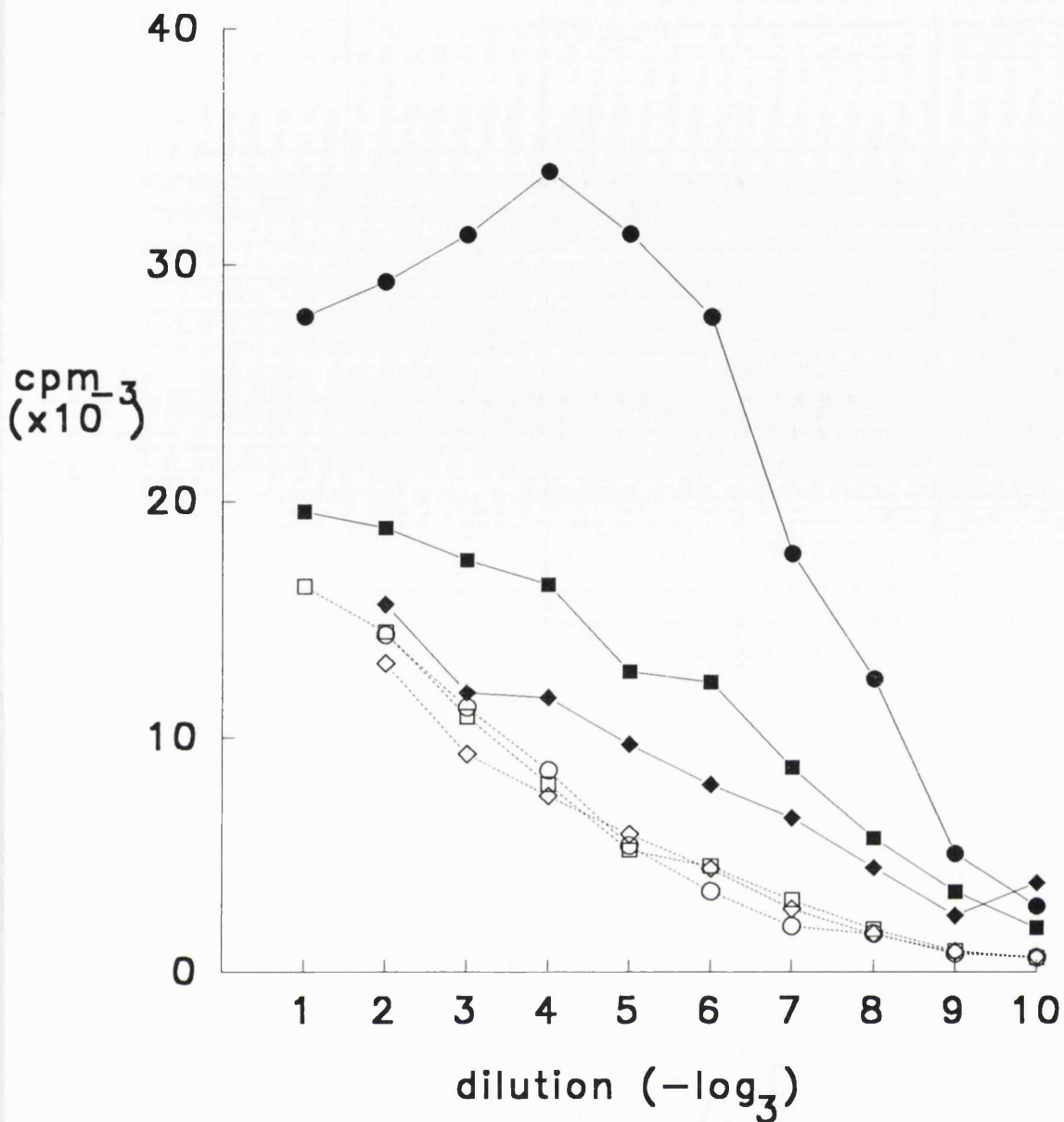
Primary Antibody Response to P1 in LEW Rat 3.

Rat 3 was immunised and boosted with 20µg P1 in the hind footpad after accepting a (DAxLEW)F1 kidney for 100 days. Titrations were performed on P1 coated PVC plates (filled symbols) or on saline control plates (open symbols). The antibody response is shown on the day of immunisation (♦), 14 days after immunisation (■) and 28 days after immunisation (●). cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 2% and 11% of the mean values obtained.
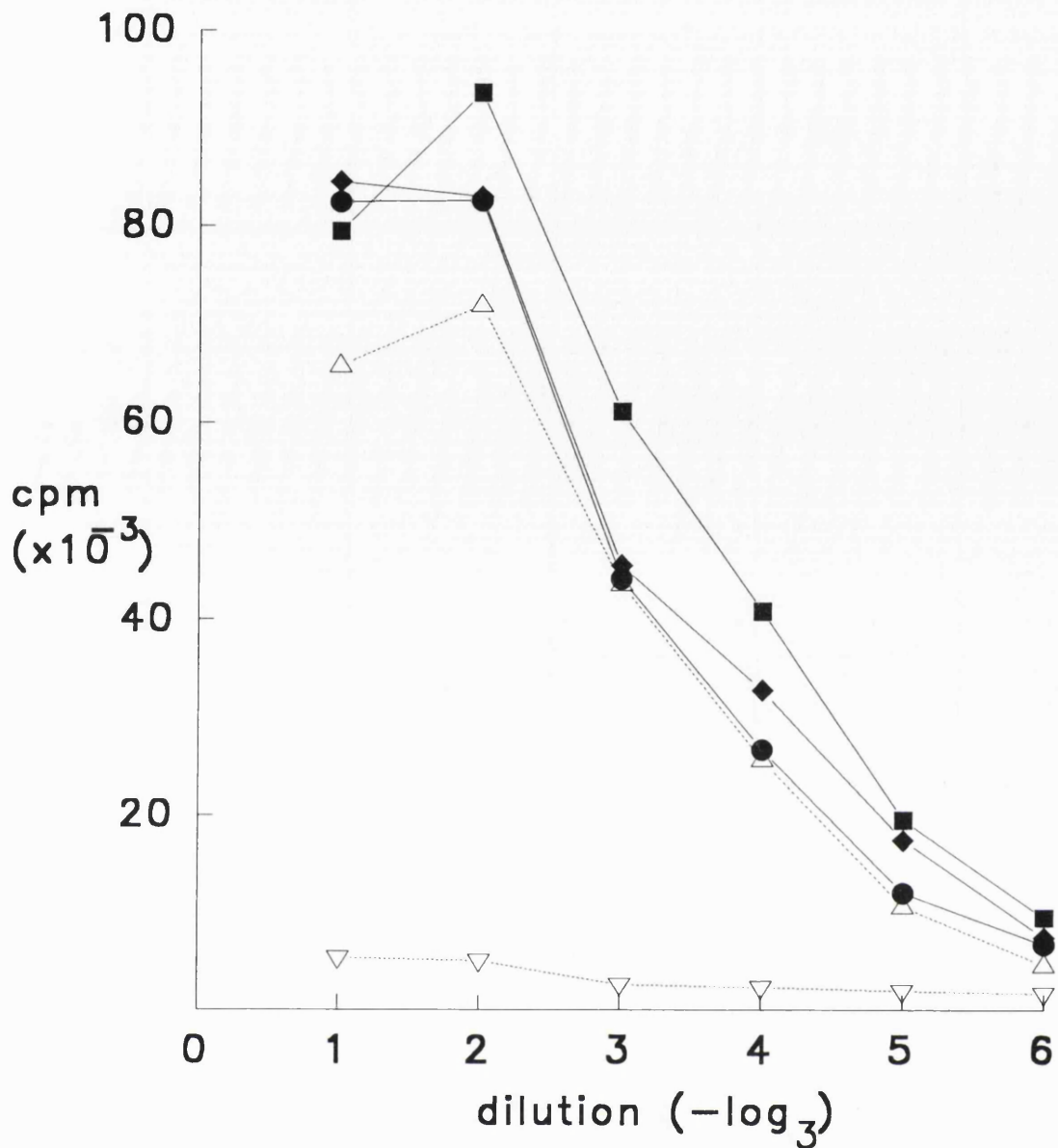
Antibody Response to DA MHC Class I in LEW Rat 3.

The response to intact donor MHC class I molecules was measured after 100 day acceptance of a (DAxLEW)F1 kidney allograft (◆), 14 days after immunisation with P1 (■) and 28 days after immunisation with P1 (●). The positive sera is the same as that for 1a (△). NRS is included as a negative control (▽). Approx. 10^8 RBC were used as targets. cpm refers to counts per minute of bound ^{125}I-RAR. Standard errors were between 0.5% and 7% of the mean values obtained.
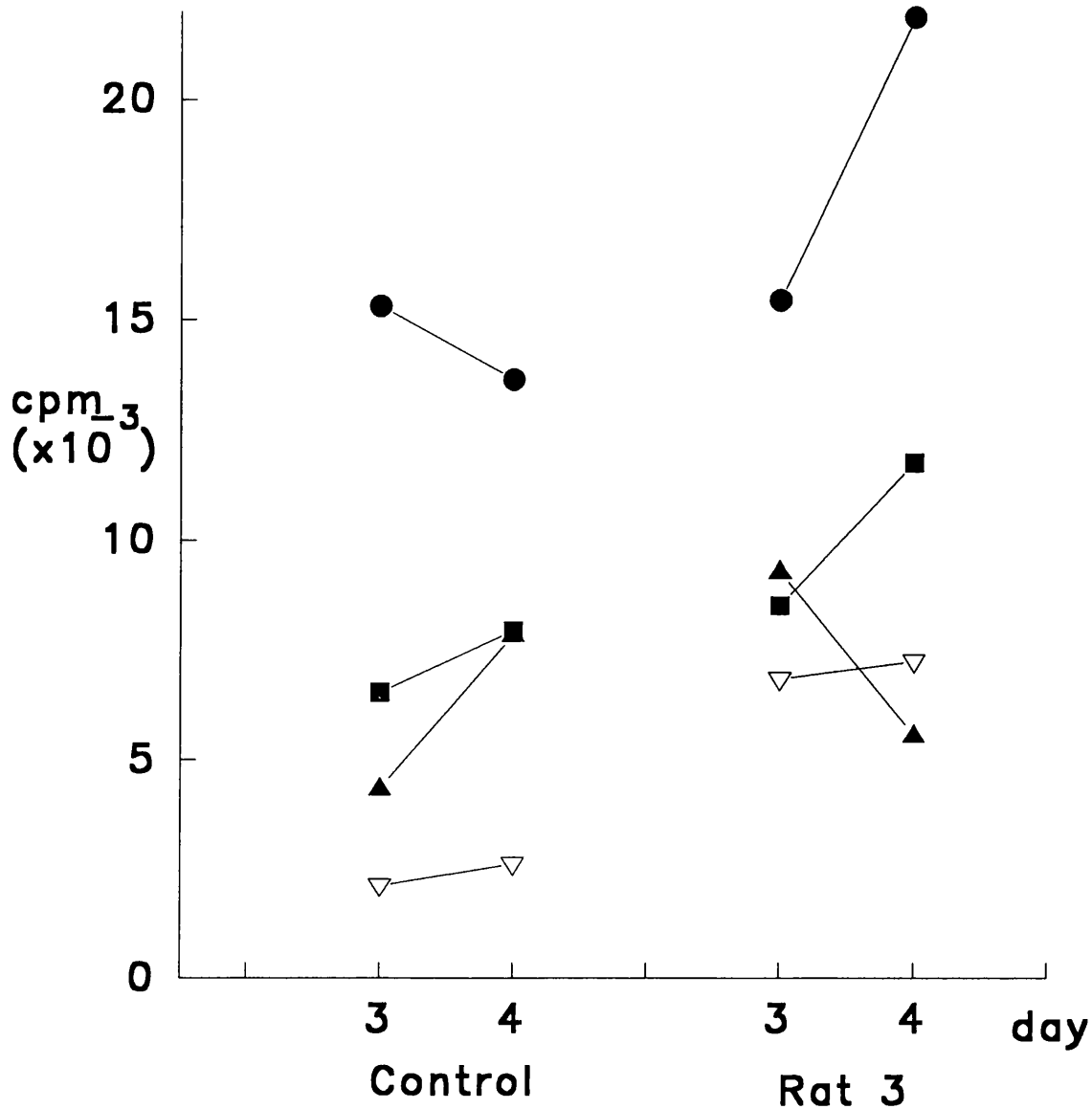
Proliferative Response of Rat 3 LN Cells to P1.

Whole LN cell preparations were dispersed at 10^5 ml^{-1} 10 days after the animal was boosted with 20μg P1. Single cell suspensions were stimulated with either 25μg ml^{-1} P1 (●), 2.5μg ml^{-1} P1 (■), 25μg ml^{-1} P2 as a control peptide (▲) and medium only (▽) for three or four days. The cells were pulsed with 20μCi ^{3}H-thymidine and harvested 24hr later. cpm refers to counts per minute of incorporated tritium. The control rat was immunised but had not received an allograft. Standard errors were between 1% and 10% of the mean values obtained.
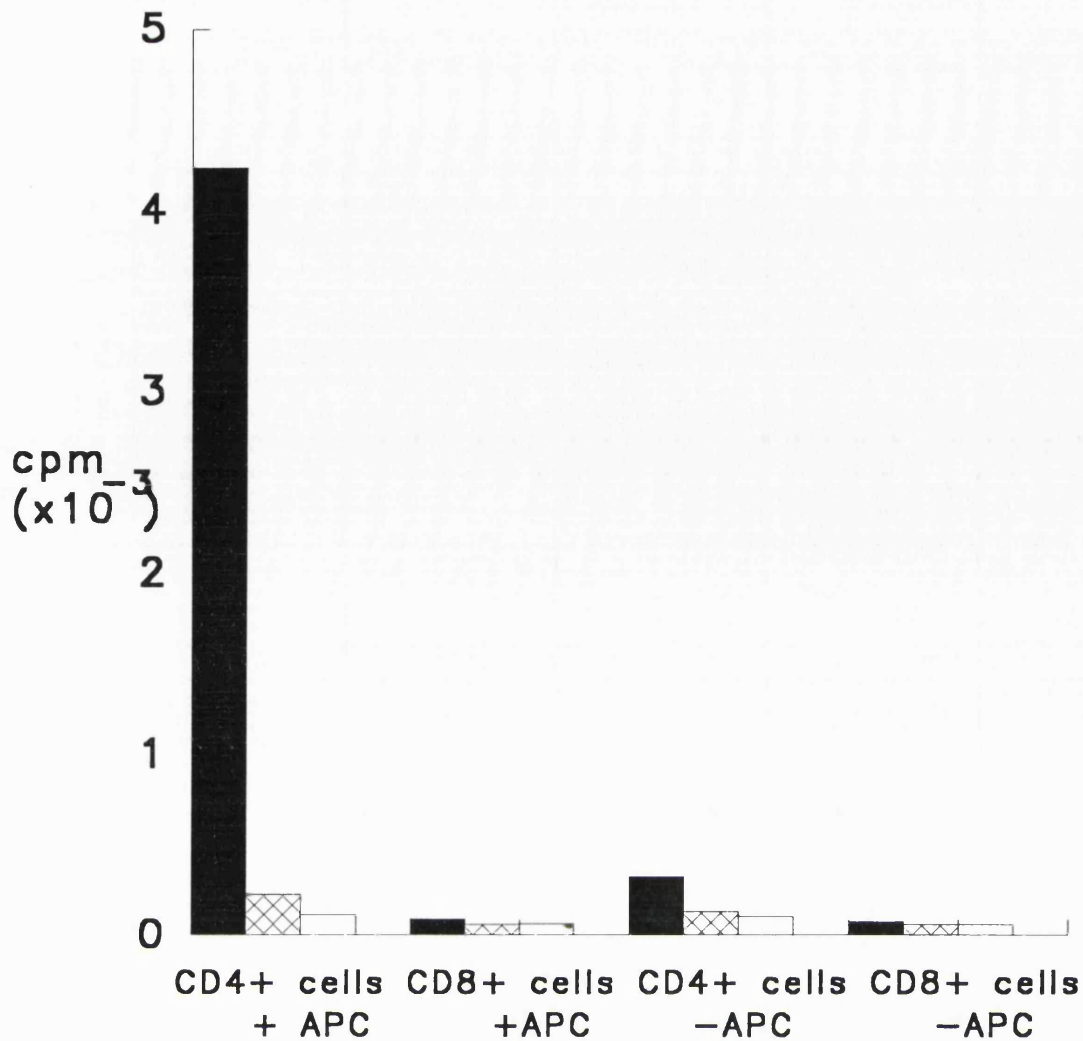
FIGURE 4d:

T Cell Subset Studies on Rat 3 LN Cells.

Rat 3 was boosted with P1 and its LN cells removed. Single cell suspensions were made and cultures were depleted of either CD8$^+$ or CD4$^+$ T cell subsets. APCs were also removed from half of the CD4$^+$ and CD8$^+$ T cell depleted cultures. Cells at 10^5 ml^{-1} were stimulated for 3 days in culture with either 25μg ml^{-1} P1 (■), 25 μg ml^{-1} irrelevant peptide Px (◨) or medium only (□). The cultures were pulsed with 20μCi ^{3}H-thymidine and harvested 24hr later. cpm refers to counts per minute of incorporated radioactivity. Standard errors were between 0.6% and 7% of the mean values obtained.

7.3 DISCUSSION:

The molecular mechanisms involved in tolerance induction (e.g Hämmerling *et al* 1993, Fowlkes and Ramsdell 1993) and the means by which B and T cells communicate to bring about activation or silencing in the periphery (Clark and Ledbetter 1994) are becoming clearer. In transplantation, however, though peripheral tolerance is often observed, the underlying mechanisms are poorly understood and donor antigen specific tolerance is clinically almost impossible to achieve. Only recently have steps been made to dissect the mechanisms involved in transplantation tolerance with regard to such phenomena as the blood transfusion effect (Fuchs and Matzinger 1992), chimerism (Starzl *et al* 1993) 'infectious' tolerance and bystander suppression (Qin *et al* 1993, Lombardi *et al* 1994). Some of these concepts may prove to be different outcomes of similar mechanisms.

The investigations in this chapter into the B and T cell responses of tolerant rats do not address specific mechanisms of tolerance maintenance or induction, but they do demonstrate the complexity of tolerance and the variety of possible outcomes even under a standard treatment regimen. About 90% of LEW rats with (DAxLEW)F1 kidney allografts develop antibodies to donor class I antigens, regardless of long term, constant exposure of the immune system to donor antigen. Despite the provision of CsA, a strong antibody response against DA class I molecules occurs but does not facilitate rejection. The regularity with which the response comes up 4-6 weeks after grafting (i.e. 2-4 weeks after the cessation of CsA therapy) is intriguing. This probably depends on the *in vivo* concentration and half-life of CsA.

It would be interesting to know what the role of antibody is in this model; whether the complement pathway is capable of being activated,

whether antibodies are having a protective effect on the allograft and which subclass of antibody is being detected.

Unfortunately, the rarity of animals that did not secrete antibodies to donor MHC class I after transplantation meant that large numbers could not be studied. Increasing the CsA dosage and duration of administration of CsA in our experiments had no effect on antibody production.

Rat 1 develops antibodies to class I molecules after $CD4^+$ T cell priming with the donor class I derived peptide P1. It represents a situation in which neither $CD4^+$ T cells or B cells are tolerant, since the former respond to P1 and the latter secrete class I antibodies. One explanation for this outcome might lie in the clonality of the T cell receptor. A particular $CD4^+$ T cell clone in this rat may have a TCR that recognises the peptide epitope (in association with MHC class II on APCs) as well as a similar epitope derived from whole donor class I (at lower stringency) once it has been activated. The 'masked' class I epitope might then be recognised because the occupancy of the upregulated class II molecules has increased enough to trigger the same T cell clone at lower stringency. In this way, rat 1 could to respond *in vitro* to P1 and provide help for B cells responsive towards whole class I (or its derivatives) once $CD4^+$ T cell help has been initiated.

In rat 2, however, peptide specific T cells may not be able to cross react with donor class I epitopes and antibody production towards donor MHC class I alloantigens will not occur despite peptide priming. Perhaps during CsA therapy, these rare rats are tolerised with respect to class I reactive $CD4^+$ T cells and it is only later, with the activation of a lower stringency clone, that donor class I antibody production becomes possible. This will still be a rare and unpredictable event. Thus individual clonotypic variation at the level of the T cell receptor might be crucial in determining the T and B cell response during rejection. The two possibilities discussed

for rats 1 and 2 are represented diagrammatically in FIGURE 5. Alternatively, T cell suppression, B cell tolerance/anergy or even differences in T_H1 and T_H2 pathways might account for this observation.

Rat 3 suggests that even with repeated challenge with P1 and the resultant activation of donor peptide specific CD4$^+$ T cells, the immune status of the animal with respect to the graft is difficult to alter. There is no graft rejection despite both T and B cell dependent alloevents operating *in vitro*. This suggests that other regulatory processes are involved in the *in vivo* maintenance of allograft survival which could very well include cytokine pathways and bystander suppression (Dallman *et al* 1993, Lombardi *et al* 1994).

The studies in this chapter are by no means exhaustive. Although it is not prudent to place too much emphasis on a few animals, this work points to the importance of the diversity of immunity and tolerance in similar animals. This may 'fine tune' the effector mechanisms involved in the immune response, even if the final outcome (graft acceptance) is the same. This may be worth considering with respect to therapeutic strategies in the future, which may need to be tailored to the individual person or animal concerned, rather than just towards a particular cellular pathway.

(a)

P1 specific APC — MHC cII / P1 — TCR — CD4+ — virgin P1 specific T cell

upregulation

no 'low stringency' activation

cl specific B cell — class I peptide — TCR — CD4+ — MHC class I specific T cell anergised during CsA therapy

inactive — X

(b)

cl specific B cell — class I peptide — TCR — CD4+ — mature, upregulated P1 specific T cell
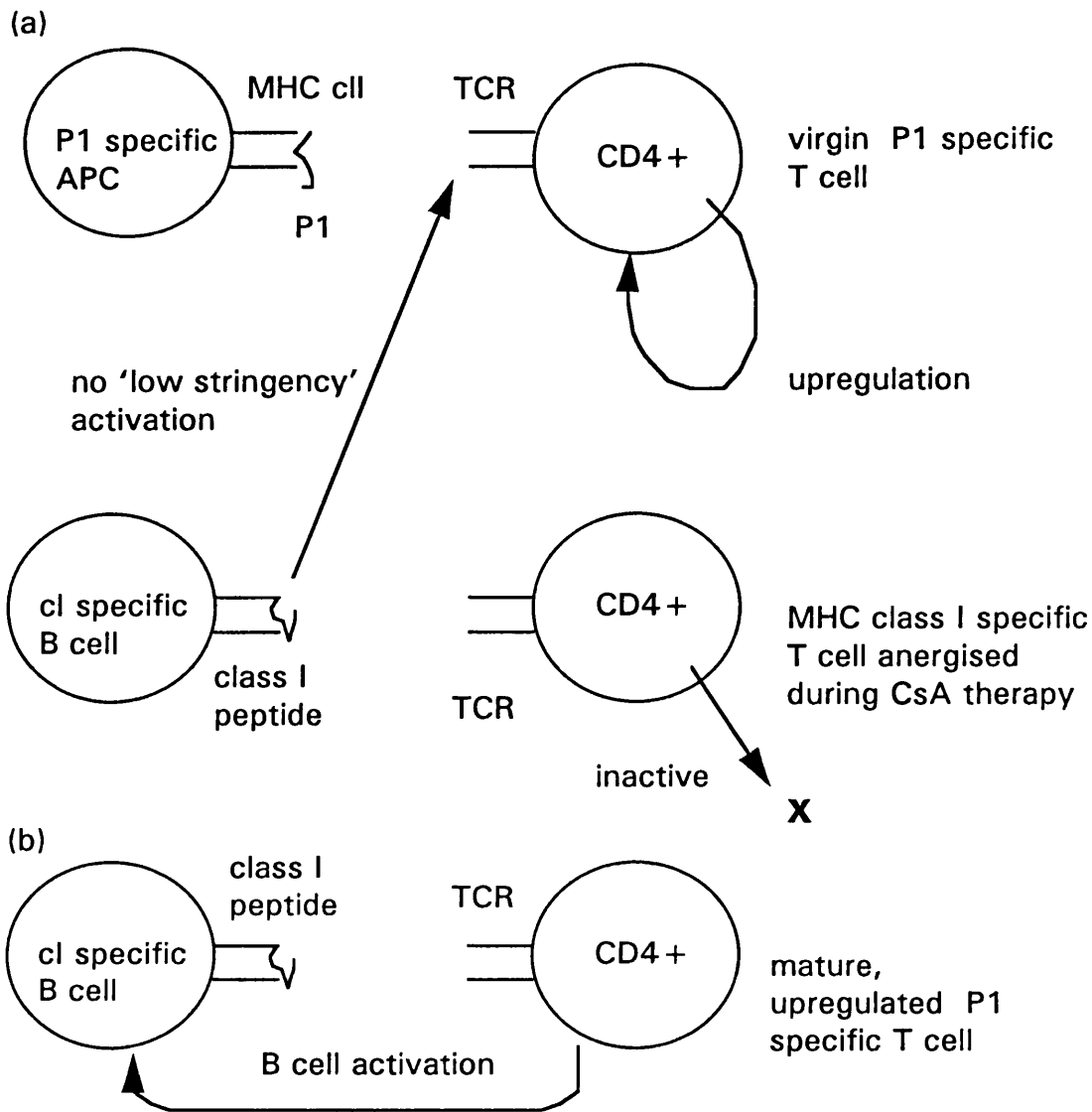
B cell activation

FIGURE 5:

Schematic Representation of B Cell Activation in Rat 2.

In (a), CsA therapy prevents T cells from helping donor MHC reactive B cells and there is no class I antibody production. P1 immunisation activates CD4+ T cells and provides P1 specific B cell help.

In (b), after P1 immunisation, mature upregulated P1 specific CD4+ T cells (which may have undergone peripheral TCR clonal selection) can now recognise lower avidity class I specific B cells and provide help for donor class I antibody production as well as P1 antibody production.

cl = class I, cII = MHC class II.

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DISCUSSION.

The work in this thesis has covered various aspects of the indirect presentation pathway using the rat as a model. I have examined both the specificity of the response (Chapter 4), and addressed some more general questions involving B and T cell peripheral tolerance to kidney transplants (Chapter 7). It has also been proven for the first time that indirect T cell allorecognition occurs during and contributes to vascularised kidney allograft rejection (Chapter 5). This was an important finding, since vascularised grafts are far more clinically relevant than skin grafts. A full understanding of the mechanisms involved in vascularised allograft rejection is required if a rational and specific approach is to be taken towards the design of novel treatment regimens.

It is pertinent to ask exactly how important the indirect response is in allograft rejection. It seems to be well established that direct presentation initiates acute rejection responses immediately after transplantation (Lechler and Batchelor 1982, Sherman and Chattopadhyay 1993) and that the T cells involved are distinct from those marshalling chronic rejection (Braun *et al* 1993a). In certain clinical situations, antibody mediated rejection (Rose 1993), cytokine responses (Dallman *et al* 1992) and graft versus host reactions (Santos *et al* 1985, Gale 1985) can all be involved in alloimmunity, and conventional antigen processing may be important in these processes. Indirect presentation has been theorised to be more important in chronic rejection, where continuous, conventional presentation of alloantigens should provide a constant source of foreign protein. This is backed up by the retransplantation model (Chapter 5). Here, kidneys depleted of their interstitial dendritic cells reject only slowly, if at all, and priming for indirect recognition accelerates the rejection response. Interstitial dendritic cells are crucial initiators of the direct arm of the

rejection pathway, being potent donor APCs. By removing them, the influence of the slower acting indirect pathway on events is clearly observed.

Investigations into chimerism in liver transplants also have an important bearing on mechanisms of chronic rejection, since the implication is that host lymphocytes and donor cells are required to maintain long term graft survival. On the other hand, mobile donor lymphocytes (along with proteins from other sources within the graft) should provide polymorphic antigens through which indirect presentation can facilitate rejection. In some ways, the outcome of chimerism is similar to what we and other investigators find in the rat kidney allograft model in which the depletion of mobile donor dendritic cells and lymphocytes enhances graft survival. Starzl's group state that it is cell migration that promotes graft survival (Starzl *et al* 1992). One could argue that it is the removal of direct recognition responses and 'professional' APCs that result in improved graft acceptance, rather than the establishment of a chimeric state. Murine islet allografts, for example, do not reject if they are pretreated with anti-dendritic cell antibodies (Faustman *et al* 1984). Nevertheless, in our long surviving animals that are not primed to indirect recognition, donor cells and host lymphocytes are presumably interacting with each other, and B cell activity against MHC class I molecules is undoubtedly present. It is also important to distinguish in these systems the difference between tolerance and prolonged or temporary graft acceptance under immunosuppression.

It is interesting to consider the role of antibodies in rejection in relation to this work (Chapters 5 and 7). Antibody mediated mechanisms are relevant in many transplantation models and in the clinic (e.g Jeannet *et al* 1970) and care must be taken to avoid applying conclusions gleaned from one experimental model to other disparate systems. In chapter 7, it

was shown that under temporary immunosuppression, rats maintain conventional single transplants indefinitely, despite the presence of graft specific antibodies and peptide specific T cells (after priming) that are at least reactive *in vitro*. It is also noteworthy that the administration of CsA does not alter donor class I antibody production in the long term, since both immunosuppressed long surviving animals (Chapter 7) and untreated 'retransplanted' animals (Chapter 5) both develop antibodies to MHC class I antigens. Neither is the response strain specific, since (PVGxLEW)F1 rats show similar antibody profiles (Chapter 6). It is, however, possible that this observation is restricted to $RT1^l$ - $RT1.A^{av1}$ mismatches. Investigations using the WAG $RT1^u$ rat as a recipient might be interesting in this regard. Perhaps the administration of CsA merely provides enough time for interstitial dendritic cells to migrate, and this then overcomes the initial barrier of acute rejection.

The mechanisms behind these states of tolerance are very intriguing. Tolerance by exhaustion, which has been described in mice with LCMV (Moskophidis *et al* 1993), could be a possibility if rejection were mediated by $CD8^+$ T cells that are subsequently exhausted by the huge antigen dose provided by the allograft. Cytotoxic mixed lymphocyte reactions could be performed to demonstrate the potential (or otherwise) of the $CD8^+$ T cell response towards allogeneic cells.

Anergy also seems an unlikely explanation for tolerance in this model, as at least some T dependent B cell responses towards the allograft remain intact (Chapter 5, 6 and 7). In other rat models (Braun *et al* 1993a and b), clonal anergy has been suggested to be involved in long term kidney allograft survival. In this work, distinct T cell populations are implicated in the acute and chronic phases of rejection. A further possibility is that suppressor T cells maintain graft stability. It is possible that graft reactive T cells are anergised by inappropriate contact with host (or indeed donor)

APCs during the initial phases of immunosuppression. This may then exert an allospecific suppressor function by 'mopping up' excess cytokines and competing against 'functional' cells for peptide (Lombardi *et al* 1994).

Another explanation for these 'tolerant' long survivors (Chapter 7) is that, after CsA therapy, the $CD4^+$ T cell - B cell interactions involved in producing antibody are re-established, but $CD4^+$ - $CD8^+$ T cell communication remains disrupted. How this occurs mechanistically is very difficult to postulate. Perhaps the sites at which these responses are generated is important. For example, many T-T interactions will occur at local lymph nodes at the site of transplantation, as well as within the graft itself. Circulating $CD4^+$ lymphocytes will encounter a complex cocktail of cytokines and adhesion molecules that might result in inappropriate cofactor contacts: abnormal $CD4^+$ helper responses may then be generated with regard to $CD8^+$ T cell activation, and a degree of tolerance may ensue. In the spleen, however, where the bulk of B cell activation takes place (away from the site of insult), more conventional T-B collaboration might be possible. This would then lead to antibody secretion.

Previous work in our laboratory suggests that different responses can be generated at different sites, since peptide specific $CD4^+$ T cells can be found in spleen but not lymph node cells after allogeneic rat kidney transplantation (Fangmann *et al* 1992a). Indeed, tissue-specific differences in the peptide profiles from splenic and thymic MHC class II molecules have been noted in the mouse (Marrack *et al* 1993). The potential for tissue specific differences in alloreactivity has also been demonstrated. Here, B cells, macrophages and dendritic cells have been shown to differ in their capacity for tolerance induction, depending on whether they originated from the spleen or thymus, when transplanted across a neonatal BALB/c Mls-1 mismatch (Inaba *et al* 1993).

Why antibodies against donor MHC class I molecules then fail to

cause graft rejection in our animals is another question. Perhaps a type of passive enhancement is occurring in which the antibodies have a protective effect on the allograft (Hart and Fabre 1982). Clinically, anti-idiotypic antibodies to human MHC molecules have been proposed to enhance the survival of liver allografts (Mohanakumar *et al* 1987), though there is no convincing explanation for these phenomena. Certainly, different Ig isotypes have distinct complement fixing abilities and Fc receptor binding potential which could influence the outcome of graft survival (Fridman 1993). It has been shown, in the mouse at least, that T_H2 type T cells result predominantly in IgE and IgG1 secretion, whereas the T_H1 subset primarily helps IgG2a secretion (Finkelman *et al* 1990). There is some evidence that different Ig subtypes can regulate the course of immunity; for example, IgE, often present in low amounts, can constantly trigger its own production, whereas a rise in IgG production may switch off IgG producing B cells. In humans, it has been demonstrated that the presence of IgG antibodies against HLA molecules correlates with cytotoxicity, whereas the presence of IgM does not (Roelen *et al* 1994). The organisation of the antigen i.e. MHC class I molecules clustered on a cell surface as opposed to individual shed molecules or fragments could have a bearing on the immune response (e.g. Bachmann *et al* 1993).

With a whole plethora of immune responses occurring in a variety of different transplantation settings, it is important (but very difficult) to establish what the important rate determining step(s) are in acute and chronic rejection. The work in this thesis is consistent with, but does not prove that, the $CD4^+$ T cell is a major influence on rejection events. A particularly popular notion at the moment (Mossman and Coffman 1989) is that the immune response to infection can take either a T_H1 type pathway (resulting in IFN gamma and IL-2 secretion and thence cytotoxic T cell activities) or a T_H2 pathway (resulting in IL-4, -5, and 6 secretion and

thence antibody production). The difference in outcome can make the difference between protective immunity (T_H2) and death (T_H1). IL-12 and IL-4 are thought to be important controllers of this balance and a link between the innate and acquired arms of immunity (Hsieh *et al* 1993). Although the situation *In vivo* is not this clear cut, there is no reason why a similar controlling role for the $CD4^+$ T cell shouldn't exist in transplantation. There have been few studies of T cell subsets in rejection, especially in the rat (e.g. Papp *et al* 1992), but it has been postulated that cytokine based therapies might help shift rejection away from the more dangerous lytic response (e.g. Abraham *et al* 1992). Any control exerted by $CD4^+$ T cells may be masked in the earlier phases of rejection by the effects of direct T cell allorecognition. So if direct recognition can be suppressed, then managing the $CD4^+$ T cell 'switch' might help prevent the onset of chronic rejection. Another interesting point is that direct recognition can cause activation of $CD8^+$ T cell clones in 'exogenous' circumstances. Thus the $CD8^+$ T cell reacts to an external protein rather than a typical viral protein. Perhaps this leads to the inappropriate generation of 'intracellular pathogen-type' responses (that have evolved to deal with viruses) rather than more relevant 'exogenous antigen-type' responses.

In the allografted, long surviving LEW animals (Chapter 7), temporary immunosuppression of one subset of T helper cells may have pushed the balance towards graft acceptance (humoral) rather than graft destruction (cellular). How this is possible in the rat but not the human is an interesting question. A clue towards whether $CD4^+$ T cells mediate such a controlling influence in these rats might be found by observing the levels of cytokines such as IL-12 and IL-4 before and after transplantation in conjunction with the timing of donor alloantibody production. There are, however, a number of technical difficulties associated with this type of approach (Dallman *et al* 1991), since it is the active concentration of

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cytokines in the cellular microenvironment that is important.

A further consideration is the increasing number of TCR$^+$ T cell subtypes that have been implicated in infectious immunity (e.g. Erard *et al* 1993, Locksley *et al* 1993). These T cells do not fit into conventional CD4$^+$ or CD8$^+$ subgroups and some may even express two TCR's of different antigen specificity (Padovan *et al* 1993). The role of such cells, as well as gamma delta T cells, in transplantation remains to be established.

Attempts to dissect the effector mechanism(s) involved in indirect presentation were unsuccessful (Chapter 6). This knowledge is crucially important if rejection by the indirect pathway is to be prevented, for it is necessary to know which cell type, cell surface molecule or cytokines need to be perturbed in order to achieve specific tolerance. The work on peptide determinants (Chapter 4) suggests that a vast array of peptides are involved in the indirect T cell allorecognition pathway. In theory, it might be possible to design an antagonistic peptide that can out compete a nested set of physiological class II binding peptides and anergise the relevant reactive T cell. However, a large series of such peptides would be required, whose physiological concentrations and binding affinities would be critical. These would have to be tailored to suit each individual patient. Thus peptide specific therapy in transplantation is likely to be both impractical (e.g. Ishioka *et al* 1994) and very costly. Other investigators (Benichou *et al* 1994) have used a mouse model to suggest that few T cell epitopes are presented during indirect T cell allorecognition and, interestingly, that the lymph node and splenic responses are different. The authors suggest that these few epitopes will be a good target for therapy. However, it would appear that these results underline the variety between different experimental systems and between lymphoid organs.

The problem in transplantation really lies in the fact that too general a therapy will have side effects and will result in non-specific

immunosuppression, whereas too specific an approach will fail to prevent all the important rejection responses occurring in a heterogeneous treatment group.

Further Investigations Into Indirect T Cell Allorecognition:

To understand the role of indirect T cell allorecognition in the context of rejection as a whole, a number of experiments have been performed in the rat that have not produced enough results to merit a Chapter in this thesis. T cells from the rat have been successfully polyclonally expanded *in vitro* for periods of greater than two weeks in an attempt to maintain long term growth in the absence of specific T cell stimulation (Chapter 2). These cells have been investigated for their susceptibility to retroviral infection. 1-5% of this pool of cells can be potentially infected by a retrovirus expressing the *lac z* reporter gene. The *lac z* gene product, ß-galactosidase, stains blue using the staining method detailed in Chapter 2. Flow cytometry using infected cells that have been double labelled with either anti-CD4 or anti-CD8 PE conjugates and FDG demonstrate that approximately equal percentages of $CD8^+$ and $CD4^+$ T cells can be infected with retrovirus. Any bias in TCR usage is unknown. Improving current culture techniques for polyspecific rat T cells would be of great use in rodent transplantation.

Numerous other studies could be performed to add to the findings contained within this thesis. The 15mer peptide studies (Chapter 4) could be extended by synthesising 'mutant' peptides with amino acid substitutions in the proposed TCR recognition region (IAKEWE). One would expect such mutant peptides to cause diminished activation of (or anergise) T cell clones from animals primed with the original 15mer peptide(s). Since the WAG rats in particular responded strongly to the N terminal regions of the original peptide P1, it might also be interesting to synthesise a larger peptide that extends beyond the current N-terminus and also to examine

the effect of indirect recognition in a DA into WAG transplantation model. Isolation of individual P1 reactive T cell clones would also be a worthwhile exercise, as these could then be specifically isolated and used in reconstitution experiments. The importance of antigen presentation in this system could also be analysed, using purified macrophages, dendritic cells or B cells from spleen, peripheral blood and lymph node, for example. The class II loci to which P1 binds could also be elucidated by incubating APCs with radiolabelled P1.

In the work on LEW long survivors of (DAxLEW)F1 allografts (Chapter 7), the generation of more 'non-antibody' secretors and the detailed analysis of more of the high responders would allow firmer conclusions to be drawn.

With perseverance, tolerance induction protocols could be devised (e.g. Chapter 6), perhaps by oral administration or intra-thymic injection of these allogeneic peptides. However, this is an avenue that we have not pursued in any detail, since tolerising to one or a few antigens in the rat may have little relevance to clinical transplantation.

CHAPTER 9: REFERENCES.

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