Analysis of Structural Features of Peptide MHC Protein Complexes

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Abstract

To help determine possible mechanisms by which MHC molecules bind a diverse but limited range of peptides, the interactions between peptides and MHC proteins have been studied using two different systems. A predictive algorithm, based on empirical analysis of known T-cell epitopes, was used to locate determinants in the L1, E6, and E7 open reading frames (ORF) of human papilloma virus (HPV) type 16. Peptides containing putative determinants were synthesised and assayed for lymphoproliferative activity in mice. The T cells elicited were highly specific for HPV type 16, but were also found to exhibit degenerate MHC restriction, indicating that some of the peptides were able to form immunogenic complexes with different MHC proteins.

The contact residues and conformational features of a panel of different peptides bound to HLA DR1Dw1 were determined by analysing the ability of analogues containing long chain biotinylated lysine substituted at each position, to bind both the MHC protein and labelled avidin. The structural and conformational features of each of the peptides when bound to the HLA molecule were unique. However, three of the peptides originally defined by HLA-DR1Dw1 restricted T cells, shared several conformational features, while the remaining three peptides defined by T cells restricted through other alleles, shared some but not all of these features. Critical contact residues identified within the determinants were used to align their sequences and revealed structural homology between the peptides at positions making contact with the MHC protein. A similar pattern of residues was found in a large number of other determinants known to bind to different MHC loci and alleles. The data suggested a common structural mechanism by which many peptides bind to MHC proteins. Knowledge of this mechanism should enable the rational design of MHC binding peptides for the development of both vaccines and autoimmune disease therapies.

Dedicated to the Hill Family

and especially to my parents.

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Abbreviations

A or Ala	Alanine
BPV	Bovine papillomavirus
BSA	Bovine serum albumin
C or Cys	Cysteine
D or Asp	Aspartate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E or Glu	Glutamate
E6	E6 open reading frame/protein
E7	E7 open reading frame/protein
EBV	Epstein-Barr virus
ELISA	Enzyme linked immunoabsorbtion assay
F or Phe	Phenylalanine
FAD	Fluorescent avidin D
FCS	Foetal calf serum
FITC	fluorescein isothiocyanate
G or Gly	Glycine
g	force of 1 gravity
H or His	Histidine
Ha 307-319	Influenza haemagglutinin residues 307-319
Ha LCB 312	Ha 307-319 with long chain biotinylated lysine at position 312. Other analogues are named similarly.
Ha LCB N	Ha 307-319 with long chain biotin at the amino terminus
HEL	hen egg lysozyme
HIV	Human immunodeficiency virus
HPLC	High pressure liquid chromatography
HPV	Human papilloma virus

HRP	Horse radish peroxidase
I or Ile	Isoleucine
K of Lys	Lysine
K_A	equilibrium association constant
k_a	rate of association constant
kbp	kilo base pairs
K_D	equilibrium dissociation constant
k_d	rate of dissociation constant
L or Leu	Leucine
L1	L1 protein/open reading frame
L1 40-63	Residues 40-63 of the L1 open reading frame. Other L1, E6, and E7 peptides named similarly.
LCB	Long chain biotin
LCMV	Lymphochoriomeningitis virus
M or Met	Methionine
Mat 17-29	Influenza matrix residues 17-29
Mat LCB 17	Mat 17-29 with LCB substituted at position 17
MHC	Major histocompatibility complex
Mr	molecular weight
Myo 68-80	Sperm whale myoglobin residues 68-80
Myo LCB 68	Myo 68-80 with LCB at position 68
Myo 110-121	Sperm whale myoglobin residues 110-121
Myo LCB 111	Myo 110-121 with LCB at position 111
N or Asn	Asparagine
NP	Influenza nucleoprotein
nuclease	*Staphylococcal aureus* ribonuclease
P or Pro	Proline
Q or Gln	Glutamine

R or Arg	Arginine
rpm	revolutions per minute
S or Ser	Serine
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
T or Thr	Threonine
TNF	Tumour necrosis factor
Tub 3-14	*Mycobacterium tuberculosis* 19kDa protein residues 3-14
Tub LCB 4	Tub 3-14 with LCB at position 4
Tween	Tween 20
V or Val	Valine
W or Trp	Tryptophan
Y or Tyr	Tyrosine

Chapter 1
Introduction

1.1. Overview

The genes of the major histocompatibility complex (MHC) express highly polymorphic class I and class II glycoproteins which have evolved the ability to bind a diverse range of peptides and display them on cell surfaces. The cell surface complex between peptide and MHC protein is the ligand recognised by the antigen specific receptor of T lymphocytes. Recognition of this complex by T lymphocytes forms the basis by which self and non-self is initially established by the cellular immune system, and effects the ability to respond to foreign antigen throughout the lifetime of the individual.

During the formation of the immune system MHC proteins influence the specificity of the developing T cell repertoire. T lymphocytes capable of interacting with self MHC molecules are positively selected by interactions involving the T cell receptor and MHC protein. They also are negatively selected by combinations of self MHC and self peptide antigen. The resultant T cell repertoire represents a fine balance between sufficient diversity to respond to antigenic challenge and a tolerance of self proteins. The range of receptors is not perfect in this respect and mechanisms exist to suppress the responses of mature self reactive T cells in the periphery. The failure of these mechanisms can lead to inappropriate immune responses, and in extreme cases, autoimmune disease.

In the mature organism, a complex of foreign antigen and self MHC protein leads to triggering of antigen specific T lymphocytes and a multitude of ensuing cellular immune responses. Class I MHC proteins present antigen to CD8+ T lymphocytes which are usually involved in cytotoxic immune reactions, such as against virally infected cells. Class II MHC proteins present antigen to CD4+ T lymphocytes which

can provide help for antibody production by B lymphocytes. Upon antigen stimulation, both groups of T cells produce a number of lymphokines involved in regulating the responses of T and B lymphocytes and other immune cells.

Although the MHC genes are the most polymorphic known, only a small number of different MHC proteins are expressed in any one individual. The result is that each MHC protein must have the capacity to bind and present to T cells a sufficiently diverse range of peptides to both, define self and stimulate antigen specific immune responses. The allelic differences in MHC proteins leads to a modification in the range of peptides bound, and this forms much of the basis for variation in immune responsiveness between individuals.

A fundamental question in immunology is the precise molecular mechanism by which the antigen combining site of an MHC protein is able to bind a diverse range of peptides, with broad specificity. Knowledge of this mechanism would increase our understanding of antigen specific immune responses, and help in the development of potentially beneficial immune agonists (vaccines) and antagonists (autoimmune disease therapies). Our current understanding of this problem has arisen from studies of the mechanism by which T cells recognise foreign antigen, and of the physical and chemical structure of MHC proteins and peptide MHC protein complexes.

1.2. MHC genes and proteins

1.2.1. Class I and Class II MHC proteins

There are two classes of MHC protein which can be functionally differentiated. Class I proteins present antigen to CD8+ T cells which often are cytotoxic and recognise endogenously synthesised antigen (Germain, 1986; Braciale et al., 1987; Townsend and Bodmer, 1989). Class II proteins present antigen to CD4+ T cells which generally are helper T cells and recognise exogenous antigen (Germain, 1986; Braciale et al., 1987; Livingstone and Fathman, 1987).

The two classes of protein can also be biochemically and structurally differentiated. The class I molecules are expressed on the surface of almost all cells in the body and consist of a polymorphic, heavy chain, transmembrane glycoprotein (Mr 44 kDa), which is tightly but non-covalently bound to a highly conserved, un-glycosylated light chain, β_2-microglobulin (β_2-m) (Mr 12 kDa) (Figure **1**.1a) (Bjorkman and Parham, 1990; Kaufman et al., 1986; Springer, 1974; Grey, 1973). The heavy chain consists of a short intracellular segment, a transmembrane segment, and three amino terminal extracellular domains of approximately 90 amino acids each which are designated α1, α2, and α3 (Malissen et al., 1982; Springer and Strominger, 1976; Orr et al., 1979a; b). β_2-m contains a single domain and does not traverse the cell membrane.

In contrast, the class II molecules are expressed on the surface of specialised antigen presenting cells, for example macrophages, B cells, dendritic cells and others, and consist of two polymorphic glycosylated membrane bound chains, α (33-35 kDa) and β (27−29 kDa) (Kaufman et al., 1986; Kappes and Strominger, 1988). The two chains are tightly bound together by non-covalent interactions and each consists of a short intracellular domain, a transmembrane segment, and two extracellular domains (α1,α2 and β1,β2) of approximately 90 amino acids (Kaufman et al., 1986) (Figure **1**.1b).

Despite these obvious differences the two classes of MHC protein perform the same basic function which is to present antigen to T lymphocytes. Similarities in the structural mechanisms by which both classes of protein perform this function are suggested by the use of the same family of T cell receptor genes by both class I and class II restricted T cells (Kronenberg et al., 1986). A common structural mechanism is also reflected by similarities in the domain structure of the two classes of proteins and in the relative structure of individual domains. Both classes of MHC have four extracellular domains of approximately 90 amino acids, with two membrane proximal

Figure 1.1.

Schematic diagram of the MHC class I and class II proteins

a) Class I protein

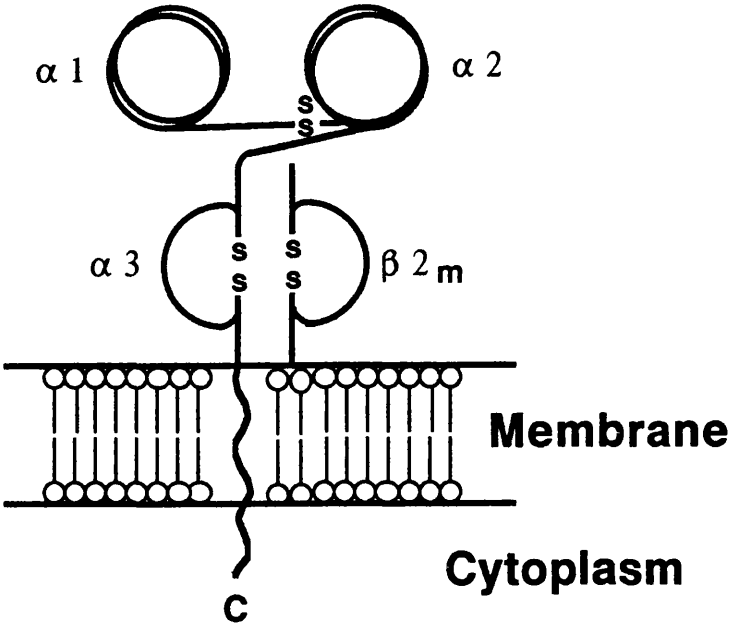
b) Class II protein

The common, four domain structure of both classes of proteins has been emphasised.

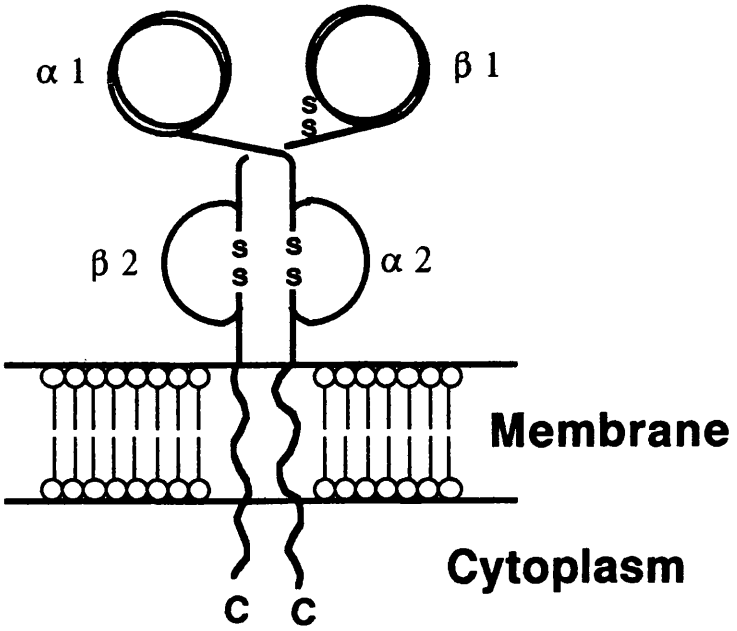
C: signifies the carboxyl terminus

s s: signifies disulphide bridges

a) **MHC Class I Protein**



b) **MHC Class II Protein**



domains and two membrane distal domains. In both cases the two membrane proximal domains ($\alpha 3/\beta_2$-m of class I and $\alpha 2/\beta 2$ of class II molecules) are relatively well conserved, share significant sequence homology with immunoglobulin constant domains, and both contain a single disulphide bridge (Orr et al., 1979a; Michaelson et al., 1980; Kaufman et al., 1986). Only one of the two membrane distal domains of each class of MHC protein ($\alpha 2$ of class I and $\beta 1$ of class II molecules) contains a disulphide bridge (Orr et al., 1979b; Kaufman, 1986). However, both membrane distal domains express the majority of the polymorphic residues of the MHC and have only weak sequence homology with each other and no significant sequence homology with other known structures (Kaufman et al., 1986; Parham et al., 1988). There are therefore significant similarities in the domain structure of class I and class II MHC proteins.

1.2.2. Major Histocompatibility Complex genes

The class I and class II MHC proteins are encoded by genes within the class I and class II sub-regions of the major histocompatibility gene complex which lies on chromosome 6 of humans (Human Leucocyte Antigen (HLA) genes) and 17 of mice (Histocompatibility-2 (H-2) genes) (Kaufman, 1986; Kappes and Strominger, 1988). The MHC expresses some of the most polymorphic genes known (Table 1.I).

The class I genes encode the polymorphic HLA A, B, and C (H-2 K, D, and L) class I protein heavy chains (Malissen et al., 1982; Bjorkman and Parham, 1990) (Figure 1.2). The gene for $\beta 2$ microglobulin lies on chromosome 15 in humans and chromosome 2 in the mouse, separate from the other MHC proteins, and is almost completely non-polymorphic (Michaelson et al, 1980).

The class II genes encode the serologically defined HLA-DR and DQ class II antigens (I-E and I-A respectively in the mouse) and the cellularly defined HLA-DP class II antigens (Figure 1.2) (Kaufman, 1986; Kappes and Strominger, 1988). The HLA-DR subregion expresses one conserved A gene and different combinations of the

Table 1.I

HLA specificities

Taken from the Nomenclature Committee Report, (1987) produced after the 10th International Histocompatibility Testing Workshop.

A	B	C	D	DR	DQ	DP
A1	B5	Cw1	Dw1	DR1	DQw1	DPw1
A2	B7	Cw2	Dw2	DR2	DQw2	DPw2
A3	B8	Cw3	Dw3	DR3	DQw3	DPw3
A9	B12	Cw4	Dw4	DR4	DQw4	DPw4
A10	B13	Cw5	Dw5	DR5	DQw5 (w1)	DPw5
A11	B14	Cw6	Dw6	DRw6	DQw6 (w1)	DPw6
Aw19	B15	Cw7	Dw7	DR7	DQw7 (w3)	
A23 (9)	B16	Cw8	Dw8	DRw8	DQw8 (w3)	
A24 (9)	B17	Cw9 (w3)	Dw9	DR9	DQw9 (w3)	
A25 (10)	B18	Cw10 (w3)	Dw10	DRw10		
A26 (10)	B21	Cw11	Dw11 (w7)	DRw11 (5)		
A28	Bw22		Dw12	DRw12 (5)		
A29 (w19)	B27		Dw13	DRw13 (w6)		
A30 (w19)	B35		Dw14	DRw14 (w6)		
A31 (w19)	B37		Dw15	DRw15 (2)		
A32 (w19)	B38 (16)		Dw16	DRw16 (2)		
Aw33 (w19)	B39 (16)		Dw17 (w7)	DRw17 (3)		
Aw34 (10)	B40		Dw18 (w6)	DRw18 (3)		
Aw36	Bw41		Dw19 (w6)			
Aw43	Bw42		Dw20	DRw52		
Aw66 (10)	B44 (12)		Dw21			
Aw68 (28)	B45 (12)		Dw22	DRw53		
Aw69 (28)	Bw46		Dw23			
Aw74 (w19)	Bw47		Dw24			
	Bw48		Dw25			
	B49 (21)		Dw26			
	Bw50 (21)					
	B51 (5)					
	Bw52 (5)					
	Bw53					
	Bw54 (w22)					
	Bw55 (w22)					
	Bw56 (w22)					
	Bw57 (17)					
	Bw58 (17)					
	Bw59					
	Bw60 (40)					
	Bw61 (40)					
	Bw62 (15)					
	Bw63 (15)					
	Bw64 (14)					
	Bw65 (14)					
	Bw67					
	Bw71 (w70)					
	Bw70					
	Bw72 (w70)					
	Bw73					
	Bw75 (15)					
	Bw76 (15)					
	Bw77 (15)					
	Bw4					
	Bw6					

The listings of broad specificities in parenthesis after a narrow specificity, e.g HLA-A23(9) is optional. The following is a listing of those specificities which arose as clear-cut splits of other specificities.

Original broad specificities	Splits
A9	A23, A24
A10	A25, A26, Aw34, Aw66
Aw19	A29, A30, A31, A32, Aw33, Aw74
A28	Aw68, Aw69
B5	B51, Bw52
B12	B44, B45
B14	Bw64, Bw65
B15	Bw62, Bw63, Bw75, Bw76, Bw77
B16	B38, B39
B17	Bw57, Bw58
B21	B49, Bw50
B21	B49, Bw50
Bw22	Bw54, Bw55, Bw56
B40	Bw60, Bw61
Bw70	Bw71, Bw72
Cw3	Cw9, Cw10
DR2	DRw15, DRw16
DR3	DRw17, DRw18
DR5	DRw11, DRw12
DRw6	DRw13, DRw14
DQw1	DQw5, DQw6
DQw3	DQw7, DQw8, DQw9
Dw6	Dw18, Dw19
Dw7	Dw11, Dw17

The following specificities are generally agreed inclusions of HLA-B specificities Bw4 and Bw6.

Bw4: B5, B13, B17, B27, B37, B38(16), B44(12), Bw47, B49(21), B51(5), Bw52(5), Bw53, Bw57(17), Bw58(17), Bw59, Bw63(15), Bw77(15).

Bw6: B7, B8, B14, B18, Bw22, B35, B39(16), B40, Bw41, Bw42, B45(12), Bw46, Bw48, Bw50(21), Bw54(w22), Bw55(w22), Bw56(w22) Bw60(40), Bw61(40), Bw62(15), Bw64(14), Bw65(14), Bw67, Bw70, Bw71(w70), Bw72(w70), Bw73, Bw75(15), Bw76(15).

The following specificities are generally agreed to be associated with DRw52 and DRw53:

DRw52: DR3, DR5, DRw6, DRw8, DRw11(5), DRw12(5), DRw13(w6), DRw14(w6), DRw17(3), DRw18(3).

DRw53: DR4, DR7, DR9

Figure 1.2.

Genetic map of the Human and Mouse MHC

a) Human MHC.

Figure is based on one by Trowsdale and Campbell (1988). Only expressed genes are shown. The DR region expresses a single A gene and at least the B1 gene. The B3 or B4 gene is also expressed in some haplotypes. There are a number of pseudogenes associated with most loci (Kappes and Strominger, 1988; Trowsdale and Campbell, 1988). There are a large number of other expressed genes within the MHC complex including the complement genes and TNF genes.

kbp: kilobase pairs

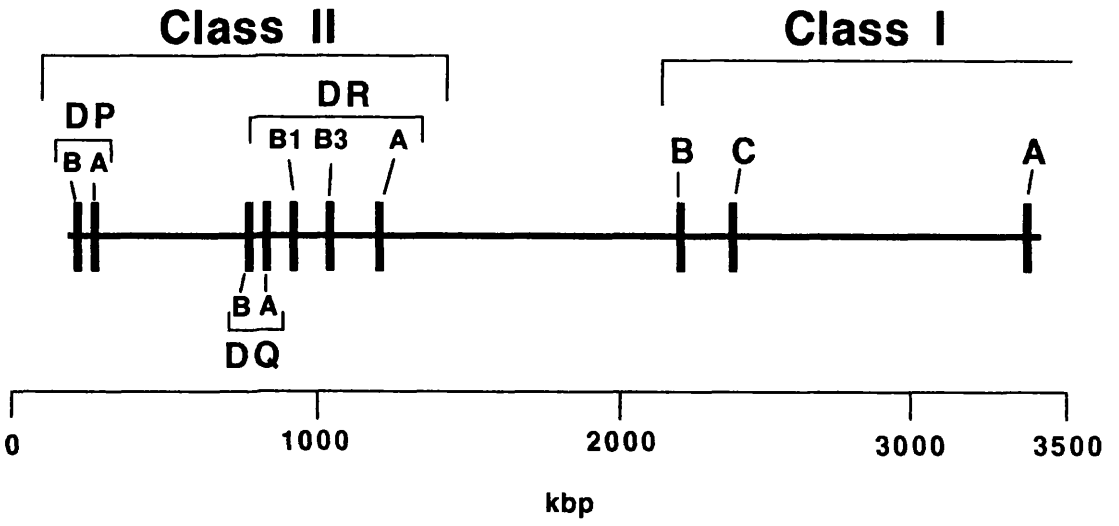
b) Mouse MHC.

Figure is based on one from Klein and Figueroa (1986). Only expressed genes are shown. In some strains of mouse there are no I-E genes.

cM: centiMorgans

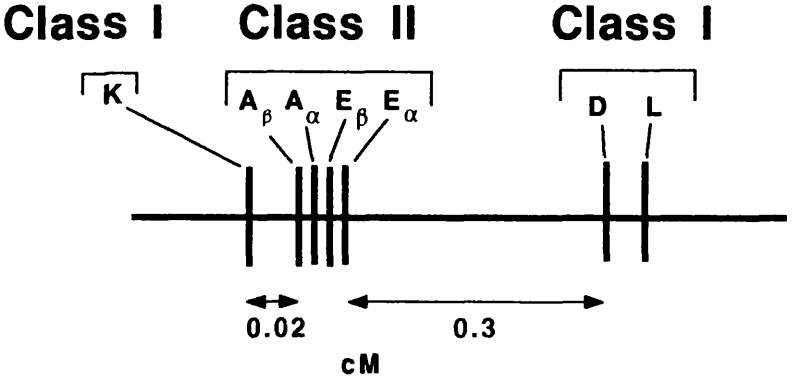
a

Human MHC



b

Mouse MHC



polymorphic B genes depending on the haplotype. For example, the DR1 haplotype expresses only the highly polymorphic B1 gene whereas the DR3 haplotype expresses the B1 and the less polymorphic B3 gene (Kappes and Strominger, 1988). The A gene encodes the α chain and the B genes different β chains of the HLA-DR heterodimer. This results in the formation of different numbers and types of HLA-DR α/β heterodimers depending on the MHC haplotype expressed. HLA-DQ and DP loci express one A gene and one B gene each, resulting in one DQ and one DP α/β heterodimer per haplotype (Kappes and Strominger, 1988). The I-E and I-A genes of the mouse express one α and one β gene each, which results in the formation of one I-$E^{\alpha/\beta}$ hetero-dimer and one I-$A^{\alpha/\beta}$ hetero-dimer in most mouse strains but certain strains lack I-E genes and therefore only express one class II molecule (Kaufman, 1986).

1.2.3. Summary

Despite functional and structural differences between class I and class II MHC proteins, similarities in their overall structural organisation, and the use of the same T cell receptor genes by both class I and class II restricted T cells, suggests a common structural mechanism by which they present antigen to T lymphocytes. Although they are the some of the most polymorphic proteins known only a limited number of each class of molecule are expressed in any one individual and hence each MHC protein must have the capacity to interact with a wide range of protein antigens. Our current understanding of the structural mechanism of this interaction has come from a large number of studies on the mechanism of antigen recognition by T cells.

1.3. Recognition of antigen by T cells

1.3.1. Restriction of T cell responses

The mechanism by which T lymphocytes recognise foreign antigen differs fundamentally from that of B lymphocytes. The cell surface immunoglobulin molecules of B cells are able to recognise free, soluble, antigen in an interaction similar

to that between other well characterised cell surface receptors and their ligands. In contrast, T cells can only recognise antigen in association with MHC molecules expressed on the surface of another cell.

In a fundamental series of experiments Zinkernagel and Doherty demonstrated that lysis of lympho-choriomeningitis virus (LMCV) infected cells by cytotoxic T cells was "restricted" to histocompatible target cells (Zinkernagel and Doherty, 1974a; b; Zinkernagel and Doherty, 1975). Their experiments indicated that the T cells recognised "self" proteins, expressed by Histocompatibility -2 (H-2) genes, which had been modified by the viral infection. Further experiments revealed that such restriction of T cell responses mapped to the H-2 K and D loci (Zinkernagel and Doherty, 1975) which are now known to express the class I molecules of the mouse MHC proteins.

Similarly, co-operative interaction between T and B cells in the production of antibody (Raff, 1970; Mitchison, 1971) and successful in vitro stimulation of T cells by macrophages was shown to require histocompatibility at the I locus of the H-2 gene complex (Katz et al. 1973; Shevach and Rosenthal, 1973; Katz et al., 1975). The inability to stimulate T cells with antigen unless there were histocompatible B cells/macrophages present was known as the Ir gene defect. Several important results were necessary to confirm that this was essentially the same phenomenon as the restriction of cytotoxic T cell responses. Demonstration that T cells from (responder x non-responder) F1 mouse strains were able to interact with B cells and macrophages from the responder strain but not those from the non-responder strain (Katz et al. 1973; Shevach and Rosenthal, 1973) indicated that the Ir gene defect was expressed by the B cells and macrophages, rather than T cells. Ia antigens, encoded by I genes of the H-2 complex, and recognised by alloantisera, were identified exclusively on the surfaces of B cells and macrophages (Benacerraf and Germain, 1978). This led to the hypothesis that T cell recognition of antigen in the context of macrophages and B cells also was restricted, this time by the presence of histocompatible Ia gene products (Benacerraf,

1978). Improved mapping of the I region and the availability of specific anti-sera enabled the inhibition of mouse T cell responses restricted through the I-A subregion of the I locus by anti-sera specific for I-A antigens (Schwartz, 1978). This helped to confirm the restriction hypothesis and identified some of the first of the class II proteins of the mouse MHC.

1.3.2. T cells recognise processed antigen

Class II restricted T cells

MHC restriction is not the only distinction between the mechanisms of antigen recognition by T and B lymphocytes. Although, genetically and structurally, the antigen specific receptors of both sets of lymphocytes are remarkably similar (Davis and Bjorkman, 1988), they interact with different forms of protein antigen. In general the immunoglobulin receptor of B cells binds conformationally intact epitopes on the surface of a protein, which often involve non-contiguous residues in the amino acid sequence, whereas the T cell receptor always recognises linear peptide fragments of protein antigen in association with an MHC molecule (Benjamin et al., 1984; Milich, 1989).

Numerous experiments have shown that B and T cells recognise different forms of antigen (Gell and Benacerraf, 1959; Maizels et al., 1980; Unanue, 1984; Livingstone and Fathman, 1987). Immunisation of mice with intact lysozyme resulted in B cells which reacted only with intact protein whereas T cells recognised both intact and fragmented protein in the presence of macrophages expressing the appropriate MHC molecule (Maizels et al., 1980). Following the immunisation of mice with intact ovalbumin, the T cells responding to intact ovalbumin in vitro were treated with 5-bromodeoxy-uridine and light, which would inhibit further proliferation by these cells. However, this treatment also resulted in the depletion of T cells capable of responding

to the denatured antigen, indicating that the same T cells recognised both intact and denatured protein (Chesnut et al., 1982).

These experiments suggested that denaturation or fragmentation of protein might be a natural process in the pathway of antigen presentation to T cells by macrophages. The active role of the macrophage was clearly demonstrated when lightly fixed macrophages were shown to be able to present fragmented (Shimonkevitz et al., 1984) or even simply denatured (Streicher et al., 1984) antigen, but not intact antigen, to T cells, whereas untreated macrophages were able to present both forms of antigen. Intact antigen presentation by macrophages was also sensitive to metabolic inhibitors, such as azide, and lysosomotropic agents, such as chloroquine and ammonium chloride (Unanue, 1984). The conclusions from a large number of experiments (Unanue, 1984) were that for antigen to be recognised by T cells in the context of class II molecules, an active process by macrophages involving denaturation and/or degradation of antigen in an acid compartment was required.

Class I restricted T cells

In class I restricted recognition (reviewed by Townsend and Bodmer, 1989) the proteins of influenza within infected cells, recognised by cytotoxic T cells, could be replaced by transfected genes, or fragments of transfected genes, expressed intracellularly (Townsend et al., 1984a; 1985). It was soon discovered that target cells could be sensitised to lysis using peptides representing the appropriate epitope (Townsend et al., 1986). The conclusion was that cytotoxic T cells also recognised fragments of protein antigen in the context of the appropriate MHC molecule and that the recognition process would share many similarities with that of class II restricted T cells. As mentioned, the fact that both class I and class II restricted T cells used the same pool of T cell antigen receptor genes helped to strengthen this conclusion (Kronenberg et al., 1986).

1.3.3. Summary

There are two main differences between antigen recognition by T and B cells. Firstly, B cells recognise free antigen in solution, whereas T cells always recognise antigen in association with an MHC protein. Secondly, B cells generally recognise conformationally intact, non-linear, epitopes, whereas T cells recognise linear fragments of protein antigen. Since the antigen receptors of both B and T lymphocytes are so similar, how does the combination of MHC protein and peptide result in a suitable ligand for the T cell receptor ?

1.4. T cells recognise a peptide MHC protein complex

The antigenic moiety recognised by the α and β chains of the T cell receptor is a highly stable complex formed between a peptide fragment of antigen and an MHC protein (reviewed Davis and Bjorkman, 1988). Evidence towards this model has come from both functional studies using T cell assays and direct peptide binding studies using purified MHC proteins (Watts and McConnell, 1987; Davis and Bjorkman, 1988).

The ability to replace almost all the known T cell determinants with simple peptide fragments of antigen (Schwartz, 1985; Livingstone and Fathman, 1987; Rothbard and Taylor, 1988; Milich, 1989), combined with the relative ease of modern synthetic peptide chemistry (Kent, 1988), has enabled the ready synthesis and modification of many T cell determinants. This has helped dramatically in the derivation of the model for how T cells recognise foreign antigen.

1.4.1. Functional experiments to demonstrate a TcR-peptide-MHC complex for T cell activation

The identification and cloning of the T cell receptor (reviewed by Davis and Bjorkman, 1988) enabled the individual α and β genes of a specific receptor to be

transfected into a second T cell with a different specificity. This resulted in complete transfer of antigen specificity in the context of a particular MHC protein and indicated that the T cell receptor alone carried the specificity for both the MHC and antigen (Dembic et al., 1986; Saito et al., 1987).

An elegant series of experiments by Schwartz's group used variations in the peptide antigen, MHC protein, and T cells involved in recognition of cytochrome c to determine that one part of a peptide antigen (residue 99 of cytochrome c) was making contact with the T cell receptor (epitope) whereas another part only a few amino acids away (residue 103) contacted the MHC protein (agretope) (Heber-Katz et al., 1983; Hansburg et al., 1983; Schwartz, 1985; Fox et al., 1987). When B10.A mice were immunised with pigeon cytochrome c residues 81-104, the T cells elicited responded to the immunising peptide, but did not respond to a substituted peptide with a Gln for the natural Lys at residue 99. However, when B10.A mice were immunised with the Gln substituted peptide, the T cells elicited responded well to the peptide with Gln at 99 but not to the natural sequence with Lys at this position. The reciprocal nature of the lack of cross-reactivity indicated that residue 99 was interacting with the receptor of the T cell clones involved. Modifications at residue 103 had quite different results. B10.A mice were responders and B10.A(5R) mice were non-responders to pigeon cytochrome c, whereas both strains responded to a carboxyl terminally truncated peptide with a lysine at residue 103. T cells from both strains of mice could recognise pigeon cytochrome c presented by B10.A antigen presenting cells, but not B10.A(5R) antigen presenting cells, whereas the deleted peptide could be presented by both. Modification of the peptide at position 103 altered the effects of changing allelic forms of the MHC and suggested that this residue contacted the MHC molecule. The data indicated that there were distinct parts to this T cell determinant, one which contacted the MHC and one which contacted the T cell receptor (Schwartz, 1985). In the light of the studies which showed that the T cell receptor expressed the specificity for both the antigen and the MHC molecule (Dembic et al., 1986; Saito et al., 1987), the simplest explanation of

the data from Schwartz's group was the existence of a tri-molecular complex between the T cell receptor, peptide and MHC protein.

1.4.3. Peptide antigen presentation

The ability of structurally related and distinct antigens to compete for T cell recognition at the level of antigen presentation (Werdelin, 1982; Rock and Benacerraf, 1983; Godfrey et al., 1984) suggested that there was a single antigen combining site on the surface of antigen presenting cells. More detailed studies by Guillet et al. showed competition during antigen presentation to T cell hybridomas (Guillet et al,. 1986; 1987). They demonstrated that the response of a hybridoma specific for bacteriophage λcI residues 12-26, restricted through I-A^d, could be inhibited by an unrelated *Staphylococcal aureus* ribonuclease (nuclease) peptide and an ovalbumin peptide which were restricted through the same allele. However, the response was not inhibited by a peptide restricted through I-E^k. This further demonstrated a single site for antigen binding on the surface of the antigen presenting cell and provided indirect evidence that the peptides were binding directly to the MHC proteins.

Several studies have indicated that peptides recognised by class I restricted T cells also interact predominantly with a single antigen binding site on the surface of the target/antigen presenting cell. Initially two related peptide determinants from HLA molecules defined in the context of K^d were shown to compete for presentation of each other (Maryanski et al., 1988). The definition of two other epitopes from the nucleoprotein of influenza recognised in the context of the same allele also were shown to be able to compete with each other and with the unrelated determinants from HLA molecules (Pala et al., 1988a; b). Recently Carreno et al. have examined competition between a number of human cytotoxic T cell determinants and a large panel of peptides (Carreno et al., 1990). They were able to show that in general, different subsets of peptides from the panel were able to compete for recognition in the context of different HLA alleles. This suggested HLA allele specificity in the interactions the peptides were

making with the antigen presenting cell. Bodmer et al. (1989) also showed competition between unrelated peptides restricted through the same MHC class I allele (D^b). Pre-incubation of target cells with mixtures of target and competitor peptides followed by washing away of free peptide resulted in competition This suggested that the competition was occurring at the level of the antigen presenting cell, and not at the level of the T cell receptor. An F1 H-2^{bxd} target cell was able to present K^d and D^b restricted determinants to mutually exclusive T cell clones indicating that both peptides formed antigenic complexes on the target cell surface. However, the K^d restricted determinant was unable to compete for presentation of the D^b restricted determinant, on either H-2^b parental target cells or F1 H-2^{dxb} target cells. These results implied that the competition for D^b presentation, which was observed with other determinants, was taking place at the level of MHC binding rather than at the level of some other non-specific peptide binding protein (Bodmer et al., 1989). A further interesting result from this set of experiments was that certain K^k, and K^d restricted cytotoxic determinants were able to compete for presentation to D^b restricted T cells. This suggested that the physical and chemical structure of these peptides allowed them to bind to more than the one class I allele originally used to define them.

1.4.5. Direct binding of peptides to MHC proteins

Class II proteins

Direct binding between peptide antigen and MHC proteins was first shown when quantities of affinity purified, detergent solubilised MHC proteins became available. The initial experiment used equilibrium dialysis to demonstrate that a fluorescently labelled lysozyme peptide (residues 46-61) bound to purified I-A^k but not I-A^d (Babbit et al., 1985). The binding data correlated with the restriction of the T cell used to define the determinant. Competition studies revealed that other I-A^k restricted peptide determinants could compete for binding of the labelled peptide, further

confirming the specificity of the binding events being measured as well as indicating a common binding site on the MHC protein for the different antigens.

Complexes between peptides and the MHC protein were of sufficient stability to allow separation from free peptide by gel filtration (Buus et al., 1986) and this enabled a large number of peptide MHC protein interactions to be studied in detail. Analysis of the interaction between a variety of peptides and Ia molecules (Babbit et al., 1985; Buus et al., 1986; Guillet et al., 1987; and Buus et al., 1987), using both purified Ia and competition for stimulation of T cell hybridomas, revealed that in general, the ability of a peptide to bind a particular MHC protein correlated with the restriction of T cells recognising it and that there was a common interaction site for all peptides binding to the same MHC molecule. However, each peptide, as well as binding to its restriction element, bound to one or more other Ia molecules examined. In the case of bacteriophage λ cI, residues 12-26, the binding to the expected restriction elements (I-A^d and I-E^k) was weaker than to another Ia molecule (I-E^d) (Buus et al., 1987; Guillet et al., 1987). Interestingly although this peptide bound strongly to I-E^d molecules, no T cells specific for this peptide restricted through this allele could be elicited (Guillet et al., 1987). Guillet et al. reasoned that the absence of a response to λcI residues 12-26 could be explained by the absence of T cells which could recognise it, since it was clearly able to bind the Ia antigen. They found strong homology between this peptide and a fragment of the I-E^d molecule and suggested that tolerance to this self peptide explained the unresponsiveness to the λcI peptide (Guillet et al. 1987).

Analyses of the binding of peptide antigen to purified HLA-DR molecules has been more recent. Studies demonstrated that the complexes were extremely stable and were formed with a single antigen combining site (Roche and Cresswell, 1990a; O'Sullivan et al., 1990). A good correlation was found between the ability of a peptide to bind a particular allele and the restriction of T cells that recognised it (O'Sullivan et al., 1990). So far no peptide has been found that does not bind to the purified form of

its restriction element. However, in common with the previous studies on mouse class II protein peptide interactions , most of the peptides analysed by O'Sullivan et al. were able to bind to HLA-DR molecules other than the original DR protein used to define the determinants.

To help determine the biological relevance of the complexes formed between peptide and MHC class II proteins, a number of the complexes have been isolated by gel filtration, incorporated into planar membranes, and then used to stimulate T cell hybridomas (Buus et al., 1986; Watts et al., 1984). The ability to stimulate using such complexes correlated with the restriction of the responses to the peptides, thus demonstrating their relevance.

Naturally processed antigen bound to class II MHC molecules

To further confirm the relevance of antigenic peptide MHC protein complexes in class II restricted presentation to T lymphocytes, two groups have used acid treatment to remove naturally processed antigen from class II molecules (Buus et al., 1988; Demotz et al., 1989). One group showed that acid treatment of I-E molecules from cells pulsed with lysozyme eluted several peaks of antigen of approximately 2000 Mr which could be added back to purified I-E to reform complexes capable of stimulating lysozyme specific T cell hybridomas (Demotz et al., 1989). The second group eluted protease sensitive material from $I\text{-}E^d$ and $I\text{-}A^d$ proteins purified from cell lines which had not been pulsed with antigen. The eluted peptides from $I\text{-}E^d$ had a mean Mr of approximately 2000, and those from $I\text{-}A^d$ a mean Mr of approximately 3500. The $I\text{-}E^d$ and $I\text{-}A^d$ peptides demonstrated allele specific ability to compete for binding of radioactively labelled peptides to purified protein (Buus et al., 1988).

These experiments helped confirm that peptide fragments of antigen bound to class II molecules are the antigenic structure recognised by the T cell receptor and also demonstrated that, in vivo, class II molecules appear to bind a range of peptides at all times.

Class I

The direct binding of peptide to class I MHC molecules has technically been more difficult to demonstrate. When iodinated peptide and affinity purified HLA molecules were mixed in solution, the percentage of class I which bound peptide was so small that clear identification of the complex required purification using an affinity column after removal of excess peptide by gel filtration (Chen and Parham, 1989). However, the assay did show a correlation between the ability of the peptide to bind a particular class I molecule and the restriction of the peptide. Another assay was developed by Levy's group in which peptides were attached to the bottom of a 96 well plate and then iodinated class I molecules added (Bouillot et al., 1989). This assay has been used by several groups, including Parham's, and the resultant binding was consistently more promiscuous than that observed with class I molecules in solution (Choppin et al., 1990; Frelinger et al., 1990; Chen et al., 1990). For example, Chen et al. tested a large panel of 64 peptides for their ability to bind to 5 different class I molecules. They found few allele specific differences in the ability of the panel of peptides to bind class I, which is in contrast to the solution phase assay where allele specificity of peptide binding was observed (Chen and Parham, 1989; Chen et al., 1990). Despite these differences, some of which may simply be due to the particular peptides and HLA molecules used (Frelinger et al., 1990), a peptide recognised in the context of a particular allele was always able to bind to that allele.

Naturally processed antigen bound to class I

Recently, to help confirm the relevance of antigenic peptide/class I protein complexes in class I restricted recognition, the form of naturally processed antigen bound to class I molecules has been examined (Rotzschke et al., 1990; Falk et al., 1990; van Bleek and Natheson, 1990). Direct acid elution of peptides from cellular proteins demonstrated that class I molecules could affect the overall cellular pool of

peptides, presumably through different class I alleles binding to different subsets of peptides and protecting them from proteolysis (Rotzschke et al., 1990; Falk et al., 1990). Acid elution of endogenous peptides from H-2 K^b purified from virally infected cells, resulted in a range of peptides with molecular weights up to approximately 1500. A single peptide (by HPLC) within this mixture was found, which exhibited allele specific binding and could sensitise target cells to lysis by virus specific T cells (van Bleek and Natheson, 1990). These experiments confirmed the relevance of peptide class I protein complexes in cytotoxic T cell recognition. They also indicated that class I proteins bound endogenous peptides at all times and could protect peptide antigen from proteolysis by cellular enzymes.

An interesting difference between the antigenic material eluted from class I and class II proteins was the recovery of a single class I restricted antigenic peptide, reactive with a particular T cell specificity, from class I proteins, whereas there were several peptides eluted from class II proteins able to stimulate the class II restricted hybridomas (Demotz et al., 1989). Whether this difference was simply due to small number of peptide MHC protein complexes examined or reflected a difference in how the two classes of proteins bound peptide will require further analysis.

1.4.6. Summary

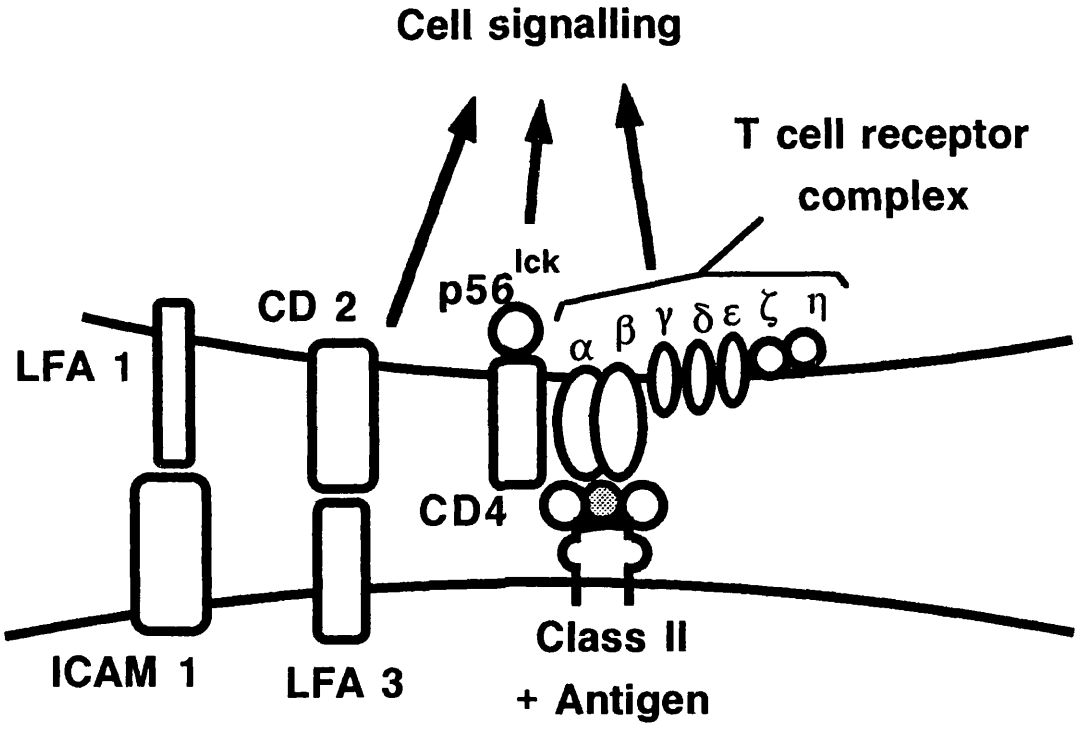
Stable complexes formed between peptide fragments of antigen and a single combining site on cell surface MHC proteins constitute a necessary and sufficient structure to allow recognition by the α and β chains of the antigen specific receptor on T lymphocytes. However, this view of antigen specific T cell recognition must be put in perspective. Figure 1.3 illustrates a recognition event between a class II restricted T cell and a peptide MHC protein complex on the surface of an antigen presenting cell. The antigen specificity is defined entirely by the T cell receptor recognition of peptide and class II protein but there are a number of other accessory molecules which are

Figure 1.3.

Presentation of antigen to a class II restricted T cell

For review of the following see Davis and Bjorkman (1988) Altman et al. (1990). The class II molecule on the antigen presenting cell binds and presents a peptide fragment of antigen to the α and β chains of the T cell receptor. The remaining chains of the T cell receptor are required for expression of the T cell receptor complex on the surface of the cell and are involved in cell signalling pathways. The class II molecule also interacts with the CD4 molecule on the surface of the T cell in an interaction, which has been shown to both increase avidity between the two cells, and to result in intracellular signals to the T cell via the association of the CD4 molecule with $p56^{lck}$. In class I restricted T cell recognition, the class I protein would interact with CD8 and the T cell receptor. The CD2 protein has been shown to be involved in T cell signalling and interacts with LFA 3. ICAM1 is a T cell surface molecule, which has been shown to increase avidity between the T cell and the antigen presenting cell by interacting with LFA 1.

T cell



Antigen Presenting Cell

involved, both in increasing the avidity of the interaction between the two cells and also in modulating signals transmitted within the T cell (Figure 1.3.) (Altmann et al., 1990).

1.5. Specificity of peptide binding to MHC proteins, T cell repertoire, and antigen processing

1.5.1. Specificity of Peptide Binding to MHC proteins

The experiments outlined above demonstrated that the antigen specific receptor of the T cell recognises a complex formed between a peptide fragment of that antigen and a self MHC protein. The specificity of the interaction between MHC and peptide antigen could clearly regulate the immune response to that antigen. However, the ability of a particular MHC protein to bind a peptide fragment of an antigen is not absolute in regulating immune responsiveness to that antigen. For example, although the bacteriophage λ cI peptide 12-26 bound to I-E^d no T cells to this peptide could be elicited from H-2^d mice (Guillet et al., 1986; 1987). Recently, Schaeffer et al. examined the ability of an overlapping set of peptides spanning the *Staphylococcal aureus* ribonuclease (nuclease) protein, to bind mouse class II molecules and to elicit T cell responses (Schaeffer et al., 1989). Of the large number of peptide Ia interactions studied, 30% resulted in binding, which demonstrated the broad specificity of the MHC binding site. However, only 70 % of the binding events resulted in a successful immune response when the peptides were used to immunise mice (Schaeffer et al., 1989). The data indicated that the immune responsiveness of an individual depends upon their T cell repertoire as well as the peptide binding capacity of their MHC proteins.

1.5.2. T cell repertoire

The broad specificity of the MHC binding site and the isolation of endogenous peptides from class II molecules (Buus et al., 1988), are consistent with the need for

self/non-self discrimination to occur largely at the level of T cell recognition and the T cell repertoire. However, the MHC also has been shown to be intimately involved in the process of shaping this repertoire by both positive and negative selection of T cell clones (Blackman et al., 1990). Maturing thymocytes have been shown to be positively selected by the presence of a particular MHC protein. The most dramatic demonstration of this was with T cell receptor transgenic mice (Kisielow et al., 1988). In these mice, large numbers of immature thymocytes were forced to express the single transgenic T cell receptor, irrespective of MHC haplotype. However, mature T cells expressing the transgenic T cell receptor only developed in mice expressing the same MHC haplotype as the mice from which the clone expressing the receptor was originally isolated (Kisielow et al., 1988). So far no evidence of a specific bound peptide for this process to occur has been found (Von Boehmer and Kisielow, 1990; Blackman et al., 1990). Immature thymocytes have also been shown to be negatively selected by the presence of a particular MHC protein, and also by the presence of a particular peptide bound to an MHC protein. For example, T cells expressing V_β 17a frequently were shown to recognise I-E class II molecules and hence to be deleted from I-E expressing mouse strains (Kappler et al., 1987a; b). Moreover, in transgenic mice which expressed a T cell receptor specific for the male H-Y antigen in the context of H-2 D^b, the thymi of female and male H-2^b mice contained the same numbers of precursor cells (CD4 - CD8 -) but male mice had a reduced thymus size and expressed almost no mature thymocytes compared to the females (Von Boehmer and Kisielow, 1990). These data indicated that MHC proteins and peptide MHC protein complexes function both at the level of antigen binding and at the level of selection of the T cell repertoire to determine an individuals ability to distinguish self from non-self.

1.5.3. Antigen Processing

The ability of an MHC protein to bind a particular fragment of antigen and the available T cell repertoire are not the only factors involved in regulating the immune

response to a whole antigen. The relative ability of different peptide fragments of the antigen to bind the MHC and/or the ability of different fragments of the protein to be processed have also been shown to be important for immune responsiveness. For example, peptides of diverse sequence known to bind the same MHC protein in vitro were shown to compete for presentation in vivo (Adorini et al., 1988). This indicated that antigenic challenge with two different antigens simultaneously or with different fragments of the same antigen could result in modulation of the responses to each by competition for binding to the MHC. Within hen egg lysozyme a hierarchy of immunodominance for a number of T cell epitopes has been established, which regulates the ability to generate an immune response to different parts of this protein (Gammon et al, 1987). For example, immunisation with the whole protein elicited responses to specific major immunodominant determinants but responses to other parts of the molecule could be elicited if individual peptides covering these determinants were used (Gammon et al., 1987). This suggests that T cell responses to a protein could be affected by the ability of different parts of the protein to be processed and/or competition for presentation at the level of MHC binding.

1.5.4. Summary

Clearly each of the processes discussed above is dependent to some degree on the broad specificity of antigen binding by the MHC protein and the presentation of peptide MHC protein complexes to antigen specific T cells. Understanding the detailed molecular mechanisms by which peptides interact with the MHC binding site to form stable complexes would enable manipulation of immune responsiveness, for example, by helping in the design of effective immune agonists (vaccine development), or of effective immune antagonists (competitive peptides to inhibit autoimmune responses). In order to understand the mechanisms by which MHC molecules bind to many different peptides, but with limited specificity, the structure of the proteins, possible mechanisms of formation of the peptide MHC protein complexes, and the chemical and

structural properties of the complexes themselves must be considered. Recently, solution of the crystal structure for HLA-A2 and Aw 68 has provided detailed information about the MHC class I peptide antigen binding site. In particular, it has provided a model for the structural basis by which the polymorphic residues of the MHC protein modulate the range of peptides bound by the site, and thereby modulate immune responsiveness.

1.6. Peptide binding sites of MHC proteins

1.6.1. Crystal structure of HLA-A2

Solution of the crystal structure for the human class I protein HLA-A2, to a resolution of 3.5Å, provided molecular detail of the four extracellular domains of the molecule, including the peptide antigen binding site of HLA-A2, and the position of polymorphic residues relative to this site (Bjorkman et al., 1987a; Bjorkman et al., 1987b). The two membrane proximal domains (α3 and β_2-m) adopted structures closely related to those of the immunoglobulin superfamily but adopted a novel packing relative to each other compared to other immunoglobulin heavy domains (Bjorkman et al., 1987a). Together, they formed a platform to support the amino terminal polymorphic α1 and α2 domains which had similar structures and formed the antigen combining site of the HLA protein (Figure 1.4) (Bjorkman et al., 1987a; Bjorkman et al., 1987b). The α1 and α2 domains each consisted of a 4 stranded anti-parallel β pleated sheet, a short helical segment, and a longer helical segment. The two domains of the molecule exhibited an approximate dyad axis of symmetry such that the 4 β strands of each domain paired up to form a single β pleated sheet of 8 strands. At diagonally opposite corners of the β pleated sheet short helical segments rose up to begin the longer helical segments which packed back down onto the edges of the sheet. This formed a cleft whose floor was made up of the 8 stranded β pleated sheet and whose walls were made up of the helical segments of each domain (Figure 1.4). The

Figure 1.4.

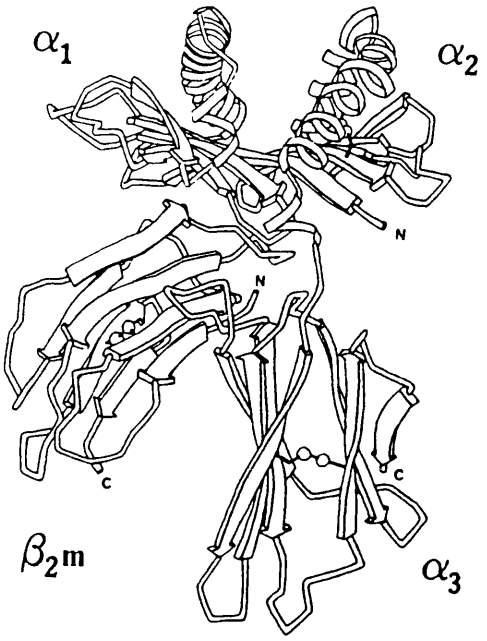
Structure of HLA-A2

Schematic diagrams of the HLA-A2 structure. β pleated sheets are shown by thick arrows and helical regions by helical ribbons. Connecting loops are shown as thinner lines and disulphide bonds as spheres. C marks the papain cleavage site at the carboxyl terminus of the crystalised fragment. Figure is taken from Bjorkman et al. (1987a).

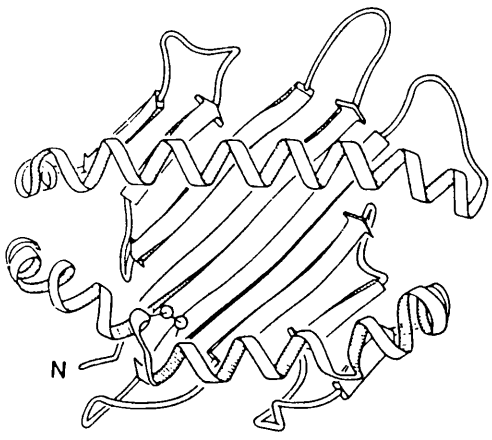
a) All four domains of HLA-A2. The membrane proximal domains are at the bottom and the polymorphic membrane distal domains are at the top. The α1 and α2 domains form the putative antigen binding site with an 8 stranded β pleated sheet (seen end on) supporting two helical segments.

b) View of the α1 and α2 domains from the direction of the T cell. The two helical regions and the β pleated sheet form the walls and floor of the putative antigen binding site respectively.

a



b



longer of the two helices in the α_2 domain was kinked in the middle at residue 162 and was joined to its β pleated sheet by a cysteine bridge between residues 164 and 101 (Figure 1.4) (Bjorkman et al., 1987a).

An intriguing feature of the original crystal structure was the presence of poorly defined electron density, as intense as the rest of the molecule but unnecessary to trace the protein backbone, in the cleft between the two helices. This electron density was concluded to be a mixture of bound peptide ligands, and the defined cleft to be the HLA antigen binding site, based on the following arguments. Firstly, the site of the cleft was distal to the antigen presenting cell membrane and hence in a position to be able to present antigen to the receptor of a cognate T cell. Secondly its size and shape was compatible with a peptide form of antigen and thirdly, the vast majority of the HLA polymorphic residues lined this cleft (Bjorkman et al., 1987a; Bjorkman et al., 1987b).

The polymorphic and conserved residues in and around the putative binding site, which could potentially interact with bound antigen, have been marked in Figure 1.5 (Bjorkman et al., 1987a; b; Garrett et al., 1989). Certain of the polymorphic residues pointed up from the strands on the floor of the cleft and hence would be expected to interact solely with the antigen and not with the T cell receptor. Others pointed in towards the binding site from the helical regions and could interact with the peptide antigen but may also form part of a surface capable of interacting with the T cell receptor (Bjorkman et al., 1987b). Still other polymorphic residues pointed up from the helical regions away from the binding site and these would be expected to interact directly with the T cell receptor (α1 residues 62, 65). A good correlation was found between the role of polymorphic residues in T cell recognition of antigen and their expected role in interacting with antigen or T cell receptor based on the structure (Bjorkman et al., 1987b).

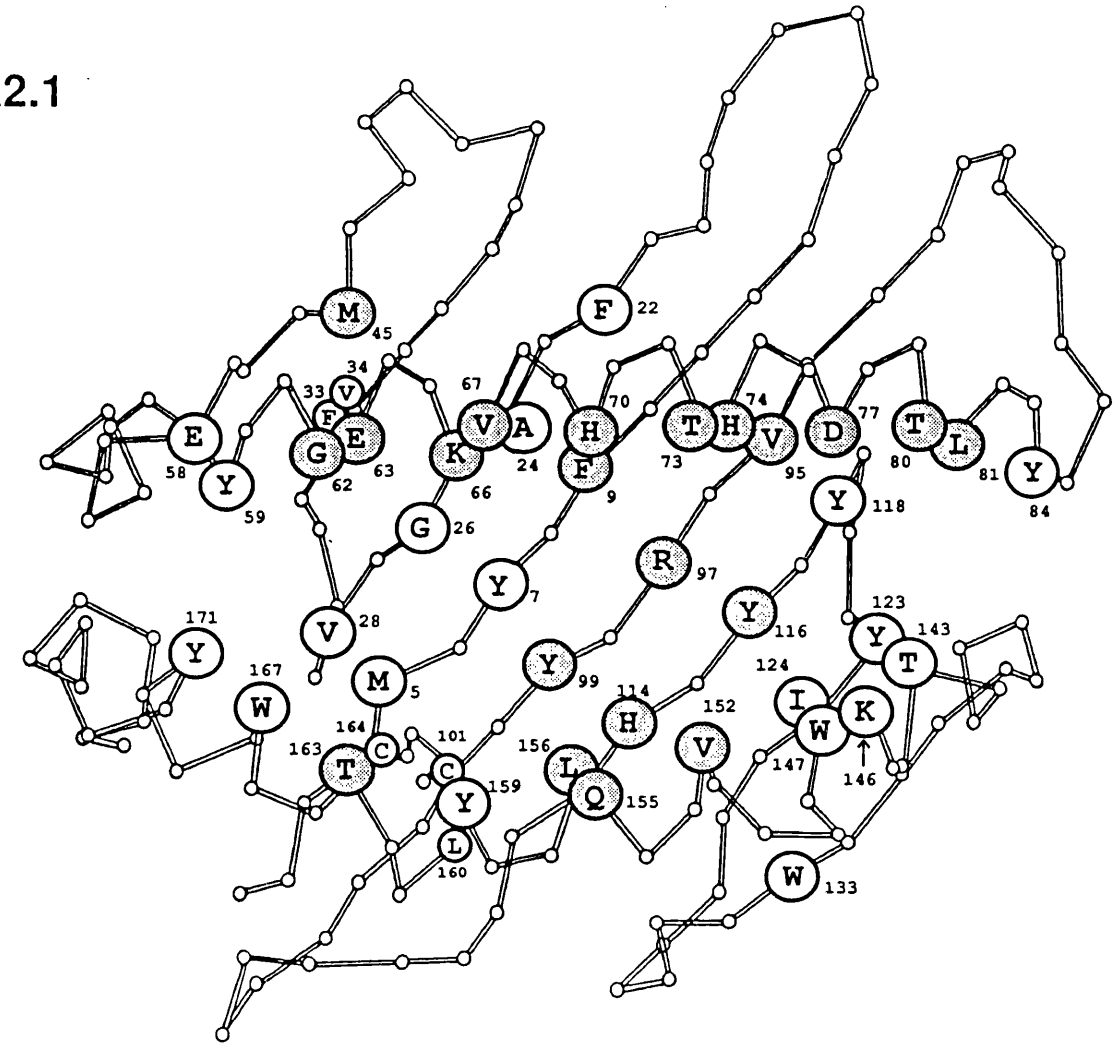
The polymorphic residues were not spread randomly throughout the structure but were localised to a limited number of residues many of which were around the

Figure 1.5.

Antigen binding site of HLA-A2

View, from the direction of the T cell, of the antigen binding site of HLA-A2.1. Residues shown are those known to be close enough to the endogenously bound material to be able to interact with it, and therefore able to form part of the antigen binding site. Shaded residues are polymorphic within class I molecules. The Cys at 101 and 164 are linked by a disulphide bridge. The residues in small circles are simply further from the T cell receptor than neighbouring residues within large circles. Figure is derived from Bjorkman et al. (1987) and data for the residues is taken from Bjorkman et al. (1987a/b), Brown et al. (1988), and Garrett et al. (1989).

HLA-A2.1


M 45
F 22
V 34
F 33
E 58
G 62
E 63
K 66
V 67
A 24
H 70
F 9
T 73
H 74
V 95
D 77
T 80
L 81
Y 84
Y 59
G 26
Y 7
R 97
Y 118
V 28
Y 171
Y 116
Y 123
Y 143
T
M 5
Y 99
W 167
H 114
V 152
I 124
W 147
K 146
C 164
C 101
T 163
L 156
Q 155
Y 159
L 160
W 133

binding site. The precise reasons for such localisations are unclear but presumably involve a mixture of selection following functionally advantageous mutational and recombinational events, and avoidance of mutations which inhibit the basic peptide binding structure (Parham et al., 1988; 1989).

As well as polymorphic residues within the binding site there were also a number of conserved residues which would be predicted to interact with bound ligand. Such residues imply that there may be certain structural features present in peptide ligands which would allow them to bind to these conserved regions of different HLA molecules. For example, these could be particular residues or groups of residues, the peptide backbone, or amino/carboxyl terminal charges (Bjorkman et al., 1987a; b).

1.6.2. Crystal Structure of HLA-Aw68

The crystal structure for a second HLA class I molecule (Aw68) has been solved to 2.6 Å and has been compared to a more recent 2.7 Å resolution structure of HLA-A2 (Garret et al., 1989; Madden et al., 1990). Comparison of the two binding sites has shown how allelic polymorphism of MHC proteins could modify the range of peptides that each binds (Garret et al., 1989).

The higher resolution of the structures enabled definition of specific interaction pockets within the binding sites, which were predicted to be able to accommodate specific amino acid side chain groups, for example hydrophobic side chains or positively charged side chains, of the bound ligand. If not all of the pockets had to be filled for a peptide to form a stable complex with the MHC protein then this would allow a broad range of peptides to bind, with some occupying one subset of pockets while other peptides could occupy a different subset. However, while making positive interactions with specific pockets the peptides would also have to avoid negative interactions with specific "bumps" and "ridges" in the binding site, and/or with particular specificity pockets (Garret et al., 1989). This model of peptide binding to

HLA implied that groups of peptides binding the same MHC molecule would share structural features important for their interaction with the MHC protein.

Comparison of the HLA-A2 and Aw68 structures revealed that the α carbon backbone of the two HLA molecules was almost superimposable despite the 13 amino acid differences between the two alleles. Eleven differences faced into the antigen binding site and individually had very little affect on its structure. However, taken together the eleven differences produced substantial modifications in the topography and chemical nature of the binding site. The alterations were principally in the shape and charge distribution of the binding site, and in a number of specific ligand interaction pockets. Differences in the specificity pockets between MHC alleles suggested that interacting peptides would have to possess different patterns of amino acid side chains to bind. The combined changes in the overall shape and size of the site, as well as the individual pockets, provided a structural basis for the allelic specificity of peptide binding by the site and the resultant differences in immune responsiveness of different MHC alleles. A number of residues which were conserved between different alleles, resulted in specificity pockets which also were conserved between alleles. This suggested, as mentioned before, that particular structural features of a ligand may allow it to interact with several different MHC alleles by interacting with specificity pockets common to the different binding sites.

1.6.3. Models of class II structures

Solution of the crystal structure for a class II MHC protein has not yet been achieved, however, the structural and functional homology between the class I and class II proteins has allowed models of class II binding sites to be derived from the details of the class I binding site (Brown et al., 1988). As already described (Introduction, 1.2), the domain structures of the class I and class II molecules are highly homologous. A class II protein can be "made" by breaking the link between the α1 and α2 domains of class I and linking the α1 domain to β_2-microglobulin. The

intramolecular dimeric interaction seen between the α1 and α2 domains of class I, in which two β pleated sheets combine to form one, has also been seen in intermolecular interactions indicating that such an interaction could be preserved between the α1 and β1 domains of class II (Bjorkman et al., 1987; Brown et al., 1988).

In order to derive an approximate model of a class II binding site from the class I protein the sequences of the α1 and β1 domains of class II were aligned with the α1 and α2 domains, respectively, of class I (Brown et al., 1988). This alignment was made primarily on the basis of a number of conserved features in all class I and II molecules. For example, a glycosylation site at residue 86 of HLA-A2 was seen to be conserved in all α1 domains of both class I and II MHC proteins. Similarly a disulphide bridge was conserved in all class I α2 and class II β1 domain sequences examined (Brown et al., 1988).

Within each of the membrane distal domains of class I and class II proteins similar patterns of polymorphic and conserved residues allowed sections of the molecules to be aligned. For example, in the class I α1 and α2 helical regions every third or fourth residue was conserved and these residues formed one face of the helices (α1; 68, 72, 75, 78, 84 and α2; 148, 153, 154, 157, 158, 161, 164, 165, 168). These conserved residues were interspersed with some of the most polymorphic segments of class I. Similar patterns of conserved and polymorphic residues also were present in the class II sequences allowing these residues to be placed into a binding site which was modelled on the dimensions of the class I site (Brown et al., 1989). Similar alignments could be made with residues of the β pleated strands (Brown et al., 1989).

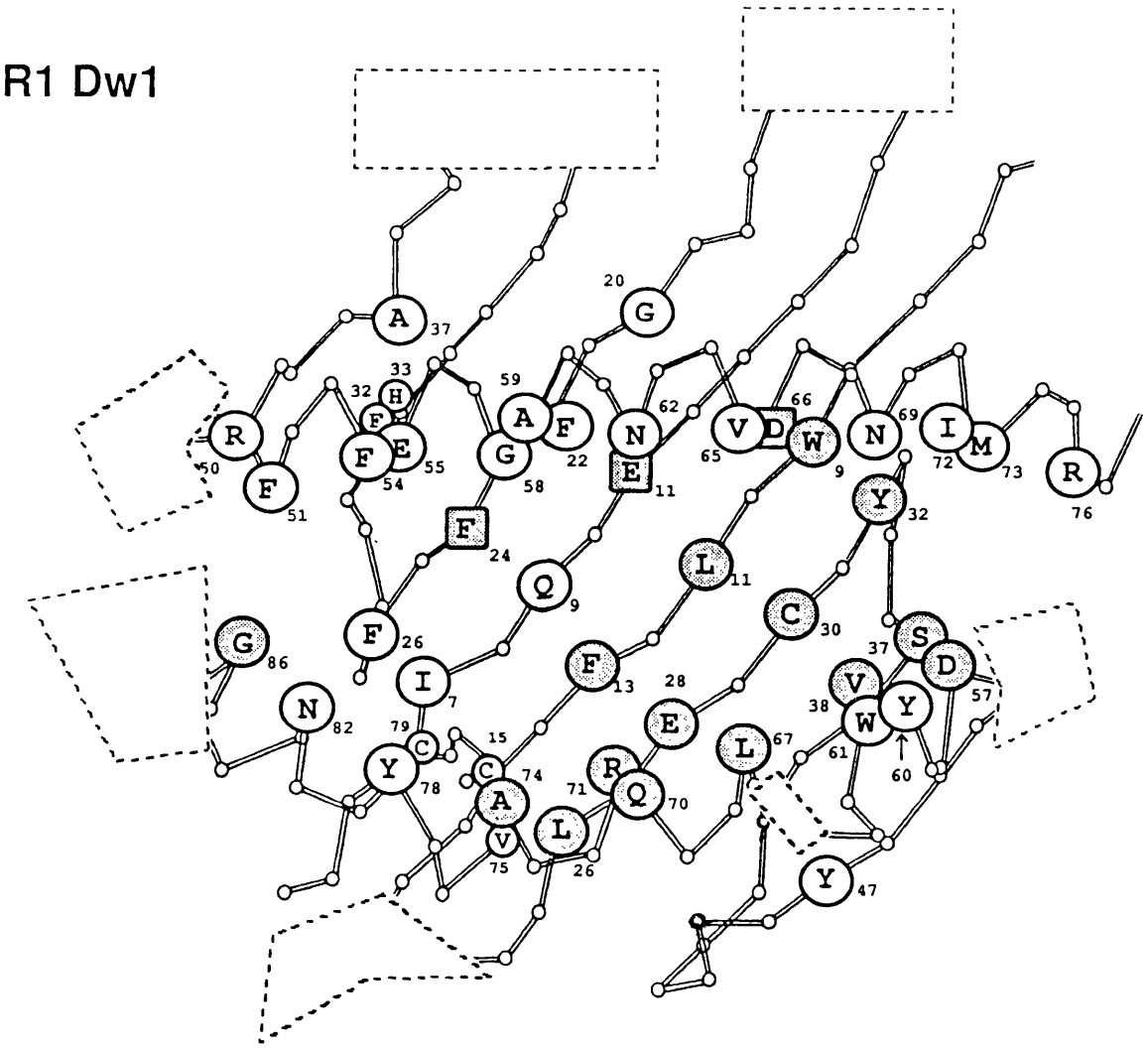
As an example, the binding site of HLA-DR1Dw1 is shown in Figure 1.6 based on the data given in the Bjorkman et al.(1987a; b), Brown et al (1988), and Garret et al (1989) papers and a Figure from Rothbard et al. (1989). As in the class I binding site there are regions of the binding site which are polymorphic and regions which are conserved. In the case of HLA-DR molecules the α chain is conserved in all

Figure 1.6.

Binding site of HLA-DR1Dw1

Residues which potentially make up the antigen binding site are indicated. Residues which are polymorphic in class I and class II sequences (Brown et al., 1988) are shaded. The residues in rounded squares are within the α chain of HLA-DR and are therefore not polymorphic within DR, although they are polymorphic within HLA-DQ and mouse class II molecules. The patterns of polymorphic and conserved residues within the MHC sequences can be used to align the binding sites between class I and class II molecules (Brown et al., 1988; see text). The figure is adapted from Rothbard et al., 1989a.

HLA-DR1 Dw1


A 37
G 20
H 33
F 32
A 59
F 22
N 62
V 66
D
W 9
N 69
I 72
M 73
R 76
R 50
F 51
F 54
E 55
G 58
E 11
F 24
Y 32
L 11
Q 9
C 30
S 37
D 57
F 26
F 13
G 86
I 7
N 82
C 79
C 15
E 28
V 38
W 61
Y 60
L 67
Y 78
A 74
R 71
Q 70
V 75
L 26
Y 47

alleles (Kappes and Strominger, 1988) and hence one end of the binding site is highly conserved in all DR proteins. The patterns of polymorphic and conserved regions of the binding site are consistent with the existence of polymorphic binding pockets which control the allele specificity of binding to the MHC, while conserved features would allow peptides able to interact predominantly with these conserved features, to bind several different alleles.

1.6.4. Summary

The molecular details of the binding site of HLA-A2 and Aw68 class I molecules have provided information about the dimensions of the peptide binding site and have suggested possible mechanisms for allele specific binding, the broad specificity of the site, and the ability of peptides to bind to more than one MHC protein. Models of the MHC class II peptide binding site, based on structural similarity between the two classes of protein, suggest that the molecular mechanisms of peptide binding in the site may be similar for class I and class II proteins. The resolution of the endogenous ligands bound to HLA-A2 and Aw68 was insufficient to determine details of the peptide-HLA complexes. To determine details of the mechanisms by which MHC proteins bind a wide range of peptides with limited specificity has required additional analyses of the complexes using both functional and direct binding experiments.

1.7. Mechanisms of formation of peptide MHC protein complexes

1.7.1. Kinetics of complex formation

As has already been discussed, isolated complexes between purified MHC molecules and peptide ligands were found to be extremely stable (Buus et al., 1986; Chen and Parham, 1989). A summary of the association and dissociation rates measured for several different complexes is shown in Table 1.II. All detailed kinetic

Table 1.II. Kinetics and Thermodynamics of Peptide MHC Protein Interactions

		Rate Constants		Equilibrium Constant	
Peptide	**Class II**	**Association ($M^{-1}s^{-1}$)**	**Dissociation (s^{-1})**	**Dissociation (μM)**	**Reference**
OVA 323-339	I-A^d	1.9	1.6×10^{-5}	8.4	Buus et al, 1986
Cyt c 88-104	I-E^k	~10	6.3×10^{-6}	~0.63	Sadegh-Nasseri and McConnell, 1989
HEL 46-61	I-A^k	0.87	--	--	Roof et al., 1990
Mat 17-31	DR1Dw1	10	2.5×10^{-6}	0.25	Jardetzky et al., 1990
Ha 306-320	DR1Dw1	120	1.6×10^{-6}	0.013	Roche and Cresswell, 1990

Key: OVA 323-339 Ovalbumin residues 323-339

Cyt c 88-104 Pigeon cytochrome c residues 88-104

HEL 46-61 Hen egg lysozyme residues 46-61

Mat 17-31 Influennza matrix residues 17-31

Ha 306-320 Influenza haemagglutinin residues 306-320

studies so far have involved class II MHC proteins. The dissociation rate constants measured in each experiment were extremely low (~10^{-6} s^{-1}), consistent with the observed stability of the complexes. However, the association constants were often slow as well (0.5 - 10 M^{-1} s^{-1}) resulting in overall equilibrium dissociation constants between 0.25 and 8.4 μM. An exception is the recent study by Roche and Cresswell (1990) on the binding of influenza haemagglutinin residues 306-320 to HLA-DR1Dw1. They demonstrated an association constant that was faster than the others shown in Table 1.I (120 M^{-1} s^{-1}) and with a low dissociation constant (1.6 x 10^{-6} s^{-1}) the overall K_D was 13 nM.

These kinetics were obtained with peptides binding to purified MHC proteins in detergent solution and therefore may be the product of in vitro conditions. However, binding of a biotinylated haemagglutinin peptide to cell surface HLA-DR1Dw1 had an association constant of ~ 1.3 M^{-1} s^{-1} and the dissociation was extremely slow (20% in 5 hours) (Busch et al., 1990). Also, the rate of binding of an iodinated influenza matrix peptide to cellular HLA-DR1Dw1 (Ceppelini et al., 1989) was consistent with the binding constants calculated by Jardetzky et al (1990) for purified HLA-DR1Dw1 . Therefore, the slow on and off rates seem to be a fundamental property of MHC peptide interactions.

The kinetics of the interaction between a fluorescently labelled pigeon cytochrome c peptide and I-E^k have been examined in detail and found to be inconsistent with a single step reaction (Sadegh-Nasseri and McConnell, 1989). Two complexes between the peptide and H-2 molecule were detected, the first was short lived and was a kinetic intermediate on the way to formation of the second stable complex. The rates of association and dissociation of the second complex were approximately 100 times slower than those of the first complex and were of the same order of magnitude as those reported for other peptide MHC protein interactions (Sadegh-Nasseri and McConnell, 1989). The kinetics indicated a slow conversion

from the intermediate to the final complex, which was consistent with possible changes in the conformation of the MHC protein, after initial binding of peptide, to form the final complex.

1.7.2. Conformations of MHC proteins

Several more direct lines of evidence indicate that conformational changes in MHC molecules may be involved in the formation of stable complexes between peptides and MHC proteins. Recently, the effect of different lipids on the binding of peptides to mouse class II molecules has been examined (Luescher et al., 1990; Roof et al., 1990). Reaction of a photoreactive compound coupled to a peptide bound to I-A^k, was predominantly with the α chain in the presence of lysophosphatidylcholine, the β chain in the presence of lysophosphatidylserine, and both chains were labelled equally in an equimolar concentration of MEGA 8 and MEGA 9 detergents (Luescher et al., 1990; Roof et al., 1990). This indicated a conformational flexibility in the class II molecule dependent on the presence of different lipids. Further studies demonstrated that the presence of different lipids could alter the rates of association of a hen egg lysozyme peptide with I-A^k. In the absence of detergent the association rate was measured as 0.87 M^{-1} s^{-1} but in the presence of lysophosphatidylserine it increased to 5.5 M^{-1} s^{-1} (Roof et al., 1990) suggesting that changes in conformation of the class II molecule could increase its ability to bind peptide. Since the lipids were all naturally occurring this implied that changes in the conformation of the class II molecule could be involved in normal peptide binding.

Analysis of mouse class II molecules by SDS polyacrylamide gels revealed the existence of two possible forms of heterodimer, termed "floppy" and "compact" (Dornmair et al., 1990b). Treatment with varying pH and temperature revealed that it was possible to convert from the "compact" to the "floppy" conformations, and from "floppy" to isolated chains (Dornmair et al., 1990b). Further experiments have revealed that each form of both I-E^k and I-A^d, including the isolated chains, can

specifically bind peptide (Dornmair et al., 1990b; Rothenhausler et al., 1990). Although the relevance of these different forms of class II to binding events which take place in vivo has not been analysed, a mechanism of binding can be imagined in which peptide interacts with a partially unfolded class II MHC protein, or even with individual chains, which then results in conformational changes and alterations in subunit interactions leading to the formation of a final stable complex.

Consistent with conformational changes in the MHC protein being involved in peptide binding is the temperature dependance of the interaction. The thermodynamics and kinetics of the interactions between, for example, antibodies and antigen can be modulated by varying the temperature, however, the formation of peptide MHC protein complexes is critically dependent on temperature. At 4° C there has been shown to be little if any detectable binding to the MHC protein (Buus et al., 1987b; Busch et al., 1991) and most assays have been performed at 37° C. This is consistent with a certain amount of thermal energy being necessary to allow the MHC protein to undergo conformational changes, upon binding peptide, to form stable peptide MHC protein complex.

A number of experiments have indicated that class I molecules also may undergo conformational changes when binding peptide. Incubation of cells, expressing HLA-A2, with an immunogenic peptide and an anti-class I antibody MA2.1 dramatically increased the amount of peptide bound by the class I molecules as measured by a cytotoxicity assay (Bodmer et al., 1989a). The antibody inhibited cytotoxicity if left in during the assay and is known to interact with at least one side of the binding site. The results suggested that by binding near the antigen combining site the antibody could stabilise a conformation important for the binding of peptide by class I molecules.

Recently, a mutant cell line, RMA-S, also has indicated that peptide binding by class I molecules might involve conformational changes. The RMA-S cell line

expressed normal amounts of non-mutant class I heavy chains and β2 microglobulin but the cell surface expression of normal heterodimers was reduced (Townsend et al., 1989). A decrease in temperature from 37° C to 26° C resulted in the formation of detectable class I heterodimers and these could be stabilised at 37° C if peptide was added to the cells (Ljunggren et al., 1990). Addition of peptide to the RMA-S cells at 37° C also increased the expression of detectable class I heterodimers (Townsend et al., 1989; Ljunggren et al., 1990). The ability to induce the formation of D^b heterodimers correlated with the ability of the peptides used, to bind D^b molecules. These experiments were unable to determine whether the stabilised class I was already present on the cell surface and simply undetectable by the available antibodies or whether the peptide induced transport of intracellular class I molecules to the cell surface. However, the experiments were consistent with peptide binding involving conformational changes and stabilisation of class I molecules.

Hence, both the kinetic data, and the experiments on conformation, were consistent with conformational changes of MHC proteins being involved in peptide binding. However, no direct experiments have been undertaken to demonstrate this either in vitro or in vivo.

1.7.3. Occupied MHC proteins

In all the binding studies undertaken so far it has proven impossible to fully saturate the isolated class II or class I proteins with peptide. For example, at equilibrium, Scatchard analysis demonstrated that only 20 % of an I-A^k preparation was available for binding by a hen egg lysozyme peptide (Roof et al., 1990). There have been two reports of greater than 50 % of class II being available for binding (Sadegh-Nasseri and McConnell, 1989; Babitt et al., 1985) but the majority of cases have reported between 1 and 20 % available (Watts and McConnel, 1985; Buus et al., 1987b; Ceppellini et al., 1989; Busch et al., 1990; Jardetzky et al., 1990; Roche and Cresswell, 1990). For class I binding the interactions between peptide and HLA in

solution indicated that only 0.3 % of the class I was available for binding (Chen and Parham, 1989), however the solid phase assay suggested that up to 40% of the class I was available (Bouillot et al., 1989). These differences may be due to assay methods used but are both consistent with the majority of the HLA molecule being unavailable for binding.

The solution of the crystal structure for HLA-A2 and Aw68 suggested that the purified HLA molecules were occupied with autologous peptides and this might explain the inability of further peptide to bind (Bjorkman et al., 1987; Garrett et al., 1989). Consistent with this was the isolation of endogenous peptides by acid treatment of class I (Rotzschke et al., 1990; Falk et al., 1990; Van Bleek and Natheson, 1990) and also class II molecules (Demotz et al., 1989; Buus et al., 1988). This suggested that binding of peptides to purified MHC proteins involved either, displacement of low affinity resident peptides, or a limited population of "empty" class I or class II molecules present in the purified material.

Recently, treatment of mouse class II molecules with reducing agent resulted in the release of endogenous peptides and protein unfolding (Dornmair and McConnell, 1990). Re-oxidation and refolding resulted in an increased peptide binding capacity of the class II protein. The experiment indicated that "empty" class II molecules could be formed and could bind peptide (Dornmair and McConnell, 1990). This did not mean that MHC proteins occupied with peptide could not bind other peptides with a concomitant displacement of the endogenous ligand. However, since only a minority of mature MHC protein was able to bind exogenous peptide suggested that the majority of stably bound peptides in a purified preparation of class II could not be displaced easily.

Evidence for existence of "empty" class I molecules has come from the experiments with the RMA-S cell line (Townsend et al, 1989; Ljunggren et al., 1990; Introduction, 1.7.2.). At 26° C these cells expressed heterodimers on the cell surface

but at a fraction of the level of the non-mutant cell line. However, the RMA-S cells at 26° C were able to act as targets in CTL assays at much lower concentrations of peptide compared to the non-mutant cell line at the same temperature. This suggested that the RMA-S cell line expressed "empty" class I proteins which were able to bind and present exogenously added peptide more efficiently than the normal cell surface class I.

1.7.4. Binding of peptide to class II proteins in vivo

By considering the role of the MHC class II protein associated invariant chain in class II maturation and peptide binding, possible mechanisms by which peptides bind class II molecules in vivo have been derived. Newly synthesised class II heterodimers are associated with a third polypeptide known as the invariant chain, which subsequently dissociates from the class II HLA antigens prior to their expression at the cell surface (Accolla et al., 1985). The dissociation seems to involve a multi-step proteolytic digestion of invariant chain, the first step produces fragments of the invariant chain which remain bound, and the second step , involving leupeptin sensitive enzymes, leads to dissociation of the remaining fragments of the invariant chain from class II molecules (Blum and Cresswell, 1988). As well as being involved in class II protein transport, a second set of experiments have demonstrated another role for the invariant chain. Comparison of peptide binding to purified mature HLA-DR5 molecules, and purified invariant chain associated HLA-DR5 molecules, revealed that the presence of the invariant chain inhibited peptide binding (Roche and Cresswell, 1990). Removal of the invariant chain from immature HLA-DR5 molecules resulted in approximately 90% of the class II protein being able to bind peptide compared to approximately 8% of the mature class II protein. Therefore, removal of the invariant chain seems to have resulted in a form of "empty" class II which was able to bind peptide (Roche and Cresswell, 1990).

These experiments were consistent with a model in which class II is synthesised and bound by the invariant chain which accompanies the class II molecules to a

compartment where the invariant chain is removed by proteolytic events. The resulting class II is then able to bind processed antigen (either endogenous or exogenous) and present it at the cell surface. Mature class II which is able to bind peptide antigen at the cell surface could either be molecules which have just lost invariant chain and been transported to the cell surface without antigen, or could still retain fragments of invariant chain which have not yet dissociated, or could have bound low affinity peptides which can easily be displaced.

1.7.5. Class I peptide binding in vivo

The RMA-S cell line (Townsend et al., 1989; Ljunggren et al., 1990) has provided an interesting insight into the mechanisms of peptide binding by class I molecules in vivo. As already mentioned, at 26° C this cell line was able to present exogenously added peptide to CTLs at much lower concentrations of peptide than the non-mutant cell line. However, at the same temperature the RMA-S cell line was unable to present endogenously synthesised minor histocompatibility antigens or influenza antigens (Ljunggren et al., 1990), whereas the non-mutant cell line successfully presented both antigens at 26° C. The lack of detectable class I on the surface of the mutant cells seemed to be due to an inability of the cell to "load" the class I intracellularly with peptides from endogenously synthesised proteins, and implied that a specific protein or group of proteins existed to perform this function (Ljunggren et al., 1990).

1.7.6. Summary

The experiments outline above have suggested that empty class I and class II molecules can be formed and can bind peptide. However, the presence of such class I and class II molecules within purified preparations of MHC proteins is unknown. Hence the limited population of MHC molecules within a purified preparation, which is able to bind peptide, remains poorly defined. Most likely it consists of either a small

number of empty MHC proteins which have co-purified with the rest of the MHC protein, or a small population of protein which contains easily displaced peptide ligand.

There is much evidence that the structural mechanisms of peptide binding by both class I and class II molecules may be similar. Firstly class I and class II proteins have significant similarities in the structures of their respective binding sites. Also, conformational changes in both classes of proteins have been shown to be involved in the mechanism of peptide binding. Finally (see later, Introduction 1.8.4) several peptides have been shown to be able to bind both class I and class II proteins. However, class I and class II molecules present T cells with different groups of determinants from different proteins during antigenic challenge, for example, during a viral infection (Germain, 1986; Braciale et al., 1987; Townsend and Bodmer, 1989). Class I molecules generally present peptide fragments of endogenous antigen, whereas class II molecules generally present peptides from exogenous antigen. This broad distinction between the sources of antigen, combined with similarities in the way that both class I and class II molecules bind peptide, suggests that the discrimination between class I and class II epitopes does not take place solely at the level of peptide MHC protein interactions.

Some of the experiments outlined above offer a possible model for how this discrimination occurs. In this model class I proteins are synthesised as empty molecules which are available to bind any peptide fragments of antigen present at the site of synthesis or within any compartment through which they pass on the way to the cell surface. In general these peptides will be from endogenously synthesised proteins. The accessibility of these peptides to the MHC proteins is regulated by a specific cellular mechanism which is involved in "loading" class I with peptide. In contrast, class II molecules are synthesised with the accompanying invariant chain which inhibits the binding of peptide until it is proteolytically cleaved and removed. As this occurs in an endosomal compartment the empty class II will tend to be exposed to degraded

exogenously added antigen. Therefore, class II and class I molecules are exposed to antigen at different locations in the cell and this accounts for different subsets of peptides, from different antigens, bound to each class of protein.

1.8. Peptide MHC Protein complexes

The experiments outlined above have provided details of the specificity of the interaction between peptides and MHC proteins and the fundamental role these interactions have in immune responsiveness. They have also described the structure of the proteins involved and possible mechanisms involved in the formation of peptide MHC protein complexes. In order to help understand the structural mechanisms which allow the MHC binding site to form stable complexes with a diverse, but limited, range of peptides, the structural and chemical features of peptide MHC complexes themselves must be examined.

1.8.1. Empirical analyses of structural features in T cell determinants

One approach to understanding the chemical and physical structure of peptide MHC complexes is to study the structural characteristics of the known T cell determinants. The demonstration that T cell antigenic determinants could be defined by linear peptide fragments of proteins (Schwartz, 1985; Livingstone and Fathman, 1987; Rothbard and Taylor, 1988; Milich, 1989), and were not conformationally intact epitopes, has greatly simplified the analysis of T cell epitopes compared to analyses of B cell epitopes. Initially two groups examined the structural features present in T cell epitopes (DeLisi et al, 1985; Rothbard, 1986; Rothbard and Taylor, 1988), both to help in the practical task of predicting further epitopes in pathogens and also to help understand possible mechanisms of peptide MHC binding and/or antigen presentation. DeLisi and Berzofsky (1985) made the assumption that T cell determinants would interact with the surface of antigen presenting cells as amphipathic helices. They then examined the sequence of sperm whale myoglobin for the presence of patterns of

residues characteristic of amphipathic helices. They discovered a correlation between such helices and the presence of known T cell epitopes, which was statistically significant.

Rothbard and Taylor (1988) took a purely empirical approach when examining a panel of known T cell epitopes. Initially, Rothbard (1986) had observed that the majority of T cell determinants occurred at sites within globular proteins, which were not on the surface of the molecule. Examination of the primary sequence of the known T cell epitopes revealed the presence of a pair of hydrophobic residues in all of the determinants. Alignment of the epitopes based on this pair of residues revealed two characteristic patterns of amino acids, of either 4 or 5 residues (Rothbard and Taylor, 1988). These patterns were shown to be statistically significant and were useful in predicting a number of other unknown determinants (Lamb et al. 1987; Rothbard et al. 1988; Gotch et al., 1987; Sakai et al. 1988). Additionally, when the epitopes analysed were segregated by the MHC allele originally used to define them, and aligned based on the pair of hydrophobic residues, then allele specific sub-patterns were observed. For example, the known I-E^d restricted determinants could be aligned to reveal a hydrophobic residue at position one, the pair of hydrophobic residues at positions 4 and 5, and a positive charge at position 8 (Rothbard and Taylor, 1988). Since the specificity of the T cells elicited by the peptides was unique, such common features suggested that each peptide was binding to the I-E^d molecule in a similar fashion via these common residues. If so, this was consistent with the peptide binding as a helix with the residues at relative positions 1, 4, 5, and 8 forming one face of the helix (Rothbard and Taylor, 1988). These observations suggested that there were common conformational and structural features of peptides binding to MHC proteins, and that therefore there may be a common structural mechanism of binding.

1.8.2. Conformation of Peptides binding to MHC proteins

Although the observations outlined above were highly provocative, to determine the possible existence of a common mechanism of peptide binding required a more direct assay of the role of the conformational and structural features of T cell determinants in peptide binding. A number of groups have examined the possible conformations of peptides which bind to MHC proteins using both functional assays and direct binding experiments. For example, an initial functional experiment involved the response of a T cell hybridoma to a bacteriophage λ cI peptide restricted through I-A^d. The response could be competitively inhibited using a completely different I-A^d binding peptide from ovalbumin (Guillet et al., 1986; 1987). The residues which were important for T cell recognition of each peptide were known. By transferring only a few amino acids from the λ cI peptide into equivalent positions in the the ovalbumin peptide, a hybrid peptide was produced, which could be recognised by the λ cI specific hybridoma (Guillet et al., 1986). Similar results could be obtained using the λ cI peptide and hybridoma, and a nuclease peptide. These experiments strongly suggested that the different peptides were binding to the MHC molecule in a similar location and orientation (Guillet et al., 1986; 1987). Consistent with this was the identification of common structural features within these peptides, which suggested a common interaction site on the MHC protein (Guillet et al., 1987).

More extensive studies using similar strategies have been used by several other groups (Rothbard et al., 1988; Lorenz et al., 1989). The four residues critical for T cell recognition of a bovine ribonuclease peptide bound to I-A^k were identified. The ribonuclease epitope was then aligned with a second I-A^k restricted epitope from influenza haemagglutinin based on the presence of an I-A^d binding motif present in both peptides (Lorenz et al., 1989). Transfer of only five residues from the ribonuclease determinant into the haemagglutinin peptide resulted in a determinant which was as potent as the original ribonuclease peptide (Lorenz et al., 1989). Rothbard et al.

aligned two HLA-DR1Dw1 restricted determinants based on the presence of common structural features at positions 1, 4, 5, and 8. When the peptides were modelled as a helices these residues formed a common facade, which was assumed to be binding the HLA-DR protein (Rothbard et al., 1988). The opposite face of the helices they assumed to be contacting the T cell receptor. Based on this alignment of the peptides they were able to transfer T cell recognition between the peptides by exchanging 7 out of the 13 residues (Rothbard et al., 1988). Although the results were consistent with the hypothesis that the determinants were binding as helices, an insufficient number of substituted peptides were synthesised to rule out the fortuitous identification of clonally specific residues, particularly when the amino acids which were not exchanged were quite homologous.

Similar experiments have also been repeated with epitopes recognised by class I restricted cytotoxic T cells (Rothbard et al., 1989c). Residues were exchanged between two K^d restricted T cell determinants, one from nucleoprotein (NP) of influenza, and one from HLA-Cw3. Transfer of 6 amino acids from NP into the HLA-Cw3 peptide resulted in partial recognition by NP specific T cells and a 7th improved the potency. The residues transferred were again consistent with both peptides adopting a helical conformation when bound to H-2 K^d (Rothbard et al., 1989c).

The success of each of these experiments strongly suggested that the different pairs of peptides must have been binding in similar locations and with similar conformations in the MHC binding site. Further, the experiments of Rothbard et al. (1989c) implied that the determinants adopted a helical structure when bound to both class I and class II proteins.

The possible conformations of peptides bound to MHC proteins have also been analysed by comparing the conformational propensity of a T cell determinant with it's ability to stimulate specific T cells or bind MHC molecules. Pincus et al. (1983) performed theoretical analyses of an epitope from pigeon cytochrome c and determined

that the principal regions of the peptide involved in T cell recognition and MHC binding formed a statistical coil (residues 99-103). However, other regions of the determinant showed a high theoretical propensity to form an α helix and the helical segments were required for the peptide to remain antigenic. Hence a helical structure was inferred to be important to enable the peptide to interact with the MHC molecule. A second group have substituted proline spacer residues into a peptide, in between the three residues identified as important for binding to K^d (Maryanski et al., 1990). Theoretically, the proline spacers should inhibit the formation of an α helical structure. The substituted peptides were still able to bind K^d, which suggested that they did not form a regular α helix when bound.

A number of groups have examined the effects of structural alterations in the peptide outside of the critical MHC binding region, on the conformational propensity of the peptide, and on its ability to bind the MHC molecule. All α helices within proteins have an inherent macrodipole due to the arrangement of hydrogen bonds along the length of the helix. Stabilisation of this macrodipole can increase the helical content of even relatively short peptides (Shoemaker et al., 1987). To increase the macrodipole of a haemagglutinin peptide, residues 307-319, (Ha 307-319) the amino terminus was acetylated and the carboxyl terminus amidated to remove the terminal charges. The resulting peptide had an increased helical propensity as measured by its circular dichroism spectrum in a mixture of water and trifluoroethanol. However, its affinity for HLA-DR1Dw1 was unchanged (Rothbard et al., 1989b; Busch, 1991). Similar modification of a peptide from hen egg lysozyme residues 46-61 failed to increase the helicity, or the peptides affinity for $I\text{-}A^k$ (Allen et al., 1989).

Carbone et al. have added varying numbers of residues of the helically constrained amino acid α-aminoisobutyric acid to a cytochrome c peptide (Carbone et al., 1987). The modified peptides had a strong helical propensity in solution as determined by circular dichroism and were recognised by a T cell clone specific for

cytochrome c. However, modification of the peptide to reduce the measured helical propensity in solution did not affect the ability of the peptide to be recognised (Carbone et al., 1987). Two other groups have examined the affects of a large number of short stretches of natural amino acids attached to the ends of peptides to affect their conformation and ability to bind an MHC protein. Sette et al (1989e) added residues which had a theoretical propensity to form particular conformational structures, for example an α helix or a β sheet, to an ovalbumin peptide. They were unable to discover any correlation between regular conformation and binding to I-A^d. Amino acid additions to a peptide from the 65 kDa protein of Mycobacterium leprae resulted in variations in α helical propensity, as measured by circular dichroism, but no correlation was found between helicity and the ability of the peptides to stimulate an antigen specific T cell clone (Anderson et al, 1990).

In general, these last few studies have indicated that peptides bound to both mouse and human, class I and class II proteins, do not adopt a regular conformation, which is in apparent contrast to the "helix swap" experiments discussed previously. However, these last studies were based on theoretical analyses, or on analytical studies of the peptides in solution. The experiments did not assess the ability of the peptides to adopt a particular conformation when bound to the MHC binding site. A number of studies have attempted to define the orientation and conformation of bound peptides by correlating the inability of the determinants containing point substitutions to stimulate T cell clones, with their capacity to bind the restriction element. (Sette et al., 1987; Allen et al., 1987; Fox et al., 1987; Lambert and Unanue, 1989; Bhayani and Peterson, 1989; Regnier-Vigoruoux et al., 1989; Ogasawara, 1989). For example, when a comparison was made between the ability of Ala monosubstituted analogues of a hen egg lysozyme peptide to be recognised by a T cell clone, or to compete for presentation of the natural sequence, then the data were consistent with the peptide binding as a regular α helix (Allen et al., 1987). In a second study, several substitutions were made at each position in an ovalbumin peptide (residues 323-329), and the analogues assayed

for their ability to stimulate an ovalbumin specific T cell clone and to compete for binding of the natural sequence to purified I-A^d (Sette et al., 1987). Many more of the analogues failed to stimulate the clone than had failed to bind I-A^d. As one might have expected, the T cell receptor clearly had a greater sensitivity to modifications of the peptide, than the MHC protein. The analysis identified residues interacting with the T cell receptor, or with the MHC, or with both and in contrast to the studies of Allen et al. (1987), the peptide was deduced to be binding in an extended β sheet conformation (Sette et al., 1987). Several other studies attempting to identify T cell receptor and MHC protein contact residues failed to find evidence that peptides were binding with a regular conformation (Fox et al., 1987; Gotch et al., 1988; Ogasawara, 1989; Regnier-Vigoroux, 1989; Milich et al., 1989; Anderson et al., 1990). More radically, two groups have argued that the same peptide may adopt more than one conformation when bound to an MHC protein (Lambert and Unanue, 1989; Bhayani and Peterson, 1989). For example, Ala substitutions at each position in a lysozyme peptide (34-45) had little affect on the ability of the peptide to bind purified I-A^k, but had differential affects on recognition by two T cell hybridomas, abrogating the ability of at least one hybridoma to respond in all cases (Lambert and Unanue, 1989). The data were consistent with no one single amino acid being critical for binding to the MHC, and they also interpreted the data to be consistent with the hybridomas each recognising a different conformation for the bound peptide (Lambert and Unanue, 1989). However, the experiments were unable to determine whether the differences in the responses of the hybridomas were simply due to differential affinities of the T cell receptors for different parts of the peptide MHC protein complex or were in fact due to the peptide binding in more than one conformation.

Experiments using monosubstitutions with natural amino acids have successfully determined the chemical requirements for peptides to bind to particular MHC proteins. However, in the majority of cases they have failed to clearly define all the residues contacting the MHC protein and hence have failed to define the

conformation of the bound peptide. In retrospect the main stumbling block has been the broad specificity of the MHC protein binding site itself, which is designed to be extremely tolerant of substitutions with natural amino acids. The strategy of our group to circumvent this problem was to use the unnaturally large long chain biotinylated (LCB) lysine as a substitution at each position in a haemagglutinin peptide (Ha 307-319) (Rothbard et al.,1989b; Busch et al., 1990). The LCB lysine is considerably larger than the largest naturally occurring amino acid tryptophan (Figure 1.7), and therefore would be expected to have a much more radical affect on binding . The LCB lysine analogues were incubated with cell surface HLA-DR1Dw1 and the interactions quantitated using fluorescein conjugated avidin, which binds to the LCB, and flow cytometry. The resultant fluorescent signals were shown to be inversely proportional to the importance of the substituted residue in the formation of the peptide MHC protein complex and helped identify the residues critical in complex formation more clearly than using substitutions with natural amino acids. From the spacing of the contact residues a single conformation for Ha 307-319 was postulated consisting of a central core folded as two turns of a helix with the amino and carboxyl termini deviating from a regular conformation (Rothbard et al., 1989b). In order to determine if this conformation was common to several different determinants or unique to Ha 307-319 the same strategy has been applied to examine the effect of LCB lysine substitutions within several unrelated peptides. The results will be considered in detail in Chapter 4 of this thesis.

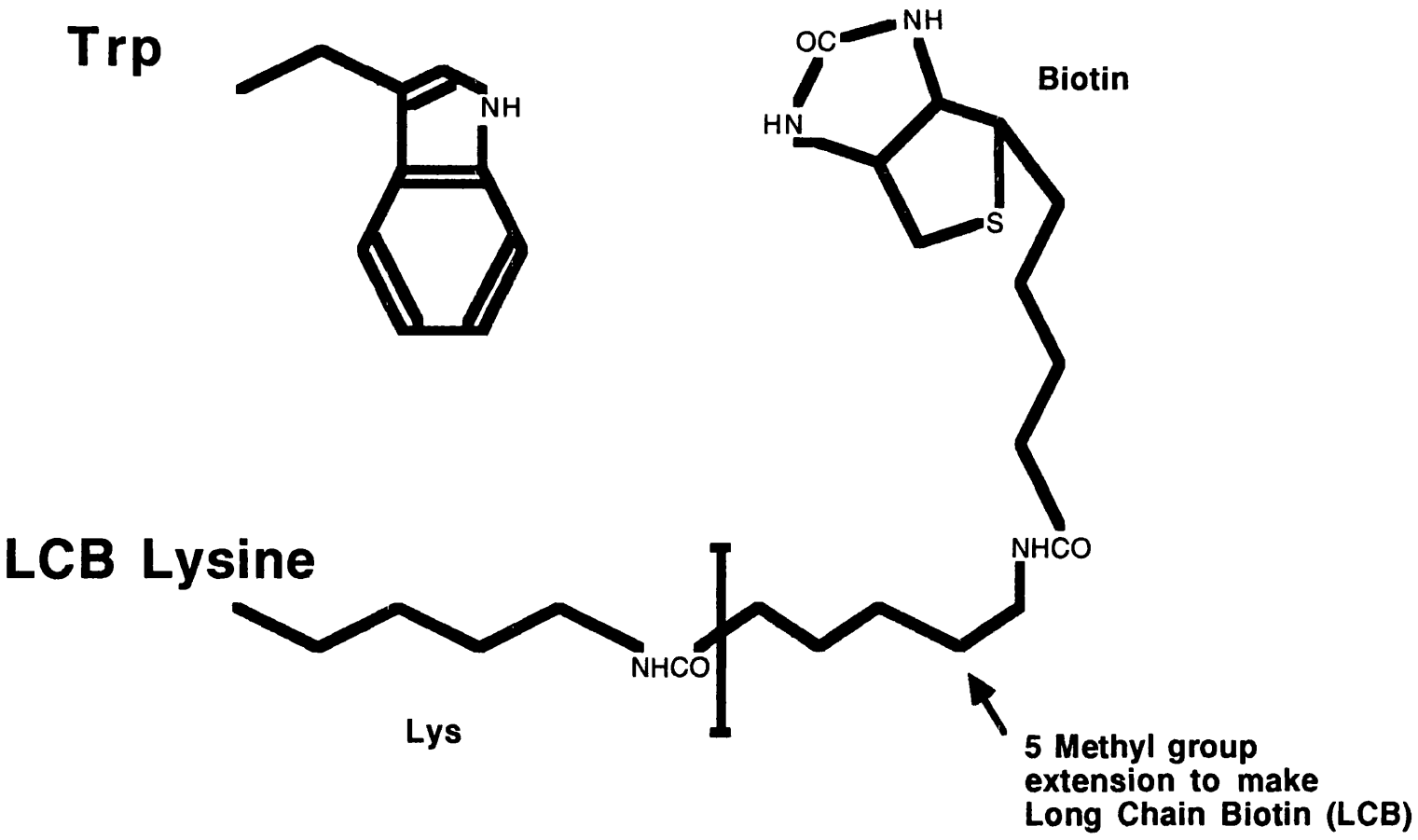
1.8.3. Structural features of peptides binding to MHC proteins

The empirical studies of DeLisi and Berzofsky (1985), and Rothbard and Taylor (1988) indicated that there were common structural features of peptides bound to MHC proteins and suggested that there may be a common structural mechanism of peptide binding. A number of studies have further analysed the structural requirements for peptides to bind MHC proteins, and have attempted to define specific structural

Figure 1.7.

Long chain biotinylated lysine

The size of LCB lysine is shown schematically in relation to the size of the largest naturally occurring amino acid, Trp.


Trp
NH
OC
NH
HN
S
Biotin
LCB Lysine
NHCO
NHCO
Lys
5 Methyl group
extension to make
Long Chain Biotin (LCB)

features of peptides, which are important for their interaction with the MHC binding site.

Solution of the crystal structure of HLA-A2 revealed a binding site compatible with a peptide of 8 amino acids bound in an extended conformation, or a peptide of 20 amino acids bound in a tightly coiled conformation (Bjorkman, 1987a; b; Bjorkman and Parham, 1990). However, the resolution of the crystal structure was unable to determine details of the peptides bound to the site. Generally, minimal peptide determinants capable of stimulating a T cell response are of approximately 7 residues in length (Livingstone and Fathman, 1987; Rothbard and Taylor, 1988; Milich, 1989), one of the shortest being a 5 amino acid cytotoxic T cell determinant from the nucleoprotein of LCMV (Whitton et al.,1989; Reddhase et al., 1989). However, for maximum stimulation the determinants are often of about 9-14 residues (Livingstone and Fathman, 1987; Rothbard and Taylor, 1988; Milich, 1989). This indicates that the critical residues within a peptide for MHC binding and/or interactions with the T cell receptor are contained within a central core of about 7 amino acids but that additional residues are necessary to maximise the MHC protein and/or T cell receptor interactions. Truncations of peptide antigens used in direct binding assays confirm these results. For example, although deletions of an ovalbumin peptide from both the amino and carboxyl termini, indicated a critical central core of 7 amino acids for binding to I-A^d, the ability of the peptide to bind was decreased with any truncated analogue shorter than about 14 residues (Sette et al., 1987).

Physical and chemical features within peptides, which are important for their interaction with MHC molecules, have been examined using large numbers of monosubstituted peptides in direct binding assays. The studies indicated that the peptide MHC protein complexes were highly insensitive to individual substitutions in the peptide (Sette et al., 1987; Lambert and Unanue, 1989; Sette et al., 1988; 1989a; Jardetzky et al., 1990; Busch and Rothbard, 1990). For example, interactions between

monosubstituted analogues of Ha 307-319 and purified HLA-DR1Dw1 (Jardetzky et al., 1990) revealed that only one position (309) was particularly sensitive to the substitutions used. However, the peptide had to be of a minimal length to bind to the MHC protein indicating that additional residues were making contributions to the binding but were not specifically required (Jardetzky et al., 1990; Busch, 1991).

These data were consistent with a number of experiments in which only the critical residues for binding within a peptide were retained, while each of the other residues were replaced by the same amino acid (Maryanski et al., 1990; Jardetzky et al., 1990). For example, Jardetzky et al. replaced all the residues in Ha 307-319 with Ala apart from the Tyr at position 309 and Lys at 316. This peptide bound with almost identical affinity to the original peptide although it required a minimal length to maintain this affinity (Jardetzky et al., 1990).

An alternative strategy to determine the important structural features of peptides binding to MHC molecules has been used by Sette's group (Sette et al., 1988; Sette et al., 1989a; c; O'Sullivan et al., 1990). For example they made monosubstituted analogues of a hen egg lysozyme (HEL) peptide and examined their ability to bind to I-E^d. They established three positions which were sensitive to substitution and hence likely to be making important contacts with the MHC protein. By truncating this peptide, and a number of other peptides, from the amino and carboxyl termini they established core regions which were important for the peptides to bind to the MHC protein. By comparing the three MHC protein contact residues, which were within the core region of the HEL peptide, with the sequences of the core regions of the other peptides they identified a structural motif characteristic of peptides binding to I-E^d (Sette et al., 1989a). Similar results identified motifs for peptides to bind to I-A^d molecules (Sette et al., 1988; Sette et al., 1989c). These motifs consisted of two or three broadly distinctive types of amino acids within a core of approximately 7 residues.

The conclusions from all these studies were that only a few residues were specifically required to enable a peptide to bind to an MHC protein but other residues were required to maximise the binding energy. These studies were consistent with the models of peptide binding proposed from the comparisons of the HLA-A2 and Aw68 crystal structure. These suggested that only a few residues from each peptide would be required to interact with particular specificity pockets in the MHC binding site. Remaining residues would interact with less specific regions of the site and would only become important if they made a negative interaction. The lack of specific structural features for a peptide to interact with an MHC protein binding site is highly consistent with the fundamental role of MHC proteins, which is to interact with a wide range of peptides with limited specificity.

The studies outlined above have successfully identified many of the chemical and structural features of peptides interacting with MHC proteins. However, much of the structural data has been hard to interpret fully because the strategies used failed to identify clearly all the amino acids in a peptide contacting the MHC protein. Unfortunately the problem is inherent in the MHC binding site, which is designed to tolerate a large number of natural amino acid substitutions within a peptide. Our own approach to establishing the structural features of a peptide important for its interactions with HLA-DR has been to first of all use LCB lysine analogues of several different peptides to establish their contact residues with the HLA molecule, and then to compare the structural features of these residues. Results of this approach are discussed in detail in Chapter 5 of this thesis.

1.8.4. Permissive binding

To investigate the physical and chemical properties of peptide MHC interactions, most of the studies considered so far have concentrated on varying the structure of the peptide. However, the natural polymorphism of the MHC can also be used to vary the structure of the MHC binding site, in the context of the same peptide.

In general, direct binding assays have demonstrated that peptides bind preferentially to one MHC molecule (Buus et al, 1987). However, several determinants were analysed which were capable of binding different alleles of I-A and I-E molecules (Buus et al., 1987; Sette et al., 1987; Sette et al,. 1989d). Analysis of the data indicated that there were greater structural similarities in the peptides binding to different alleles of the same locus rather than those binding different loci (Sette et al., 1989d). Hence peptides binding to I-A^d were more likely to bind I-A^k than to bind I-E^d and peptides binding different alleles of the same locus (I-A^d and I-E^d) were found to be using distinct but overlapping parts of the determinant (Sette et al., 1989d). Recently, Busch et al. have demonstrated that several determinants originally defined in the context of a single class II allele can bind to a wide range of HLA-DR alleles (Busch et al., 1990). Each peptide bound with high affinity to a different subset of the HLA alleles and varied in the extent of their permissive binding.

Functional studies have also demonstrated that certain peptides could be presented by both mouse and human class II proteins of different alleles (Panina-Bordignon et al.,1989; Sinigaglia et al., 1988). For example Sinigaglia et al. identified a T cell epitope within the circumsporozoite protein of malaria, which could be recognised in the context of more than 7 different DR alleles and elicited responses in more than 7 different strains of mouse (Sinigaglia et al., 1988).

Further functional experiments have demonstrated that class I restricted T cell determinants could also elicit class II restricted responses in mice (Perkins et al., 1989). More recently, Hickling et al. have used a cell surface binding assay to show that biotinylated analogues of a number of determinants, defined by class I restricted T cells, could bind to a range of HLA-DR alleles (Hickling et al., 1990). The results indicated that the structural features which enabled peptides to bind class I molecules would also allow a peptide to bind to class II molecules. Therefore, as previously

discussed, details of the mechanisms by which the two classes of proteins bind immunogenic peptides are likely to be the same.

The above analyses have relied on the natural polymorphism of the MHC binding site and have lead to the general conclusion that the structural features of peptide can allow it to bind to both mouse and human, and class I and class II MHC molecules. Detailed analyses of the effects of alterations in the MHC binding site have been limited (Rothbard and Gefter, 1991). Natural polymorphisms between alleles occur at multiple residues and hence the importance of individual changes in the MHC binding site cannot be defined. Site directed mutagenesis can be used to change individual residues, however, mutations that affect individual peptide MHC protein interactions, and mutations that affect the overall conformation of the binding site cannot easily be distinguished. Recent experiments by Busch et al. have illustrated the effect a single amino acid change can have on the overall binding site. They were able to show that the structural requirements for binding of Ha 307-319 to several HLA-DR proteins were dependent on the presence of a Val or a Gly at position 86 of the β chain (Busch et al., 1991). This single amino acid change in the DR molecule altered several of the interactions of the peptide with the MHC protein. These alterations could not be rationalised by considering only a local change in the MHC protein structure and indicated a change in the overall structure of the MHC binding site.

In conclusion the flexibility of the MHC binding site is such that the same peptide can bind to more than one MHC molecule as well as many different peptides being able to bind to the same MHC molecule. The ability of the same peptide to bind to different MHC proteins could be due to several different mechanisms. Firstly, the peptide may bind with different conformations and in different locations within the binding sites of the different alleles. Secondly, a peptide whose structure allows it to interact predominantly with conserved regions of different MHC binding sites may be able to bind with a common conformation and in a common location. To help address

this question Rothbard et al. have used LCB lysine analogues of Ha 307-319 to analyse the conformation of this peptide when bound to HLA-DR1Dw1, and HLA-DR4 and its subtypes (Rothbard et al, 1989a). Comparison of the conformations of the peptide bound to HLA-DR1Dw1 and HLA-DR4Dw10 suggested that the peptide was binding to each MHC protein with related but distinct conformations. However, the conformations of the peptide on the other HLA-DR4 alleles considered were remarkably similar to that adopted by the peptide when bound to HLA-DR1Dw1. This indicated that the peptide was interacting through similar residues and binding in a similar way to the different MHC proteins. In turn, this suggested that certain structural features of the peptide could be interacting predominantly with conserved features of the HLA-DR binding site. The definition of conserved structural features within MHC binding sites, which may be important in the formation of peptide MHC protein complexes, is considered in more detail in Chapter 5 of this thesis.

1.9. Scope of this thesis

The analysis of T cell determinants by Rothbard and Taylor identified common structural features within different epitopes, which were consistent with them interacting in a similar fashion with the MHC binding site. To initiate a more detailed analysis of further determinants and also to help understand the role of immune responses in the regulation of diseases associated with HPV type 16 infection, for example, cervical neoplasias, the predictive algorithm of Rothbard and Taylor was used to identify T cell epitopes in the proteins of HPV type 16 (Chapter 3). The epitopes were refined and their crossreactivity with other HPV types analysed. The class II restriction of the responses elicited by the determinants was also established and considered in detail.

A functional analysis of HPV type 16 determinants was limited in what it could contribute to our understanding of peptide MHC protein interactions. The development, in our laboratory, of an assay to quantitate the interactions of peptides

with cell surface HLA-DR molecules allowed the direct examination of conformational and structural features of peptides bound to HLA proteins. Therefore, the rest of the thesis (Chapters 4 and 5) describes the use of this binding assay to explore the interactions between HLA-DR and diverse peptides to help understand the structural mechanisms behind the broad specificity of the MHC protein binding site.

Chapter 2
Materials and Methods

2.1. Peptide synthesis and chemical modification

2.1.1. General

All reagents for synthesis were obtained from Applied Biosystems (Foster City, CA) or Bachem. Reagents for preparation, purification, and analysis were HPLC or analytical grade from Burdick Jackson or equivalent.

2.1.2. Synthesis

All peptides were synthesised on an Applied Biosystems 430A or 431A synthesiser (Applied Biosystems) using resins, tBoc or Fmoc protected amino acids, and coupling reagents from either Bachem or Applied Biosystems. Peptides were cleaved from the resin and the side chain protecting groups simultaneously removed using, anhydrous hydrofluoric acid for peptides synthesised using tBoc chemistry, or trifluoroacetic acid for peptides synthesised using Fmoc chemistry, with protecting group 'scavengers' where appropriate. Peptides were then extracted into 10% acetic acid (v/v) and lyophilised.

2.1.3. Analysis

Peptides were routinely analysed by high pressure liquid chromatography (HPLC) on a Hewlett Packard HP1090 with chemstation using a C18 column (Vydac, or Applied Biosystems) and a linear gradient of 1.25% acetonitrile per minute in 0.08% trifluoroacetic acid in water. Peptides less than 90% pure following synthesis were purified by preparative HPLC on C8 columns (Applied Biosystems) using a 1% per minute gradient with the above buffer system. Amino acid analysis was also routinely performed using acid hydrolysis and phenyl thiol carbamyl (PTC) derivatised amino

acids, on an Applied Biosystems 420A hydrolysis/derivitization system, 130A separation system, and 920A data analysis module.

2.1.4. Biotinylation

Peptides for biotinylation were synthesised with either a free α amino group or with a lysine residue at the position in the sequence where a long chain biotin (LCB) group was required. Where necessary, to avoid non-specific biotinylation of the free α amino group normally present on the peptide, the group was acetylated using acetic anhydride before the peptide was cleaved from the resin. Also to avoid non-specific biotinylation of lysine residues normally present in the sequence these were replaced with arginine residues during the synthesis.

After cleavage from the resin and lyophilisation peptides were biotinylated using NHS LCB (sulphosuccinimidyl-6-(biotinamido)hexanoate, Pierce). 20 mg of peptide was suspended in 4 ml of Dulbecco's phosphate buffered saline (PBS) (see below). 20 mg of NHS LCB (approximately 5 fold excess) was dissolved in the minimum volume of dimethyl sulphoxide (DMSO) / PBS (50% v/v), added to the peptide solution and stirred overnight at room temperature. The biotinylated peptide was isolated by reverse phase preparative HPLC and lyophilised. Biotinylation was confirmed using a spot test with dimethylaminocinnamaldehyde (0.1% w/v dimethylaminocinnamaldehyde, 1% H_2SO_4 in ethanol), which forms a red biotin adduct (McCormick and Roth, 1970).

2.2. Cell culture

2.2.1. Cell lines used

The cell lines used, their MHC haplotype, and source, are detailed in Table 2.I

2.2.2. Plasticware

Tissue culture flasks were from Costar, Corning, or Falcon and plates were from Nunclon or Costar.

Table 2.I. Cell Lines

Cell line	HLA Haplotype							Source
	DR	Dw	DQ	DP	A	B	C	
IBW4	1	1	5	4	3	35	4	TAL
MAJA	1	1	5	3?4	2,3	35	4	TAL
PGF	15 *	2	6	4	3	7	nt	TAL
RJ225	NE	NE	NE	NE	1	35	4	TAL
DR2Dw2A L cells	2	2A	NE	NE	NE	NE	NE	JT
DR2Dw2B L cells	2	2B	NE	NE	NE	NE	NE	JT

* PGF expresses the B1 gene DR2Dw2B and a second B gene DR2Dw2A (Marsh and Bodmer, 1989)

NE Not Expressed

nt not tested

TAL Tissue Antigen Laboratory, Imperial Cancer Research Fund (I.C.R.F.)

JT John Trowsdale and Dave Sansom in the Laboratory of Immunogenetics, I.C.R.F. Reference: Wilkinson et al., 1988.

Typing information is from Steve Marsh, Tissue antigen laboratory, I.C.R.F.

2.2.3. Media and supplements

General

All chemicals were tissue culture grade from Gibco or Sigma.

Complete IMDM

Mouse T-cell media was essentially as described previously by Corradin et al. and Chain et al. (Corradin et al 1977, Chain et al 1987). Briefly the media was made from powdered Iscoves modified Dulbecco's medium with 25mM Hepes and L-glutamine from Gibco BRL using double glass distilled water. The following supplements were added:

$FeCl_3$	(0.18 µg/ml)
Indomethacin	(1µg/ml)
Sodium selenite	(5ng/ml)
Na Pyruvate	(1mM)

which were from Sigma (Sigma) and:

Non Essential amino acids	
Penicillin/Streptomycin	(50IU/ml/50ug/ml)
Glutamine	(2mM)
2-mercaptoethanol.	(50µM)
Transferrin	(5µg/ml)
Foetal calf serum	(5%)

which were from Gibco BRL. This media will be abbreviated as '**complete IMDM**'.

Foetal Calf Serum

Foetal calf serum was batch tested for mouse T-cell proliferation assays and was heat inactivated at 56°C for one hour before use.

Complete RPMI

Epstein Barr virus transformed human B cell lines were maintained in liquid RPMI 1640 media from Gibco BRL with following supplements:

Penicillin/Streptomycin	(50IU/ml/50ug/ml)
Glutamine	(2mM)
Foetal Calf Serum	(5%)

which were from Gibco BRL. This media will be abbreviated as '**complete RPMI**'.

Complete DMEM

Mouse hybridomas producing monoclonal antibodies and L cell transfectants were maintained in liquid DMEM media from Gibco BRL with appropriate selective supplements where necessary (see below) and the following general supplements:

Penicillin/Streptomycin	(50IU/ml/50ug/ml)
Glutamine	(2mM)
Foetal Calf Serum	(5%)

which were from Gibco BRL. This media will be abbreviated as '**complete DMEM**'

PBS

Phosphate buffered saline (**PBS**) was made up according to Dulbecco's formula:

NaCl	8g/l
KCl	0.2g/l
Na_2HPO_4	2.16g/l
KH_2HPO_4	0.2g/l
pH 7.2	

Trypsin

0.25 % (w/v) trypsin in PBS

Versene

0.5 mM EDTA in PBS

2.2.4. Growth of Cell Lines

Epstein Barr virus transformed lymphoblastoid (EBV) B cell lines

EBV B cell lines were grown in complete RPMI medium at a density of between 2 x 10^5 and 10^6 cells / ml in sterile tissue culture flasks in a 5% CO_2 humidified incubator and diluted with fresh medium every 3 days. Their density and viability was assessed by visual inspection and by staining with a 1:1 dilution of 0.4% trypan blue in PBS and counting using a haemocytometer.

Growth of Hybridoma Cell Lines

Hybridomas producing monoclonal antibodies were maintained similarly to B cell lines except that they were grown in complete DMEM.

Growth of L cells and L cell transfectants.

L cells were grown at 37°C in a 10% CO_2 humidified incubator, in 10 cm tissue culture petri dishes, in complete DMEM, until confluent. At confluency they were washed three times with PBS, and 2 mls of a 50 % (v/v) solution of trypsin in versene added at room temperature, or 37°C, until the cells were loosened from the petri dish. 10 mls of complete DMEM was added and the cells split approximately 1:10 - 1:20 into 10 mls of fresh media.

Transfectants were generously provided by D.Sansom from the Laboratory of Immunogenetics, Imperial Cancer Research Fund. They were grown in the same way as L cells except they were split 1:5 - 1:10 and were grown in media supplemented with the following selection reagents (Wilkinson et al., 1988):

hypoxanthine	10^{-4} M
methotrexate	10^{-5} M
thymidine	1.6×10^{-5} M

2.2.5. Ficoll Enrichment

Viable cells were enriched to greater than 95% when necessary by Ficoll density gradient centrifugation. Typically 10mls of cells were layered onto 10mls of Ficoll (Histopaque, Sigma) and centrifuged at 2500rpm for 20 minutes in a Sorvall RT6000B centrifuge. The interface was collected and washed in complete medium, once at 1700 rpm for 7 minutes and again at 1500 rpm for 5 minutes. The cells were then counted and placed back into culture.

2.2.6. Cryopreservation

Stocks of cells were kept frozen in liquid nitrogen vapour at -296° C. Exponentially growing cell cultures were harvested and then spun down at 1500rpm for 5 minutes and carefully resuspended at approximately 10^7 cells / ml in ice cool 90%

FCS, 10% dimethylsulphoxide. The suspension was immediately transferred to cooled screw top freezing vials (Nunc), wrapped in tissue paper and transferred to -70°C overnight before transfer to liquid nitrogen the next day. Cells were thawed rapidly by gentle shaking the vials in a 37°C water bath till the pellet was just thawed when it was then transferred to a sterile tube and complete medium was added slowly to 15mls. The cell suspension was spun down at 1500rpm for 5 minutes, washed once again in 15 mls of complete medium and placed into culture.

2.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.3.1. General

All buffers and reagents were obtained from Sigma or from Bio Rad. Gel plates and apparatus were from Aladdin Enterprises or Bio Rad.

2.3.2. Method

SDS PAGE was performed essentially after the method of Laemmli et al 1970. Resolving gels were 1 mm thick and contained 10 or 12% total acrylamide monomer with an acrylamide to bisacrylamide ratio of 36.5 : 1 in 0.1% SDS, 300mM Tris, pH 8.6. Stacking gels were 20 mm high and contained 4% total acrylamide monomer in 0.1% SDS, 125mM Tris HCl, pH 6.8. Samples were boiled for 4 minutes in 1% SDS, 3% glycerol, 68mM Tris HCl pH 6.8 sample loading buffer with or without reducing agent (1mM of 2-mercaptoethanol) before loading on gel. Gels were run in 100mM glycine, 50mM Tris, 0.1% SDS, pH 8.3 running buffer at 25mA for 1.5 hours or until dye front reached bottom of gel.

All gels were run with protein molecular weight standards from BioRad. Standards were lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (42.7 kDa), bovine serum albumin (66.2 kDa), and phosphorylase b (97.4 kDa).

2.3.3. Gel Staining

Protein gels were routinely stained with Coomassie blue solution (25% ethanol, 0.25% Coomassie Blue) for 0.5 hours and then destained with several washes of 20% ethanol, 6.25% acetic acid in water.

2.4. Protein Determination

Protein determinations were performed by three methods. Firstly, by the UV absorbance at 280nm using a quartz cuvette in a Hewlett Packard Diode Array Detector UV/VIS spectrophotometer with chemstation . Secondly, by BCA assay (Pierce) as per the manufacturers instructions. Briefly, the reagents were mixed and added to the protein samples for 30 minutes at 37° C. Then the absorbance was read at 562nm with an ELISA plate reader (Molecular Devices). A standard curve of BSA, over the appropriate concentration range, was used with each assay. The third technique involved an antigen specific ELISA for HLA-DR determination.

The ELISA's were routinely performed by Maya Tanaka. Briefly 100µl of first antibody at 200µg/ml was added, in carbonate/bicarbonate buffer at pH 9.6, to ELISA plate wells (Falcon), and incubated at 37°C for one hour. The plate was washed twice with PBS/0.05% Tween 20 (Tween) and blocked with 200µl of PBS/0.1%BSA/ 0.05% Tween for 1 hour at room temperature. Plates were washed, and then 100 µl of samples and standards in PBS/0.1%BSA/1%OG added, over a concentration range, and incubated at room temperature for 2 hours. The plates were washed and 100µl of horse radish peroxidase (HRP) conjugated second antibody added in PBS/0.1% BSA/0.05% Tween for 1 hour at room temperature. The plates were washed and 100µl was added of a 0.16% OPD solution (w/v) in, 0.012% H_2O_2 in PBS (v/v). The plate was kept in the dark for approximately 15-30 minutes, or until the substrate had developed, and then the reaction stopped with 4.5N H_2SO_4. The plate was read with an ELISA plate reader (Molecular Devices) at 450nm.

2.5. Staining and FACScan analysis of cells using monoclonal antibodies

2.5.1. General

Bovine serum albumin was RIA grade fraction V from Sigma.

2.5.2. Staining of cells

Non-adherent cells were washed twice with 10 mls of PBS (spinning at 1500 rpm for 5 minutes). 2 x 10^5 cells were then plated out in 100µl of PBS in each well of a 96 well assay plate (Microtest III assay plates, Falcon), spun down in a plate carrier at 1400 rpm for 2 minutes, and resuspended in 100µl of ice cold PBS containing 0.1% bovine serum albumin (BSA) and the antibody of choice. Antibodies were used at saturating concentrations, generally between 10 and 25 µg/ml. The cells were then incubated on ice for 30-45 minutes before being spun down at 1400 rpm for 2 minutes using a 96 well plate carrier and washed twice with 200µl of PBS/0.1% BSA (spinning at 1400 rpm for 2 minutes). If the antibody itself was not directly conjugated to fluorescein then the cells were resuspended in 100µl of PBS/0.1% BSA containing fluorescent goat anti-mouse antibody (Becton Dickinson) at a dilution of 1:25 and incubated for a further 30-45 minutes before being washed twice with 200µl of PBS/0.1% BSA. At the end of the incubations the cells were resuspended in 200 µl of PBS/0.1% BSA and transferred to plastic test tubes (2052, Falcon) suitable for the FACScan flow cytometer (Becton Dickinson). If required, then 25µl of a 50µg/ml solution of propidium iodide (Sigma) was added at this time. This stained dead cells and enabled them to be gated out of subsequent analysis on the FACScan.

2.5.3. Analysis

Cells were then analysed using a FACScan flow cytometer, with an argon laser (488nm), 1024 channels, and running the standard FACScan research software. Normally 5000 events were collected from each, ungated, sample. In general

histograms of fluorescence 1 (fluorescein) channel number against cell number showed a single symmetrical peak. After gating the data, where necessary, to remove dead cells from the analysis, the mean fluorescence 1 channel number was calculated. Cell death was assessed by the fluorescence 3 signal due to propidium iodide staining (see above Chapter 2.5.2). The fluorescence 1 amplifier was normally set to logarithmic mode. This meant that the resultant channel number represented the log of the fluorescent intensity emitted by the cell. Therefore to enable quantitative comparisons of the fluorescent signals emitted by different cells the channel number for fluorescence 1 was converted to a 4 decade linear scale using the following formula:

Fluorescence signal = 10^(Channel number x 4 / 1023)

2.6. Antibody preparation.

2.6.1. Antibodies used

The antibodies used and their reported specificities are detailed in Table 2.II.

2.6.2. General

Buffers and chemicals were obtained from Sigma unless otherwise stated. Dialysis tubing was Spectra/por (12-14000 molecular weight cut off) from Spectrum Medical Industries. Protein A sepharose was CL 4B from Pharmacia LKB.

2.6.3. Antibody purification

Antibodies were prepared from tissue culture supernatants by first pre-clearing by spinning at 3000g for 20 minutes at 4°C. Supernatant was then buffered with 1/10 th volume of 1M Tris pH 8 and 1 litre loaded onto a 5 ml protein A sepharose column at 0.5-1 ml / minute. Alternatively the pre-cleared supernatant was ammonium sulphate precipitated at 4°C by slowly adding an equal volume of precooled saturated ammonium sulphate solution at pH 7 and stirring for 6 hours. The precipitated protein was then spun down at 10,000g for 15 minutes, resuspended in PBS and then dialysed

Table 2.II. Antibodies

Name	Specificity	Isotype	Source	Ref.
L243	HLA-DR	IgG2a	ATCC	Lampson and Levy, 1980
L227	HLA-DR*	IgG1	ATCC	Lampson and Levy, 1980
LB3.1	HLA-DR	IgG2b	ATCC	Gorga et al., 1986
SPVL3	HLA-DQ	IgG2a	HS	Spits et al., 1984
14.4.4S	I-$E^{k/d}$	IgG2b	ATCC	Ozato et al., 1980
HB3	I-A^{d}	unknown	ATCC	Kappler et al., 1981
H40	I-A^{k}	unknown	BC	None

Key:

* L227 reacts with immature and denatured DR β chains in immunoprecipitation and with most DR and some DQ and DP molecules when used in flow cytometry.

ATCC American Type Culture Collection

HS Hergen Spits, DNAX

BC Benny Chain, University College

against PBS three times before being precleared and loaded onto the protein A sepharose column. A loaded protein A sepharose column was washed with several column volumes of 100mM Tris pH 8, several column volumes of 10mM Tris at pH 8 and then eluted with 100mM glycine at pH 3 into tubes containing 1/10th fraction volume of 1M Tris at pH 8. The purified antibody fractions were detected by measuring the UV absorbance at 280nm or by BCA assay, the purified antibody fractions were pooled and then, either dialysed overnight against several changes of PBS, or simultaneously exchanged into PBS and concentrated using an Amicon concentrator (Amicon). The concentration of purified antibody was then calculated from the UV absorbance at 280nm assuming 1 OD is equivalent to 750µg/ml of antibody. Antibodies were stored at greater than 1mg/ml, at -70° C in small volumes.

2.6.4. Antibody integrity

Principally this was assessed by two methods. Firstly a reducing and non-reducing SDS polyacrylamide gel of the antibody was run, stained with Coomassie blue, and the antibody checked for contaminants and correct molecular weight. Secondly the antibodies were used to stain cells expressing the relevant antigen (Chapter 2.5) and the antibody was titrated over a wide range to compare its affinity with that of a standard preparation of antibody. Standard antibodies were in-lab stocks and, where available, commercially available antibodies (Becton Dickinson).

2.7. T cell Proliferation Assays

2.7.1. General

Mice were maintained at animal facilities at University College, London University, or the Imperial Cancer Research Fund.

2.7.2. Preparation of peptide antigen

Peptides were accurately weighed out and then dissolved at 1mg/ml in PBS in sterile tubes. When peptides would not solubilise then small amounts of acid or alkali

were added (depending on the peptide) to improve the solubility. Peptide solutions were then filtered using 0.22μm cellulose acetate filters (Corning). Control immunisations (see below) were made with PBS plus acid or alkali and in proliferation assays the highest concentration of peptide used was a 1:10 dilution of the starting solution and the pH of the media was unaffected by the acid or alkali. The molar concentrations of the peptide solutions were calculated based on their molecular weight and assuming all the peptide was solubilised.

2.7.3. Immunisation and harvesting lymph node cells

Mice were immunised with 50μl of a 1:1 emulsion of peptide and complete Freunds adjuvant (Difco) in the base of the tail. Eight days later the mice were sacrificed by cervical dislocation and the para-aortic and inguinal lymph nodes removed into PBS containing 5% foetal calf serum (FCS). The lymph nodes were pressed gently through a nylon mesh and the cells washed several times with 10 mls of PBS/5%FCS (spun at 1000rpm for 10 minutes with each wash) before being resuspended in complete IMDM.

2.7.4. Primary lymphoproliferative assay

Peptide antigen was plated out in triplicate in 10 fold serial dilutions in 96 well plates (Nunc) in complete IMDM. Lymph node cells were added to the plates at 3 x 10^5/well in complete IMDM. The responses of the cells to the peptides was assayed by measuring proliferation determined by the incorporation of ^{3}H-Thymidine (Amersham) added in PBS at 1μCi per well during the final six hours of a 3 day culture. Cells were then harvested onto filter mats (Skatron cell harvester), liquid scintillation fluid added and the filters counted (LKB Pharmacia beta plate counter).

2.7.5. Antibody blocking

To determine the MHC restriction of the proliferative responses lymph node cells were stimulated with 15μM of peptide and sterile filtered, protein-A sepharose

purified antibody was added at 10 fold serial dilutions of 50 µg/ml. Antibodies used included 14.4.4S (anti-I-$E^{k/d}$), HB3 (anti-I-A^{d}), and H40 (anti-I-A^{k}) (Table I).

2.8. Assay to determine binding of peptides to cell surface HLA-DR

2.8.1. General

All reagents were from Sigma, Vector labs, or Becton Dickinson. Fluorescent avidin D (FAD) from Vector labs contains a form of hen egg avidin which has been purified to decrease its non-specific binding characteristics which are as low as those of commercially available streptavidin from *Staphylococcus aureus* (Vector). FAD is purified avidin conjugated with fluoroscein iso-thiocyanate. The BSA used in all two layer FAD assays (see below) was crystalline grade, essentially immunoglobulin free, from Sigma.

2.8.2. Preparation of peptide solutions

All peptides and LCB analogues were dissolved in PBS at approximately 1mg/ml and then sonicated in a water bath sonicator to help solubility. The solutions were filtered with 0.22µm cellulose acetate filters (Corning) and stored in sterile polystyrene tubes or clean glass scintillation vials. Particularly insoluble peptides were initially dissolved in a small volume of DMSO which was diluted 100 fold into PBS before filtering as before to make a stock solution. Stocks were stored at 4°C in small volumes.

2.8.3. Concentration of peptide stocks

The concentration of filtered peptide solutions was determined by several different methods. If the peptide contained a single tyrosine residue then the concentration could be determined by measuring the UV absorbance at 278 nm assuming an absortion coefficient of 1.4 $M^{-1}cm^{-1}$.

For peptides which did not contain tyrosine residues analytical HPLC analyses were run and the integrated absorbance at 214, which is largely due to peptide bonds (Cantor and Schimmel, 1980), calculated. The integrated absorbance at 214 was divided by the number of peptide bonds (including those within the LCB group of biotinylated peptides) present in the peptide and this number compared to that from a standard peptide, at known concentration, run under the same conditions. The standard was normally a tyrosine containing peptide which could be analysed by UV spectrophotometry. Wherever possible the standard was a tyrosine analogue of the peptide being analysed because comparison between completely different peptide sequences lead to greater inaccuracies due to weak absorbances at 214nm by amino acid side chains (Cantor and Schimmel, 1980).

Concentrations were confirmed for all peptide stocks by quantitative amino acid analysis using 500 pmols of a non hydrolysable nor-leucine standard in each sample. Concentrations were checked regularly as certain of the less soluble peptides tended to form aggregates with prolonged storage.

2.8.4. Binding assay

Assays were performed in a 96 well assay plate (Microtest III assay plates, Falcon) using 2×10^5 cells per well in duplicate. EBV B cells were spun down, washed in complete RPMI and then added in 50µl of complete RPMI to 150µl of PBS containing the LCB analogue of interest at the appropriate concentration. L cells were washed twice with PBS and then removed from plates using 2-3 mls of versene at 37°C. The cells were washed twice in complete DMEM and then added to 96 well assay plates in the same way as EBV B cells, only in complete DMEM. The cells were incubated with peptide for 4 hours at 37°C in a humidified CO_2 incubator before being washed twice with 200µl of PBS/0.1% BSA (spinning at 1400rpm for 2 minutes) and incubated with 100µl of a solution of FAD in PBS, at 10µg/ml, at 4°C for 30 minutes. For some LCB analogue/cell mixtures the cells were then simply washed twice with

PBS/0.1% BSA and resuspended in 200µl of PBS/0.1% BSA, this staining procedure was abbreviated as a single layer FAD assay (Figure 2.1a). For greater sensitivity in the assay the cells were washed twice with PBS/0.1% BSA and incubated with 100µl of a solution of biotinylated anti-avidin (Vector) in PBS, at 10µg/ml, at 4°C for 30 minutes. The cells were then washed again and a second incubation with FAD performed before the cells were washed twice and resuspended in 200µl of PBS/0.1% BSA. This second staining procedure was abbreviated as a two layer FAD assay (Figure 2.1b). For both the single layer and two layer FAD assays 25µl of propidium iodide at 50µg/ml was then added to each sample and they were transferred to plastic test tubes (2052, Falcon) suitable for the FACScan. In each assay, cell samples were also stained with directly fluoresceinated L243 antibody (Becton Dickinson) to assess levels of HLA-DR expression.

Cells were analysed as for antibody samples (Chapter 2.5.3), with 5000 ungated events collected. Ungated histograms of cell number against fluorescence 1 (fluorescein) routinely contained two peaks, sometimes overlapping. Gating of the data by propidium iodide staining (fluorescence 3) to include only live cells, resulted in histograms of cell number against fluorescence 1, which showed a single symmetrical peak, indicating that one of the peaks in the ungated data was due to dead cells. Mean channel numbers were calculated from gated data and converted to fluorescence as previously detailed (Chapter 2.5.3).

2.8.5. Competition and Antibody Inhibition assays

Competition assays were performed as per the normal binding assay but to the 50µl of cells was added 50 µl of the LCB analogue peptide in PBS, and 100µl of competitor peptide in PBS at the appropriate concentrations. Similarly, antibody inhibition experiments were performed as above with 100µl of affinity purified antibody in PBS. In both cases the competitor and antibody were titrated over a range.

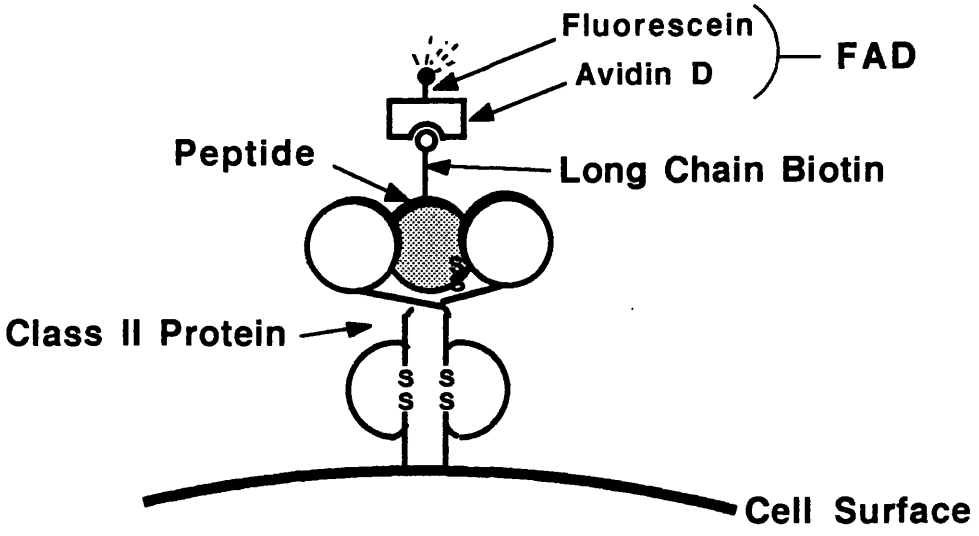
Figure 2.1.

Schematic diagram of cell surface binding assay

a) Single layer FAD assay. A peptide containing Long Chain Biotinylated (LCB) lysine is bound to a class II protein on the surface of the cell. The fluorescently labelled avidin D binds to the biotin group on the LCB and the fluorescent intensity of the cell is increased.

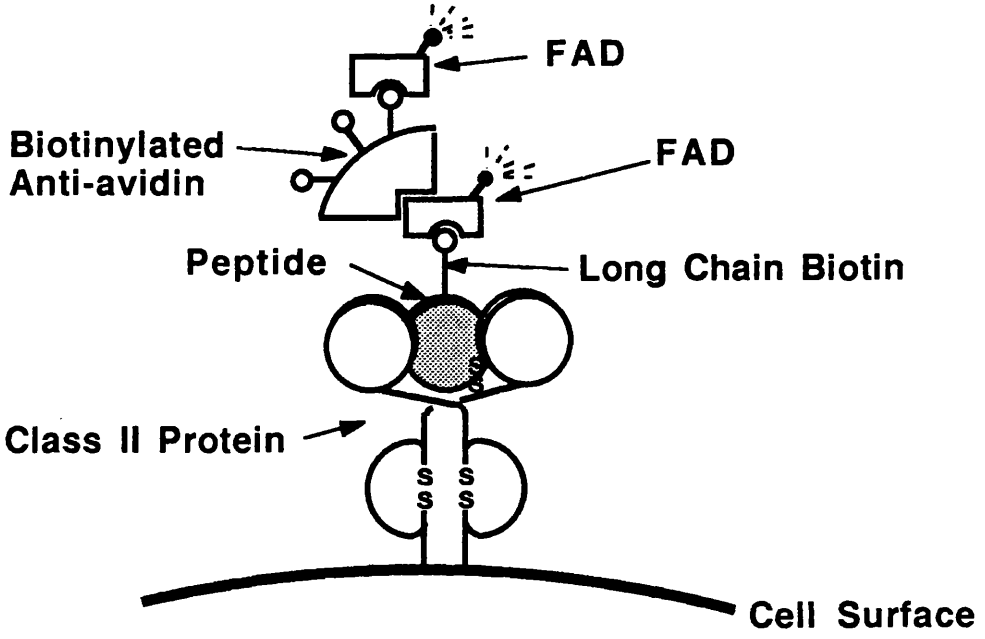
b) Two layer FAD assay. The two layer assay is similar in principle to the single layer assay, however, after the first layer of FAD has been added then a layer of biotinylated anti avidin is added, followed by a second layer of FAD.

a) Single Layer FAD Assay



b)

Two Layer FAD Assay



2.8.6. Protease Inhibitors

Assays with protease inhibitors were performed as per the normal binding assay but to the 50µl of cells was added 100µl of LCB analogue peptide solution and 50 µl of the following inhibitors: Leupeptin (3mM), pepstatin (400µM), and TPCK (400µM). The concentrations used were based on work by Berzofsky et al. (1989). The specificities of the inhibitors are indicated in Table 2.III.

2.9. Assay to determine binding of peptides to purified HLA-DR

2.9.1. General

Columns and column reagents were from BioRad or LKB Pharmacia. All chemicals were from Sigma except for octylglucoside which was from Calbiochem.

2.9.2. Preparation of Peptides

All peptides and peptide stocks were prepared in PBS as for the cellular binding assay (Chapter 2.8.2 and 2.8.3).

2.9.3. Class II Purification

Purified HLA-DR was routinely provided by Lisa Paborsky, Immulogic Pharmaceutical Corporation. The purification was essentially as described (Gorga et al., 1986). Briefly, EBV B cell lines were grown in complete RPMI and roller bottles (Costar), and then 10^{10} - 10^{11} cells were harvested using a SteriCell harvester (DuPont), washed in PBS and lysed in PBS, 1% NP40, 1mM phenylmethylsulphonyl fluoride. The lysate was precleared using IgG sepharose and the lysates passed over L243- or LB3.1-cellulose columns. After buffer exchange into 1% octylglucoside (OG) in PBS the proteins were eluted at 4°C using 50mM glycine, pH 11.5, 1% OG and immediately neutralised with 1M glycine, pH 2. The buffer was exchanged, using

Table 2.III. Protease Inhibitors

	Protease Inhibitor		
Enzyme	**Leupeptin 750 μM**	**TPCK 100 μM**	**Pepstatin 100 μM**
Papain	++	-	-
Plasmin	++	-	-
Cathepsin B	++	-	-
Cathepsin L	+	-	-
Trypsin	++	-	-
Chymotrypsin	-	++	-
Pepsin	-	-	++
Cathepsin D	-	-	++

Protease inhibitors used in cell surface binding assay along with their known specificities.

"++" Inhibits enzyme at the concentration shown.

"+" Partially inhibits enzyme at the concentration shown.

"-" Does not inhibit enzyme at the concentration shown.

Data is taken from Berzofksy et al., 1989, and from Boehringer Mannheim Biochemicals.

an Amicon ultrafiltration unit, to 10mM Tris pH 7.5 and the protein stored at 4°C. Protein concentration was routinely determined by BCA assay against a BSA standard curve and by ELISA using a standard DR preparation. The ELISA used LB3.1 as the first antibody and L227 as the HRP conjugate and hence only measured dimer present in the preparation.

2.9.4. Analysis of class II

Class II preparations were routinely analysed by PAGE and found to contain monomer, heterodimer, and small amounts of actin. Relative purity of preparations for dimer could be assessed by comparing the concentration based on ELISA with those based on the BCA assay as well as by visual inspection of gels.

2.9.5. Binding assay

Complexes between LCB peptide and HLA-DR were formed by co-incubation in 250µl of PBS/1% OG, overnight, at 37°C in round bottom 96 well plates (Costar tissue culture plates). Complexes were separated from free peptide by incubation with an antibody coated 96 well plate. The plate (Immulon 2 Stripwell) was a standard high protein binding stripwell ELISA plate and was prepared by a first incubation with 200µl of 10-50µg/ml LB3.1, at 4°C, for 1 hour or overnight. The plate was then washed 3 times using PBS/0.05% Tween (all washing steps were identical) and blocked with 300µl of 5%FCS in PBS, at 4°C, for 1 hour or overnight. After three more washes 100µl of 5%FCS/1%OG in PBS was added to each well and then 100µl of the peptide/HLA-DR incubation mix was added, in duplicate, to each well of the antibody coated plate and incubated for 4 hours at 4°C. The plate was washed three times and 200µl of ^{125}I-streptavidin added in PBS/0.1% BSA overnight at 4°C before washing the plate a final three times. Each well of the ELISA plate was then broken off counted in a gamma counter.

Initial experiments titrated both the peptide and HLA-DR over a wide concentration range. For HLA-DR1Dw1 a concentration of 10nM was found to not saturate the detection system used and is therefore used in all the experiments shown.

Chapter 3

Prediction and identification of T cell determinants within HPV type 16 proteins

3.1. Introduction

T and B lymphocytes differ fundamentally in the mechanism by which they recognise antigen. The antigen receptor of B cells (immunoglobulin) generally binds free, soluble, antigen and recognises conformationally dependent epitopes on the surface of an intact antigen (Benjamin et al., 1984; Rothbard, 1986). In contrast the antigen receptor of T cells recognises a linear peptide fragment of antigen which is bound to an MHC protein on the surface of another cell (Benjamin et al.,1984; DeLisi and Berzofsky, 1985; Berzofsky, 1986; Rothbard, 1986; Livingstone and Fathman, 1987; Rothbard and Taylor, 1988; Milich, 1989; Introduction, 1.3 and 4). As T cell epitopes consist of relatively short linear fragments of protein, this has made the identification and detailed analysis of these epitopes much simpler than that of conformationally dependent B cell epitopes.

3.1.1. Identification of T cell epitopes

T cell epitopes within a protein can be defined using a number of techniques (Livingstone and Fathman, 1987; Milich, 1989). Initial attempts were dependent on naturally occurring homologues of particular proteins, for example myoglobins or cytochromes (Livingstone and Fathman, 1987; Solinger et al., 1979). Species specific differences between these proteins were localised to only a few amino acids and hence differences in T cell responses could be localised to these areas of the protein. Similarly, recombinant and naturally occurring strains of viruses were used to localise T cell immune responses to a particular viral antigen, and/or segment of that antigen (Townsend et al.,1984b; Townsend and Skehel, 1984). Enzymatic cleavage was also used to localise T cell determinants to particular fragments of proteins, although there

was always the risk that the specificity of the proteolytic enzymes would lead to the loss of an epitope (Berzofsky et al., 1977; Fritz et al.1983; Shimonkevitz et al., 1984; Livingstone and Fathman, 1987). The use of recombinant DNA technology allowed a much more controlled and detailed analysis of potential epitopes using truncated and mutated forms of protein antigens (Townsend et al., 1984a; Townsend et al., 1985; Lamb et al., 1987; Whitton et al.,1988). In all of these cases, the epitopes were generally refined and analysed in detail using synthetic peptides (Townsend et al., 1986; Livingstone and Fathman, 1987; Lamb et al., 1987; Milich, 1989). However, synthetic peptides could also be used directly to define T cell determinants within an antigen, if a sufficient number of peptides of appropriate length were synthesised spanning the protein (Bixler et al.,1985; van der Zee, 1989; Schaeffer et al., 1989).

Responses of both human and murine T cells, primed to whole antigen in vivo, can be analysed in vitro using synthetic peptide determinants. However, in the murine system the immunogenic responses to the peptides can also be examined. Peptides to be tested can be emulsified in complete Freund's adjuvant and injected into mice. If the peptide is immunogenic, a class II restricted T helper cell response normally is elicited (Hackett et al., 1985; Milich, 1989). Alternatively, peptides can be tested by attempting to elicit cytotoxic T cells (CTL) with in vitro priming, although to date the resultant CTLs have not always been able to lyse targets expressing the whole antigen (Carbone et al., 1988). In the murine system, the immunogenicity of the peptides can also be tested against a number of different homogeneous genetic backgrounds in inbred strains of mice which have not had any prior exposure to the whole antigen. This can be an advantage over human studies where prior exposure to related and possibly cross reactive antigens, for example virus strains, is often unknown.

Eliciting a T cell response to a peptide by direct immunisation does not necessarily lead to the identification of an immunodominant T cell determinant (Gammon et al., 1987). For example, immunisation of mice with peptides from hen

egg lysozyme resulted in T cells with three different response patterns. Firstly, certain peptides elicited T cells which reacted in vitro with the peptide but not whole antigen, and were known as cryptic determinants. Secondly, other peptides produced T cells which reacted with the peptide, and only weakly with the complete antigen, and were known as minor determinants. Thirdly certain peptides elicited T cells which reacted well with both the peptide and the whole protein, and were known as major or immunodominant determinants. Immunisation with whole hen egg lysozyme resulted in T cells which reacted with the immunodominant determinants and very weakly with minor determinants but not at all with cryptic determinants (Gammon et al., 1987). If direct immunisation with peptides is used to determine T cell determinants within proteins of a known pathogen then establishing the immunodominance of the defined epitopes is of great importance (Milich, 1989). Immunodominant peptide determinants may be able to form part of subunit vaccines against the pathogen, but minor or cryptic determinants would be of little use for this.

The ease with which T cell determinants within an antigen are defined can, theoretically, be increased if a predictive algorithm, based on known determinants, is used to help decide which parts of the molecule should be examined first. DeLisi and Berzofsky (1985) identified structural features in a number of T cell determinants which led them to believe that many determinants would bind as amphipathic helices. Rothbard and Taylor (1988) identified common structural features within the primary sequence of a large number of known determinants which they showed to be statistically significant (Introduction, 1.8). Although neither group were able to define the precise structural reason for the existence of such features their functional significance was demonstrated by the ability of algorithms based on the features to identify determinants in a number of systems including malaria, HIV, influenza, and *M. tuberculosis* (Berzofsky et al., 1987; Lamb et al., 1987; Gotch et al., 1987; Rothbard et al., 1988). Experiments described in this chapter have used the algorithm of Rothbard and Taylor (1988), to identify a number of T cell determinants within

human papillomavirus (HPV) type 16. The experiments were designed to fulfill two aims. Firstly to set up a system in which to examine the structural features of the determinants identified and hence, improve the algorithm and our understanding of peptide MHC protein interactions. Secondly, the experiments would help in our understanding of the immune responses to a pathogen believed to be important in human cancers.

3.1.2. Epidemiology of Human Papillomaviruses

Human papillomaviruses are small DNA viruses which infect only squamous epithelium and mucosa at specific anatomical sites (reviewed by zur Hausen, 1989; Galloway and McDougall, 1989; zur Hausen and Schneider, 1987 and Pfister, 1987). Different HPV types are not classified serotypically but by DNA sequence homology assayed by cross-hybridisation with the known types (Coggin and zur Hausen, 1979). Sixty types of HPV have been molecularly cloned (de Villiers, 1989) and they can be segregated into groups associated with particular clinical lesions (Table 3.I and Broker and Botchan, 1986). For example, HPV types 1 and 4 are associated with benign lesions such as plantar warts on the hands and feet whereas types 16, 18, 31, 33 and others are associated with neoplasias and carcinomas of the genital tract, including cervical cancer.

Cervical cancer is a major disease worldwide with over 500,000 new cases and 300,000 deaths each year (Cuzick and Boyle, 1988). In developing countries it is the second most prevalent form of cancer amongst women, with breast cancer being the first. Human papillomaviruses are believed to be important in the generation of many epithelial and mucosal malignant tumours, and HPV DNA has been isolated from over 90% of all anogenital cancers (Durst et al., 1983 and Gissmann et al., 1983). HPV type 16 is the predominant virus type associated with malignancies of the cervix, being found in almost 50% of these lesions (Durst et al., 1983; zur Hausen, 1989). In addition to the epidemiological data, functional studies have demonstrated that

Table 3.I. Human Papillomaviruses

HPV Type	Disease	Oncogenic Potential
1	deep plantar and palmar warts	benign
2	common warts	benign
3, 10, 28	juvenile warts, some intermediate warts associated with epidermodysplasia verruciformis and genital infections	rarely malignant
4	plantar and common warts	benign
5, 8, 46, 47	macular lesions in epidermodysplasia verruciformis, in patients with immune deficiency; also in immunosuppressed transplant patients	30% progress to malignancy
6, 11	Condylomata acuminata, low grade dysplasia (CIN I, CIN II), laryngeal papillomas	usually benign
7	common warts of meat and animal handlers	benign
9, 12, 14, 15	epidermodysplasia verruciformis	rarely malignant
17, 19-25, 36, 15, 13, 32	oral focal epithelial hyperplasia (Heck's disease)	benign
16, 18, 31, 33, 35, 39, 51, 52, 56	high grade dysplasia, intraepithelial neoplasias and carcinomas of genital mucosa: Bowenoid papulosis	high malignant association
26 27	cutaneous wart, immune deficient patient cutaneous wart, renal transplant recipient	unknown
29	common wart	unknown
30, 40	laryngeal carcinoma	malignant
34	non-genital Bowen's disease	benign
37	keratocarcinoma	benign
38	melanoma	malignant
41	condylomata and cutaneous flat warts	benign
42-44	genital warts	benign

Table shows the association of HPV types with clinical conditions and whether the associated lesions are likely to become malignant (adapted from Broker and Botchan, 1986).

transfection with HPV type 16 DNA is able to immortalise human foreskin keratinocytes (Durst et al., 1987; Pirisi et al., 1987), and can lead to the transformation of primary epithelial cells (Baby rat kidney cells) when co-transfected with a second oncogene such as an activated *ras* gene (Matlashewski et al., 1987).

However, despite the strong association of HPV with cervical dysplasia and the ability of HPV DNA to transform cells in vitro, it is clear that infection does not always lead to disease (zur Hausen, 1989; de Villiers et al., 1987) Studies examining DNA from cervical biopsies of both normal and symptomatic individuals indicated that as many as 60% of women may be infected with the virus (Young et al., 1989; Gergely, 1987; zur Hausen, 1989), but only about 3% of infected women would develop disease (zur Hausen, 1989) and the mean latency time between infection and the onset of cancer could be between 20 and 50 years (zur Hausen, 1989). These data suggested that other co-factors were involved in the evolution of a malignancy associated with HPV infection. Early onset of sexual relations (Brinton, 1986), the use of oral as opposed to barrier contraceptives (W.H.O., 1985), smoking (Clarke et al., 1982; Sasson et al., 1985), and hormonal influences (Mitrani-Rosenbaum et al., 1989; Vessey, 1986), are all associated with cervical cancer, and may result in the necessary co-factors to produce disease.

The above data has strongly suggested that HPVs are important in the development of epithelial malignancies. Also, they seem to be one of the few well defined components of the mechanism of induction of these diseases (zur Hausen, 1989). They therefore make an obvious target for possible therapies to modulate the induction and development of such malignancies.

3.1.3. Natural Immunity to HPV infection

Studies on the immunobiology of HPVs have been hampered by the lack of an in vitro culture system for producing large quantities of the virus. Hence antigen has been obtained from clinical samples or by recombinant and synthetic technologies. A

large number of studies have identified antibodies specific for HPV antigen in infected individuals (Galloway and McDougall, 1989; Steele and Gallimore, 1990). Recent examination of the humoral responses to HPV types associated with genital lesions has been dependent on the production of synthetic antigens , either bacterial fusions proteins, or synthetic peptides (Galloway and Jenison, 1990; Davies et al, 1991). All of the open reading frames of HPV types 6 and 16 have been expressed as bacterial fusion proteins and used to analyse sera from HPV infected individuals (Jenison et al, 1988; Galloway and Jenison, 1990). Antibodies to HPV type 6 antigens were found in approximately 70% of patients and antibodies to HPV type 16 antigens in the sera of 50% of the patients (Galloway and Jenison, 1990). The prevalence of antibody responses to HPV antigens may be even higher than these figures indicate since the fusion proteins may not adopt the same conformation as the intact eucaryotic protein and hence reactivities with the intact protein may not have been detected. Consistent with this are the experiments of Steele and Gallimore, who demonstrated that in ELISA assays, the reactivity of sera from patients with skin warts to intact HPV type 1 virions, was considerably more frequent than reactivity to disrupted virions (Steele and Gallimore, 1990). Despite the obvious presence of HPV specific antibodies in sera from patients, little correlation has been shown between antibody titres and the clinical status of the disease (Kirchner, 1986; Jenison et al., 1988; Cubie and Norval, 1988; Steele and Gallimore, 1990). The only evidence for the role of humoral responses in modulating papillomavirus infections comes from studies of bovine papillomavirus (BPV) type 1. Pilacinski et al. (1986) were able to show that antibodies to the major capsid protein (L1) could inhibit infectivity in vitro, and that immunisation of cattle with recombinant L1 protein could protect against infection with BPV type 1 in vivo (Pilacinski et al., 1986).

Immunosuppressed individuals, for example renal-allograft and cancer patients, have been shown to have an increased incidence of warts and other HPV induced lesions (Porreco, 1975; Morison, 1975; Lutzner, 1985; Gassenmaier, 1986). This has

suggested that a normal cellular immune response may be important in regulating HPV infection and is consistent with the enhanced lymphoid cell infiltration observed in regressing hand warts (Tagami et al 1985; Iwatsuki et al., 1986). Stimulation of peripheral blood lymphocytes from patients with cervical dysplasia using disrupted and intact HPV virions provided some direct evidence that there was a specific cellular immune response to HPV, although the detailed antigen specificity of these response was not analysed (Cubie and Norval, 1988). Recently fusion proteins from HPV types 16 and 18 have been used to stimulate T cells from patients with cervical intraepithelial neoplasia (Cubie et al., 1989). This has provided more direct evidence for a specific cellular immune response to HPV antigens.

3.1.4. Aims and Summary

The evidence described above has indicated that there is an active immune response to HPV in infected individuals and that immunocompromised patients exhibit an increase in HPV associated disease. Therefore, control of HPV infection by immunological intervention may be possible and may form a route by which to modulate epithelial malignancies associated with various HPV types. However, such therapies require a far more detailed understanding of the immunology of HPV than is currently available. As mentioned, one of the principle reasons for the scarcity of studies on the immunology of the human papillomaviruses is the absence of an in vitro culture system for producing experimentally useful quantities of the viruses. Hence, in order to make any in depth study of their immunobiology antigen must be produced by recombinant or synthetic means.

As part of a larger study of the immune responses to HPV type 16, including human T cell responses (Strang et al., 1990), potential T cell determinants within a number of open reading frames (ORF) of HPV type 16 were identified using the predictive algorithm of Rothbard and Taylor (1988) (Taylor, 1988). HPV type 16, like all papillomaviruses, is a relatively small DNA virus of only 7900 base pairs which

have been completely sequenced (Seedorf et al., 1985). The genome contains 8 predicted ORFs (Figure 3.1) and therefore it would have been possible to use the predictive algorithm to examine the whole virus for the presence of potential T cell epitopes. However, to make the analysis simpler three of the ORFs (L1, E6, and E7) were selected for these studies, based on the following. The predicted major capsid protein of HPV type 16 is encoded by the L1 ORF (Pelcinski et al., 1984; Seedorf et al., 1985) and T helper cell responses to capsid or envelope proteins of other viruses have often been observed (Milich, 1989). Also, as already discussed, the L1 protein of BPV type 1 has been shown to be effective in inducing antibodies capable of inhibiting BPV type 1 infection in vitro, and in immunising cattle against infection in vivo. This suggests that L1 may be an important target for possible immunotherapeutic strategies. The E6 and E7 proteins of HPV type 16 have been shown to be sufficient for the transformation of primary cells in vitro, and their expression is also maintained in long term cell lines derived from biopsies taken from cervical cancer patients (Storey et al., 1988; Androphy et al., 1987; Seedorf et al., 1987; Smotkin and Wettstein, 1986). Since the E6 and E7 genes are likely to be present in cells which will become malignant, it would be important to determine possible immune responses to these proteins.

Thirteen peptides containing predicted T cell epitopes from the HPV type 16 E6, E7, and L1 ORFs were synthesised, mixed with complete Freund's adjuvant, and used to immunise inbred strains of mice. This method of immunisation normally results in class II restricted T cell responses. Such responses often involve T helper cells which are of primary importance because they provide help for B cells to produce antibody and can also help in the production of cytotoxic T cells. Knowledge of such T helper epitopes could therefore be important in the development of sub-unit vaccines to help elicit both memory B and T cells (Milich, 1989). Five determinants able to generate lymphoproliferative responses were successfully identified, three of which were shown to elicit responses restricted through class II molecules of more than one allele and

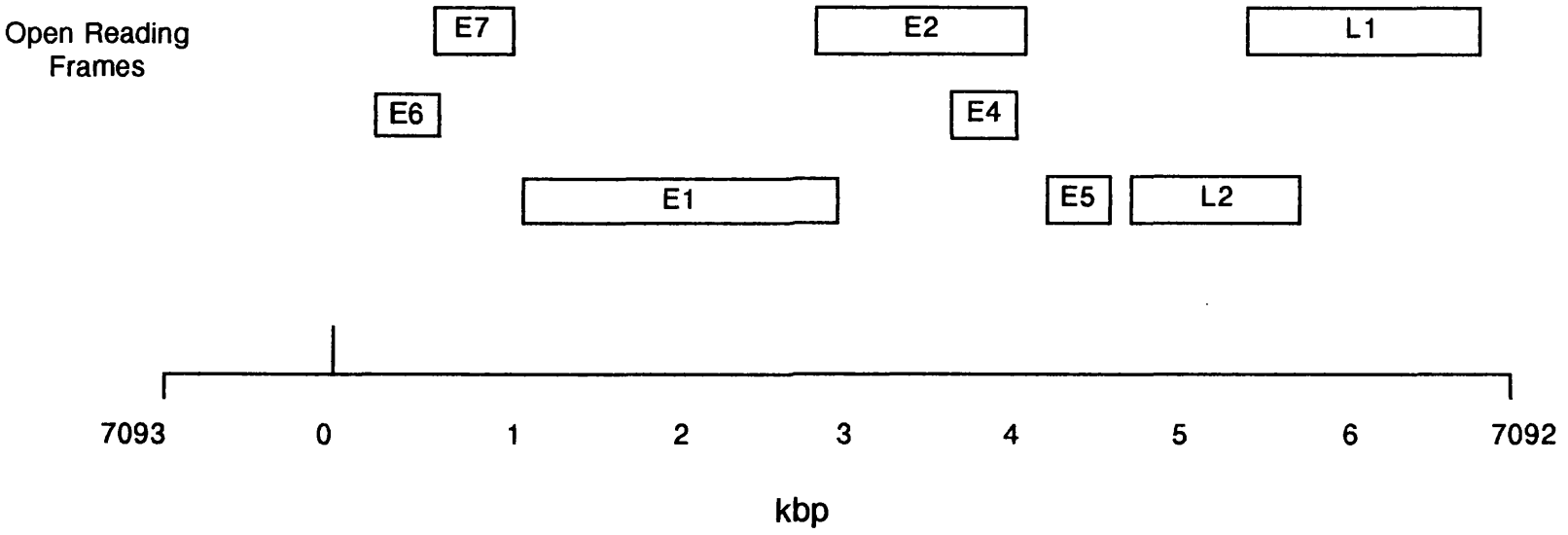
Figure 3.1.

Genome of HPV type 16

Map of the HPV type 16 genome showing the predicted ORFs based on three possible translation frames of the sequence by Seedorf et al. (1985).

kbp = kilo base pairs

HPV 16


Open Reading Frames
E7
E2
L1
E6
E4
E1
E5
L2
7093
0
1
2
3
4
5
6
7092
kbp

isotype. The responses elicited were found to be highly specific for HPV type 16 and showed no cross reaction with other HPV types. In a parallel study it was interesting to note that some of these same determinants were able to elicit responses from human peripheral blood lymphocytes indicating the epitopes were highly permissive in their ability to form immunogenic complexes with different class II molecules.

3.2. Results

3.2.1. Prediction of HPV type 16 determinants

The algorithm defined by Rothbard and Taylor (1988; Taylor 1988) was used to analyse the E6, E7, and L1 ORFs of HPV type 16 and resulted in the identification and synthesis of 13 peptides containing potential T cell epitopes (Table 3.II) (Materials and Methods, 2.1). The algorithm was based on two patterns of amino acids within known T cell determinants identified in the context of both MHC class I and class II proteins. One pattern consisted of a charged residue or glycine, followed by two hydrophobic residues, followed by a polar or a glycine residue. The second pattern consisted of a charged residue or glycine, followed by two hydrophobic residues, followed by a hydrophobic residue or proline, followed by a polar residue or glycine (Rothbard and Taylor, 1988). The data used to define these patterns is summarised in Table 3.III. To predict T cell epitopes the algorithm examined all linear sequences of four amino acids and scored them according to their similarity to the previously identified determinants (Rothbard and Taylor, 1988; Taylor, 1988). For example, if the first position of the four amino acids being examined contained Glu, then 18 was added to the score for these four amino acids, whereas His added 7, and a Tyr added zero (Table 3.III). Similarly if the second position contained an Ala then 16 was added to the score and Ile added 9 (Table 3.III). Patterns of four amino acids were initially scored and then if the fourth position contained a hydrophobic residue or a proline then the fifth position was also added into the score (Rothbard and Taylor, 1988; Taylor, 1988).

Table 3.II.

Sequences of peptides containing predicted epitopes within HPV type 16 open reading frames

	L1 Residues	Scores
29-44	L W L P S [E A T V Y] L P P V P V	49
40-63	P P V P V S [K V V S] T D [E Y V A R] T N I Y Y H A	37,43
91-106	V S G L Q Y [R V F R] I G L P D P	26
209-224	G D C P P L [E L I N] T V I Q D G	40
219-244	T V I Q D G [D M V H] T G F [G A M] [D] [F T T] L Q A N K S	26, 34, 21
279-294	E Q M F V R [H L F N] R A G T V G	26
295-310	E N V P D [D L Y I K] G S G S T A	28
382-397	K N T N F K [E Y L R] H G E E Y D	40
	E6 Residues	
27-39	Q T T I H [D I L L E] C V Y	35
46-57	R R [E V Y] [D] [F A F R] D L	34, 35
71-84	D K C L [K F Y S] Q I S E Y R	28
88-101	Y S L Y [G T T L E] Q Q Y N K	34
	E7 Residues	
82-97	L L M G T L [G I V C] P I C S Q K	31

Epitopes predicted by the algorithm of Rothbard and Taylor within three ORF's (L1, E6, and E7) of HPV type 16.
The patterns identified by the algorithm are boxed.
The scores associated with each pattern are caclulated from the data shown in Table II and are shown in the second column.
Amino acid residues shown contain either highest scoring patterns or more than one pattern in close proximity.
5 or 6 amino acids are included on either side of the patterns shown to improve chances of identifying the determinants (see text).

Table 3.III. Amino acids within 64 T cell determinants

Position >	1		2		3		4		5	
	Amino acid	Occur-rence	Amino acid	Occur-rence	Amino acid	Occur-rence	Amino acid	Occur-rence	Amino acid	Occur-rence
	D	7	A	16	A	11	A	4	A	5
	E	18	V	9	V	11	V	6	L	2
	K	14	L	9	L	13	L	3	I	3
	H	7	I	9	I	9	I	2	F	3
	R	7	F	6	F	6	F	1	Y	2
	G	11	Y	5	Y	3	Y	4	T	7
			M	4	M	3	T	1	E	7
			T	5	W	1	D	4	K	7
			W	1	T	7	E	10	H	3
							K	5	R	5
							H	4	N	4
							R	4	Q	1
							N	4	S	6
							Q	4	P	1
							S	5	G	6
							P	1		
							G	2		
	53 charged, 11 glycine		64 hydrophobic		64 hydrophobic		20 hydrophobic, 27 charged, 14 polar 1 pro, and 2 gly		22 hydrophobic 22 charged, 11 polar 1 pro, and 6 gly	

Occurrence of amino acids within the sequence of 64 different T cell determinants. The determinants were aligned based on the presence of two hydrophobic residues within all the sequences and then the amino acids at relative positions 1, 4, and 5 were determined.
(Adapted from Rothbard and Taylor; 1988)

The initial analysis by the algorithm resulted in identification of a large number of possible patterns within the ORFs. However, the scores associated with each pattern varied considerably. To maximise the chances of identifying regions of the proteins which contained T cell epitopes, patterns were selected either because they had a high score or because several patterns were relatively close together in the sequence. Eight regions of the L1 protein, 4 regions of the E6 protein, and one region of the E7 protein were selected (Table 3.II). The minimal length of an MHC class II restricted T cell epitope which still gives a response consists of approximately 7-8 residues (Livingstone and Fathman, 1987; Rothbard and Taylor, 1988; Milich, 1989). However, a peptide of this length rarely results in maximal stimulation, while peptides of 10-12 amino acids tend to give a maximum stimulatory signal (Livingstone and Fathman, 1987; Rothbard and Taylor, 1988; Milich, 1989). Therefore peptides, which were the lengths of the regions shown in Table 3.II, were synthesised for this study, containing five or six amino acids either side of the patterns of 4 or 5 residues in order to produce all possible 10 amino acid peptides which might contain the patterns. These peptides are illustrated in Figure 3.2 relative to the size of the whole ORFs.

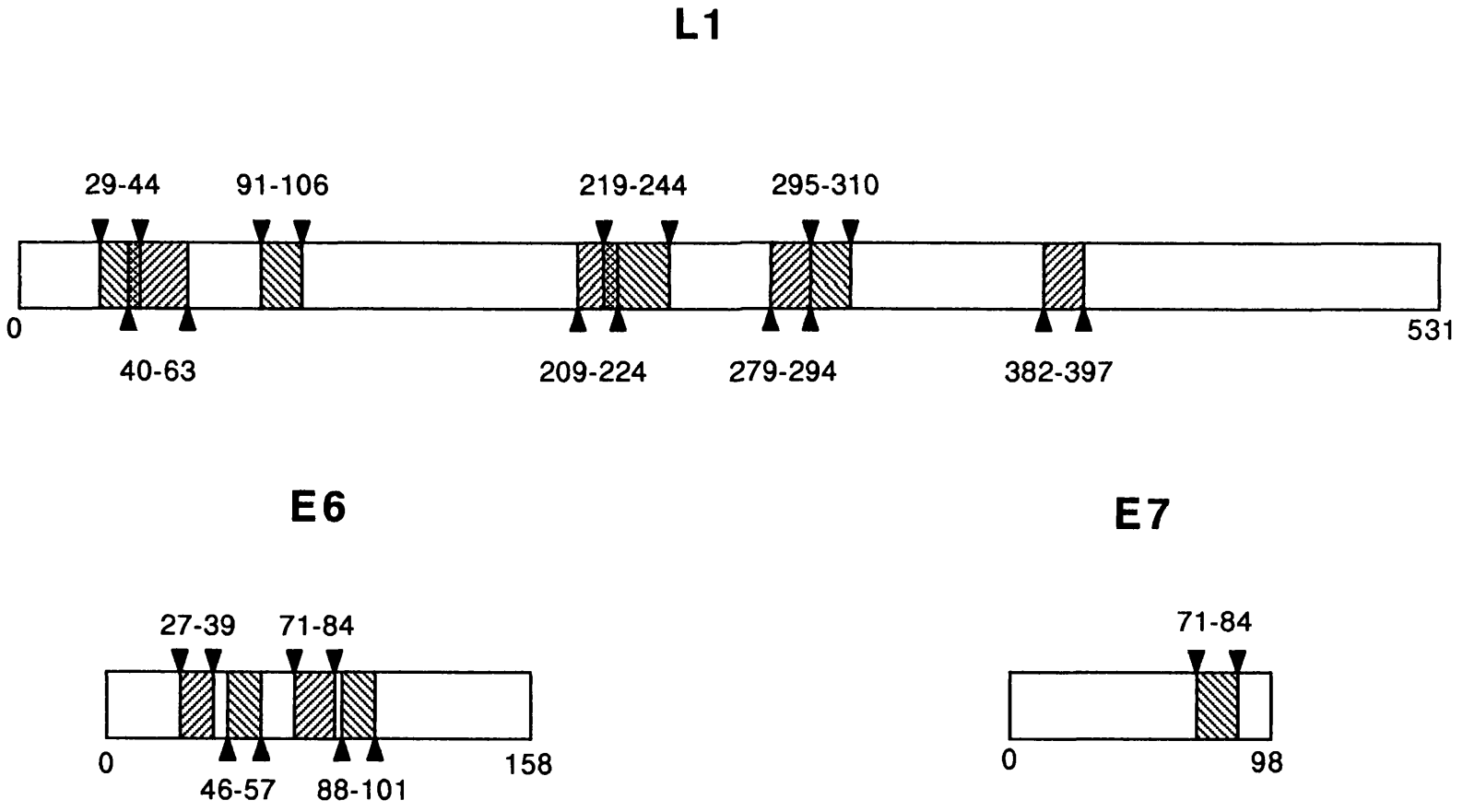
3.2.2. Lymphoproliferative responses to predicted determinants from the L1 protein of HPV type 16

Initially 9-12 nmols of each of the eight L1 peptides shown in Table 3.II, emulsified in complete Freund's adjuvant, were used to individually immunise groups of 3 - 5 Balb/c x CBA (H-2^d x H-2^k) mice in the base of the tail (Materials and Methods, 2.7). This amount of peptide has been able to elicit T cell responses in several other systems (Fox et al 1987; Milich, 1989). The use of F1 mice increased the number of MHC alleles present in the animals and hence the chances of seeing a response elicited by a particular peptide. Eight days after immunisation draining lymph nodes were removed, pooled within each group, and assayed in triplicate wells in a three day proliferation assay for a specific response to the immunising peptide

Figure 3.2.

Predicted T cell determinants within HPV type 16 proteins

Positions and relative sizes of the peptides containing predicted T cell determinants within the L1, E6, and E7 ORFs of HPV type 16.


L1
29-44
91-106
219-244
295-310
0
531
40-63
209-224
279-294
382-397
E6
27-39
71-84
0
158
46-57
88-101
E7
71-84
0
98

(Materials and Methods, 2.7). Three of the eight L1 peptides induced responses (Figure 3.3). In all groups the cells were shown to give a strong proliferative response when stimulated with con A (greater than 100,000 c.p.m.) indicating that none of the peptides were inducing T cell anergy, at least in the majority of T cells present. The signal obtained with no peptide present in the culture was always approximately the same between different groups assayed on the same day and was normally less than 5000 c.p.m. (Figure 3.3). Peptide L1 40-63 gave the maximal response which was shown to titrate steadily down towards background with 10 fold decreasing concentrations of peptide. The peptide L1 91-106 induced about a third of the proliferative response of L1 40-63 at the maximum concentration of each peptide used in the assay. Also, L1 40-63 elicited the same response as the maximal response elicited by L1 91-106 at 100 fold lower concentrations of peptide. The third responding peptide, L1 279-294, produced a response of similar magnitude to L1 91-106. Immunisation with the responding peptides failed to elicit T cells capable of being stimulated by other peptides from L1, over a wide concentration range, indicating that the responses were specific (Figure 3.4).

In the above experiments groups of mice were immunised with individual peptides and the differences in proliferative responses may not represent their true immunodominance within L1. This could only be defined if the mice were immunised with the whole L1 molecule, or the peptide specific T cells stimulated with whole L1 protein. In the absence of available intact L1 protein, the relative immunodominance of the three immunostimulatory L1 peptides was established. Balb/c x CBA (H-2^d x H-2^k) mice were immunised with a mixture of 9 nmols of each of the three L1 peptides, and primed lymph node cells were assayed in vitro with all eight individual L1 peptides over an equivalent molar concentration range (Figure 3.5). Once again peptide L1 40-63 elicited the best immunostimulatory response, the peptide L1 91-106 induced a weaker response and the peptide L1 279-294 induced a distinctly weaker response than 91-106. None of the other peptides from L1 stimulated the T cells indicating the

Figure 3.3.

Responses of F1 mice to HPV type 16 L1 peptides

Proliferative responses of (Balb/c x CBA) F1 primary lymph node cell cultures to the eight priming peptides of HPV type 16 L1. Each peptide was immunised into one of eight groups of mice. Results are mean of triplicates and error bars give the standard deviation of the triplicate. Background is the mean background for all eight groups of cultures. The three immunostimulatory L1 peptides are marked with a "*" in the legend

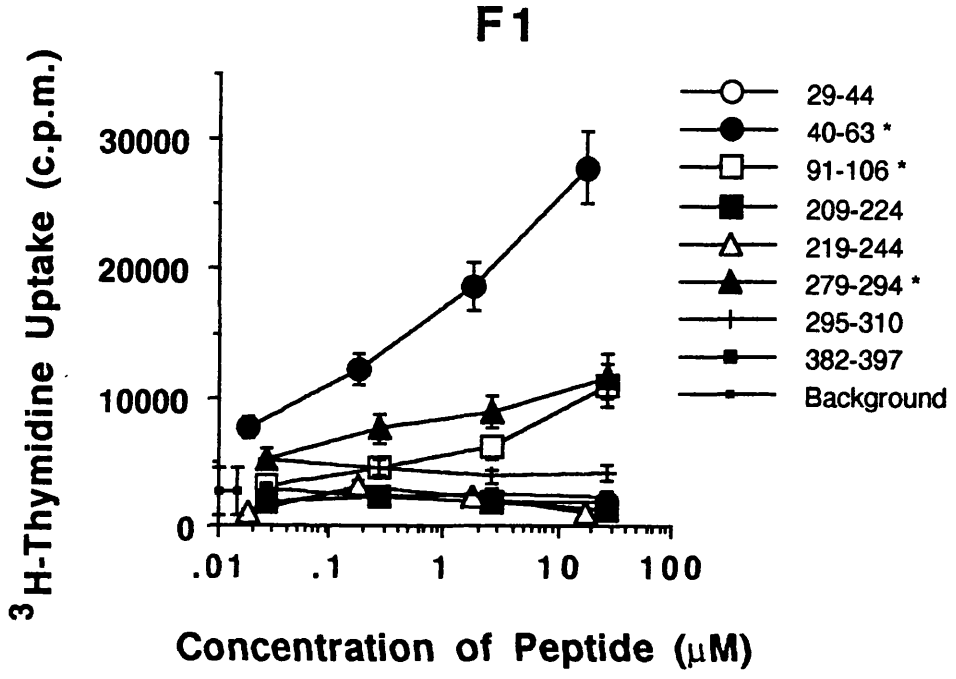

F1
^{3}H-Thymidine Uptake (c.p.m.)
30000
20000
10000
0
.01
.1
1
10
100
Concentration of Peptide (μM)
29-44
40-63 *
91-106 *
209-224
219-244
279-294 *
295-310
382-397
Background

Figure 3.4.

Specificity of T cell responses to HPV type 16 L1 peptides

Proliferative responses of (Balb/c x CBA) F1 primary lymph node cell cultures to priming peptides of HPV type 16 L1 and to all other L1 peptides. **a)** Responses of lymph node cells from mice primed to L1 40-63 and stimulated in vitro with all eight L1 peptides. **b)** Responses of lymph node cells from mice primed to L1 91-106 and stimulated in vitro with all eight L1 peptides. **c)** Responses of lymph node cells from mice primed to L1 279-294 and stimulated in vitro with all eight L1 peptides. Results are mean of triplicates and error bars give the standard deviation of the triplicate.

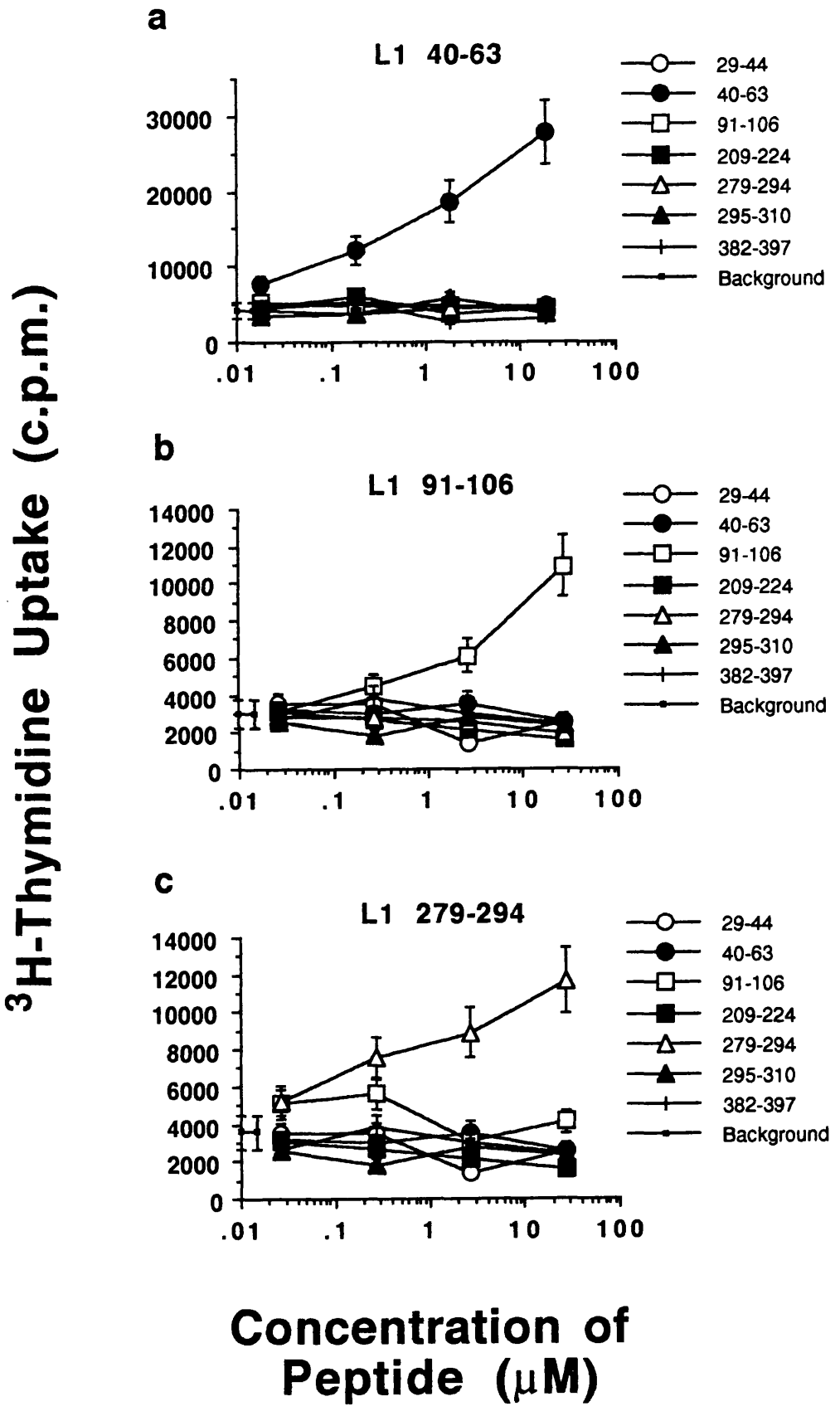
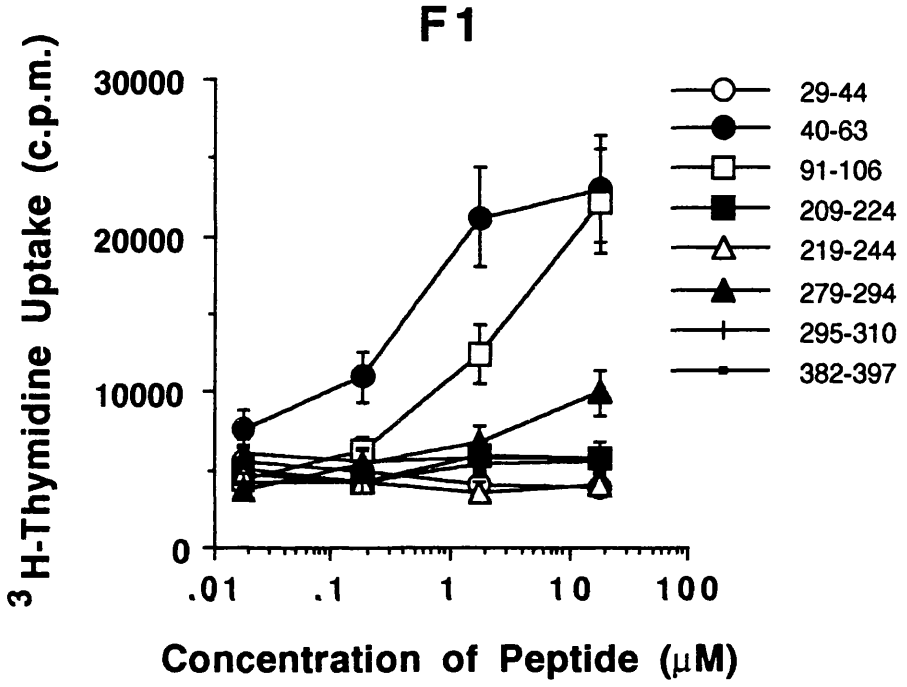

a
L1 40-63
30000
20000
10000
0
.01
.1
1
10
100
29-44
40-63
91-106
209-224
279-294
295-310
382-397
Background
b
L1 91-106
14000
12000
10000
8000
6000
4000
2000
0
.01
.1
1
10
100
29-44
40-63
91-106
209-224
279-294
295-310
382-397
Background
c
L1 279-294
14000
12000
10000
8000
6000
4000
2000
0
.01
.1
1
10
100
29-44
40-63
91-106
209-224
279-294
295-310
382-397
Background
^{3}H-Thymidine Uptake (c.p.m.)
Concentration of Peptide (μM)

Figure 3.5.

Responses of F1 mice to co-immunisation with HPV type 16 L1 peptides

Responses of primary lymph node cells from (Balb/c x CBA) F1 mice primed with an equimolar (9 nmols) mixture of L1 40-63, 91-106, and 279-294 and challenged in vitro with all eight individual peptides from L1. Results are mean of triplicates and error bars give the standard deviation of the triplicate.


F1
30000
20000
10000
0
.01
.1
1
10
100
^{3}H-Thymidine Uptake (c.p.m.)
Concentration of Peptide (μM)
29-44
40-63
91-106
209-224
219-244
279-294
295-310
382-397

specificity of the responses. The results therefore indicated that although L1 40-63 remained the immunodominant epitope when all the peptides were co-injected, the response to L1 91-106 was clearly greater than that to L1 279-294. Although these experiments addressed the relative immunodominance of these peptides in isolation, they still did not determine whether responses to these peptides were immunodominant within the whole L1 protein (see Discussion, 6.1).

To determine the contribution of the individual alleles present in the Balb/c x CBA (H-2^d x H-2^k) mice, in the responses to the peptides from the L1 protein, parental Balb/c and CBA mice were immunised with each of the eight peptides and the resultant lymph node cells assayed as before (Figure 3.6). Peptide L1 40-63 elicited the maximum response of all the L1 peptides in both Balb/c and CBA mice and peptides L1 91-106 and 279-294 induced a weaker response compared to L1 40-63 in both strains of mice. The CBA mice also gave a consistent, although weak response, to L1 residues 295-310. In summary, the same three immunogenic peptides were able to induce responses in Balb/c and CBA mice as well as in the F1 offspring and the same peptide elicited the greatest response in all three cases.

3.2.3. Lymphoproliferative responses to predicted determinants from the E6 and E7 proteins of HPV type 16

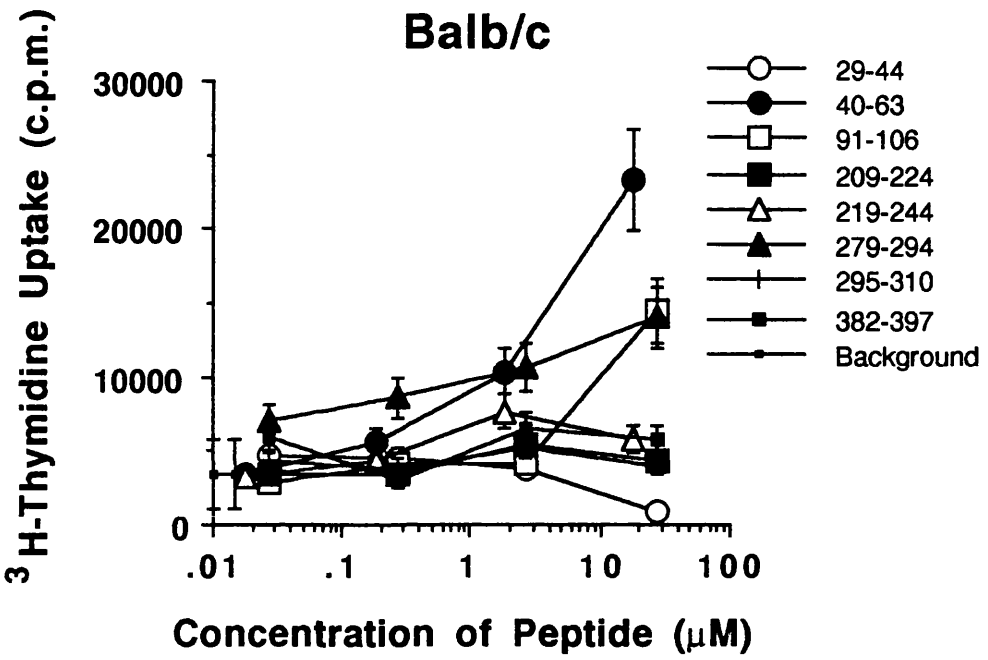
Four peptides were synthesised from the E6 protein which were predicted to contain T cell epitopes (Table 3.II). Since both Balb/c and CBA mice had responded to the same peptides from the L1 protein of HPV type 16, this time the four E6 peptides were assayed only in Balb/c mice. A solution of all four peptides in PBS was emulsified in complete Freund's adjuvant, and 12 nmols of each peptide was immunised into each of a group of 4 Balb/c (H-2^d) mice . Eight days later draining lymph node cells were assayed as before with the individual peptides. E6 71-84 was the only peptide to elicit a response in Balb/c (H-2^d) mice (Figure 3.7a). The response was specific and titratable with peptide concentration. The cells responded to the

Figure 3.6.

Responses of Balb/c and CBA mice to HPV type 16 L1 peptides

Proliferative responses of **a)** Balb/c, or **b)** CBA, primary lymph node cell cultures to the eight priming peptides of HPV type 16 L1. Each peptide was immunised into one of eight groups of mice. Results are mean of triplicates and error bars give the standard deviation of the triplicate. Background is the mean background for all eight groups of cultures.

a


Balb/c
30000
20000
10000
0
.01
.1
1
10
100
^{3}H-Thymidine Uptake (c.p.m.)
Concentration of Peptide (μM)
29-44
40-63
91-106
209-224
219-244
279-294
295-310
382-397
Background

b

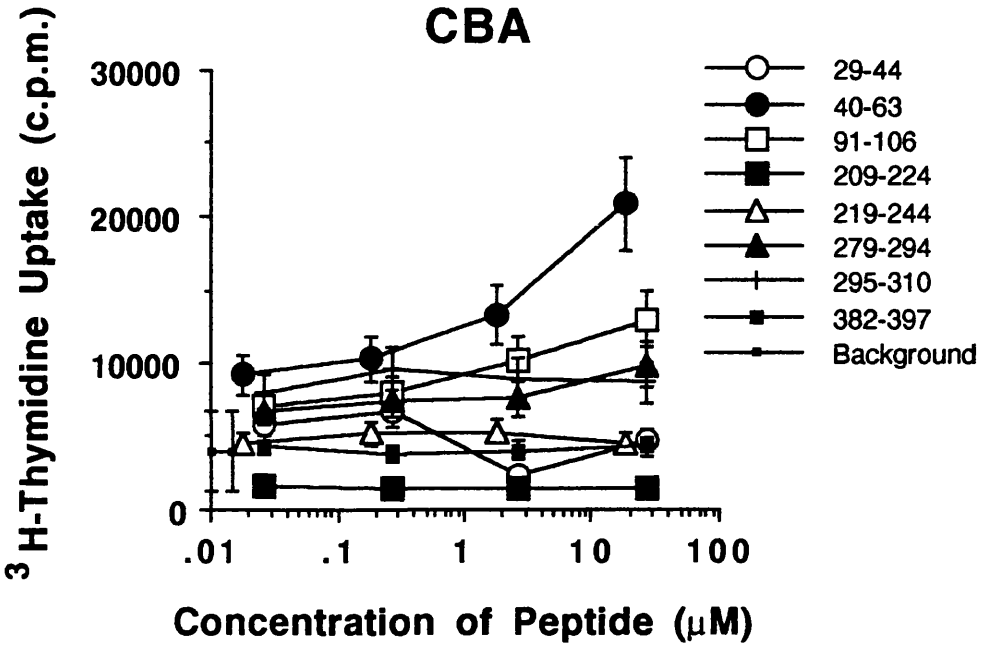
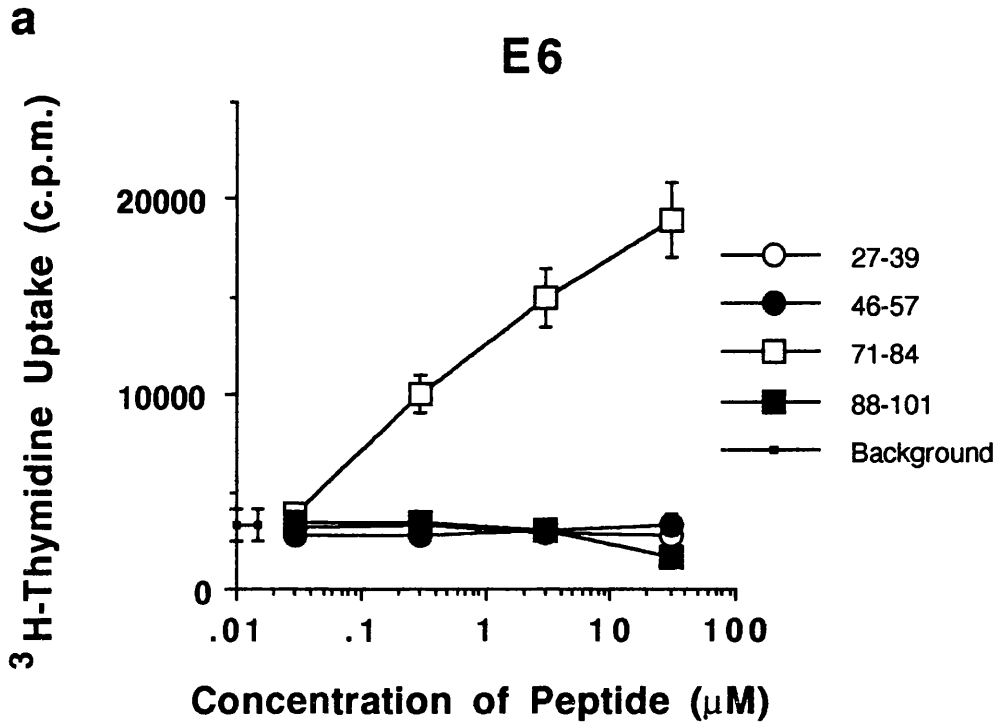

CBA
30000
20000
10000
0
.01
.1
1
10
100
^{3}H-Thymidine Uptake (c.p.m.)
Concentration of Peptide (μM)
29-44
40-63
91-106
209-224
219-244
279-294
295-310
382-397
Background

Figure 3.7.

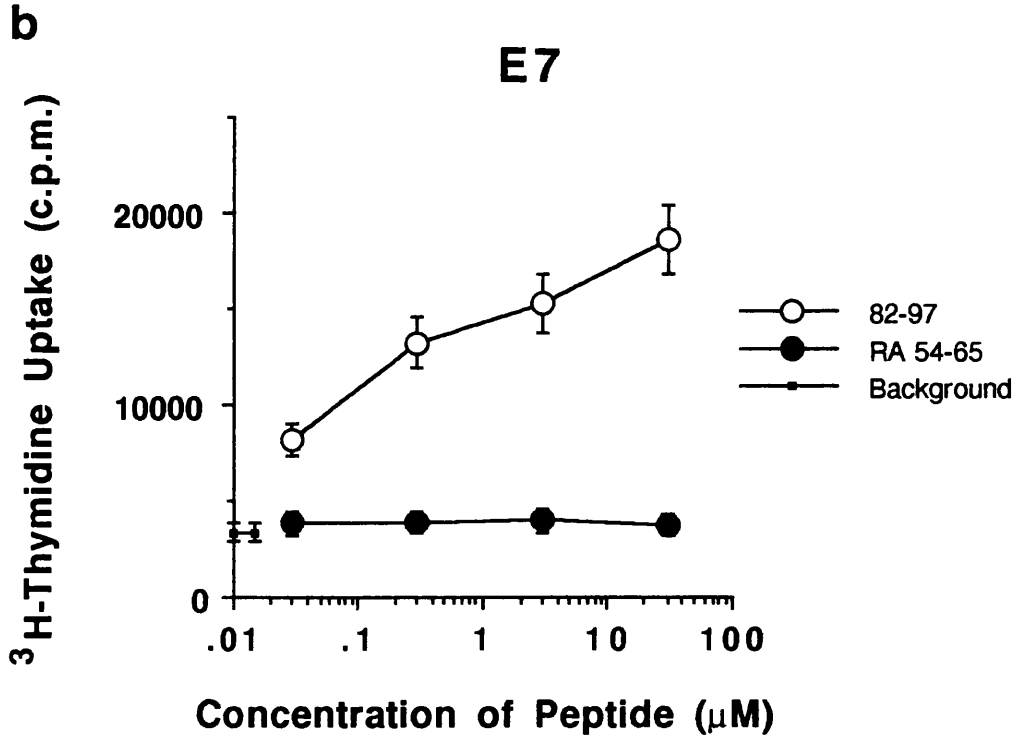
Responses of Balb/c mice to peptides from E6 and E7 of HPV type 16

Responses of primary lymph node cells from Balb/c mice, **a)** primed with an equimolar (9 nmols) mixture of E6 27-39, 46-57, 71-84, and 88-101 and challenged in vitro with individual peptides from E6, and **b)** primed with the E7 82-97 peptide and challenged in vitro with the priming peptide and ragweed allergen (RA) 54-65. Results are mean of triplicates and error bars give the standard deviation of the triplicate.

a


E6
20000
10000
0
.01
.1
1
10
100
^{3}H-Thymidine Uptake (c.p.m.)
Concentration of Peptide (μM)
27-39
46-57
71-84
88-101
Background

b


E7
20000
10000
0
.01
.1
1
10
100
^{3}H-Thymidine Uptake (c.p.m.)
Concentration of Peptide (μM)
82-97
RA 54-65
Background

positive control with con A and the proliferation without peptide is indicated as the background in the figure.

Only one peptide was selected from the E7 protein of HPV type 16 (Table 3.II), and 12 nmols of this peptide were used to immunise Balb/c mice. Eight days later the lymph node cells were assayed as for the other peptides and a proliferative response, titratable with peptide concentration, was observed (Figure 3.7b). The absence of a response to ragweed allergen, residues 54-65, by these lymph node cells was used to indicate specificity.

3.2.4. Summary

A total of 13 peptides from the L1, E6, and E7 proteins of HPV type 16 were predicted to contain T cell epitopes. Of these, 5 were shown to be able to elicit responses in Balb/c mice, and 3 to be able to elicit responses in Balb/c and CBA mice. (see later in Table 3.VII).

3.2.5. Proliferative responses to truncated peptides from L1 of HPV type 16

Since the peptides from the L1 protein of HPV type 16, which elicited responses, were of greater length than the minimal determinants defined in a number of antigens (Livingstone and Fathman, 1987; Milich, 1989) they may well have contained more residues than were necessary to stimulate the responding T cells. To determine more precisely the regions of the three immunogenic L1 peptides recognised by the T cells, the three were individually used to immunise groups of mice and then truncated sets of each peptide were assayed for their ability to induce in vitro proliferative activity (Table 3.IV). T cells from Balb/c (H-2^d) and CBA (H-2^k) mice specific for L1 residues 40-63 responded to analogues of this peptide, truncated at the amino terminus, as shown in Figure 3.8. T cells restricted through H-2^d gene products were stimulated equally by peptides L1 40-63, 48-63, and 50-63 and none of the other truncated

Table 3.IV.

Truncated analogues of HPV type 16 L1 peptides

L1 Residues		Responses	
		H-2d Balb/c	H-2k CBA
40-63	P P V P V S K V V S T D E Y V A R T N I Y Y H A	++	++
48-63	V S T D E Y V A R T N I Y Y H A	++	+
50-63	T D E Y V A R T N I Y Y H A	++	-
52-63	E Y V A R T N I Y Y H A	-	-
54-63	V A R T N I Y Y H A	-	-
50-61	T D E Y V A R T N I Y Y	-	-
51-61	D E Y V A R T N I Y Y	-	-
91-106	V S G L Q Y R V F R I G L P D P	++	++
91-105	V S G L Q Y R V F R I G L P D	++	++
91-104	V S G L Q Y R V F R I G L P	+	+
91-103	V S G L Q Y R V F R I G L	-	-
92-103	S G L Q Y R V F R I G L	-	-
93-103	G L Q Y R V F R I G L	-	-
279-294	E Q M F V R H L F N R A G T V G	++	++
282-294	F V R H L F N R A G T V G	-	-
284-294	R H L F N R A G T V G	-	-
279-292	E Q M F V R H L F N R A G T	-	-
280-292	Q M F V R H L F N R A G T	-	-
281-292	M F V R H L F N R A G T	-	-
282-292	F V R H L F N R A G T	-	-

Truncated analogues of the three immunostimulatory peptides from L1 of HPV type 16.

Responses shown are from either Balb/c or CBA mice immunised with the full length peptide.

"++" indicates response equivalent to that of the immunising peptide.

"+" indicates an intermediate response.

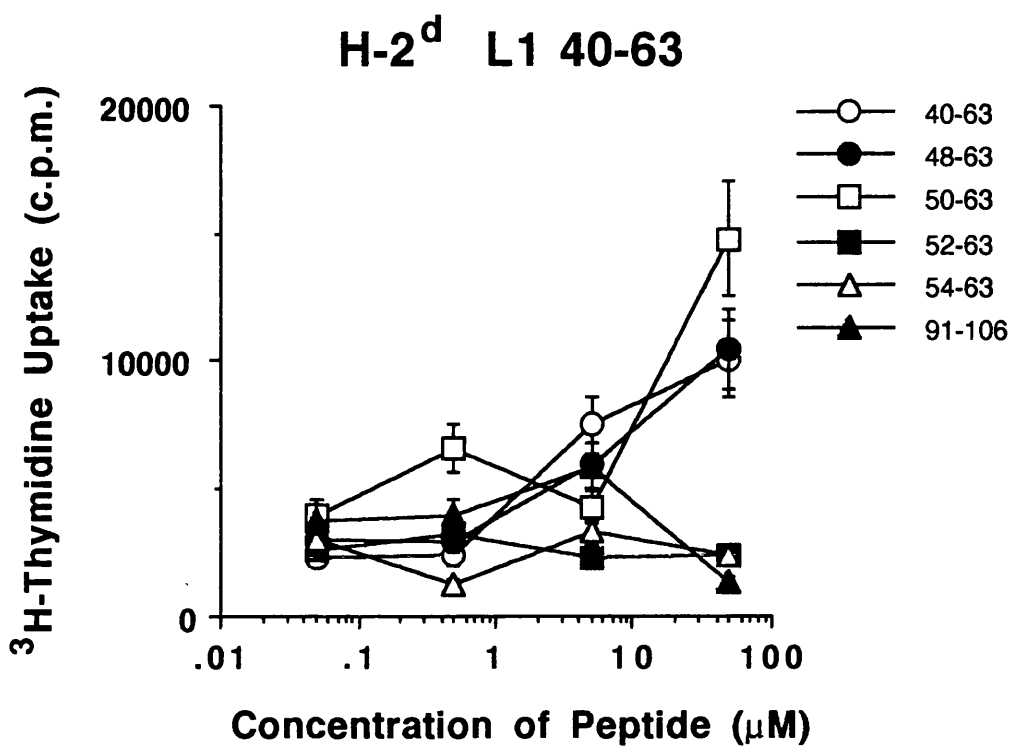
"-" indicates no response to the stimulating peptide.

Figure 3.8.

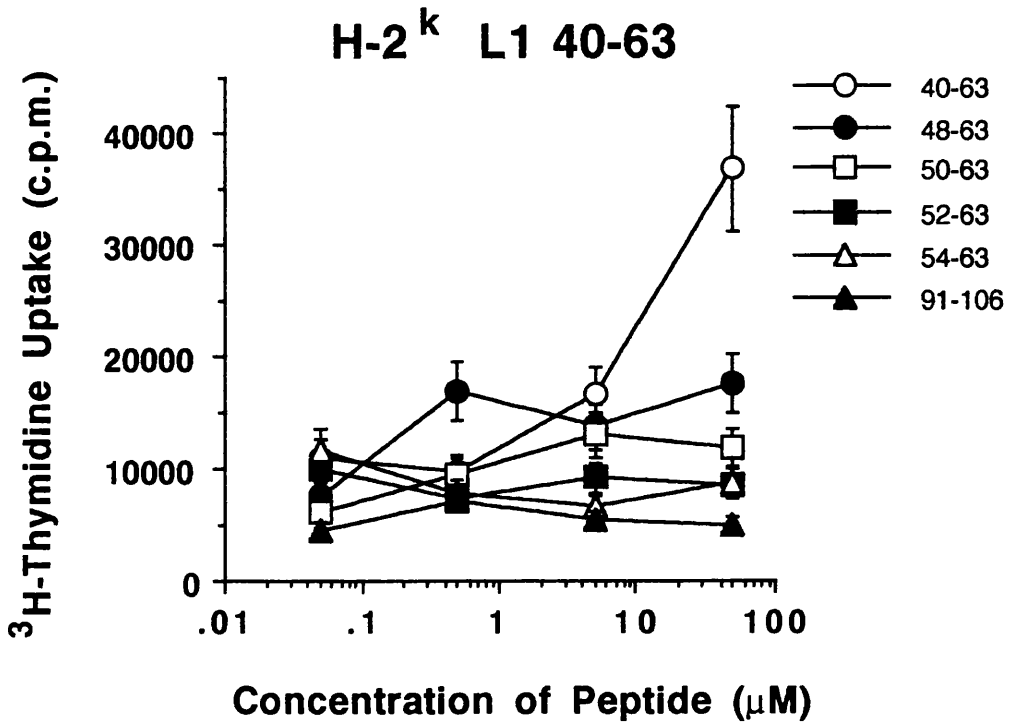
Responses to truncated analogues of HPV type 16 L1 40-63

Proliferative responses of primary lymph node cultures from **a)** Balb/c, and **b)** CBA mice primed with L1 40-63 and stimulated in vitro with the priming and the truncated analogues of L1 40-63 shown in Table 3.IV. Results are mean of triplicates and error bars give the standard deviation of the triplicate.

a


H-2d L1 40-63
20000
10000
0
.01
.1
1
10
100
3H-Thymidine Uptake (c.p.m.)
Concentration of Peptide (μM)
40-63
48-63
50-63
52-63
54-63
91-106

b


H-2k L1 40-63
40000
30000
20000
10000
0
.01
.1
1
10
100
3H-Thymidine Uptake (c.p.m.)
Concentration of Peptide (μM)
40-63
48-63
50-63
52-63
54-63
91-106

peptides were able to elicit responses from these T cells. In contrast, T cells from CBA ($H\text{-}2^{k}$) mice specific for L1 40-63 showed a weak response to L1 48-63 in one experiment but no response to any of the other truncated peptides. A summary of these data, and also of data for truncations of L1 91-106 and L1 279-294, is shown in Table 3.IV. T cells specific for L1 91-106 from both Balb/c and CBA mice responded similarly . Truncation at the carboxyl terminus by one amino acid had no affect on recognition, but removal of one more residue significantly reduced recognition, and peptide 91-104 was not recognised at all. T cells specific for L1 279-294 from both Balb/c and CBA mice also responded similarly. Removal of two amino acids from the carboxyl terminus or three amino acids from the amino terminus abolishes recognition by the T cells specific for the full length peptide (Table 3.IV). In some cases peptides shorter than those which gave no stimulation also were assayed, to see if intermediate peptides were simply resulting in particular peptide MHC protein complexes which could not be recognised.

In summary, 50-63 represents the minimal length determinant, as defined by this assay system, for $H\text{-}2^{d}$ restricted L1 40-63 responsive T cells. A different determinant is recognised by $H\text{-}2^{k}$ restricted T cells, but the position of this determinant has not been defined by these experiments. T cells specific for L1 91-106, restricted by both $H\text{-}2^{d}$ and $H\text{-}2^{k}$, recognise an epitope within residues 91-105 although further experiments would be required to more accurately define the amino terminus. T cells specific for L1 279-294, restricted by both $H\text{-}2^{d}$ and $H\text{-}2^{k}$, recognise an epitope which lies within the residues of the full length peptide and must include residues 282-293.

3.2.6. Cross-reactivity with L1 peptides from other HPV types

The L1 proteins of the human papillomaviruses are highly conserved amongst different HPV types (Giri and Danos, 1986). The T cell responses to HPV type 16 L1 peptides were therefore examined to determine their cross reactivity with any of the other known and sequenced HPV types. Since the three L1 peptides (40-63, 91-106,

and 279-294) elicited good responses in Balb/c (H-2^d) mice this strain was used for these studies. T cells specific for L1 40-63 were able to respond to residues L1 50-63, and T cells specific for L1 91-106 and L1 279-294 responded well to the full length peptides. Therefore the sequences of L1 residues 50-63, 91-106, and 279-294 were used to search homologous sequences within L1 proteins from other sequenced HPV types. A single homologous region was identified in each L1 protein examined and these are shown in Table 3.V. The peptides corresponding to these regions were synthesised and then tested for their ability to stimulate T cells specific for the prototype peptides from the L1 protein of HPV type 16. Balb/c (H-2^d) mice were immunised with HPV type 16 peptide L1 40-63 and eight days later the lymph node cells assayed for proliferation against HPV type 16 L1 50-63 and the analogous peptides from other HPV types . There was no response from the HPV type 16 L1 50-63 specific T cells to any of the analogue peptides from other HPV types over a wide concentration range (Figure 3.9). Identical results were obtained when the analogues of HPV type 16 L1 91-106 and 279-294 were tested for their ability to stimulate T cells specific for these peptides. The data are summarised in Table 3.V and demonstrate that all the peptide responses were highly specific for HPV type 16.

3.2.7. MHC Restriction of the T cell responses to HPV type 16 L1 peptides

Since the three peptides, 40-63, 91-106, and 279-294, from the L1 protein of HPV type 16 were able to elicit responses in both Balb/c (H-2^d) and CBA (H-2^k) mice the peptides must be able to form immunogenic complexes with different MHC proteins. To determine which MHC molecules were involved the MHC restriction of the T cell responses to the three immunostimulatory peptides from the HPV type 16 L1 protein was examined in detail. Each of the three peptides were immunised individually into Balb/c or CBA mice and the draining lymph node cells assayed for in vitro proliferative activity in the absence or presence of anti-class II monoclonal antibodies.

Table 3.V.

Amino acid sequences of, and responses to, analogues of HPV type 16 L1 peptides

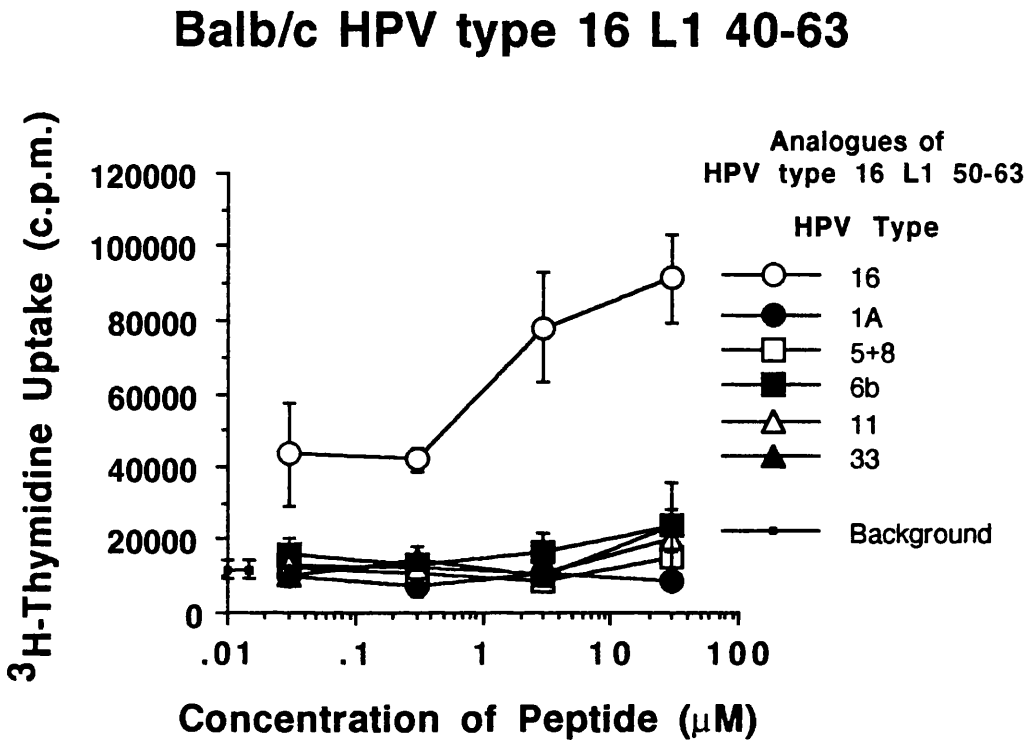
HPV type	Residues of L1	Sequence	Response
16	50-63	T D E Y V A R T N I Y Y H A	+
1a	20-33	- - - - - T - - - L F - - -	-
5/8	25-38	- - - - I Q - - - - - - - -	-
6b	23-36	- - A - - T - - - F - - -	-
11	23-36	- - A - - K - - - - F - - -	-
18	85-98	- - D - - T P - S - F - - -	-
33	24-37	- - - - - S - - S - - - Y -	-
16	91-106	V S G L Q Y R V F R I H L P D P	++
5/8	66-81	- - - N - H - - - - L K - - - -	-
6/11	61-74	- - - Y - - - - - K V V - - - -	-
18	117-130	- - A Y - - - - - - V Q - - - -	-
33	65-78	- - - - - - - - - - V R - - - -	-
16	279-294	E Q M F V R H L F N R A G T V G	++
1a	257-272	- - - Y T - - F - T - G - S - -	-
5/8	252-267	- - C Y A - - F - V - G - K T -	-
6b	249-264	- - - - A - - F - - - - - E - -	-
11	250-265	- - - - A - - F - - - - - - - -	-
18	314-329	- - L - A - - F W - - - - - M -	-
33	253-268	- - - - - - - F - - - - - - L -	-

The HPV type 16 sequences were compared with all the known sequences in the NBRF protein data bank using the proramme FIND, from the University of Wisconsin Software Package, and allowing up to 8 mis-matches between the template and the retrieved sequences.
There may be more HPV types with homologous sequences to the HPV type 16 L1 peptides, however these were the only sequences available within the data base.
A dash indicates identity with the HPV type 16 sequence.
Responses are of T cells from Balb/c mice, immunised with the full length L1 peptides (L1 40-63, 91-106, 279-294), to the peptides shown.
Sequences: 1a (Danos et al., 1982); 5/8 (Fuchs et al., 1986);
6b (Schwarz et al., 1983); 11 (Dartman et al., 1986);
18 (Cole and Danos, 1987); 33 (Cole and Streeck, 1986)

Figure 3.9.

Cross reaction of HPV type 16 specific T cells with other HPV types

Proliferative responses of primary lymph node cells from Balb/c mice primed to L1 40-63 and stimulated in vitro with HPV type 16 L1 50-63 and analogous peptides from other HPV types (Table 3.V). Results are mean of triplicates and error bars give the standard deviation of the triplicate.


Balb/c HPV type 16 L1 40-63
^{3}H-Thymidine Uptake (c.p.m.)
120000
100000
80000
60000
40000
20000
0
.01
.1
1
10
100
Concentration of Peptide (μM)
Analogues of
HPV type 16 L1 50-63
HPV Type
16
1A
5+8
6b
11
33
Background

Inhibition of the proliferative response in the presence of a particular antibody would indicate the response is restricted through the MHC molecule the antibody was reactive with. The assays were performed with an anti-I-$E^{d/k}$ (14.4.4S), an anti-I-A^d (HB3), and an anti-I-A^k (H40) antibody, which had each been affinity purified on protein A-sepharose (Materials and Methods, 2.7.5). Each assay was performed with all three antibodies with one acting as a control antibody reactive with a haplotype irrelevant to the experiment. The peptides were used in the assays at a constant concentration of 15μM resulting in a suboptimal response which would be easier to inhibit than a maximal response, but also produces a sufficient signal to easily measure the degree of inhibition. The antibodies were added at concentrations of 5, 0.5 and 0.05 μg/ml to determine whether the inhibition was titratable. The results from a typical series of experiments are shown in Figures 3.10 and 3.11. Whenever the inhibition was not clearly titratable the experiments were repeated twice. The data from two experiments for each peptide, immunised into mice of each haplotype, are summarised in Table 3.VI. The maximum inhibition was obtained with the maximum concentration of antibody used (5μg/ml) and hence the summary Table 3.VI shows the data for this concentration of antibody. When the peptide L1 40-63 was used to immunise CBA (H-2^k) mice the resultant in vitro responses could be inhibited to different degrees by both an anti-I-$E^{k/d}$ and an anti-I-A^k antibody. The small degree of inhibition seen with the anti-I-A^d antibody must be non-specific since there are no I-A^d molecules present in the assay. When T cells from Balb/c (H-2^d) mice immunised with peptide L1 40-63 were assayed then the responses could be inhibited by both the anti-I-$E^{k/d}$ and the anti-I-A^d antibody. Responses to peptide L1 91-106 could be inhibited by both the anti-I-$E^{k/d}$ and the anti-I-A^k antibody when T cells from CBA (H-2^k) mice were assayed but only by the anti-I-$E^{k/d}$ antibody when the T cells from the Balb/c (H-2^d) mice were assayed. The responses to the peptide L1 279-294 were inhibited in a similar way to those of the peptide L1 91-106. None of the responses were ever inhibited 100%, even when

Figure 3.10.

Anti-class II antibody inhibition of H-2^d restricted T cell responses to HPV type 16 L1 peptides

Proliferative responses of primary lymph node cells from Balb/c mice primed with **a)** L1 40-63, **b)** L1 91-106, and **c)** L1 279-294 and challenged in vitro with the priming peptide in the presence or absence of protein-A sepharose purified anti-Ia antibodies. Antibodies used were 14.4.4S (anti-I-E$^{d/k}$), HB3 (anti-I-A^d), and H40 (anti-I-A^k) (Materials and Methods, Table 2.II.). Results are mean of triplicates and error bars give the standard deviation of the triplicate.

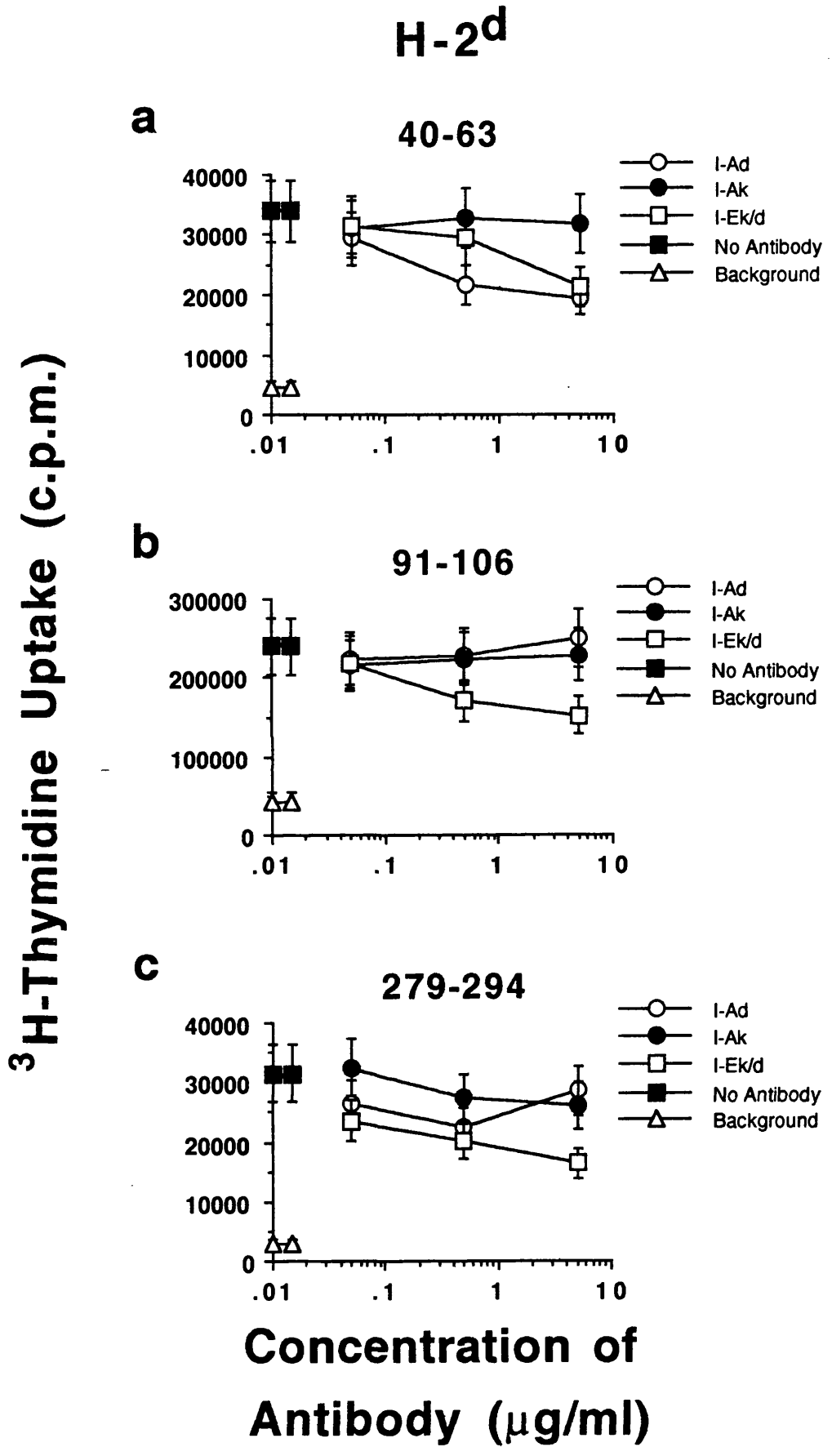

H-2d
a
40-63
40000
30000
20000
10000
0
.01
.1
1
10
I-Ad
I-Ak
I-Ek/d
No Antibody
Background
b
91-106
300000
200000
100000
0
.01
.1
1
10
I-Ad
I-Ak
I-Ek/d
No Antibody
Background
c
279-294
40000
30000
20000
10000
0
.01
.1
1
10
I-Ad
I-Ak
I-Ek/d
No Antibody
Background
3H-Thymidine Uptake (c.p.m.)
Concentration of Antibody (μg/ml)

Figure 3.11.

Anti-class II antibody inhibition of H-2^k restricted T cell responses to HPV type 16 L1 peptides

Proliferative responses of primary lymph node cells from CBA mice primed with **a)** L1 40-63, **b)** L1 91-106, and **c)** L1 279-294 and challenged in vitro with the priming peptide in the presence or absence of protein-A sepharose purified anti-Ia antibodies. Antibodies used were 14.4.4S (anti-I-E$^{d/k}$), HB3 (anti-I-A^d), and H40 (anti-I-A^k) (Materials and Methods, Table 2.II.). Results are mean of triplicates and error bars give the standard deviation of the triplicate.

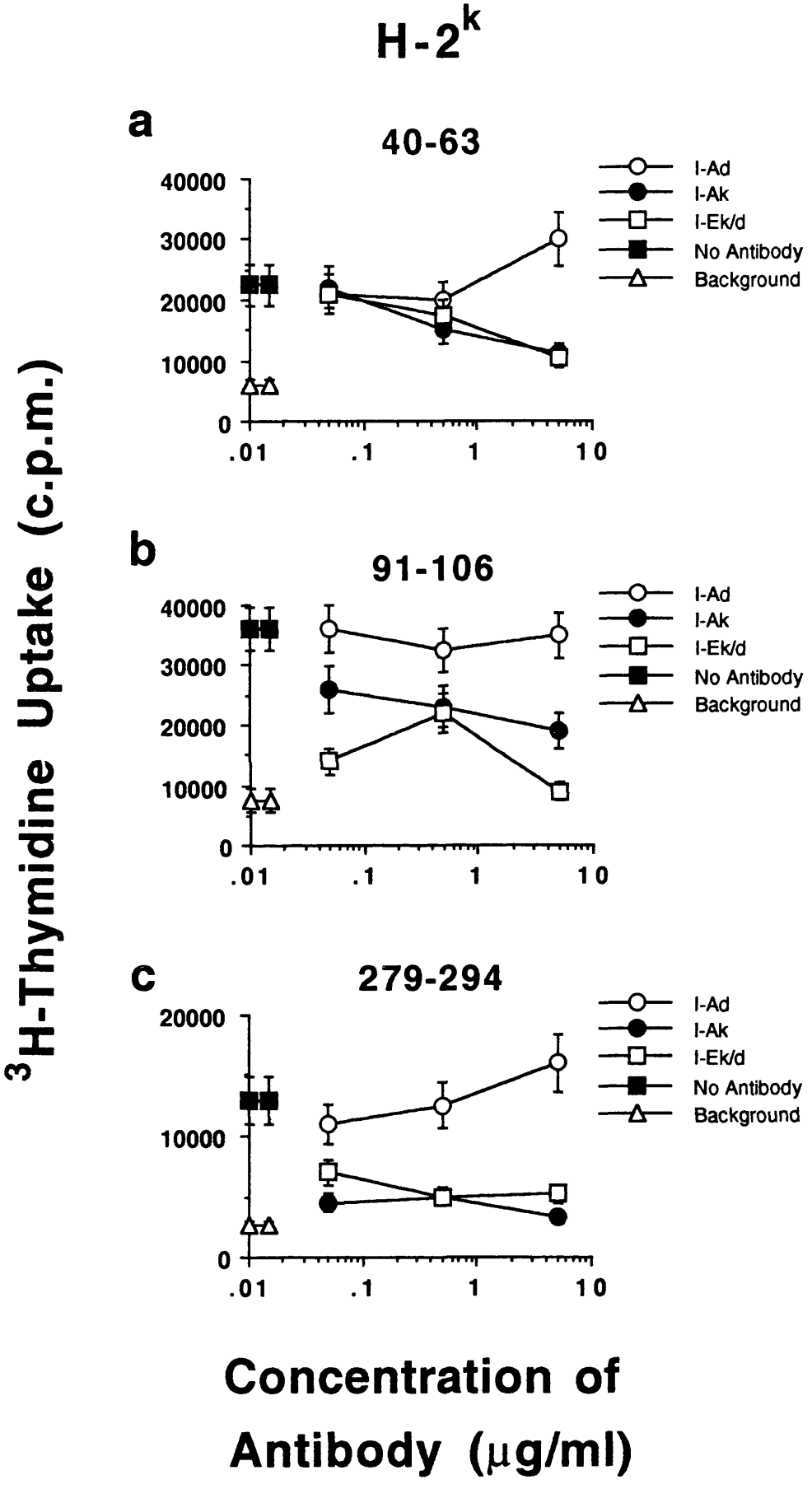

H-2k
a
40-63
b
91-106
c
279-294
I-Ad
I-Ak
I-Ek/d
No Antibody
Background
40000
30000
20000
10000
0
.01
.1
1
10
3H-Thymidine Uptake (c.p.m.)
Concentration of Antibody (μg/ml)

Table 3.VI.

Antibody inhibition of proliferative responses to HPV type 16 L1 peptides

Experiment	Haplotype	Peptide	Antibody Specificity		
			I-E$^{k/d}$	I-A^{k}	I-A^{d}
			% Inhibition		
	CBA				
1	H-2^{k}	40-63	33	61	14
2			54	50	0
1		91-106	75	34	0
2			75	49	3
1		279-294	21	69	0
2			61	75	0
	Balb/c				
1	H-2^{d}	40-63	38	7	43
1		91-106	37	4	0
2			49	0	0
1		279-294	43	6	4
2			48	17	10

The results of two experiments are shown for each peptide used to immunise each mouse haplotype (except L1 40-63 immunised into Balb/c (H-2^{d}) mice).
The concentration of stimulating peptide was 15μM
The concentration of inhibitory antibody was 5 μg/ml
The percentage inhibition was calculated using the following formla: (1-(cpm in the presence of antibody - background) / (cpm in the absence of antibody - background)) x 100.

higher concentrations of antibody were used, although such lack of complete inhibition is normal for such antibody inhibition experiments.

The data indicated that responses elicited by peptide L1 40-63 were restricted by all four possible class II restriction elements (I-E^k, I-E^d, I-A^k, and I-A^d) whereas both the responses to L1 91-106 and L1 279-294 were restricted by I-E^k, I-E^d and I-A^k. These data are summarised in Table 3.VII. and indicate that the peptides are capable of forming immunogenic complexes with several different MHC proteins.

3.3. Discussion

3.3.1. Success of predictive algorithm

The predictive algorithm of Rothbard and Taylor (1988; Taylor, 1988) was used to analyse three ORFs from HPV type 16. Thirteen peptides, covering 24% of the protein analysed, were synthesised and their immunogenicity in mice determined (Table 3.VII). Five of the thirteen peptides elicited responses in the mouse strains used. The peptides which were shown to elicit responses in the mouse strains tested did not correlate with the peptides containing the patterns with the highest scores based on the predictive algorithm. However, the significance of this is hard to assess since the algorithm is certainly not allele specific and the predicted HPV type 16 peptides which failed to elicit responses in the experiments described may induce responses in other strains of mice not tested in this study. Clearly the use of the predictive algorithm resulted in the successful prediction of T cell determinants within HPV type 16 without the need to synthesise peptides spanning the vast majority of the ORFs. However, the statistical significance of the success rate is hard to assess without knowledge of all the other determinants within the proteins analysed and whether the determinants identified were immunodominant. An estimate can be made of the statistical significance by considering the experiments by Schaeffer et al. (1989) who analysed the T cell responses to *Staphylococcal aureus* ribonuclease by synthesising overlapping peptides

Table 3.VII.

Summary of Immunostimulatory Responses Elicited by Peptides from the L1, E6, and E7 ORFs of HPV type 16

ORF and Residues	Mouse Strain / H-2 molecule expressed			
	Balb/c (H-2^d)		CBA (H-2^k)	
L1 Residues	I-A^d	I-E^d	I-A^k	I-E^k
29-44	-		-	
40-63	+	+	+	+
91-106	-	+	+	+
209-224	-		-	
219-244	-		-	
279-294	-	+	+	+
295-310	-		-	
382-397	-		-	
E6 Residues				
27-39	-		nt	
46-57	-		nt	
71-84	+		nt	
88-101	-		nt	
E7 Residues				
82-97	+		nt	

Key: "+" response restricted by particular MHC molecule or, response elicited in a particular strain of mouse.

"-" response not restricted by particular allele or not elicited from particular strain of mouse

"nt" not tested

spanning the entire protein. Approximately 21% of the peptides analysed were able to elicit responses in the strains of mice tested (Schaeffer et al., 1989). Five out of 13 peptides (39%) of the peptides tested from HPV type 16 were able to elicit responses which suggested that the algorithm was almost twice as effective as randomly synthesising peptides at identifying T cell epitopes. This is probably an underestimate because Schaeffer et al. (1989) used more than two strains of mice in their study. Although T cell determinants within HPV type 16 have clearly been identified, the experiments did not address whether there were still further T cell epitopes within these proteins or the immunodominance of any of these determinants.

The existence of class II restricted epitopes in the capsid protein, L1, of HPV type 16 was consistent with the presence of T helper cell epitopes in the capsid proteins of other viruses (Milich et al., 1989). This was also consistent with the recent demonstration that sera from patients with HPV type 16 cervical neoplasias will react with peptides from the HPV type 16 L1 protein in ELISA based assays (Dillner et al., 1990). However, there was no evidence that the identified L1 T cell determinants were indeed generating T helper cells, or that they were the immunodominant epitopes which would be relevant to a viral infection.

The generation of proliferative responses to the peptides from the E6 and E7 proteins of HPV type 16 is not surprising considering the method of immunisation used. The relevance of such responses to the E7 protein is suggested by the recent demonstration that sera from patients with cervical neoplasias showed reactivity with peptides from E7, and an E7 fusion protein (Jochmus-Kudielka, 1989; Muller, 1990). This indicates that T helper epitopes within this protein may be relevant although, as for the L1 protein determinants, the epitopes defined here may not be the immunodominant ones within the E6 and E7 proteins.

3.3.2. Immunodominance of the epitopes defined

In order to help determine whether the responses elicited by the HPV type 16 peptides within this study would be relevant during a viral infection their immunodominance needed to be established (Gammon et al. 1987). To discover whether the determinants identified were immunodominant within a protein required the complete protein. If immunisation with the protein produced T cells reactive with the peptides or if peptide specific T cells were stimulated by the protein, this would indicate their immunodominance. If immunisation with the protein produced T cells which were not reactive with the peptides or if peptide specific T cells were not stimulated by the protein, then this would indicate that the determinants were minor or cryptic.

At the time of the studies described intact E6, E7, and L1 proteins were unavailable to determine the immunodominance of the peptides. As an initial step in analysing the immunodominance the three peptides from the L1 protein, they were co-immunised into mice and the resultant T cell responses analysed with individual peptides. The peptide L1 40-63 gave the greatest proliferative response, as it had when the peptides were immunised individually into mice, however,the response to peptide L1 279-294 was now less than that of L1 91-106. One simple explanation would be that the affinity of the peptides 40-63 and 91-106 for the restriction elements involved in generating the responses (I-E^{k+d} and I-A^{k}) is greater than the affinity of L1 279-294 and hence competition at the level of MHC binding is taking place. Peptide 40-63 is restricted through all four alleles in the F1 mice whereas peptide 91-106 is only restricted through three which may explain why the response to 40-63 is consistently the highest. Establishing the relative immunodominant status of the three peptides still does not indicate whether any of them are immunodominant within the whole L1 protein. To address this problem these peptides have recently been used to generate T cell lines which were shown to react with the whole L1 protein on solubilised nitrocellulose (Davies et al., 1990). Such responses indicated that these epitopes may

be immunodominant although further analysis is required to establish whether there are other major determinants within the L1 protein.

3.3.3. Cross reactivity with other HPV types

As previously discussed (Chapter 3.2.6) the L1 proteins of the papillomaviruses are highly conserved between different types and hence it was of interest to examine whether or not T cells responding to the immunogenic L1 peptides would respond to equivalent areas in other HPV types. The homologous peptide sequences identified contained up to eight amino acid differences from the HPV type 16 sequence. In several cases these were quite conservative substitutions. For example HPV type 1a L1 20-33 has three amino acid differences from HPV type 16 L1 50-63; a Thr for an Ala, a Leu for an Ile, and a Phe for a Tyr. The first substitution replaces a small hydrophobic residue with a small polar residue, the second replaces a branched hydrocarbon side chain with another branched hydrocarbon side chain, and the third substitution replaces an aromatic side chain with another aromatic side chain. However, despite such conservative substitutions, the T cells elicited by the peptides from L1 were highly specific for the immunising peptide (Figure 3.9) and there was no cross reactivity with any of the homologous peptides from other HPV types (Table 3.V). The specificity of these responses was consistent with the high degree of antigen specificity which is associated with the T cell receptor. However, since in general very few residues in a T cell determinant make highly specific contacts with the MHC binding site (Rothbard, 1989; Sette et al., 1989a; Introduction, 1.8) if these residues were making interactions solely with the MHC protein concerned one might expect them to be tolerated. This suggested that these divergent residues in the analogues were making interactions with particularly important contact sites in the MHC, or contacting directly the T cell receptors of major T cell populations elicited in the primary response to the immunising peptide.

When human T cell lines, specific for these same peptides, were generated from asymptomatic individuals (Strang et al 1990) a line specific for HPV type 16 L1 40-63 could be stimulated by both HPV type 16 L1 50-63 and by analogue peptides from HPV types 1a, 6b, and 33, indicating that there were cross reactive T cells present. Cross reactivities were also seen in a human T cell line specific for HPV type 16 L1 91-106 with peptides from HPV type 18 and 33. Hence, human T cell lines specific for HPV type 16 L1 peptides were able to respond to some of the other HPV types in contrast to the murine T cells which failed to respond to peptides from any of the L1 molecules from other HPV types. The differences in cross reactivity between the human and the murine studies could be due to the use of only a limited number of subjects and strains in these studies, or could be a difference between the use of primary cultures and T cell lines. Alternatively the human subjects may have been primed by an HPV type other than HPV type 16 and the T cells were sufficiently cross reactive to be grown out in the lines with HPV type 16 peptides. Clearly, more work is required to resolve these issues.

3.3.4. HPV type 16 L1 peptides bind to multiple Class II alleles and isotypes

The responses to the three immunogenic peptides from the L1 protein of HPV type 16 were found to be restricted through multiple MHC alleles using inhibition with anti-class II antibodies (Table 3.VI). Such permissive interactions are unusual for normal T cell recognition of peptide antigen (Schwartz, 1986), however, there are several other examples of such peptides (Cease et al.,1987; Sinigaglia, 1988; Panina-Bordignon, 1989). Antibody inhibition of the proliferative responses unfortunately did not reveal the proportion of the response which was restricted through a particular allele or isotype since the antibody inhibition would have depended on both the relative affinity of the T cell receptor for the class II and the antibody affinity for the class II. Transfectants expressing individual alleles may be the most accurate way of assessing

this, however, transfectants have generally been used to stimulate established lines and clones, and only primary responses to con A have been demonstrated in the presence of class II transfectants (Germain, 1984; Norcross et al., 1984; Lechler et al., 1985). An alternative would be to use antigen presenting cells from mice mismatched at all other H-2 loci other than the one of interest.

Despite this limitation, the antibody inhibition studies indicated that the immunogenic L1 peptides defined in this study were each able to interact with several different MHC proteins. Therefore, as might be expected, these peptides do not contain any of the allele specific sub-patterns present in the epitopes analysed by Rothbard and Taylor (Rothbard and Taylor, 1988). Sette et al. have analysed a number of other murine T cell epitopes which have been shown to be able to bind to more than one MHC protein. Their group determined that there was greater similarity in the structural requirements for a peptide to bind to different alleles of the same isotype than to bind to different MHC isotypes (Sette et al. 1989d). This makes it comparatively likely that the same region of a peptide will be able to bind to, for example both I-A^d and I-A^k, rather than I-A^d and I-E^d. They also determined that peptides which bound to more that one MHC isotype contained I-A and I-E binding regions which were distinct but overlapping parts of the determinants they examined (Sette et al 1989d). The L1 peptides exhibited degenerate binding to both I-A and I-E molecules of the same allele and to I-A and I-E molecules of different alleles (Table 3.VII) and were therefore examined for possible overlapping I-A and I-E binding motifs as defined by Sette et al. Examination of the L1 peptides 91-106 and 279-294 failed to reveal either of the I-E or I-A binding motifs. The I-A^d binding motif identified by Sette et al. is based on the sequence Val His Ala Ala His Ala present in ovalbumin residues 327-332 (Sette et al., 1988; Sette et al. 1989d) and in L1 40-63 a sequence with some homology to this is present within residues 45-50; Ser Lys Val Val Ser although Ser at position 1 of the pattern has little structural homology with Val at this position in the ovalbumin peptide. Therefore the presence of overlapping I-A and I-E motifs, as defined by Sette et al.,

cannot help to explain how these L1 peptides were able to bind to different MHC proteins.

Interestingly as well as being able to stimulate murine T cell responses the same L1 peptides from HPV type 16 were also able to stimulate DR restricted responses from human T cells and must therefore be able to form antigenic complexes with human HLA-DR molecules (Strang et al., 1990). Several other T cell epitopes have been identified which are capable of inducing responses restricted through multiple mouse and also human alleles. Such promiscuous determinants have been identified in HIV (Cease et al.,1987), malaria (Sinigaglia, 1988), and tetanus toxin (Panina-Bordignon, 1989). The mechanism by which such peptides bind to different MHC proteins is uncertain. If they were to use a common mechanism of binding then this might be revealed by common structural features within the peptides. However, comparison of these peptides and the HPV type 16 determinants from L1 failed to reveal any distinctive structural characteristics present in these determinants which are not present in many other T cell epitopes.

3.3.5. Conclusions

The structural features characteristic of T cell epitopes, identified by Rothbard and Taylor, clearly were useful in predicting T cell determinants within HPV type 16. Five determinants able to elicit immunostimulatory responses in inbred mice were identified from three ORFs. The cross reactivity with other HPV types of the responses to three of the peptides was established, and suggested that the responses were highly specific for HPV type 16. The responses to the same three peptides were found to be restricted through several class II molecules.

The functional correlation of the common structural features identified by Rothbard and Taylor (1988) within the T cell determinants analysed is not certain. They could indicate common interactions between different peptides and different MHC proteins, or could be involved in for example, a common pathway of antigen

processing. A direct binding assay is required to identify structural features within a peptide important for its interaction with an MHC protein. Analysis of such structural features would help establish possible mechanisms by which the MHC binding site interacts with its peptide ligands. A simple and efficient assay to measure the direct binding of LCB lysine analogues of any peptide, to HLA-DR molecules on cell surfaces, had been developed in our laboratory (Busch et al., 1990). The next two chapters describe experiments in which LCB lysine analogues of a number of different T cell determinants were used to define their important conformational and structural features when bound to HLA-DR molecules.

Chapter 4

Conformational features of peptides bound to HLA-DR

4.1. Introduction

The molecular mechanism by which MHC proteins bind a diverse but limited range of peptides is poorly understood (reviewed Rothbard and Gefter, 1991). There are two possible extreme mechanisms by which the MHC protein antigen combining site could interact with a large number of different peptide ligands. Firstly, different peptides could bind with many different conformations and in many different locations in the binding site. Alternatively, different peptides could bind with a preferred conformation and in a preferred location within the site. To determine possible mechanisms of peptide binding a number of groups have examined the conformations of peptides which interact with MHC proteins.

Different studies have produced conflicting results as to whether peptides adopt a regular and/or common conformation when bound to MHC proteins. Some groups have found evidence for the importance of α helical conformations, whereas several others have failed to find evidence to support such a model (Introduction, 1.8). There were a number of weaknesses associated with the various approaches used such that strong conclusions were hard to draw from many of these studies. Several analyses considered the theoretical propensity of peptides to adopt a particular conformation based on amino acid composition, others used circular dichroism to determine the propensity of peptides to adopt a particular conformation in mixtures of tri-fluoroethanol and water (Pincus et al., 1983; Carbone et al., 1987; Sette et al., 1989e; Allen et al., 1989; Anderson et al., 1990; Maryanski et al., 1990; Busch, 1991). Attempts were then made to correlate the conformational propensities determined, with the affinity of the peptides for the MHC protein. A fundamental weakness of these

studies, was that they did not examine the conformations of the peptides while bound to the MHC molecule. To determine the relative conformations of pairs of bound peptides a number of groups exchanged residues, predicted to be contacting the T cell receptor, between different determinants and were able to transfer T cell recognition (Guillet et al., 1986; Rothbard et al., 1988; Lorenz et al., 1989; Introduction 1.8). These studies suggested that different peptides were binding in a similar location and with a similar conformation within the binding site. However, in a number of the studies the sequence homology between the pairs of peptides was such that the results could not be conclusive. Alternative strategies determined the conformations of peptides bound to the MHC protein by examining the ability of monosubstituted analogues to both stimulate a peptide specific T cell clone and to bind the relevant MHC protein (Sette et al., 1987; Allen et al., 1987; Fox et al., 1987; Lambert and Unanue, 1989; Bhayani and Peterson, 1989; Regnier-Vigoruoux et al., 1989; Ogasawara, 1989). These studies were effective at establishing the chemical requirements at each position in the peptide for binding the MHC protein. However, the ability of the MHC protein to tolerate a large number of different peptides led to a failure to identify all the peptide residues contacting the MHC binding site, and hence the conformation could not always be fully elucidated.

The strategy of our group to circumvent these problems was to use the unnaturally large long chain biotinylated (LCB) lysine as a substitution at each position in an influenza haemagglutinin peptide residues 307-319 (Ha 307-319) (Rothbard et al., 1989; Busch et al., 1990; Introduction, 1.8). This substitution allowed both the quantitation of peptide binding to cell surface HLA-DR, using fluorescein conjugated avidin, and also revealed MHC contact residues in the peptide more clearly than substitutions with natural amino acids. From the spacing of the contact residues with HLA-DR1Dw1 a single conformation for the bound peptide was postulated consisting of a central core folded as two turns of a helix with the amino and carboxyl termini deviating from any regular conformation.

4.1.1. Aims and Summary

In order to determine whether the conformation of Ha 307-319 bound to HLA-DR1Dw1 was characteristic of this peptide alone, or common to many determinants, biotinylated analogues of several different T cell epitopes, including Ha 307-319, were used to examine their relative conformations and orientations when bound to HLA-DR1Dw1. Six separate peptides were studied; three defined by HLA-DR1Dw1 restricted T cells, and three identified by T cells restricted through alleles other than HLA-DR1Dw1. The similar patterns of fluorescent signals observed when the former three peptides were studied indicated that they shared several conformational features when bound. In contrast, when the latter three peptides were examined, the data indicated that they shared some but not all of the conformational features characteristic of the first three peptides bound to HLA-DR1Dw1. The differences in the affinity of the peptides for HLA-DR1Dw1 allowed the conclusion that there was no simple correlation between the ability of a peptide to adopt a particular conformation when bound, and its affinity for the MHC protein. Finally, a peptide from the 65 kDa protein of *M. leprae* was shown to adopt a distinctly different conformation when bound to HLA-DR1Dw1 and HLA-DR2Dw2A. Interestingly the conformation adopted when this peptide was bound to HLA-DR2Dw2A (the MHC allele originally used to define this epitope) was very similar to the conformation of the first three peptides bound to HLA-DR1Dw1.

4.2. Results

4.2.1. LCB lysine analogues of three different peptides binding to cells expressing HLA-DR1Dw1

Initially the relative conformations and orientations of two other peptides, known to be recognised by T cells restricted by HLA-DR1Dw1, were compared with those of Ha 307-319. These were, influenza matrix residues 17-29 (Mat 17-29) (Rothbard et al., 1988) and the *M. tuberculosis* 19 kDa protein residues 3-14 (Tub 3-14) (Lamb et al., 1988) (Table **4.I.**). Their sequences were diverse relative to each other and Ha 307-319 but were still known to form antigenic complexes with HLA-DR1Dw1. Although several of the following experiments have previously been described in detail for the analogues of Ha 307-319 (Rothbard et al, 1989b; Busch et al, 1990; Busch, 91) they were included in each experiment to allow valid comparisons to be made. For each of the three peptides a set of analogues was synthesised in which the amino acids of the natural sequence were individually substituted by LCB lysine (Table **4.II**; Materials and Methods, 2.1). The LCB lysine substituted peptides were incubated individually with an EBV B cell line, IBW4, expressing HLA-DR1Dw1, to screen for their ability to bind class II molecules on the cell surface. The cells were incubated in PBS containing 2.5% FCS and 10μM of the analogue peptide for 4 hours at 37° C. Cell surface bound peptide was quantitated by staining with fluorescent avidin D (FAD) and flow cytometry on a Becton Dickinson FACscan (Materials and Methods, 2.8). A well defined pattern of fluorescence was associated with each set of peptide analogues (Figure **4.1**) and the fluorescent intensity clearly depended on both the peptide examined and the position of LCB lysine within each peptide.

The LCB lysine substituted peptides used were acetylated at their amino terminus and contained arginine substituted for the natural lysine (Materials and Methods, 2.1). The replacement of lysine with arginine was a highly conservative

Table 4.I.

Peptides recognised by T cells restricted through HLA-DR1Dw1

Peptide Name	Sequence												
	307	308	309	310	311	312	313	314	315	316	317	318	319
Ha 307-319	P	K	Y	V	K	Q	N	T	L	K	L	A	T
	17	18	19	20	21	22	23	24	25	26	27	28	29
Mat 17-29	S	G	P	L	K	A	E	I	A	Q	R	L	E
	3	4	5	6	7	8	9	10	11	12	13	14	
Tub 3-14	R	V	K	R	G	L	T	V	A	V	A	G	

Key :

Ha 307-319 - Influenza haemagglutinin residues 307-319 (Lamb et al., 1983)

Mat 17-29 - Influenza matrix residues 17-29 (Rothbard et al., 1988)

Tub 3-14 - M.Tuberculosis 19 kDa protein residues 3-14 (Lamb et al., 1988)

Table 4.II.

Examples of LCB lysine analogues of peptides shown in Table 4.I.

LCB Lysine Analogue	Sequence
Ha LCB 307	Ac 307 K(LCB) 308 R 309 Y 310 V 311 R 312 Q 313 N 314 T 315 L 316 R 317 L 318 A 319 T
Ha LCB 308	Ac 307 P 308 K(LCB) 309 Y 310 V 311 R 312 Q 313 N 314 T 315 L 316 R 317 L 318 A 319 T
Mat LCB 17	Ac 17 K(LCB) 18 G 19 P 20 L 21 R 22 A 23 E 24 I 25 A 26 Q 27 R 28 L 29 E
Mat LCB 18	Ac 17 S 18 K(LCB) 19 P 20 L 21 R 22 A 23 E 24 I 25 A 26 Q 27 R 28 L 29 E
Tub LCB 3	Ac 3 K(LCB) 4 V 5 R 6 R 7 G 8 L 9 T 10 V 11 A 12 V 13 A 14 G
Tub LCB 4	Ac 3 R 4 K(LCB) 5 R 6 R 7 G 8 L 9 T 10 V 11 A 12 V 13 A 14 G

Key: Ac - Acetylated amino terminus. LCB - long chain biotin.

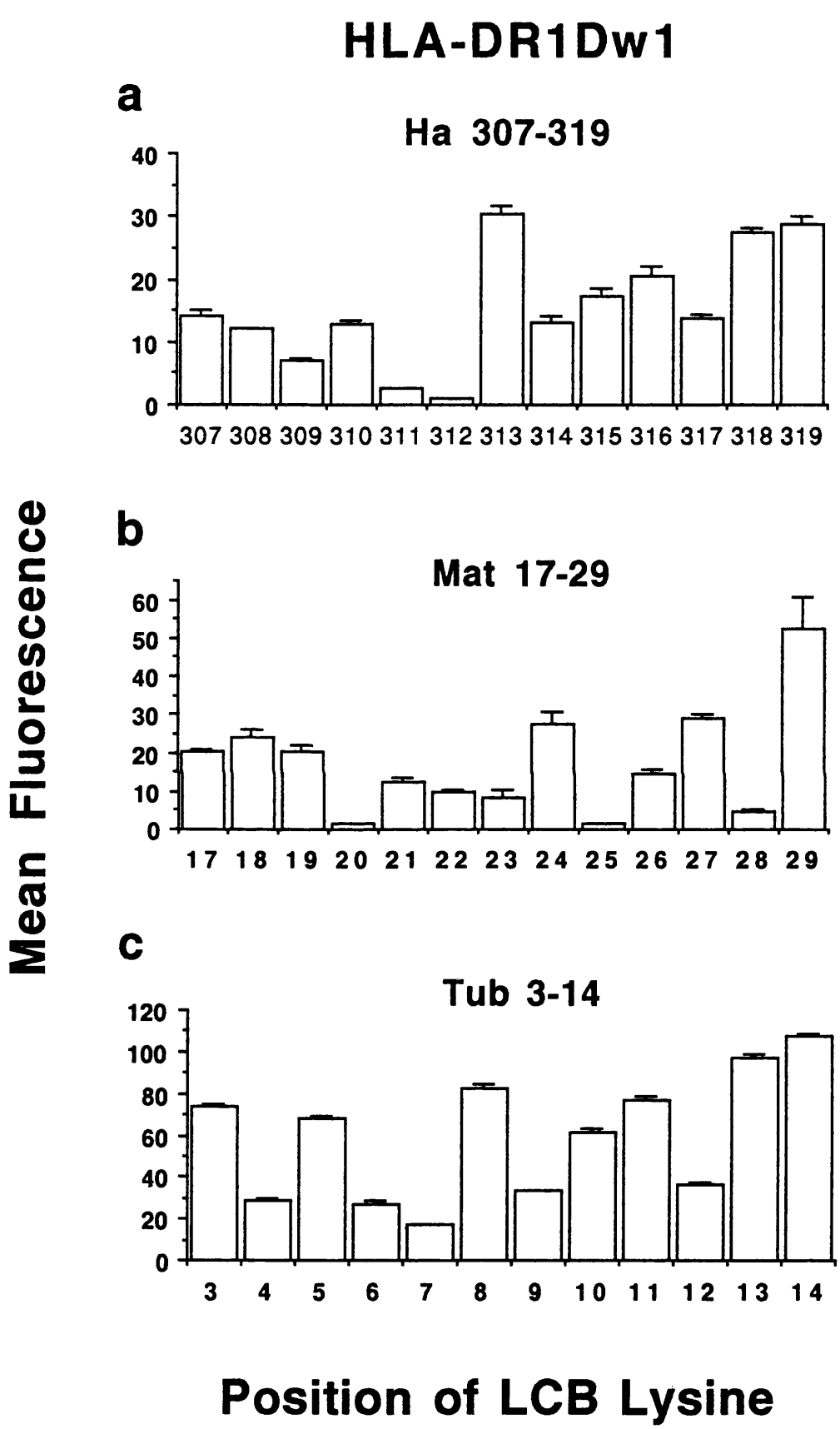
Ha LCB 307 - Ha 307-319 with LCB lysine at position 307. Other analogues are named similarly.

Amino acids are in single letter code and an Arg substituted for a natural Lys is in outline.

Figure 4.1.

LCB lysine analogues of Ha 307-319, Mat 17-29, and Tub 3-14 bound to IBW4

Mean fluorescent signals generated by LCB lysine analogues of **a)** Ha 307-319, **b)** Mat 17-29, and **c)** Tub 3-14, bound to IBW4 cells expressing HLA-DR1Dw1. Each of the analogues was at 10 μM. The Ha 307-319 analogues were assayed with a single layer FAD assay and the Mat 17-29 and Tub 3-14 analogues with a two layer FAD assay (see Materials and Methods, 2.8.4). Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples.


HLA-DR1Dw1
a
Ha 307-319
40
30
20
10
0
307 308 309 310 311 312 313 314 315 316 317 318 319
b
Mat 17-29
60
50
40
30
20
10
0
17 18 19 20 21 22 23 24 25 26 27 28 29
c
Tub 3-14
120
100
80
60
40
20
0
3 4 5 6 7 8 9 10 11 12 13 14
Mean Fluorescence
Position of LCB Lysine

substitution (Taylor, 1986) and would therefore not be expected to effect the relative ability of the analogues to bind. Recently this has been tested by synthesising analogues of HA 307-319, containing LCB lysine, on the resin using appropriate protecting groups, thereby removing the need to acetylate the amino terminus or to replace natural lysines with arginines (Busch, 1991). These analogues bound to HLA molecules identically to the set containing arginine substitutions indicating that the arginines are unlikely to affect the ability of the peptides to bind HLA-DR1Dw1 (Busch, 1991). The EBV B cell line used for these experiments had a consistently high level of MHC class II expression (see below) and as it grew in suspension it was extremely easy to use in the assay. The viability of the cells at the beginning of the assay was normally 90-95% by trypan blue staining. The presence of 2.5% FCS in the assay decreased the loss of cell viability during the assay, and peptides present in the FCS had been shown to have no affect on the ability of the analogue peptides to bind (Busch, 1991). Analogues of Ha 307-319 were assayed at 10μM using a single layer FAD assay (Materials and Methods, 2.8.4), as previously this has been shown to give a good signal with these analogues (Busch, 1991). For Mat 17-29 and Tub 3-14, 10μM was the highest concentration that the most insoluble analogue from each peptide could be conveniently used in the assay. The LCB lysine greatly reduced the solubility of certain analogues and hence the maximum concentration at which they could be used was low. For example, the analogue of Tub 3-14 with LCB lysine at position 8 (Tub LCB 8) could be used at a maximum concentration of 10 μM after being made up in solution and diluted into the assay. The binding of the the Mat 17-29 and Tub 3-14 analogues was assayed with a two layer FAD assay (Materials and Methods, 2.8.4) to maximise the signals observed. After staining with FAD, propidium iodide was added to stain dead cells. From each sample, 5000 events were recorded using the flow cytometer and analysis revealed that cell viability, normally, was approximately 80-90%. Histogram analysis of the gated data with fluorescence 1 (fluorescein) against cell number resulted in a single symmetrical peak. The mean fluorescence channel

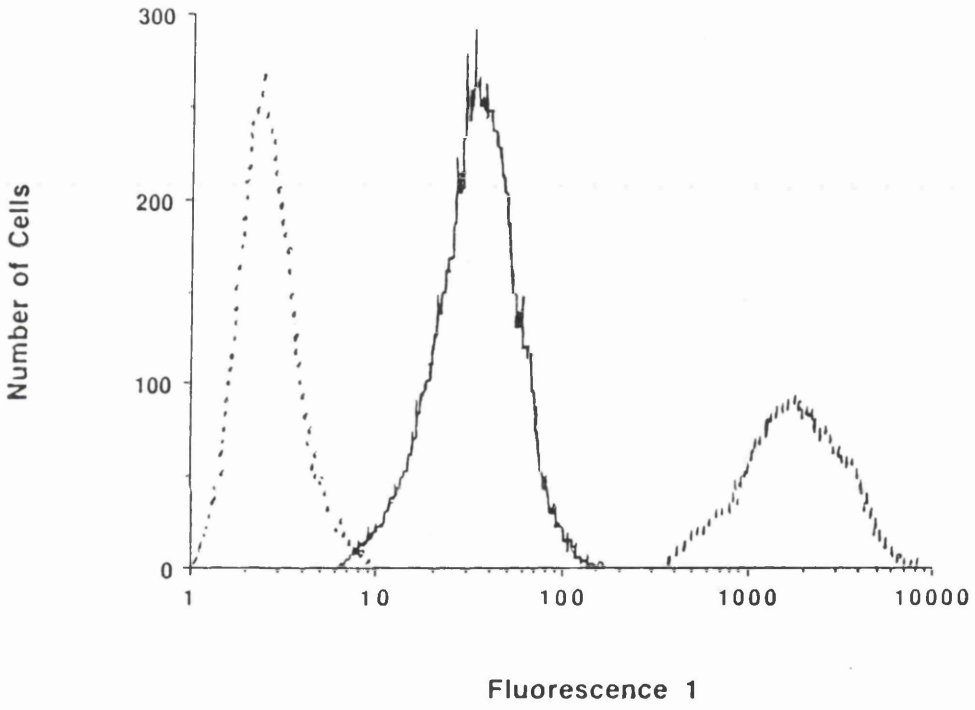
Figure 4.2.

Histogram analysis of cells from a typical cell surface binding assay

Typical histogram analysis of fluorescence 1 (fluorescein) intensity of IBW4 cells treated in the following ways: **a)** incubation with no peptide under standard binding assay conditions and then a two layer FAD assay (Materials and Methods, 2.8.4). **b)** incubation with Tub LCB 9 at 10μM under standard binding assay conditions and then a two layer FAD assay. **c)** incubation with no peptide under standard binding assay conditions followed by staining with directly fluoresceinated L243 anti-DR antibody.

Key:

a) ·····	2.2	mean fluorescence (reagent background)
b) ———	31	mean fluorescence (peptide signal)
c) – – – – –	1,973	mean fluorescence (antibody signal)


300
200
100
0
Number of Cells
1
10
100
1000
10000
Fluorescence 1

number of the peak was calculated and converted to mean fluorescence intensity (Materials and Methods, 2.5.3). A typical gated histogram analysis is shown in Figure 4.2, the three peaks include the peptide binding signal, the signal due to the staining reagents alone, and the signal due to antibody staining with directly fluorescein conjugated anti-DR antibody L243 . The signal due to staining reagents was subtracted from all signals before they were plotted in Figure 4.1. The signal due to the anti-DR antibody is considerably greater than the signal due to peptide alone and is consistent with previous results using this binding assay (Busch et al., 1990; Busch, 1991).

4.2.2. Binding of LCB lysine analogues is specific for HLA-DR1Dw1

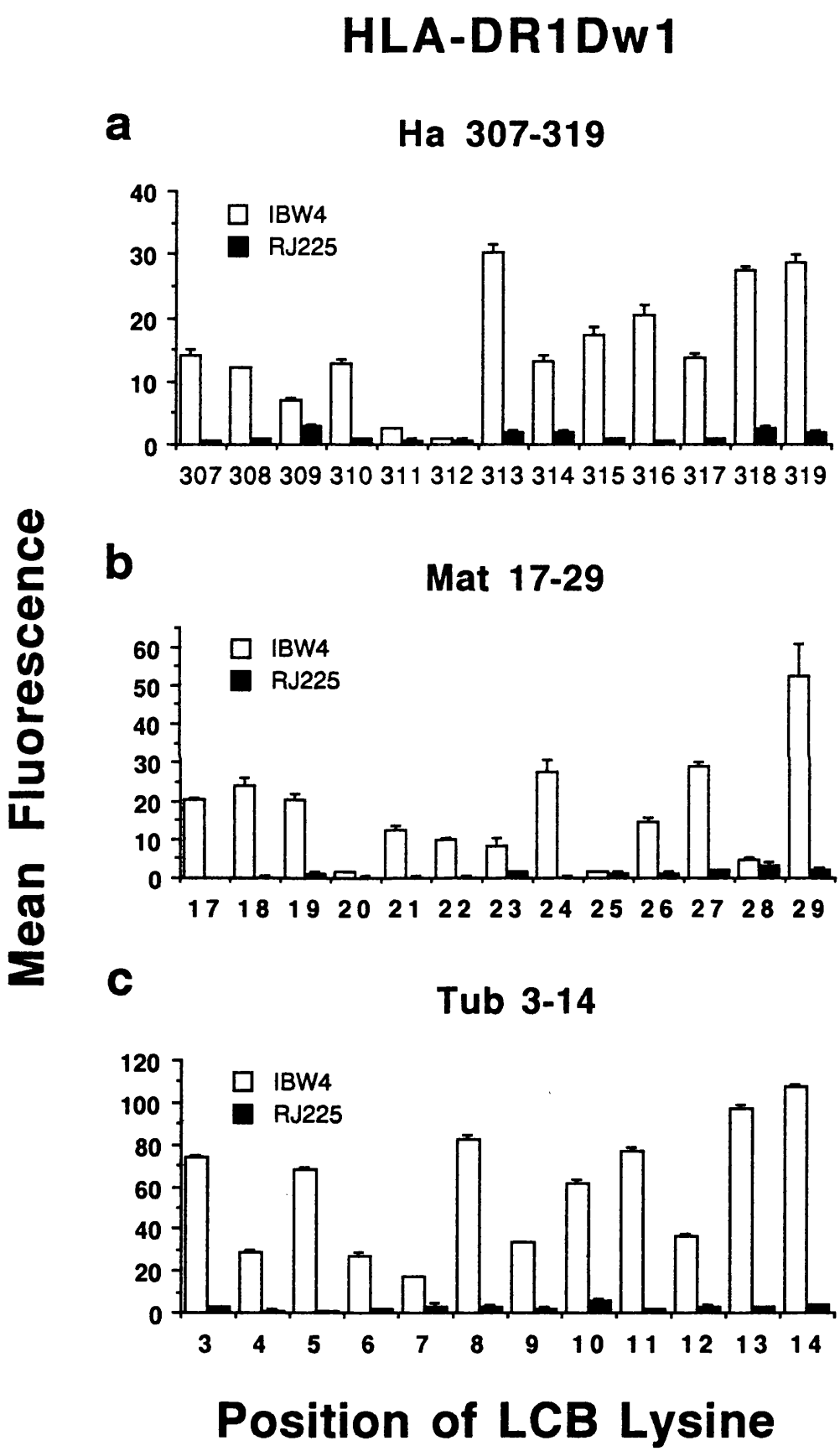
The fluorescent signals seen in Figure 4.1 represent the ability of the analogues to bind to the surface of the IBW4 cell line. However, the signals could be due to non-specific binding to the cell surface or interactions with molecules other than HLA-DR. To demonstrate that the biotinylated peptides were binding specifically to HLA class II molecules the analogues were incubated with a B cell line deficient in class II expression (RJ225) as well as with the B cell line IBW4. The RJ225 cell line is derived from an EBV positive Burkitt lymphoma cell line Raji and fails to express DR, DQ, or DP molecules on its surface due to a mutation in a trans-acting factor required for expression of these genes (Accolla, 1983; Yang et al., 1988; Koch et al., 1988). Data obtained when the analogues of the three peptides were incubated with RJ225 cells were compared to those obtained in the same experiment with IBW4 (Figure 4.3). The signals obtained with the RJ225 cell line were consistently a small fraction (< 10%) of the peak signals obtained with the IBW4 cell line, and the data therefore strongly suggested that the fluorescent signals seen with the IBW4 cell line were due to interactions of the analogues with cell surface MHC class II molecules.

To demonstrate that the LCB analogues were interacting specifically with HLA-DR1Dw1, an anti-DR antibody (LB3.1) was used to attempt to inhibit the fluorescent signals observed with the IBW4 cell line. An analogue of Ha 307-319 with LCB at the

Figure 4.3.

LCB lysine analogues of Ha 307-319, Mat 17-29, and Tub 3-14 bound to IBW4 and RJ225

Mean fluorescent signals generated by LCB lysine analogues of the **a)** Ha 307-319, **b)** Mat 17-29, and **c)** Tub 3-14 peptides, bound to, IBW4 cells expressing HLA-DR1Dw1, and RJ225 cells which do not express DR or DQ molecules. Each of the analogues was at 10 μM. The Ha 307-319 analogues were assayed with a single layer FAD assay and the Mat 17-29 and Tub 3-14 analogues with a two layer FAD assay. Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples with the reagent background subtracted.


HLA-DR1Dw1
a
Ha 307-319
IBW4
RJ225
40
30
20
10
0
307 308 309 310 311 312 313 314 315 316 317 318 319
b
Mat 17-29
IBW4
RJ225
60
50
40
30
20
10
0
17 18 19 20 21 22 23 24 25 26 27 28 29
c
Tub 3-14
IBW4
RJ225
120
100
80
60
40
20
0
3 4 5 6 7 8 9 10 11 12 13 14
Mean Fluorescence
Position of LCB Lysine

amino terminus (Ha LCB N) had previously been shown to give a fluorescent signal approximately equal to the greatest signals from the other analogues of this peptide (Rothbard et al., 1989; Busch, 1991). Incubation of Ha LCB N (10 μM) with IBW4 resulted in a fluorescent signal which could be inhibited by the affinity purified anti-DR antibody LB3.1 (Figure 4.4a). The signal titrated down towards background with increasing concentrations of LB3.1. However, at concentrations of antibody greater than 10μg/ml the four hour incubation at 37° C resulted in cell toxicity to the extent that the results of the binding assay were highly irreproducible. To establish what proportion of the cell surface class II was bound by antibody under these conditions IBW4 cells were incubated with the same peptide and with 10μg/ml of LB3.1 for four hours at 37° C and then stained with fluorescent goat anti-mouse antibody for one hour at 4° C to give a signal of 1634 fluorescence units. The signal was 80% of that obtained when the cells were incubated with peptide and 10μg/ml of LB3.1 for four hours at 37° C, and then a saturating amount of LB3.1 (25μg/ml) was added at 4° C for 1 hour and the cells stained in the same way (2058 fluorescence units). This indicated that at 10μg/ml, at 37° C, LB3.1 was only binding to 80% of the class II on the cell surface and would therefore be expected to inhibit a maximum of 80% of the peptide binding signal. The titration of the signal shown in Figure 4.4a, is shown as % inhibition in Figure 4.4b. The maximum inhibition was 65 % indicating that approximately 85% or more of the signal was due to a specific interaction of the peptide with HLA-DR1Dw1.

Although 10μg/ml of LB3.1 does not fully saturate the class II on the surface of the IBW4 cell line, this concentration was used to inhibit the binding of LCB lysine analogues from the three other peptides in order to to maximise inhibition of the signals while maintaining reproducibility of results. Selected analogues from each of the peptides, which gave a peak of fluorescence, were chosen to see if these signals could be specifically inhibited by LB3.1 and not by an anti-HLA-DQ antibody (SPVL3). When LB3.1 was used, the degree of inhibition of the fluorescent signal was between

Figure 4.4.

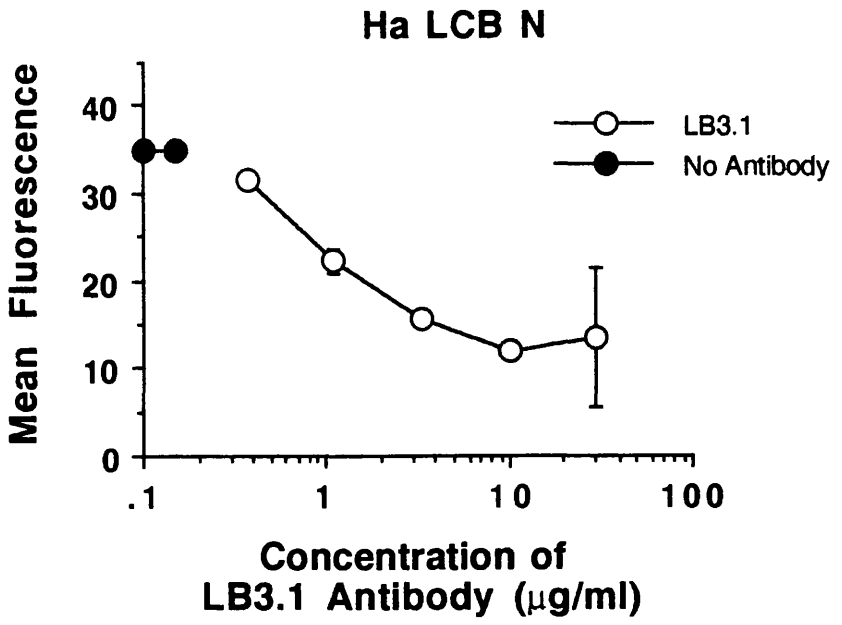
Anti-DR antibody inhibition of the binding of Ha LCB N to HLA-DR1Dw1

a) Effect of the anti-DR antibody LB3.1 on the fluorescent signal generated by Ha LCB N when co-incubated with IBW4 cells expressing HLA-DR1Dw1. Ha LCB N was at 10 μM and the binding determined with a single layer FAD assay. Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples with the reagent background subtracted.

b) Data from **a)** replotted as percentage inhibition using the following formula

% inhibition = (1 - (mean fluorescence with antibody / mean fluorescence without antibody)) x 100.

a


Ha LCB N
Mean Fluorescence
40
30
20
10
0
.1
1
10
100
LB3.1
No Antibody
Concentration of
LB3.1 Antibody (µg/ml)

b

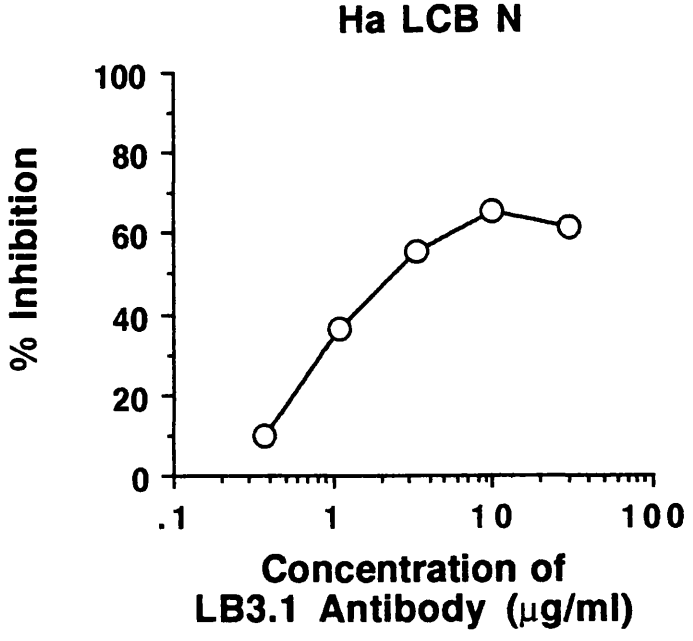

Ha LCB N
% Inhibition
100
80
60
40
20
0
.1
1
10
100
Concentration of
LB3.1 Antibody (µg/ml)

Table 4.III.

Antibody inhibition of the binding of LCB lysine analogues of Ha 307-319, Tub 3-14, and Mat 17-29, to HLA-DR1Dw1

Peptide	Position of LCB	% Inhibition
Ha 307-319	310	74
	313	78
	316	80
Mat17-29	21	69
	24	ND
	27	80
Tub 3-14	5	81
	8	83
	11	71

Antibody inhibition of the binding of LCB analogues to IBW4 cells expressing HLA-DR1Dw1, was performed as described in Materials and Methods, 2.8.5. Ha 307-319 and Tub 3-14 analogues were at 10μM, and Mat 17-29 analogues were at 20μM. Antibody inhibition was at 10μg/ml of the anti-DR antibody LB3.1.

70 and 80 % for each of the analogues examined indicating that the vast majority of the signal was due to interactions with DR molecules on the cell surface (Table **4.**III). Inhibition experiments using the anti-DQ antibody SPVL3 resulted in less than 5% inhibition of any of the signals over a range of antibody concentration (Data not shown). The above data therefore helped to confirm that the fluorescent signals observed when the LCB lysine analogues of the Ha 307-319, Mat 17-29, and Tub 3-14 peptides were incubated with IBW4 were due to interactions with cell surface HLA-DR1Dw1.

4.2.3. Fluorescent signal is proportional to the affinity of the peptides for HLA-DR1Dw1

The variation in the fluorescent signals observed was dependent on the position of LCB lysine within a peptide. This was consistent with not all amino acids contributing equally to the formation of the peptide MHC protein complex. LCB lysine substituted for residues unimportant in binding should not have interfered with the formation of the complex and should have resulted in a high fluorescent signal. In contrast, substitution of a critical residue should have dramatically decreased peptide binding and the resultant fluorescent signal. This assumption was true only if the LCB lysine reduced the affinity of the peptide for HLA-DR1Dw1, and did not, for example, modify the peptides ability to adopt the correct conformation for binding, or its susceptibility to cellular proteases. Several experiments had been performed which indicated that the LCB lysine had no affect on the conformational propensity of the Ha 307-319 peptides in solution (Busch et al., 1990; Busch, 1991; see Discussion). The results suggested that the LCB lysine substitutions within the Mat 17-29 and Tub 3-14 analogues were unlikely to affect the conformational propensity of these analogues in solution.

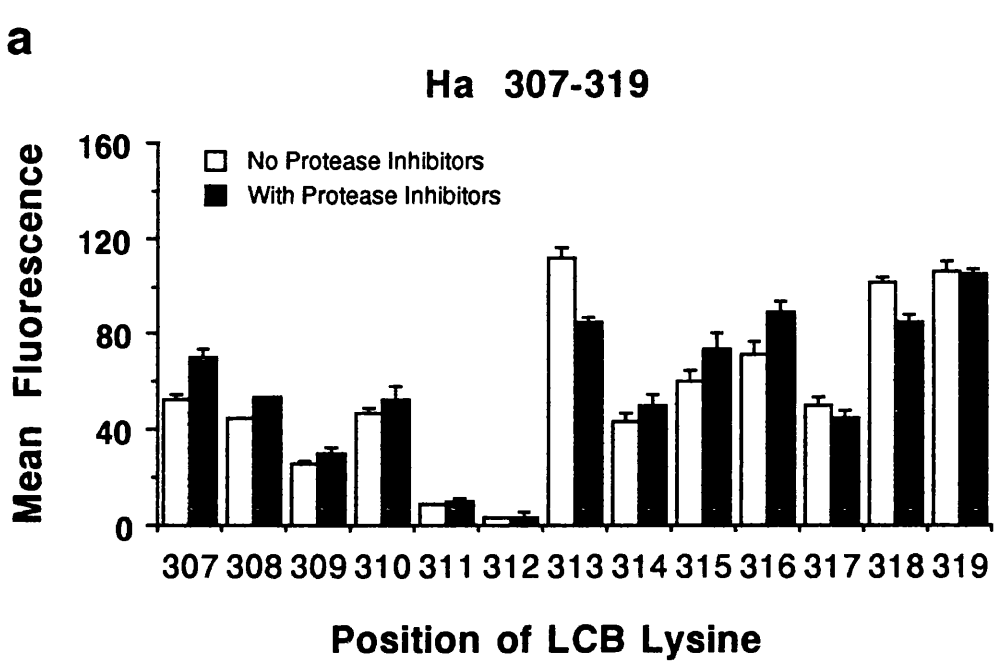
To determine if proteases were affecting the integrity of the peptide analogues in the assay, and hence the relative signals shown, the assays were repeated with the Ha

Figure 4.5.

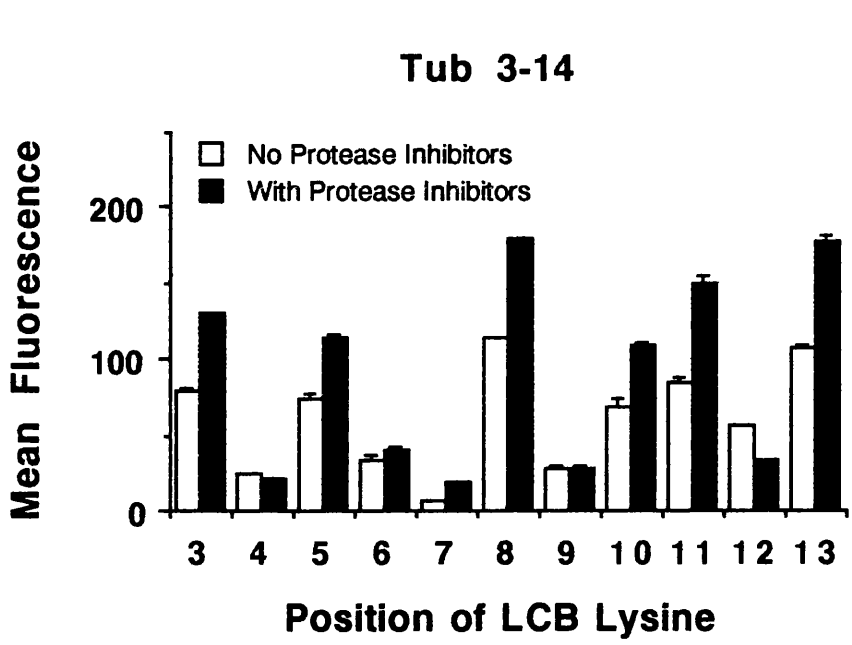
LCB lysine analogues of Ha 307-319 and Tub 3-14 bound to IBW4 with and without protease inhibitors

Mean fluorescent signals generated by LCB lysine analogues of **a)** Ha 307-319, and **b)** Tub 3-14, bound to IBW4 cells expressing HLA-DR1Dw1 in the absence or presence of the following protease inhibitors: leupeptin (750μM), TPCK (100μM), pepstatin (100μM). Both peptides were at 10μM and the binding determined with a two layer FAD assay. Mean fluorescence is in arbitrary units and each signal is the mean of triplicate samples with the reagent background subtracted.

a


Ha 307-319
No Protease Inhibitors
With Protease Inhibitors
Mean Fluorescence
160
120
80
40
0
307 308 309 310 311 312 313 314 315 316 317 318 319
Position of LCB Lysine

b


Tub 3-14
No Protease Inhibitors
With Protease Inhibitors
Mean Fluorescence
200
100
0
3 4 5 6 7 8 9 10 11 12 13
Position of LCB Lysine

307-319 analogues in the presence of a number of protease inhibitors (Figure 4.5a). The combination of leupeptin, TPCK, and pepstatin inhibit a broad cross section of normal cellular proteases (Materials and Methods, 2.8.6), including the cathepsins believed to be important in antigen processing (Berzofsky et al., 1989). The absolute peak intensities were slightly different in the presence of the protease inhibitors, however, there was little difference in the relative signal intensities observed. This indicated that the position of LCB lysine was not having a differential effect on the susceptibility of the peptides to proteolysis. A similar experiment was performed with analogues of Tub 3-14 and the data for this is shown in Figure 4.5b. Again the proteases had little effect on the relative signal intensities indicating that the LCB lysine, if it was affecting the susceptibility of the peptide to proteases, was effecting each of the analogues in a similar fashion. Unfortunately due to the lack of availability of the Mat 17-29 analogues similar experiments could not be performed with these peptide. Overall the experiments indicated that the position of the LCB lysine along the length of the peptides had no differential affect on the ability of the peptides to form a particular conformation or their susceptibility to proteases.

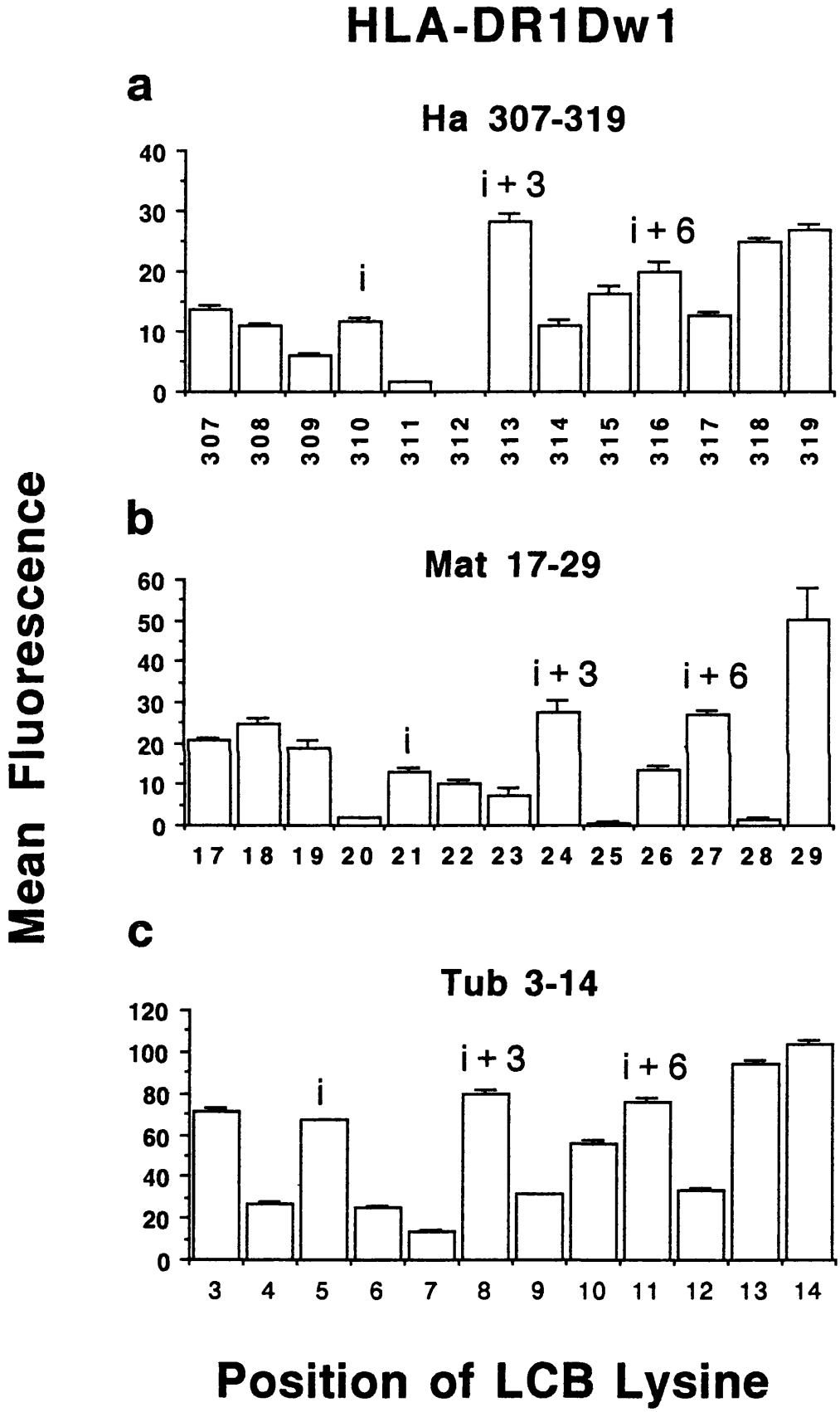
4.2.4. Similarities in the patterns of fluorescence associated with each peptide

The combined data strongly argued that the variations in fluorescent signals observed when the analogues of the Ha 307-319, Tub 3-14, and Mat 17-29 peptides were incubated with IBW4, were due to variations in the effect of LCB lysine on the ability of the analogues to bind cell surface HLA-DR1Dw1. The fluorescent patterns are shown in Figure 4.6, with the non-specific binding observed when the peptides were incubated with the RJ225 cell line, subtracted from the signal for each peptide analogue. The fluorescent pattern associated with each peptide was unique. However, a significant number of qualitative similarities between the patterns were apparent. With all three peptides, three relative peaks of fluorescence were present at relative

Figure 4.6.

LCB lysine analogues of Ha 307-319, Mat 17-29, and Tub 3-14 bound to HLA-DR1Dw1

Mean fluorescent signals generated by LCB lysine analogues of the **a)** Ha 307-319, **b)** Tub 3-14, and **c)** Mat 17-29 peptides, bound to IBW4 cells expressing HLA-DR1Dw1 with the fluorescent signals due to binding to RJ225 cells subtracted from each signal. Relative peaks of fluorescence are marked at relative positions i, i + 3, and i + 6. Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples with the reagent background subtracted.


HLA-DR1Dw1
a
Ha 307-319
i + 3
i + 6
i
40
30
20
10
0
307 308 309 310 311 312 313 314 315 316 317 318 319
b
Mat 17-29
i + 3
i + 6
i
60
50
40
30
20
10
0
17 18 19 20 21 22 23 24 25 26 27 28 29
c
Tub 3-14
i + 3
i + 6
i
120
100
80
60
40
20
0
3 4 5 6 7 8 9 10 11 12 13 14
Mean Fluorescence
Position of LCB Lysine

positions i, i + 3, and i + 6 (Figure 4.6), whereas relatively low fluorescent signals were seen at positions i - 1, i + 1, i + 2, i + 4 and i + 7 in all cases. The similarities in the fluorescent signals observed with each of the three peptides indicated similar patterns of contacts with HLA-DR1Dw1, with a position which gives a relatively low fluorescent signal in the pattern making a more important contact than positions either side giving a higher fluorescent signal.

4.2.5. LCB lysine analogues of Ha 307-319 binding to purified HLA-DR1Dw1

An assay able to determine the binding of LCB analogues of a peptide to purified HLA-DR was used to determine whether the same pattern of critical contacts described in the previous section, was observed when LCB lysine analogues of Ha 307-319 were bound to purified HLA-DR1Dw1. Initially affinity purified MHC protein at 10nM, was incubated with Ha LCB N over a wide concentration range to determine a convenient concentration to use for these studies. The peptide MHC protein complexes were formed overnight at 37° C and then separated from free peptide by incubation of the complexes with an anti-HLA-DR antibody coated onto ELISA plate wells (Materials and Methods, 2.9). Free peptide was washed away and the number of peptide MHC complexes formed was quantitated by incubation with ^{125}I-streptavidin. The signal obtained due to non-specific binding of the ^{125}I-streptavidin to HLA-DR, or to any of the analogue peptides, was approximately equal and the higher background was subtracted from all the signals obtained in the assay. The titration of peptide concentration resulted in a titration of the amount of bound ^{125}I-streptavidin (Figure 4.7a). The data indicated that a concentration of biotinylated peptide of 10nM would give a signal close to maximum without saturating the DR or the detection system being used. Therefore, the other analogues of Ha 307-319 were assayed at this concentration for their ability to bind purified HLA-DR1Dw1 (Figure 4.7b). The relative signal intensity at several positions was quantitatively different from that

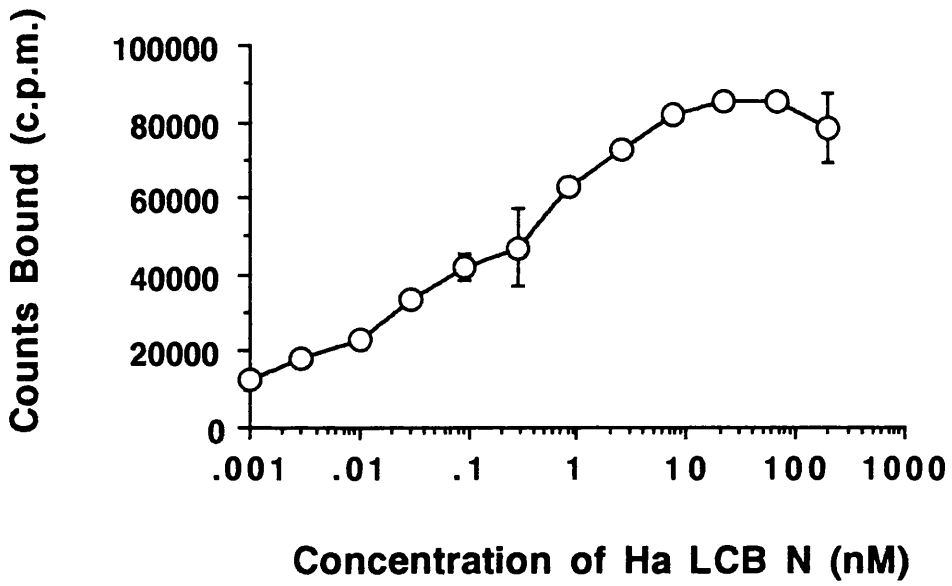
Figure 4.7.

LCB lysine analogues of Ha 307-319 bound to purified HLA-DR1Dw1

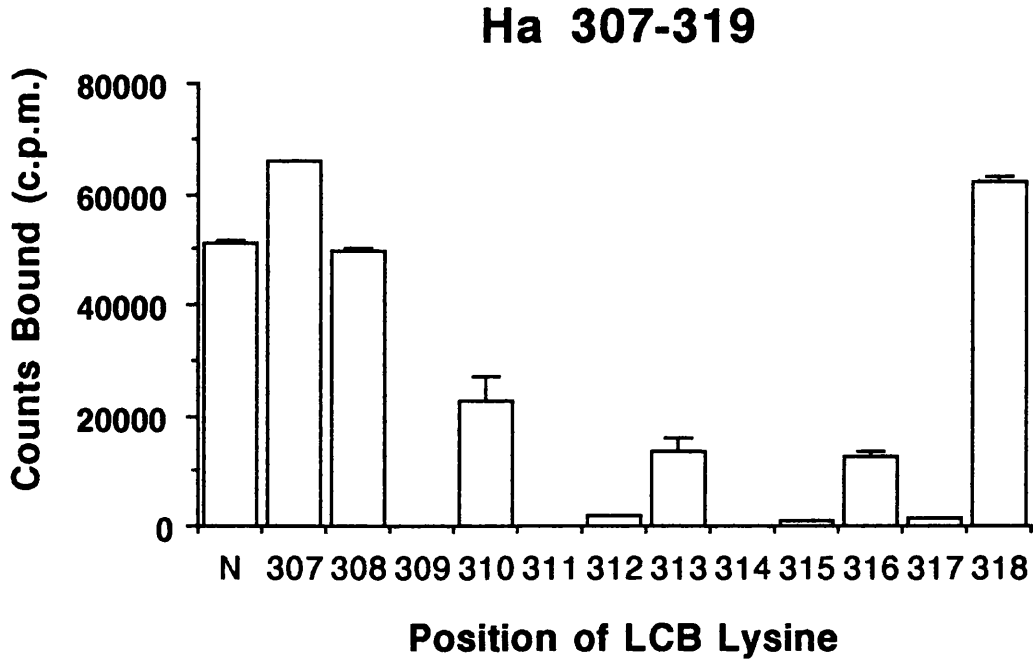
a) Binding of ^{125}I-streptavidin to Ha LCB N bound to purified HLA-DR1Dw1 (10nM). Peptide MHC protein complexes were captured using the anti-DR antibody LB3.1 (Materials and Methods, 2.9.5). Background binding of Ha LCB N is subtracted from each signal (3400 c.p.m.). Each signal is the mean of duplicate samples.

b) Binding of ^{125}I-streptavidin to LCB lysine analogues of Ha 307-319 (10nM) bound to purified HLA-DR1Dw1 (10nM). Peptide MHC protein complexes were captured using the anti-DR antibody LB3.1 (Materials and Methods, 2.9.5). Background binding of each analogue is subtracted from each signal. Each signal is the mean of duplicate samples.

a


HLA-DR1Dw1
Counts Bound (c.p.m.)
100000
80000
60000
40000
20000
0
.001
.01
.1
1
10
100
1000
Concentration of Ha LCB N (nM)

b


Ha 307-319
Counts Bound (c.p.m.)
80000
60000
40000
20000
0
N
307
308
309
310
311
312
313
314
315
316
317
318
Position of LCB Lysine

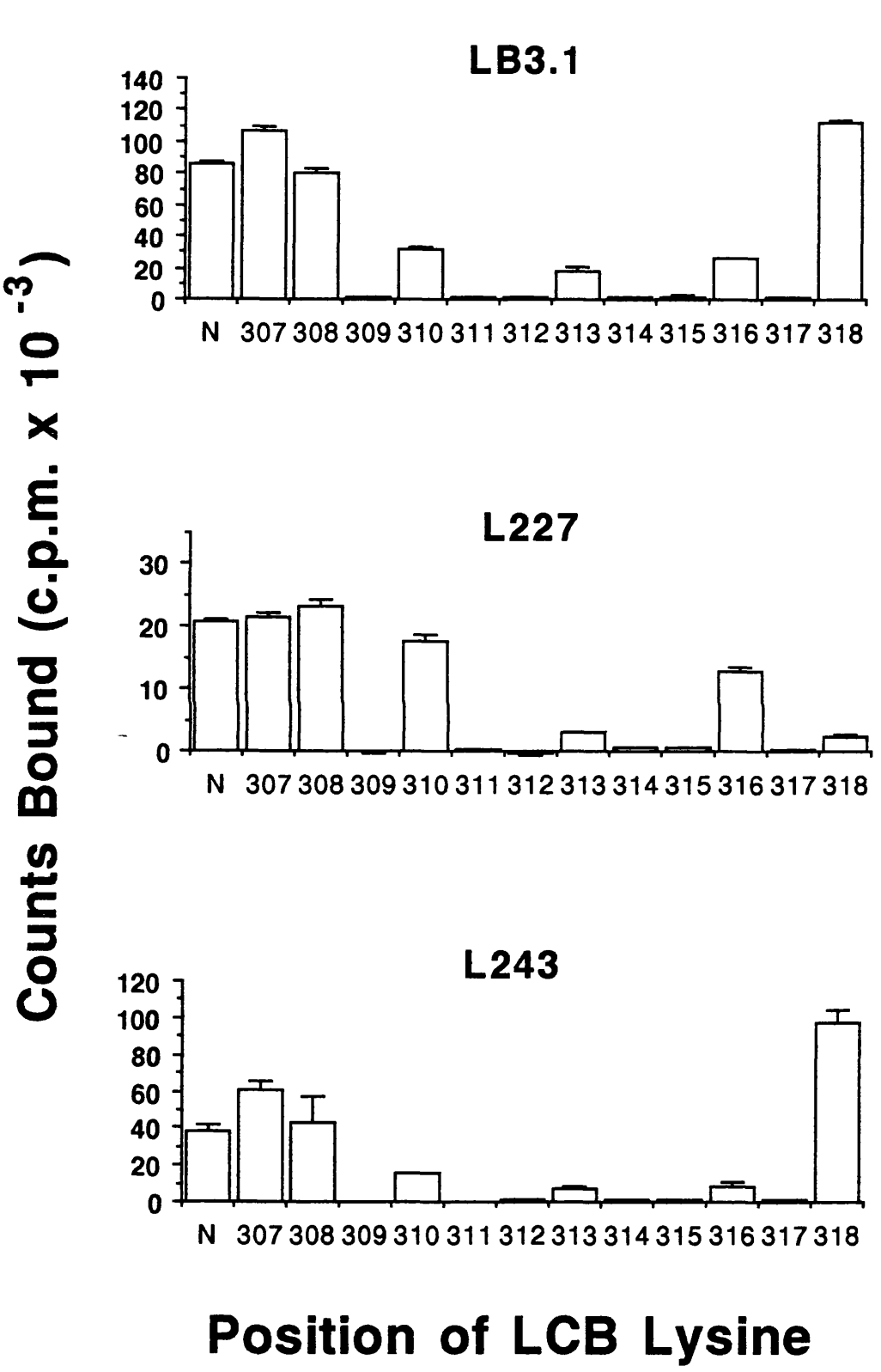
obtained when the analogues were incubated with cell surface HLA-DR1Dw1. For example, the signal at position 313 with cell surface HLA-DR1Dw1 was greater than the signal at position 307, whereas with purified protein the signal at position 313 was considerably less than that at 317. However, the pattern of relative peak and minimum intensities was strikingly similar. There were small intensity, relative signal peaks, at positions i, i+3, and i+6 (310, 313, and 316), and low signals at positions i - 1, i + 1, i + 2, i + 4 and i + 7, which was similar to the pattern of signals seen in the cell surface binding assay with the LCB lysine analogues of Ha 307-319.

The differences in the signals obtained with cell surface HLA-DR1Dw1 and purified HLA-DR1Dw1 could have been due to the configuration of the assay used. In the assay with purified molecules the class II peptide complexes were separated from free peptide by being bound to an anti-DR antibody, which was in turn bound to a polystyrene plate. Firstly, the LCB group on some of the analogues may have affected the ability of the antibody to bind , and secondly, the antibody and polystyrene plate may have affected the ability of the ^{125}I streptavidin to bind the LCB group on the peptide MHC protein complexes. Such steric obstruction would not have been a problem in the cell surface binding assay. If this hypothesis was true then different anti-DR antibodies, binding to different parts of the DR molecule, would be expected to modify the relative signal intensities seen along the length of the peptide in the assay. Therefore, three different antibodies (LB3.1, L243, and L227), specific for DR molecules (Materials and Methods, 2.6.1), were compared for their ability to produce signals in the antibody capture assay (Figure 4.8). The data acquired using the L227 and L243 antibodies resulted in quantitative differences from the signals obtained with LB3.1 (Figure 4.8). The two antibodies also gave relative signal intensities at a number of positions which were different from those seen with LB3.1. For example, when using L227 the signal seen with Ha LCB 318 was considerably lower, relative to Ha LCB N, than when using LB3.1 when they were approximately similar. Interestingly however, in each case the only positions within the central core of the

Figure 4.8.

Complexes between LCB lysine analogues of Ha 307-319 and purified HLA-DR1Dw1 separated from free peptide using three different anti-DR antibodies

Binding of ^{125}I-streptavidin to LCB lysine analogues of Ha 307-319 (10nM) bound to purified HLA-DR1Dw1 (10nM). Peptide MHC protein complexes were captured using **a)** LB3.1, **b)** L227, and **c)** L243 anti-DR antibodies (Materials and Methods, 2.6.1 and 2.9.5). Background binding of each analogue on a particular antibody is subtracted from each signal. Each signal is the mean of duplicate samples.


Ha 307-319
LB3.1
140
120
100
80
60
40
20
0
N 307 308 309 310 311 312 313 314 315 316 317 318
L227
30
20
10
0
N 307 308 309 310 311 312 313 314 315 316 317 318
L243
120
100
80
60
40
20
0
N 307 308 309 310 311 312 313 314 315 316 317 318
Counts Bound (c.p.m. x 10 -3)
Position of LCB Lysine

patterns to show a relative signal peak were 310, 313, and 316 (i, i+3, and i+6) and there was only ever a weak signal seen above background at positions i - 1, i + 1, i + 2, i + 4 and i + 7. These data were therefore consistent with Ha 307-319 making a similar pattern of contacts with purified HLA-DR1Dw1, as with the cell surface HLA-DR1Dw1.

4.2.6. Sperm Whale Myoglobin peptides binding to HLA-DR1Dw1

The combined data for the LCB lysine substituted Ha 307-319, Tub 3-14, and Mat 17-29 peptides bound to HLA-DR1Dw1 indicated that there were shared features in the patterns of signals obtained for each set of analogues. To determine if the shared features of the patterns were common to other peptides, including those not defined by T cells restricted through HLA-DR1Dw1, the complexes of two other determinants with cell surface HLA-DR1Dw1 were examined. T cell determinants consisting of residues 68-80 and 110-121 of sperm whale myoglobin (Myo 68-80 and Myo 110-121) were originally defined with I-E^d, and I-E^k restricted T cells respectively. Analogues of each, containing LCB lysine monosubstitutions, were prepared as before (Table 4.IV). The peptides were incubated with HLA-DR1Dw1 expressing cells (IBW4) and the complexes quantitated by staining with the two layer FAD assay and analysis by flow cytometry (Figure 4.9). As with the other peptides the signals observed varied depending both on the position of LCB lysine in the analogues and on the peptide. When these analogues were incubated with the class II negative cell line RJ225 the signals were all a small fraction of the peak signals seen on IBW4 indicating that the fluorescent signals were due to interactions with cell surface class II MHC proteins (Figure 4.9). The peak signals in the patterns could also be inhibited by the anti-HLA-DR antibody LB3.1 (Table 4.V) using a similar assay to that used for the analogues of Ha 307-319, Mat 17-29, and Tub 3-14. The inhibition seen was between 60 and 80 % demonstrating that the majority of the fluorescent signals seen were due to interactions with HLA-DR1Dw1 on the cell surface. Myo LCB 113 was not used

Table 4.IV.
Peptides from Sperm Whale Myoglobin

Peptides (Restriction)	Sequence
Myo 68-80 (I-E^k)	68 V, 69 L, 70 T, 71 A, 72 L, 73 G, 74 A, 75 I, 76 L, 77 K, 78 K, 79 K, 80 G
Myo110-121 (I-E^d)	110 A, 111 I, 112 I, 113 H, 114 V, 115 L, 116 H, 117 S, 118 R, 119 H, 120 P, 121 G
LCB lysine analogues	**Sequence**
Myo LCB 68	Ac 68 K(LCB), 69 L, 70 T, 71 A, 72 L, 73 G, 74 A, 75 I, 76 L, 77 R, 78 R, 79 R, 80 G
Myo LCB 110	Ac 110 K(LCB), 111 I, 112 I, 113 H, 114 V, 115 L, 116 H, 117 S, 118 R, 119 H, 120 P, 121 G

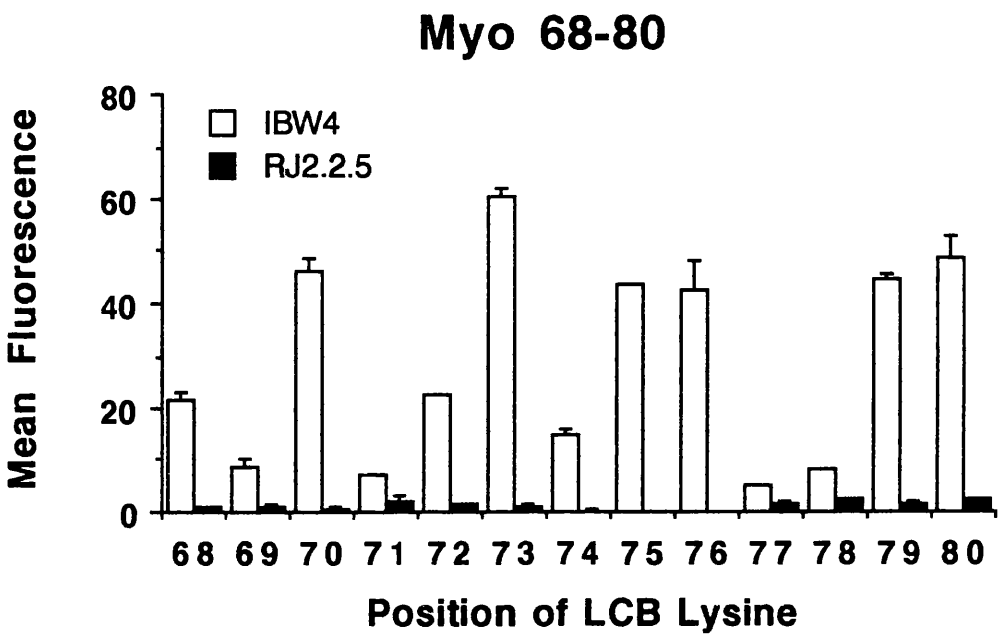
Key: Ac - Acetylated amino terminus. LCB - long chain biotin.
Restriction - Class II molecule by which T cells originally used to define epitope, were restricted.
Myo 68-80 - Sperm whale myoglobin residues 68-80.
Myo 110-121 -Sperm whale myoglobin residues 110-121.
Myo LCB 68 - Myo 68-80 with LCB lysine at position 68. Other analogues are named similarly.
Amino acids are in single letter code and an Arg substituted for a natural Lys is outlined.

Figure 4.9.

LCB lysine analogues of Myo 68-80 and Myo 110-121 bound to IBW4 and RJ225

Mean fluorescent signals generated by LCB lysine analogues of **a)** Myo 68-80 (5μM), and **b)** Myo 110-121 (20μM), bound to, IBW4 cells expressing HLA-DR1Dw1, and RJ225 cells which do not express DR or DQ molecules. Binding was determined with a two layer FAD assay. Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples with the reagent background subtracted.

a


Myo 68-80
IBW4
RJ2.2.5
Mean Fluorescence
0
20
40
60
80
68 69 70 71 72 73 74 75 76 77 78 79 80
Position of LCB Lysine

b

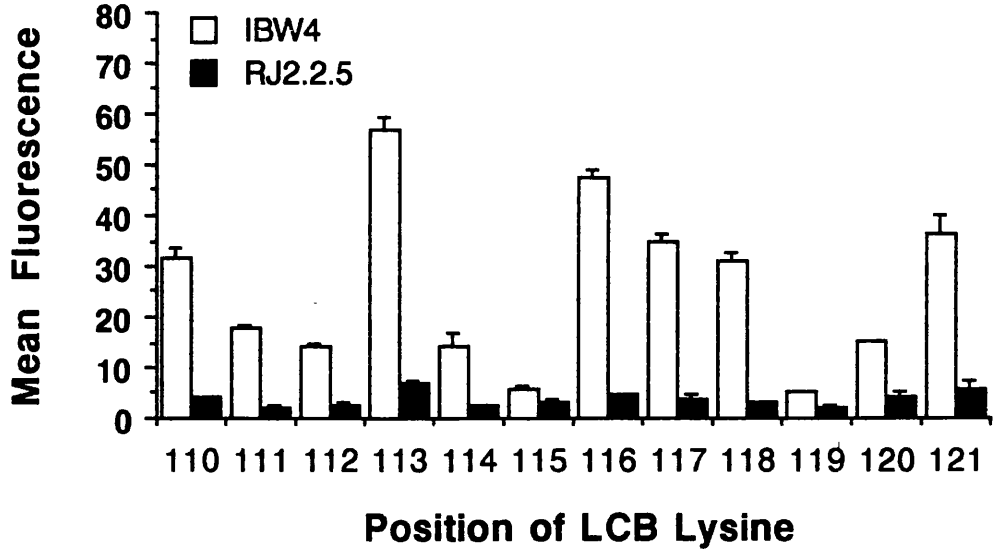

Myo 110-121
IBW4
RJ2.2.5
Mean Fluorescence
0
10
20
30
40
50
60
70
80
110 111 112 113 114 115 116 117 118 119 120 121
Position of LCB Lysine

Table 4.V.

Antibody inhibition of the binding of LCB lysine analogues of Myo 68-80 and Myo 110-121, to HLA-DR1Dw1

Peptide	Position of LCB	% Inhibition
Myo 68-80	70	60
	73	80
	76	74
Myo 110-121	113	ND
	116	64

Antibody inhibition of the binding of LCB analogues to IBW4 cells expressing HLA-DR1Dw1, was performed as described in Materials and Methods, Chapter 2.8.5. Myo 110-121 analogues were at 20μM, and Myo 68-80 analogues were at 5 μM. Antibody inhibition was at 10μg/ml of the anti-DR antibody LB3.1.

because it was not available. The data argued that the variation in fluorescent signals resulted from differences in the ability of the analogue peptides to bind to cell surface HLA-DR1Dw1.

Interestingly, despite the fact that these determinants were originally defined in mouse systems, residues corresponding to relative positions i-1 to i+3 of Myo 68-80 and Myo 110-121 showed fluorescent signals (Figure 4.10) similar to those observed in Figure 4.6 for Ha 307-319, Mat 17-29, and Tub 3-14. However, differences were seen at the carboxyl terminal portion of the patterns for the myoglobin peptides. At position 119 (i+6) of Myo 110-110 a particularly low fluorescent signal was seen whereas at the equivalent position in Ha 307-319, Mat 17-29, and Tub 3-14 there was a peak in the fluorescent signals. At position 75 (i+5) in Myo 68-80 the fluorescent signal was consistently seen to be equivalent or higher than that at position 76 (i+6) whereas this position showed a lower fluorescent signal in the first three peptides examined. There were clearly similarities in the patterns of important contacts the Myo 68-80 and Myo 110-121 peptides were making with HLA-DR1Dw1 when compared to those made by Ha 307-319, Mat 17-29, and Tub 3-14, however,there were also differences which seemed to be focused at the carboxyl termini of the peptides.

4.2.7. Relative affinity of peptides binding to HLA-DR1Dw1

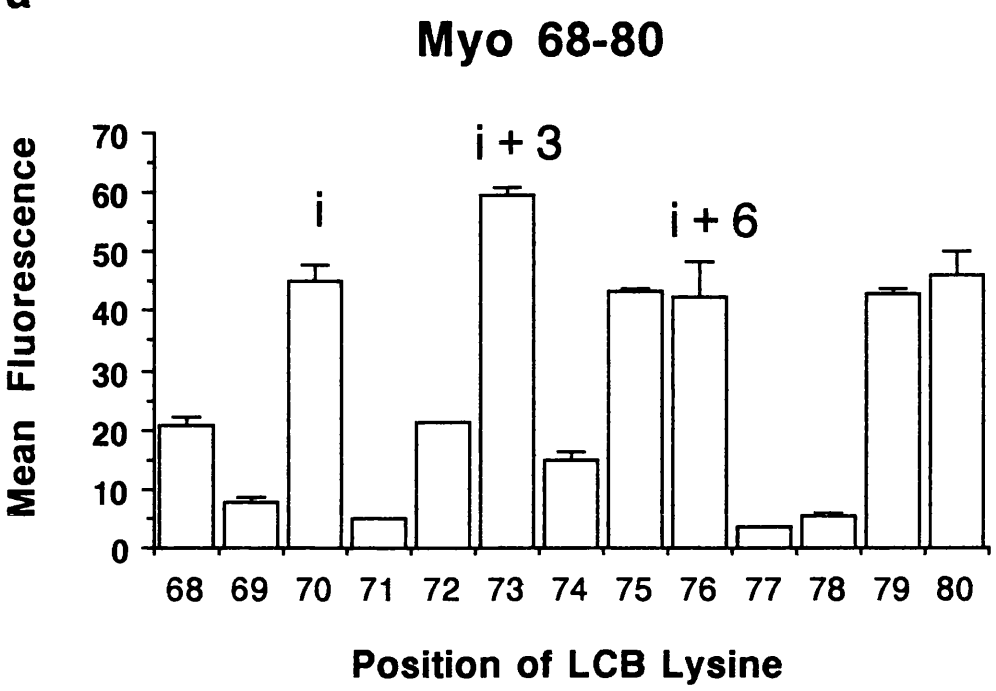
In order to determine whether the differences in the contacts these five peptides made with the MHC protein correlated with the relative affinity of the peptides for HLA-DR1Dw1, the ability of selected analogues to bind to the cell surface MHC protein was compared over a concentration range. The analogues were selected to be representative of the peak fluorescent signals obtained with each pattern while also being soluble over as wide a concentration range as possible. The different analogues were incubated with the IBW4 cell line over 4 hours at 37° C and stained and analysed as before (Figure 4.11). The titration curves showed that the Myo 68-80 analogue had the highest affinity, followed by the Ha 307-319 analogue and the Tub 3-14 analogue,

Figure 4.10.

LCB lysine analogues of Myo 68-80 and Myo 110-121 bound to HLA-DR1Dw1

Mean fluorescent signals generated by LCB lysine analogues of the a) Myo 68-80 (5μM) and b) Myo 110-121 (20μM), bound to IBW4 cells expressing HLA-DR1Dw1, with the fluorescent signals due to binding to RJ225 cells subtracted from each signal. Relative peaks of fluorescence are marked at relative positions i, i + 3, and i + 6. Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples with the reagent background subtracted.

a


Myo 68-80
Mean Fluorescence
70
60
50
40
30
20
10
0
i
i + 3
i + 6
68 69 70 71 72 73 74 75 76 77 78 79 80
Position of LCB Lysine

b

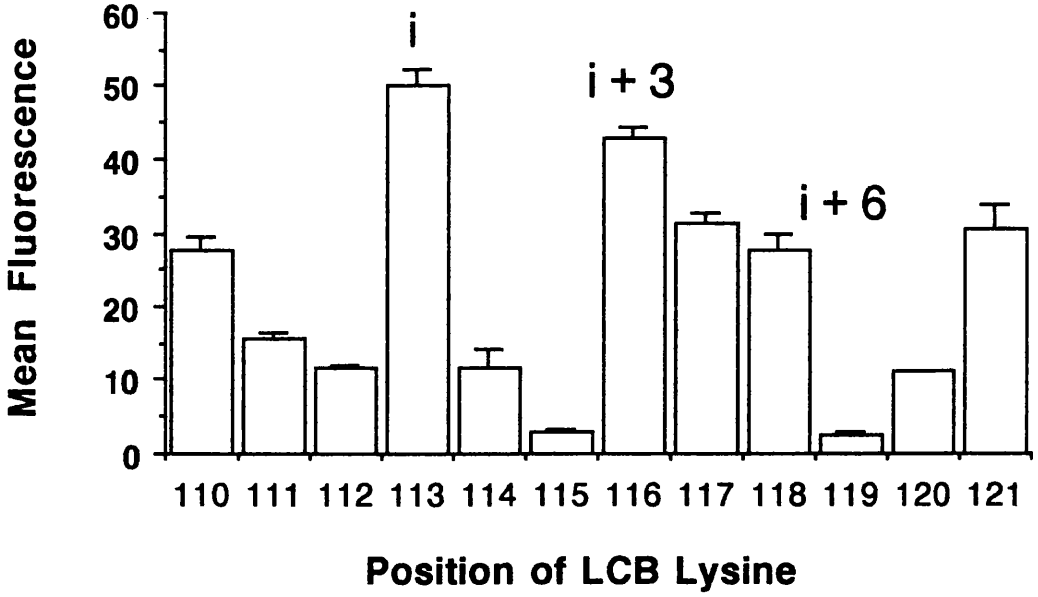
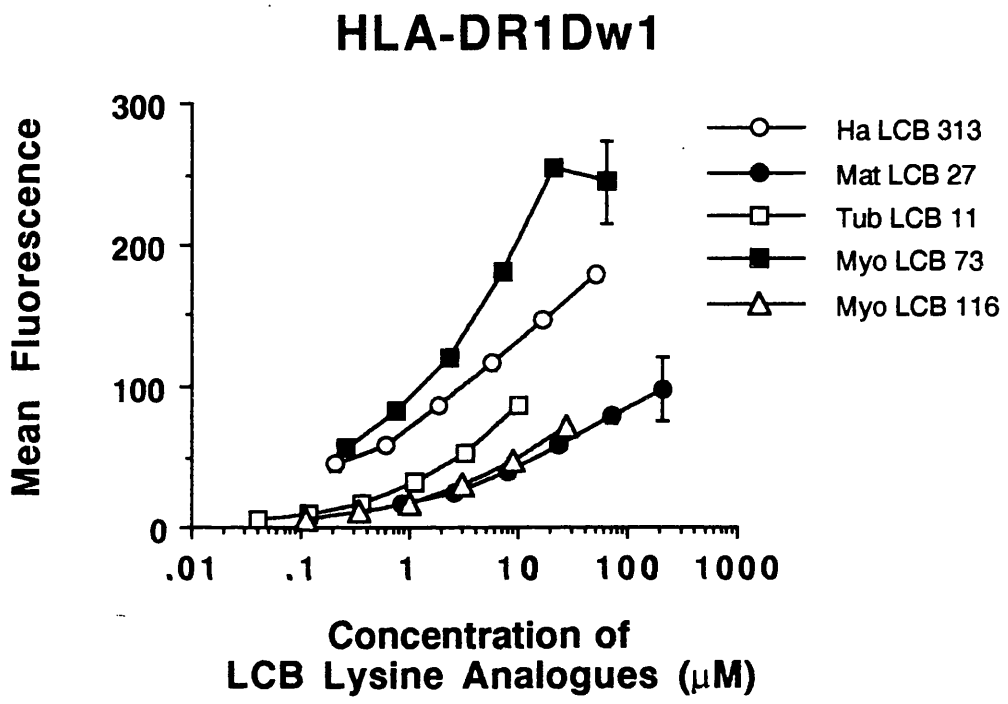

Myo 110-121
Mean Fluorescence
60
50
40
30
20
10
0
i
i + 3
i + 6
110 111 112 113 114 115 116 117 118 119 120 121
Position of LCB Lysine

Figure 4.11.

Relative affinity of LCB lysine analogues of Ha 307-319, Mat 17-29, Tub 3-14, Myo 68-80, and Myo 110-121

Mean fluorescent signals generated by titration of LCB lysine analogues of Ha 307-319, Mat 17-29, Tub 3-14, Myo 68-80, and Myo 110-121 bound to IBW4 cells expressing HLA-DR1Dw1. Analogues used each gave a peak fluorescent signal in the patterns associated with each peptide (Figure 4.6 and 4.10). Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples with the reagent background subtracted.


HLA-DR1Dw1
Mean Fluorescence
300
200
100
0
.01
.1
1
10
100
1000
Concentration of
LCB Lysine Analogues (μM)
Ha LCB 313
Mat LCB 27
Tub LCB 11
Myo LCB 73
Myo LCB 116

while the Mat 17-29 and Myo 110-121 analogues had the lowest affinity. Therefore, there was no obvious correlation between the pattern of contacts made by a peptide with HLA-DR1Dw1 and its affinity for the MHC protein.

4.2.8. Binding of M. leprae 65 kDa protein residues 418-427 to HLA-DR1Dw1 and HLA-DR2Dw2

The last peptide to be analysed for its interactions with HLA-DR1Dw1 expressing cells corresponded to residues 418-427 from the 65 kDa protein of M. *leprae* (Lep 418-427) (Table **4**.VI.A). When LCB lysine analogues of this peptide were incubated with IBW4 a different pattern of fluorescence to any of those seen so far was observed (Figure **4**.12a). The pattern of fluorescence contained two peaks at relative positions i and i + 3 which were localised at the carboxyl half of the peptide but low fluorescence was seen when analogues containing LCB lysine in the first 4 residues were assayed.

The peptide from the *M. leprae* 65 kDa protein was originally defined using HLA-DR2Dw2A restricted T cells. To determine how the peptide bound to HLA-DR2Dw2A, the analogues of the 65 kDa peptide were incubated with a B cell line (PGF) expressing this allele and assayed for binding (Figure **4**.12b). The resultant fluorescent signals were characterised by a distinct pattern of high and low fluorescent signals whose regularity closely resembled that seen with Ha 307-319, Mat 17-29, and Tub 3-14 bound to HLA-DR1Dw1, and was clearly different from those seen when Lep 418-427 was bound to HLA-DR1Dw1. The cell line PGF expresses both HLA-DR2Dw2A and HLA-DR2Dw2B (Bell et al., 1987), and therefore the pattern of fluorescence could be due to interactions with either or both of these DR proteins. The assay was therefore repeated on L cells transfected with HLA-DR2Dw2A or -DR2Dw2B genes (Wilkinson et al., 1988). An almost identical pattern was seen on the cells expressing HLA-DR2Dw2A as observed on the B cell line PGF, whereas there was little or no detectable binding to the L cells expressing HLA-DR2Dw2B (Figure

Table 4.VI.

Peptide from the 65 kDa protein of M. leprae, residues 418-427

Peptide LCB lysine analogue	Sequence
Lep 418-427	418 419 420 421 422 423 424 425 426 427 L Q A A P A L D K L
Lep LCB 418	418 419 420 421 422 423 424 425 426 427 Ac K Q A A P A L D R L | LCB

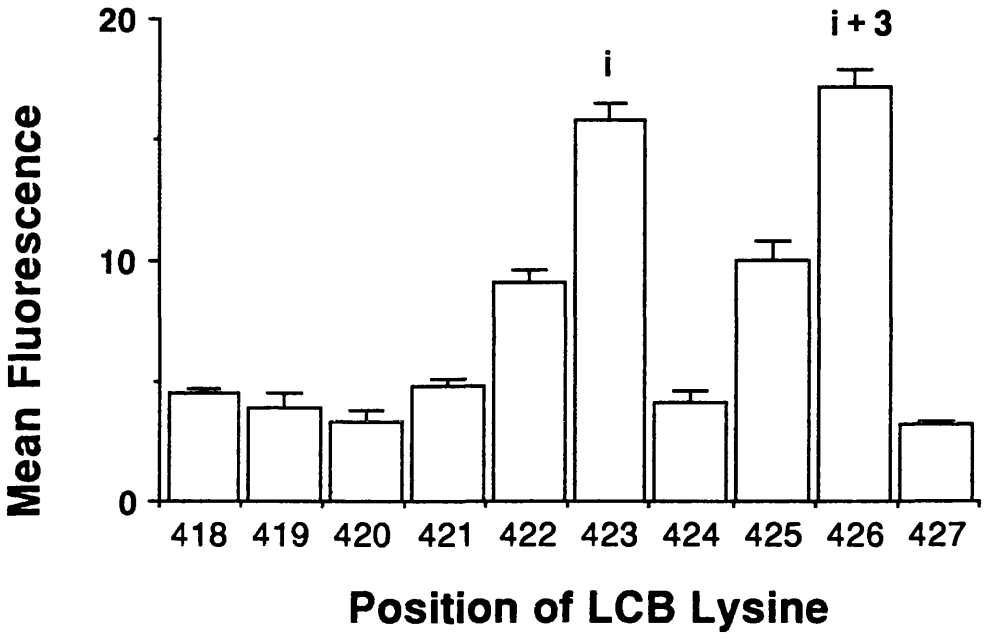
Key: Ac - Acetylated amino terminus. LCB - long chain biotin.
Lep 418-427 - M. leprae 65 kDa protein residues 418-427.
Lep LCB 418 - Lep 418-427 with LCB lysine at position 418.
Amino acids are in single letter code and an Arg substituted for a natural Lys is outlined.

Figure 4.12.

LCB lysine analogues of Lep 418-427 bound to IBW4 and PGF

Mean fluorescent signals generated by LCB lysine analogues of Lep 418-427 (20μM) bound to **a)** IBW4 cells expressing HLA-DR1Dw1, and **b)** PGF cells expressing HLA-DR2Dw2A and HLA-DR2Dw2B. Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples with the reagent background subtracted.

a


IBW4 (HLA-DR1Dw1)
Mean Fluorescence
20
10
0
i
i + 3
418 419 420 421 422 423 424 425 426 427
Position of LCB Lysine

b

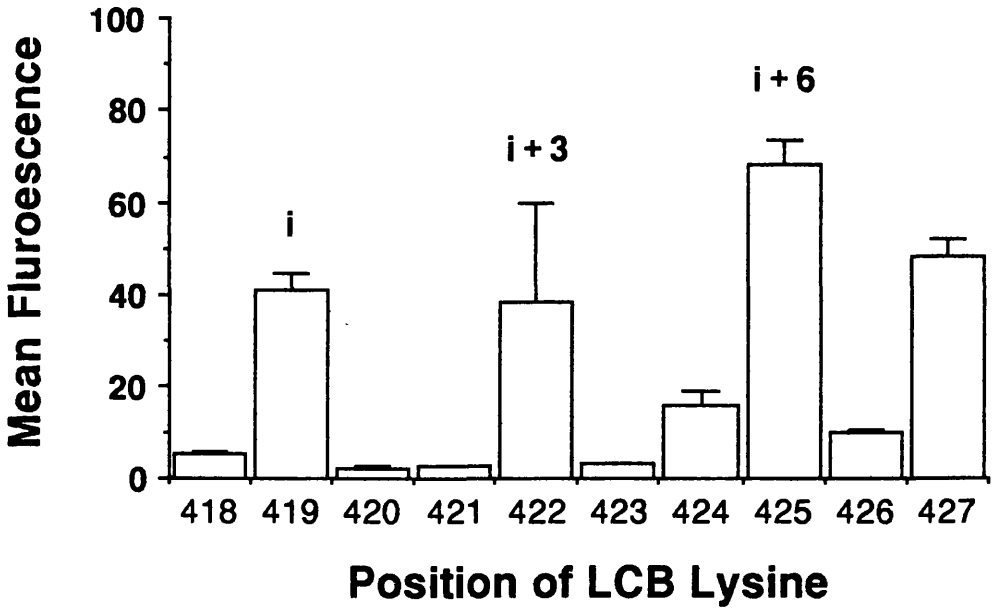

PGF (HLA-DR2Dw2A/B)
Mean Fluroescence
100
80
60
40
20
0
i
i + 3
i + 6
418 419 420 421 422 423 424 425 426 427
Position of LCB Lysine

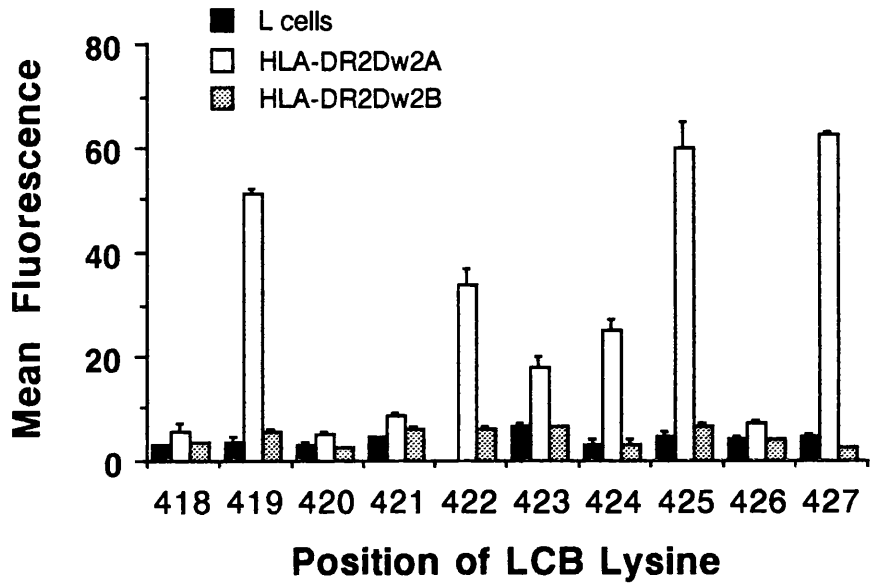
Figure 4.13.

LCB lysine analogues of Lep 418-427 bound to HLA-DR2Dw2A and HLA-DR2Dw2B

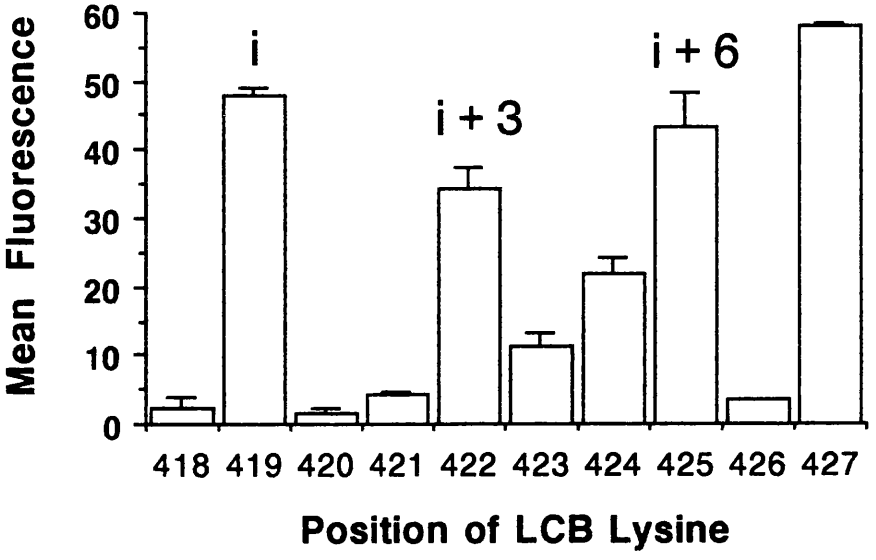
a) Mean fluorescent signals generated by LCB lysine analogues of Lep 418-427 (20μM) bound to L cells, L cells transfected with HLA-DR2Dw2A, and L cells transfected with HLA-DR2Dw2B. Binding was determined with a 2 layer FAD assay. Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples with the reagent background subtracted.

b) Mean fluorescent signals from a) generated by LCB lysine analogues of Lep 418-427 (20μM) bound to L cells transfected with HLA-DR2Dw2A, with the signal due to binding to L cells subtracted. Relative peaks of fluorescence are marked at relative positions i, i + 3, and i + 6.

a


Lep 418-427
L cells
HLA-DR2Dw2A
HLA-DR2Dw2B
Mean Fluorescence
80
60
40
20
0
418 419 420 421 422 423 424 425 426 427
Position of LCB Lysine

b


Lep 418-427
Mean Fluorescence
60
50
40
30
20
10
0
i
i + 3
i + 6
418 419 420 421 422 423 424 425 426 427
Position of LCB Lysine

4.13). The expression of HLA-DR2Dw2B was slightly greater than the expression of HLA-DR2Dw2A on the transfectants and the data therefore indicated that the leprosy peptide bound to HLA-DR2Dw2A with a different pattern of contact residues to those seen when bound to HLA-DR1Dw1.

4.3. Discussion

To examine the important features of a number of different peptide MHC protein complexes analogues of several T cell determinants, with LCB lysine substituted at each position, were used. Incubation of the peptide analogues with cells expressing HLA-DR1Dw1 resulted in the formation of cell surface peptide MHC protein complexes which were quantitated by staining with FAD and flow cytometry to reveal a characteristic pattern of fluorescence associated with each of the T cell determinants. In order to analyse the significance of the patterns of fluorescence associated with each peptide, in terms of interactions between the peptides and HLA-DR1Dw1, the specificity of the binding of the LCB lysine analogues to cell surface DR molecules was established.

4.3.1. Specificity of the binding of LCB lysine analogues to cell surface HLA-DR1Dw1

A number of experiments have been performed to determine whether the signals seen in this assay system involved direct binding of LCB lysine analogues to molecules on the cell surface or whether the binding events involved internalisation of peptides into, for example, an acid or other intracellular compartment (Busch et al., 1990; Busch, 1991). When fixed EBV B cells were used to determine the ability of Ha LCB N to bind cell surface HLA-DR1Dw1 the signal was identical to that obtained with unfixed cells (Busch, 1991). This implied that the binding to HLA-DR1Dw1 was taking place with molecules present on the surface of the cell and did not need to involve internalisation of the peptide (Busch, 1991). Also, experiments have examined

the effect of the metabolic inhibitor azide, and lysosomotropic agents, such as chloroquine and ammonium chloride, on the ability of a number of LCB lysine substituted analogues to bind and no effect was seen (Busch et al, 1990; Busch, 1991). These data indicated that the binding events measured in this assay did not involve active internalisation of the peptides nor their specific interaction with class II in an acidic environment. The data were consistent with the binding events measured occurring solely on the cell surface.

As well as binding to cell surface HLA-DR1Dw1 the LCB lysine analogues could have been binding non-specifically to the cell surface, or to other cell surface proteins, for example structurally related DQ or DP molecules. The DR1Dw1 haplotype does not express a B3 or B4 gene product (Kappes and Strominger, 1988) and therefore these molecules could not be involved. The resultant signals when the analogues were incubated with the class II negative cell line RJ225 strongly suggested that the fluorescence was not due to non-specific interactions with the cell surface but to interactions with class II molecules on the surface of the IBW4 cell line. The RJ225 cell line was derived from the B cell line Raji, and not from IBW4, therefore it was possible that the signals were due to interactions with class I molecules specific to IBW4 or to additional cell surface peptide binding proteins specific to IBW4. Since the mutation in RJ225 was in a trans-acting factor affecting expression of MHC class II genes it was unlikely that this mutation would affect cell surface expression of other proteins suggesting that the analogues were unlikely to be binding additional cell surface molecules (Accolla, 1983; Yang et al., 1988; Koch et al., 1988; Doyle et al., 1990). Historically it has proven extremely difficult to demonstrate direct binding of peptides to class I molecules (Chen and Parham, 1989) suggesting that the possibility of the analogues binding to these molecules was also small.

However, the best confirmation that the signals observed were due to interactions between the LCB lysine analogues and HLA-DR1Dw1 was the ability of an

anti-HLA-DR antibody (LB3.1) to inhibit the fluorescent signals. Under the conditions of the antibody inhibition assay used, staining of cells with fluorescent rabbit anti-mouse antibody indicated that the antibody bound only 80% of the available cell surface class II. Data from Cresswell's group have shown that DR molecules on the surface of EBV B cell lines do not endocytose detectably over a 12 hour period at 37° C, even in the presence of an anti-DR antibody (Davis and Cresswell, 1990). This indicated that the figure of 80% of the class II bound by the antibody in the assay was not due to endocytosis of class II, but simply due to the temperature, and concentration of antibody used. Assuming only 80% of the cell surface class II is bound by antibody in the inhibition experiments implied that 85 - 100% of the signals in Figure 4.1 were due to interactions with HLA-DR1Dw1 (Table 4.III., V.). The mechanism of inhibition in this assay was unknown. The antibody could have been inhibiting the ability of the peptide to bind to the class II molecule during the assay or could have inhibited binding of the fluorescent avidin D to the LCB group on the peptide. When an alternative anti-HLA-DR antibody (L243), which was also capable of inhibiting the binding of LCB lysine analogues in the cell surface assay, was added after the formation of peptide MHC protein complexes, it did not affect the ability of fluorescent avidin to bind to the LCB group on the peptide (Busch, 1991). Regardless of the mechanism for the inhibition of the fluorescent signal by the antibody, the results still demonstrated the specificity of the binding of the LCB lysine analogues to cell surface HLA-DR1Dw1.

The variations in fluorescent signal along the length of each peptide were consistent with the hypothesis that different residues were of varying importance in the formation of the complex between the peptide and the MHC protein. This would only be true if the LCB lysine were affecting solely the affinity of the peptide for HLA-DR1Dw1. Previously, the effects of LCB lysine on the possible conformations of Ha 307-319 have been examined (Rothbard et al., 1989; Busch, 1991). The conformational propensity of each of the LCB lysine analogues in a solution of tri-fluoroethanol and water was determined by circular dichroism (Rothbard et al., 1989;

Busch, 1991). No difference was seen in any of the absorption spectra, which indicated that the different LCB lysine analogues had the same conformational propensity. This suggested that LCB lysine analogues of Mat 17-29, and Tub 3-14 were unlikely to have modified conformational propensities.

A further possible explanation for the low fluorescent signals seen within each pattern was that the peptide was still binding but that the biotin group was inaccessible to the avidin. If the biotin group were completely obscured then an increase in the concentration of the biotinylated peptide in the assay should not have increased the fluorescent signal measured. Experiments with LCB lysine analogues of Ha 307-319 have demonstrated that the low fluorescent signals increased with increasing concentrations of peptide indicating that the biotin was not completely obscured (Busch, 1991). Although similar experiments have not been performed using the analogues of Tub 3-14 and Mat 17-29, none of the positions in any of the peptides had zero fluorescence, indicating that the biotin was unlikely to be completely obscured in any of the low fluorescent signals. Even if the accessibility of the biotin group was the reason for the lack of a signal with certain of the analogues, the 'masking' of the biotin was most likely due to this residue position being in close contact with the HLA-DR protein.

Further experiments were performed on the susceptibility of the Ha 307-319 and Tub 3-14 analogues to cellular proteases and allowed the conclusion that the proteases examined were not differentially affecting the ability of the biotinylated analogues to bind to HLA-DR1Dw1. In summary, the simplest rationalisation of the above data was that the fluorescent signals were proportional to the lack of importance of each individual peptide residue in the formation of the cell surface peptide-HLA-DR1Dw1 complexes.

4.3.2. Binding of LCB lysine analogues to purified HLA-DR1Dw1

LCB lysine analogues of Ha 307-319 were used to determine whether the residues identified as important for contacting cell surface HLA-DR1Dw1 could also be identified as important for contacting purified HLA-DR1Dw1. Interestingly the antibody used to separate peptide MHC protein complexes from free peptide was the same as that used to inhibit the binding of the LCB lysine analogues to cell surface HLA-DR1Dw1. This was not necessarily a paradox since, although the antibody may have inhibited the ability of the LCB lysine analogues to bind class II on the cell surface, the analogues would not necessarily inhibit the binding of the antibody. The relative signal intensities between certain positions in the peptide were clearly different in the cell surface assay when compared to the purified protein assay (Figure 4.6 / 7). To determine whether the differences depended on the configuration of the assays, three different anti-DR antibodies were used to capture the complexes between peptide and purified HLA-DR1Dw1. The results indicated that the antibodies could modify the relative signal intensities of certain positions, however, within each of the patterns there were always small intensity relative peaks seen in the central cores of the patterns at positions 310, 313, and 316 (relative positions i, i+3, and i+6). These were the same positions as the relative peaks seen in the cell surface assay. Also, the presence of LCB lysine at positions i - 1, i + 1, i + 2, i + 4 and i + 7 using purified protein resulted in only a weak, if any, signal above background and these same positions were all determined to be important for contacting cell surface HLA-DR1Dw1. The data were consistent with Ha 307-319 making a similar pattern of contacts with purified HLA-DR1Dw1 and the cell surface MHC protein.

4.3.3. Conformations of Peptides Bound to HLA-DR1Dw1

The combined data relating to the specificity of the interactions of the different LCB lysine analogues with HLA-DR1Dw1 indicated that the signal intensity observed at a particular position was inversely proportional to the importance of that residue in the formation of the peptide HLA-DR1Dw1 complex. For Ha 307-319, Mat 17-29, and Tub 3-14 the relative positions of the MHC protein contact residues were remarkably similar. This could most easily be visualised if the patterns were normalised, by dividing each signal by the mean fluorescence signal across the whole pattern, and then the mean of the three signals from each peptide at equivalent positions plotted as in Figure 4.14a. The common features of the fluorescent patterns associated with the determinants indicated that they made similar patterns of important contacts with HLA-DR1Dw1 along the length of the peptides. From the relative spacing of the contact residues the conformations of the bound peptides could be inferred and compared. The fluorescent pattern associated with each peptide could have represented the sum of a number of different conformations of the bound peptide however, the positions with a low fluorescent signal were important in all possible cases. If each peptide was binding in a single conformation then the spacing of the critical contacts between the peptides and HLA-DR1Dw1 was consistent with the central core of all three binding in a helical conformation similar to that previously proposed for Ha 307-319 alone (Figure 4.14b). In this conformation relative positions i, i+3, and i+6 were pointing out of the site whereas intermediate positions contacted the MHC protein. If the central cores of the peptides were modelled as α helices then the residue at position i+3 pointed directly out of the binding site but the residues at positions i and i+6 pointed at equal and opposite angles from this residue (Figure 4.15a). These positions represented peaks in the fluorescent signal which implied that these residues made fewer direct interactions with the MHC and were more accessible to the FAD than adjacent residues. These data were perfectly consistent if the peptide was modelled into

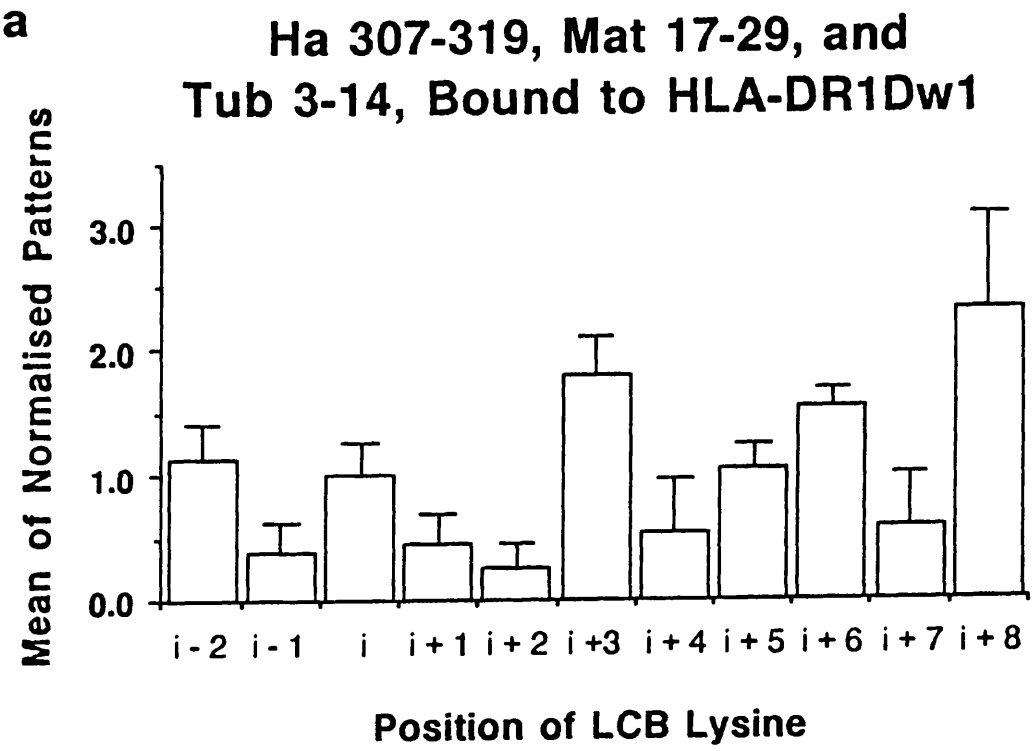
Figure 4.14.

Conformational model of Ha 307-319, Mat 17-29, and Tub 3-14 bound to HLA-DR1Dw1

a) Mean pattern of signals associated with Ha 307-319, Mat 17-29, and Tub 3-14. The signals from each pattern were normalised by dividing each signal by the mean fluorescence from residue position i - 2 to residue position i + 8. Then, at each position the mean of the three normalised signals (one from each peptide) was calculated and used to plot the figure.

b) Conformational model for Ha 307-319, Tub 3-14, and Mat 17-29 bound to HLA-DR1Dw1 based on the patterns of fluorescence in Figure 4.6 and the mean patterns shown in **a)**. Relative positions i, i + 3, and i + 6 point out of the binding site towards the T cell receptor, intermediate positions contact the MHC protein to a greater or lesser degree. The relative positions of the T cell receptor and MHC protein are shown.

a


Ha 307-319, Mat 17-29, and
Tub 3-14, Bound to HLA-DR1Dw1
Mean of Normalised Patterns
3.0
2.0
1.0
0.0
i-2 i-1 i i+1 i+2 i+3 i+4 i+5 i+6 i+7 i+8
Position of LCB Lysine

b

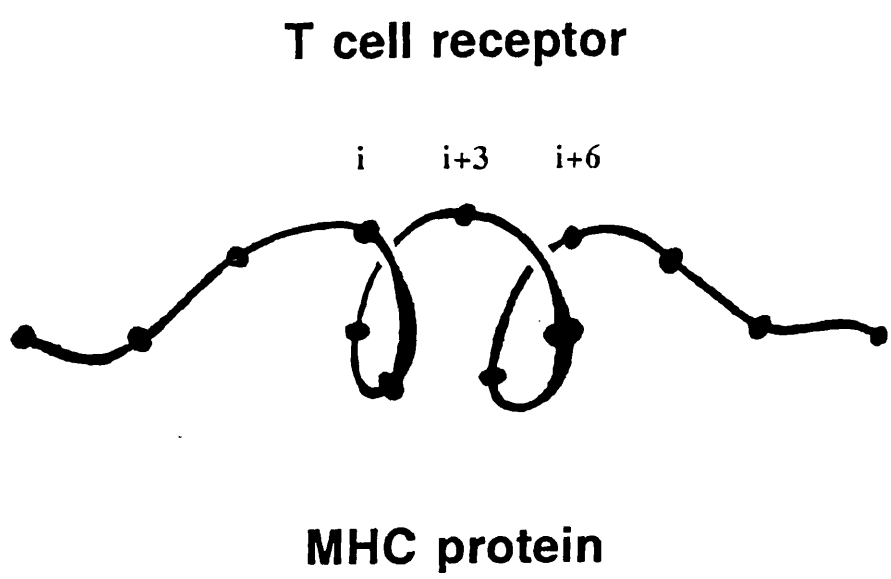

T cell receptor
i
i+3
i+6
MHC protein

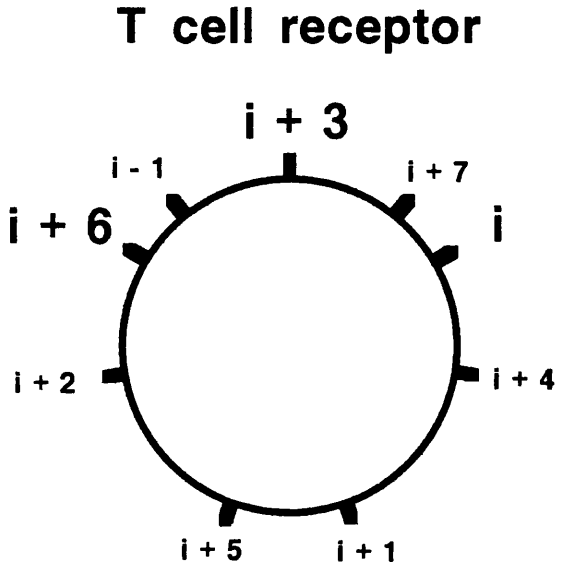
Figure 4.15.

Putative positions of i, i + 3, and i + 6 relative to HLA-DR binding site

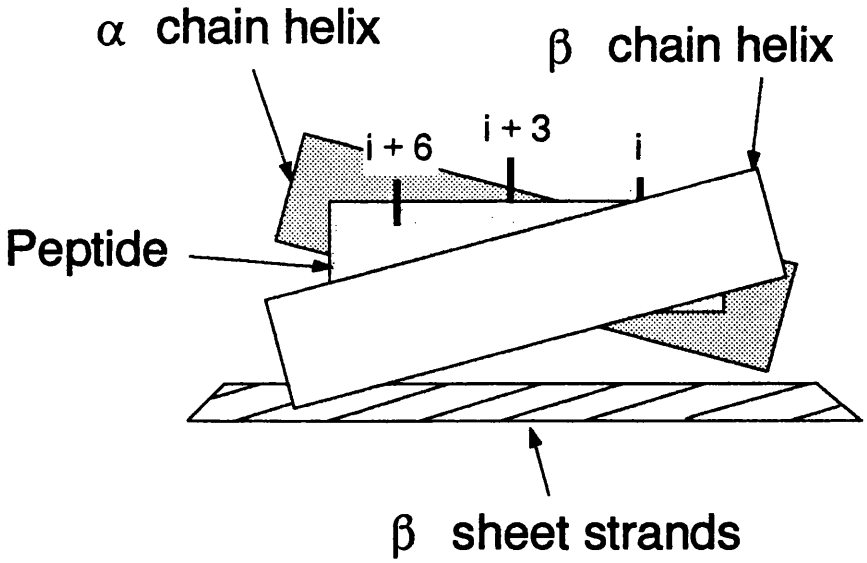
a) Helical wheel representation of a peptide folded as an α helix looking along the axis of the helix from the amino terminus of the peptide. There is 100° between the angles adopted by each residue pointing away from the axis and hence if relative position i + 3 points straight up out of the binding site relative position i "points off to the right" and i + 6 "points off to the left".

b) Schematic figure of an MHC protein binding site with a peptide placed in the site with its amino terminus towards the right of the figure. The relative tilt of the long helical regions of the α and β chains of the MHC protein are shown. With the peptide modelled as shown the tilt of the helices means that relative positions i, i + 3, and i + 6 are equally exposed to the solvent environment (adapted from Busch and Rothbard, 1990).

a


T cell receptor
i + 3
i - 1
i + 7
i + 6
i
i + 2
i + 4
i + 5
i + 1
MHC protein

b


α chain helix
β chain helix
i + 6
i + 3
i
Peptide
β sheet strands

the binding site as shown in Figure Figure 4.15b and one considered the relative angle of the helical regions of the HLA protein that made up the sides of the binding site (Figure 4.15b). This relative tilt resulted in an equal steric availability of all three peak residues and indicated that they would be making fewer contacts with the MHC protein compared to the adjacent residues.

The patterns of contacts made by Myo 68-80 and Myo 110-121 with HLA-DR1Dw1 were similar to those made by Ha 307-319, Mat 17-29, and Tub 3-14 at the amino termini of the peptides, but were different at the carboxyl termini of the peptides (Figure 4.6 and 4.10). Postulated conformations reflected these similarities and differences. Myo 110-121 could be modelled with one turn of a helix at the amino terminus of the core region of the peptide while the carboxyl terminus of the core deviated from a regular conformation (Figure 4.16a). Myo 68-80 could be modelled, again, with one turn of the helix at the amino terminus but then the fluorescent pattern was consistent with the peptide folded as two additional turns of a helix (Figure 4.16b). Therefore, although each peptide-MHC protein complex was clearly unique they shared conformational features, with greater similarity at the amino than at the carboxyl terminus of the peptides.

4.3.4. Affinity and Conformation

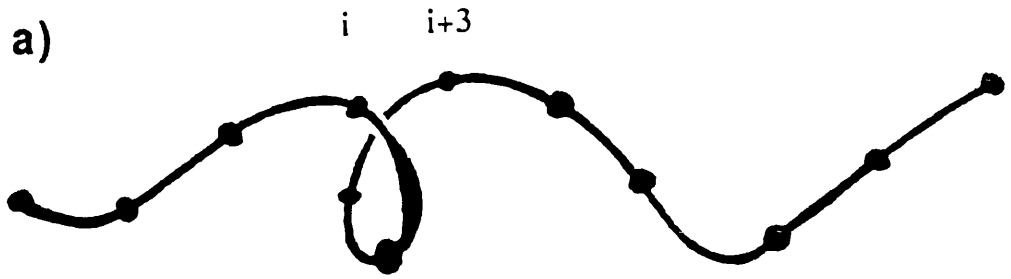
When the affinity of five of the peptides for HLA-DR1Dw1 was compared there was no correlation between the relative affinity and the conformations that each peptide were able to adopt. For example, Ha 307-319 and Mat 17-29 had different affinities and yet shared similar conformations. Although Ha 307-319, Tub 3-14, and Mat 17-29 shared a similar conformation and made a similar number of contacts with the MHC protein, the structural differences between them were obviously important in determining their relative affinity. Interestingly however, the peptide whose conformation allows it to make the most contacts with HLA-DR1Dw1, Myo 68-80, has

Figure 4.16.

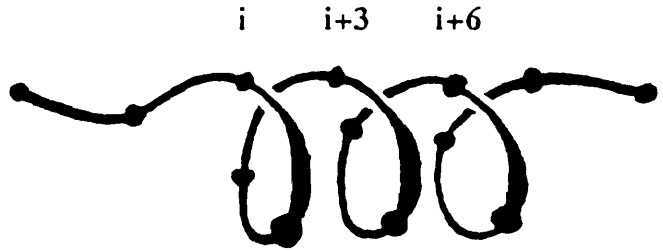
Conformational models for Myo 68-80, Myo 110-121, and Lep 418-427 bound to HLA-DR1Dw1

Conformational models for peptides binding to HLA-DR1Dw1 based on the patterns of fluorescence shown in Figures **4.**10 and **4.**12. Relative positions i, i+3, and i+6 are shown with the MHC protein orientated below the peptide models whereas the T cell receptor is above. The different conformations are consistent with the fluorescent signals associated with **a)** Myo 110-121, **b)** Myo 68-80, and **c)** Lep 418-427.

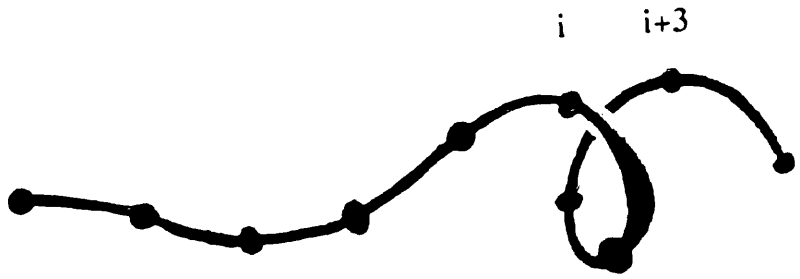
a)



b)



c)



the highest affinity of all the peptides, although this presumably depends upon the physical and chemical structure of the individual contact residues.

4.3.5. Lep 418-427 bound to HLA-DR1Dw1 and HLA-DR2Dw2A

Lep 418-427 showed yet further conformational variation when bound to HLA-DR1Dw1 with the amino terminus of the core region having no defined conformation while the carboxyl terminal fluorescent signal was consistent with a single turn of a helix (Figure 4.16c). Interestingly Lep 418-427, which was originally defined by HLA-DR2Dw2A restricted T cells, adopted a distinctly different conformation when bound to HLA-DR2Dw2A, and one that was remarkably similar to Ha 307-319, Tub 3-14, and Mat 17-29 bound to HLA-DR1Dw1. Since this conformation was adopted by these peptides when bound to the HLA allele which was originally used to define these determinants it appears to be characteristic of peptide/HLA-DR complexes known to generate T cell responses. The formation of this particular conformation may therefore result in a general peptide HLA-DR complex that is required for the binding of a T cell receptor molecule. This suggests that the conformation of a peptide binding to DR molecules may be important in determining its immunogenicity.

4.3.6. Conclusions

The data indicated that the six different peptides analysed did not bind to the same HLA molecule with identical regular conformations. However, the conformations that three out of six peptides adopted when bound to HLA-DR1Dw1 were remarkably similar and the data obtained with all six peptides was consistent with a single turn of a helix in one part of the peptide. There was no simple correlation found between the ability of a peptide to adopt a particular conformation and the affinity of the peptide for HLA-DR1Dw1. The conformation of the three peptides Ha 307-319, Mat 17-29, and Tub 3-14 when bound to HLA-DR1Dw1, and of Lep 418-427 when

bound to HLA-DR2Dw2 revealed a conformation which may be characteristic of an immunogenic peptide MHC protein complex.

Chapter 5
Structural features of peptides bound to HLA-DR

5.1. Introduction

The binding site of MHC proteins is able to accommodate a diverse but limited range of peptide ligands. The structural requirements which enable a peptide to bind to the MHC have so far been poorly defined. If there is a common structural mechanism by which different peptides interact with an MHC protein then this should be revealed by common structural features which are important for the interactions of the peptides with the MHC binding site. Only a few such structural features would be expected within the sequences of the wide range of peptides able to bind to a particular MHC protein. Identification of these features would simplify any subsequent analysis of the molecular mechanism by which the MHC protein binding site interacts with it's peptide ligands.

Empirical analysis of the structural features present in many T-cell determinants led Rothbard and Taylor to identify allele specific patterns of amino acids which suggested that different peptides were binding with a similar conformation and in a similar location in the MHC binding site (Rothbard and Taylor, 1988). Direct binding studies have confirmed the existence of common structural features of peptides binding to MHC proteins. By analysing the "core" regions of a number of peptides required for their binding to the MHC protein, Sette's group identified a number of motifs characteristic of peptides binding to I-E^d and I-A^d molecules (Sette et al., 1988; 1989a; c; d; Introduction, 1.8). The ability to define patterns of common structural and chemical characteristics within the MHC protein binding regions of different peptides suggests that they bind in similar locations in the binding site. However, the structural basis for the motifs is hard to define unless the actual MHC protein contact residues

within the motif are accurately defined. The use of monosubstituted peptides in direct binding assays to define MHC protein contact residues is limited by the inherent ability of MHC molecules to bind a large number of diverse peptides. Such studies have been useful in defining the chemical properties at different positions required for binding to the MHC protein but have often failed to clearly identify all the residues which contact the MHC protein (Sette et al., 1988; 1989a; c; d; Rothbard et al, 1989b; Jardetzky et al., 1990; O'Sullivan et al., 1990).

5.1.1. Aims and Summary

Experiments described in Chapter 4 of this thesis and, previously (Rothbard et al., 1989a; b; Busch, 1989), demonstrated that monosubstitutions with LCB lysine could be used to define the important contacts between MHC proteins and antigenic peptides. In Chapter 4 comparison of the patterns of fluorescence associated with the analogues of a number of determinants indicated that there were a limited number of important contacts made between different peptides and HLA-DR1Dw1. Despite differences in the spacing of some of these contacts, in five of the six peptides examined there were important contacts at relative positions i-1, i+1, and i+2 interspersed by non-contact residues at i and i+3 (Figure 4.6, 4.10, 4.14, 4.16). This was consistent with at least one turn of a helical conformation within the bound peptide and indicated similarities in the patterns of contacts made by different peptides with the MHC protein. If the peptides were interacting with a similar site in the MHC molecule then they should share similar structural features at some of the positions critical for binding and also should share similar structural requirements for binding at these positions.

In an attempt to identify common structural features involved in the interactions of the T cell determinants described in Chapter 4 with HLA-DR1Dw1, they were aligned based on the contacts they made with the MHC protein. Three of the peptides shared structural homology at three positions which contacted HLA-DR1Dw1. A

hydrophobic residue at relative position i - 1, a small residue at i + 4 and a branched hydrocarbon side chain at position i + 7. Five of the determinants shared structural features at two residue positions. A hydrophobic residue at relative position i - 1 and a small residue at i + 4. The structural requirements at relative position i - 1 for binding of three of the peptides to HLA-DR1Dw1 were examined and found to be very similar, suggesting that the peptides were binding in a similar location in the HLA-DR1Dw1 binding site. Interestingly, when the sequences of a large number of determinants, originally defined in the context of a number of different MHC alleles and isotypes, were examined, a pattern of a hydrophobic residue at i - 1 and a small residue at i + 4 was found in the majority of sequences.

5.2. Results

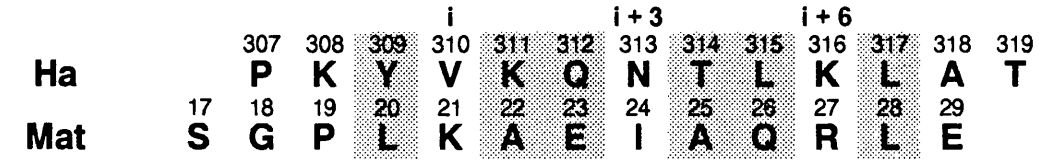
5.2.1. Alignment of Ha 307-319 and Mat 17-29 Peptide Sequences

The residues within a number of T-cell determinants that directly contacted HLA-DR1Dw1 were identified using peptide analogues containing monosubstituted LCB lysine (Chapter 4). To compare these residues the sequences of the peptides were aligned based on the patterns of critical contact residues they made with the MHC protein (Table 5.I). However, several of the patterns were symmetrical within the central core of the peptides and hence the peptides could be aligned in a parallel or anti-parallel fashion (Table 5.I.A and B). For example, the two possible alignments of the Mat 17-29 sequence relative to the Ha 307-319 sequence are shown in Table 5.I.A. In a parallel alignment contact residues showed structural homology at relative positions i - 1, i + 4, and i+7 in the two peptides. Both peptides had a large hydrophobic residue at position i - 1 (Tyr and Leu), a small residue at position i + 4 (Thr and Ala), and Leu is present in both peptides at position i + 7. In an anti-parallel alignment contact residues again showed structural homology at positions i - 1 (Tyr and Leu) and i + 7 (Leu and Leu) but there was no longer homology at position i + 4 (Thr and Glu). The structural

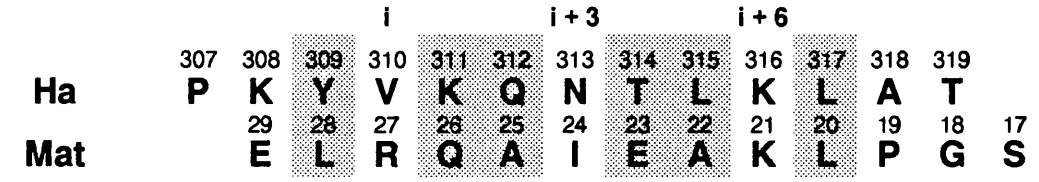
Table 5.I. Parallel and anti-parallel alignments of Ha 307-319, Mat 17-29, and Tub 3-14

A.

Parallel alignment

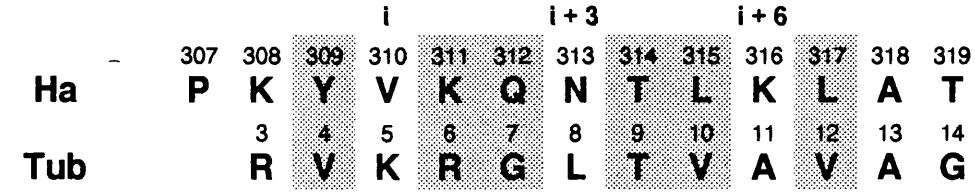


Anti-parallel alignment

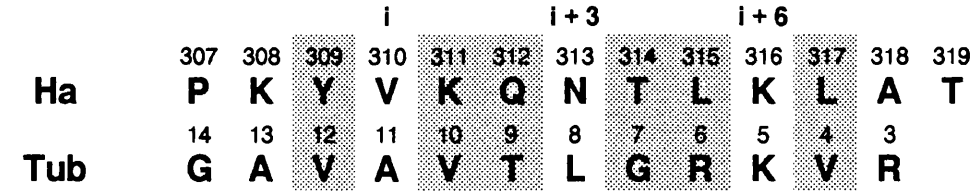


B.

Parallel alignment



Anti-parallel alignment



Based on the patterns of fluorescence in Chapter 4, the important residues contacting HLA-DR1Dw1 are shaded

homology was greater with the parallel alignment, but the data was insufficient to determine the functional significance of these homologies in terms of interactions with the MHC protein because the relative orientation and location of the two bound peptides was unknown.

5.2.2. Hybrid Ha 307-319/Mat 17-29 Peptides Binding to HLA-DR1Dw1

To test the possible relative orientation and location of the two peptides analogues of Mat 17-29 were synthesised containing substitutions from Ha 307-319 at contact residues based on the parallel and anti-parallel alignments (Table 5.II). Ha 307-319 bound with higher affinity to HLA-DR1Dw1 than Mat 17-29 (Figure 4.8), and hence if the two peptides were binding in a similar location, and the substitutions made were based on the correct relative orientation, the affinity of Mat 17-29 for HLA-DR1Dw1 should have been increased. Substitutions in the parallel alignment were made at position i - 1 (Leu to Tyr) and i + 1 (Ala to Arg) in Mat 17-29. These substitutions were chosen because the Tyr at position 309 had been shown to be important for binding of Ha 307-319 in several studies (Busch, 1991; Jardetzky et al., 1990). While the Arg at position 311 was markedly different in structure from the Ala at position 20 in Mat 17-29, and from the affects of LCB lysine was an important contact residue in Ha 307-319. Substitutions using the anti-parallel alignment were made at relative positions i - 1, i +1, and i + 2 on the basis of similar arguments (Table 5.II). Ha 307-319, and Mat 17-29 and its substituted analogues, were synthesised with LCB at the amino terminus and assayed for their ability to bind to cell surface HLA-DR1Dw1. Each analogue was incubated at 5μM with the IBW4 cell line (HLA-DR1Dw1) for four hours at 37° C, stained with the two layer FAD assay, and analysed by flow cytometry. The Ha LCB N gave approximately four times the fluorescent signal of Mat LCB N (Figure 5.1). In all cases, the substitutions into Mat 17-29 based on the parallel alignment improved the ability of this peptide to bind whereas similar

Table 5.II.

Substitutions from Ha 307-319 into Mat 17-29 based on a parallel and anti-parallel alignment

					i			i + 3			i + 6			
		307	308	309	310	311	312	313	314	315	316	317	318	319
Ha	LCB -	P	R	Y	V	R	Q	N	T	L	R	L	A	T
		17	18	19	20	21	22	23	24	25	26	27	28	29
Mat	LCB -	S	G	P	L	R	A	E	I	A	Q	R	L	E
Mat	LCB -	S	G	P	Y	R	A	E	I	A	Q	R	L	E
Mat	LCB -	S	G	P	L	R	R	E	I	A	Q	R	L	E
Mat	LCB -	S	G	P	Y	R	R	E	I	A	Q	R	L	E

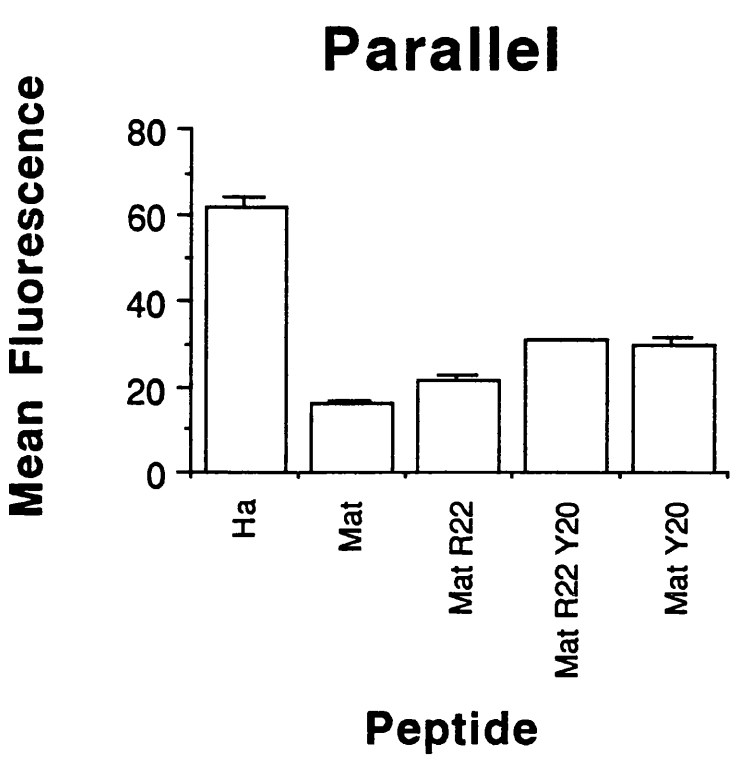
					i			i + 3			i + 6					
		307	308	309	310	311	312	313	314	315	316	317	318	319		
Ha	LCB -	P	R	Y	V	R	Q	N	T	L	R	L	A	T		
		29	28	27	26	25	24	23	22	21	20	19	18	17		
Mat		E	L	R	Q	A	I	E	A	R	L	P	G	S	-LCB	
Mat		E	Y	R	R	A	I	E	A	R	L	P	G	S	-LCB	
Mat		E	L	R	R	Q	I	E	A	R	L	P	G	S	-LCB	
Mat		E	L	R	Q	Q	I	E	A	R	L	P	G	S	-LCB	

Figure 5.1.

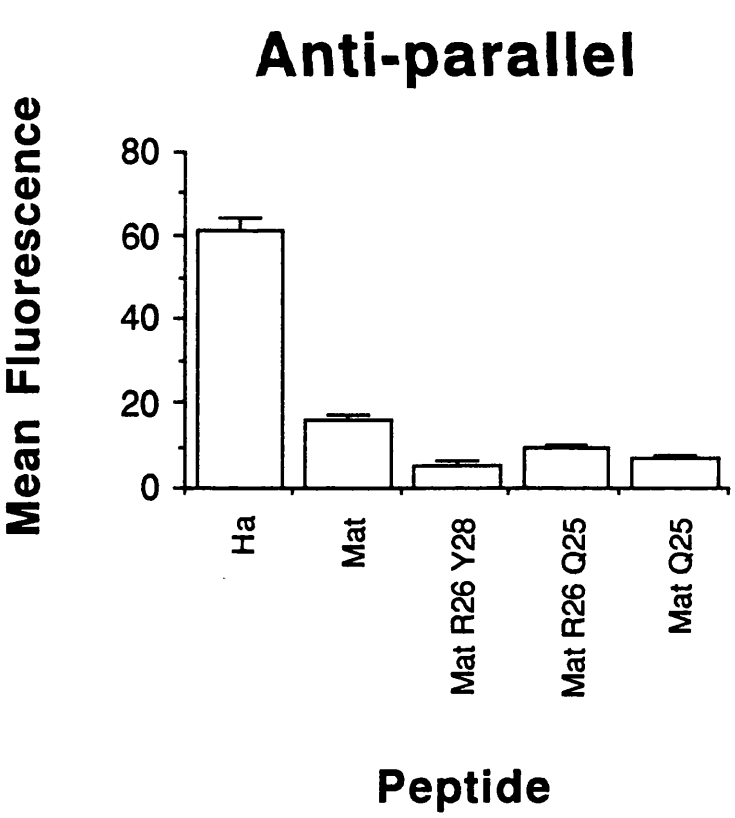
Ha 307-319 / Mat 17-29 hybrid peptides binding to HLA-DR1Dw1

Mean fluorescent signals generated by Ha LCB N, Mat LCB N, and Mat 17-29 analogues with LCB N shown in Table 5.II., bound to IBW4 cells expressing HLA-DR1Dw1. Each of the analogues was at 5 μM and binding was determined with a two layer FAD assay. Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples.

a


Parallel
Mean Fluorescence
80
60
40
20
0
Ha
Mat
Mat R22
Mat R22 Y20
Mat Y20
Peptide

b


Anti-parallel
Mean Fluorescence
80
60
40
20
0
Ha
Mat
Mat R26 Y28
Mat R26 Q25
Mat Q25
Peptide

substitutions based on the anti-parallel alignment reduced the fluorescent signal (Figure 5.1). The data argued that Ha 307-319 and Mat 17-29 were binding in a parallel orientation and similar location in the HLA-DR1Dw1 binding site. None of the substituted Mat 17-29 analogues bound as well as the Ha LCB N analogue indicating that other residues were contributing to the binding as well as those substituted.

5.2.3. Alignment of Ha 307-319 and Tub 3-14 Peptide Sequences

Based on important contact residues with HLA-DR1Dw1 the Tub 3-14 peptide could also be aligned relative to Ha 307-319 in either a parallel or anti-parallel direction (Table 5.I.B). In a parallel alignment there was structural homology at positions i - 1, i + 1, i + 4, i + 5, and i + 7 with hydrophobic residues at i - 1 (Tyr and Val), i + 5 (Leu and Val), and i + 7 (Leu and Val), along with a small residue at i + 4 (Thr), and a basic or positive residue at i + 1 (Lys and Arg). In the anti-parallel alignment there was structural homology at relative positions i - 1, i + 4, and i + 7, with a hydrophobic residue at i - 1 (Tyr and Val) and i + 7 (Leu and Val) and a small residue at i + 4 (Thr and Gly). The similarities based on the parallel alignment suggested that these two peptides could be binding in a parallel orientation and in a similar location in the HLA-DR1Dw1 binding site. However, although there were fewer positions of structural homology based on the anti-parallel alignment, further testing was required to help determine the relative orientation and location of these two peptides bound to HLA-DR1Dw1.

5.2.4. Substituted Tub 3-14 Peptides Binding to HLA-DR1Dw1

The high degree of structural homology between Ha 307-319 and Tub 3-14, and the similarity in their relative affinity for HLA-DR1Dw1 meant that the approach used to determine the relative orientation of Ha 307-319 and Mat 17-29 would not have been as effective with this second pair of peptides. Therefore, their relative orientation was determined by comparing their structural requirements for binding to HLA-

DR1Dw1. Previously the binding of Ha 307-319 to HLA-DR1Dw1 had been shown specifically to require a large hydrophobic residue at position 309 while all other positions were remarkably tolerant to a variety of substitutions (Jardetzky et al., 1990; Busch, 1991). If the patterns of contacts the two peptides made with the MHC molecule were used to align them in a parallel fashion residue 309 corresponds to residue 4 in the Tub 3-14 sequence (Table 5.III). A number of different substitutions were made at position 4 in the Tub 3-14 peptide, including hydrophobic and polar amino acids (Table 5.III). Rather than biotinylating these peptides, their ability to bind to HLA-DR1Dw1 was compared by assaying their ability to compete for binding of Ha LCB N. The competitors were each acetylated at the amino terminus as this has been shown to increase the ability of Tub 3-14 to compete (data not shown) and therefore makes the assay technically easier to perform. Cells expressing HLA-DR1Dw1 (IBW4) were incubated with Ha LCB N at a constant concentration of 10μM, and the competitor peptides were assayed over a concentration range. The resultant binding of Ha LCB N was determined using a two layer FAD assay and analysis by flow cytometry, as before. The resultant fluorescent signal without any competitor was 42, and all data was converted to percentage competition before being plotted (Figure 5.2). The data demonstrated that substitution of a polar or charged amino acid at position 4 of Tub 3-14 strongly decreased the ability of the peptide to compete whereas a hydrophobic residue at this position had little effect. These results were identical to the structural requirements at position 309 for binding of Ha 307-319 (Jardetzky et al., 1990; Busch 1991). If the Tub 3-14 peptide was aligned in an anti-parallel direction relative to Ha 307-319 then position 4 aligns with residue 317 of Ha 307-319 and substitutions of polar and charged residues at this position in Ha 307-319 do not dramatically affect its ability to bind HLA-DR1Dw1 (Rothbard et al., 1988; Jardetzky et al., 1990; Busch, 1991). The shared chemical and structural requirements at position 309 in Ha 307-319, and 4 in the Tub 3-14 peptide, for binding to HLA-DR1Dw1 were

Table 5.III.

Substitutions at position 4 In Tub 3-14

				i			i + 3			i + 6			
	307	308	309	310	311	312	313	314	315	316	317	318	319
Ha	**P**	**K**	**Y**	**V**	**K**	**Q**	**N**	**T**	**L**	**K**	**L**	**A**	**T**
		3	4	5	6	7	8	9	10	11	12	13	14
Tub	**Ac**	**R**	**V**	**K**	**R**	**G**	**L**	**T**	**V**	**A**	**V**	**A**	**G**
	Ac	**-**	**Y**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
	Ac	**-**	**F**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
	Ac	**-**	**S**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
	Ac	**-**	**D**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
	Ac	**-**	**K**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**

Substitutions used to determine the relative orientation and location of Ha 307-319 and Tub 3-14 bound to cell surface HLA-DR1Dw1.

All Tub 3-14 analogues were acetylated to improve the percentage competition seen.

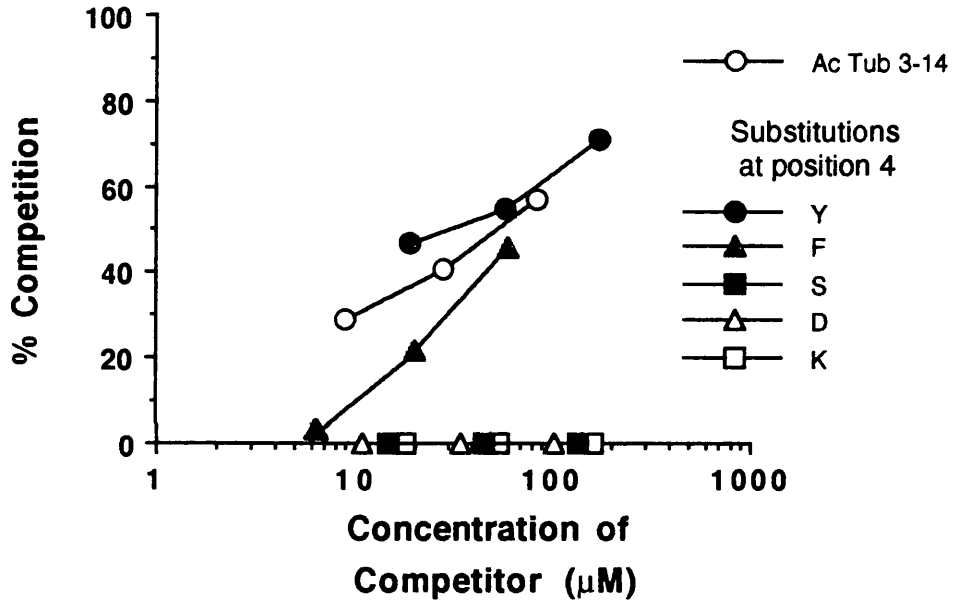
Shaded residues are important contacts with the MHC protein.

Dashes indicate identity with the residues above.

Figure 5.2.

Analogues of Tub 3-14, substituted at position 4, binding to HLA-DR1Dw1

Ability of acetylated (Ac) Tub 3-14 and analogues substituted at position 4 (Table 5.III.) to compete for binding of Ha LCB N to IBW4 cells expressing HLA-DR1Dw1. Ha LCB N was at 10μM. The analogues are shown by the amino acid substituted at position 4 of Ac Tub 3-14. Mean fluorescence is in arbitrary units and each signal is the mean of duplicate samples.


100
80
60
40
20
0
% Competition
1
10
100
1000
Concentration of
Competitor (μM)
Ac Tub 3-14
Substitutions
at position 4
Y
F
S
D
K

consistent with them binding in a parallel relative orientation and similar location in the MHC protein binding site.

5.2.5. Binding of Substituted Ha 307-319, Mat 17-29, and Tub 3-14 Peptides to Purified HLA-DR1Dw1

To further confirm the relative orientation and location of Ha 307-319, Tub 3-14, and Mat 17-29 when bound to HLA-DR1Dw1 the structural requirements for binding of all three were compared. Based on a parallel alignment, a number of substitutions at relative position i - 1 were made in each peptide (Table 5.IV) and the ability of the analogues to compete for the binding of Ha LCB N to affinity purified HLA-DR1Dw1 was compared (Materials and Methods, 2.9). Purified HLA-DR1Dw1 (10nM) was incubated with Ha LCB N at 10 nM, and the competitor peptides were added over a wide concentration range. The peptide MHC protein complexes were allowed to form at 37° C overnight and then separated from free peptide by incubation with the anti-DR antibody LB3.1, as before. The complexes were quantitated by addition of ^{125}I-streptavidin (Figure 5.3). The results with the analogues of Ha 307-319 were very similar to those obtained previously with the cell surface binding assay (Rothbard et al., 1988; Busch, 1991). Substitutions at position 309 with bulky hydrophobic residues were tolerated but substitutions with polar or charged residues greatly diminished the capacity of Ha 307-319 to compete for binding of Ha LCB N. Position 20 of Mat 17-29 had the same structural requirement to compete for binding of Ha LCB N as position 309 of Ha 307-319 . Also, substitution with Tyr at position 20 increased the capacity of Mat 17-29 to bind, relative to the natural Leu, as it had in the cell surface binding assay. Substitution at position 4 of Tub 3-14 with a Tyr also increased the affinity of this peptide, and substitutions with charged residues greatly decreased the affinity of this peptide for HLA-DR1Dw1. The data provided further evidence that Ha 307-319, Tub 3-14, and Mat 17-29 were bound in a parallel relative orientation and similar location within the HLA-DR1Dw1 binding site.

Table 5.IV.

Substitutions in Ha 307-319, Mat 17-29, and Tub 3-14 at relative position i - 1

				i			i + 3			i + 6			
	307	308	309	310	311	312	313	314	315	316	317	318	319
Ha	P	K	Y	V	K	Q	N	T	L	K	L	A	T
	-	-	V	-	-	-	-	-	-	-	-	-	-
	-	-	L	-	-	-	-	-	-	-	-	-	-
	-	-	S	-	-	-	-	-	-	-	-	-	-
	-	-	K	-	-	-	-	-	-	-	-	-	-

	17	18	19	20	21	22	23	24	25	26	27	28	29
Mat	S	G	P	L	K	A	E	I	A	Q	R	L	E
	-	-	-	Y	-	-	-	-	-	-	-	-	-
	-	-	-	S	-	-	-	-	-	-	-	-	-
	-	-	-	D	-	-	-	-	-	-	-	-	-

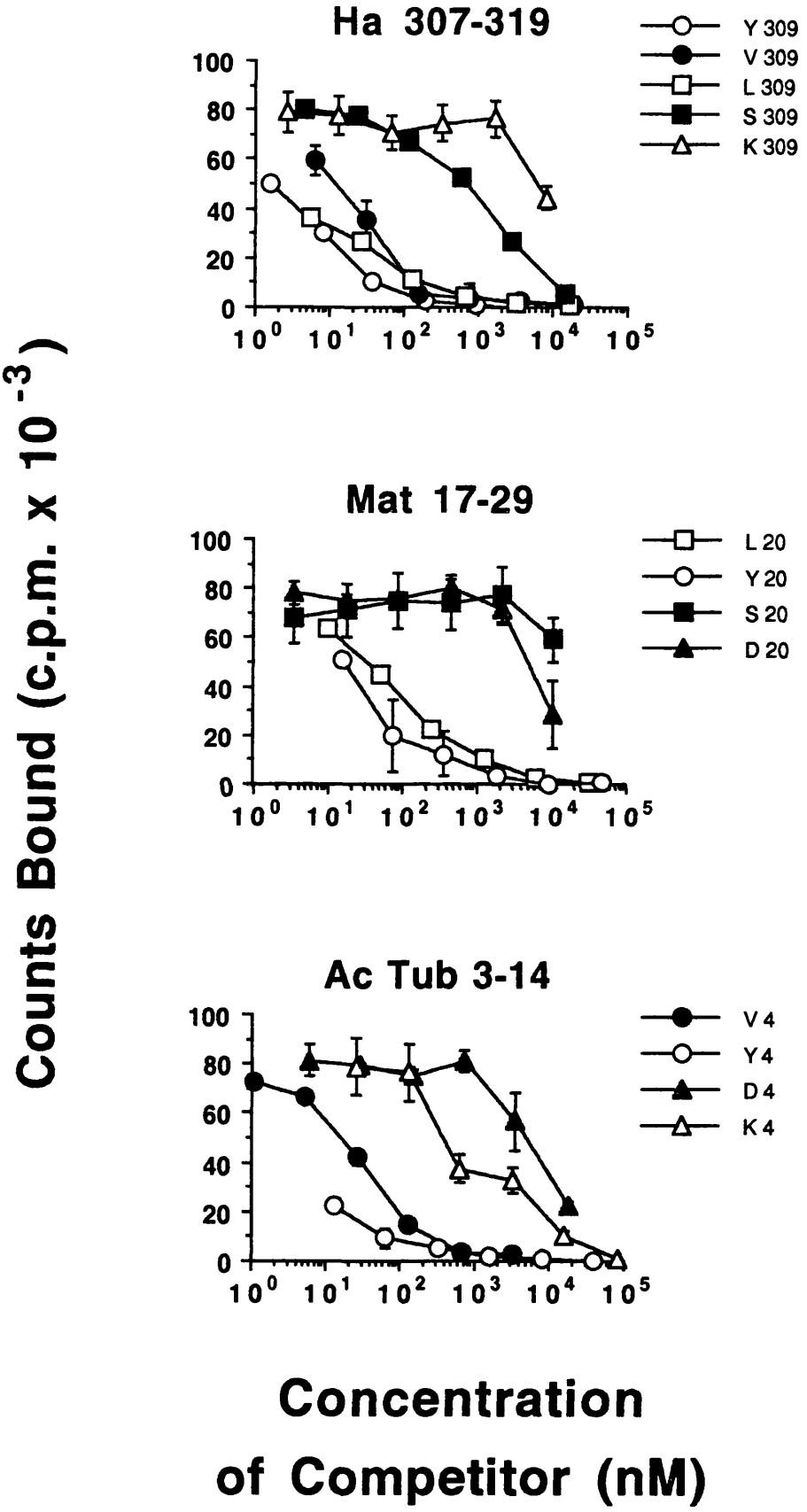
	3	4	5	6	7	8	9	10	11	12	13	14
Tub	R	V	K	R	G	L	T	V	A	V	A	G
	-	Y	-	-	-	-	-	-	-	-	-	-
	-	D	-	-	-	-	-	-	-	-	-	-
	-	K	-	-	-	-	-	-	-	-	-	-

Substitutions at position 309 of Ha 307-319, 20 of Mat 17-29, and 4 of Tub 3-14 to determine their relative orientation and location when bound to purified HLA-DR1Dw1.
Shaded residues contact the MHC protein.
Dashes indicate identity with the residue above.

Figure 5.3.

Analogues of Ha 307-319, Mat 17-29, and Ac Tub 3-14, with substitutions at position i - 1, binding to HLA-DR1Dw1

Analogues shown in Table 5.IV. were used to competitively inhibit the binding of Ha LCB N (10nM) to purified HLA-DR1Dw1 (10nM). Competitors were used over a wide concentration range and are denoted by the substitutions at, **a)** position 309 of Ha 307-319, **b)** 20 of Mat 17-29, and **c)** 4 of Ac Tub 3-14. Each point is the mean of a duplicate. Counts Bound are due to ^{125}I-streptavidin binding to Ha LCB N. Background binding of Ha LCB N was 3400 c.p.m. which was subtracted from all data before plotting.


Ha 307-319
Y 309
V 309
L 309
S 309
K 309
Mat 17-29
L 20
Y 20
S 20
D 20
Ac Tub 3-14
V 4
Y 4
D 4
K 4
Counts Bound (c.p.m. x 10^-3)
Concentration of Competitor (nM)

5.3. Discussion

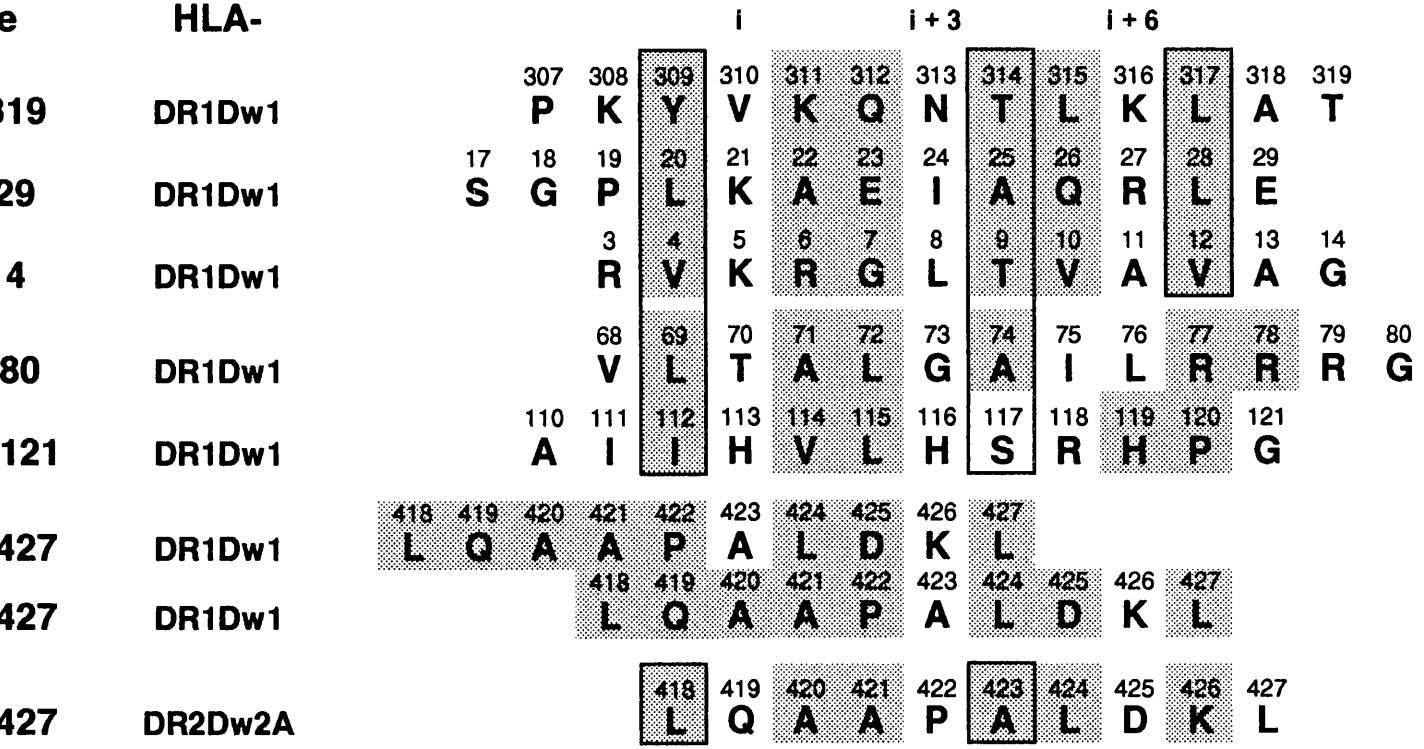
5.3.1. Comparison of the structural features of the residues of Ha 307-319, Tub 3-14, and Mat 17-29 which contact HLA-DR1Dw1

To determine possible similarities in the structural requirements for the Ha 307-319, Tub 3-14, and Mat 17-29 peptides to bind to HLA-DR1Dw1, the sequences of the peptides were aligned and compared based on the patterns of contacts they made with HLA-DR1Dw1. The patterns of contacts were symmetrical over the central core and therefore the sequences of the peptides could be aligned in either a parallel or anti-parallel fashion. Considering the structural requirements of each peptide for binding to HLA-DR1Dw1 suggested that all three peptides were binding with the same parallel orientation and in a similar location in the MHC binding site. The sequences of the three peptides are aligned in a parallel fashion in Table 5.V.

Interestingly the sequences of Ha 307-319 and Mat 17-29 have previously been compared and aligned based on sequence homology (Rothbard et al., 1988). The patterns of amino acids that Rothbard and Taylor (1988) had identified within the primary sequence of a large number of T cell determinants had allowed them to align a number of epitopes to reveal allele specific sub-patterns. The Ha 307-319 and Mat 17-29 sequences were aligned in a similar fashion based on two hydrophobic residues within each sequence (Figure 5.4a). This alignment also revealed positions of homology at two other residues within the sequences. If both peptides were modelled as helices the four homologous residues formed one face of the helices and were predicted to interact with the MHC protein (Figure 5.4a). The orientation of the peptides was confirmed by exchanging residues from one peptide into the other and transferring clonally specific T cell recognition, however, the number of residues required to transfer recognition resulted in hybrid peptides that were so homologous to the parent peptide that the results were not conclusive. In Table 5.V. the Ha 307-319

Table 5.V.

Alignment of Six Peptides Based on Contacts with HLA-DR



Contacts with HLA-DR, based on patterns of fluorescence (Figure 4.6, 4.10, 4.12, 4.13), are shaded. Homologous residues are outlined.

Figure 5.4.

a.

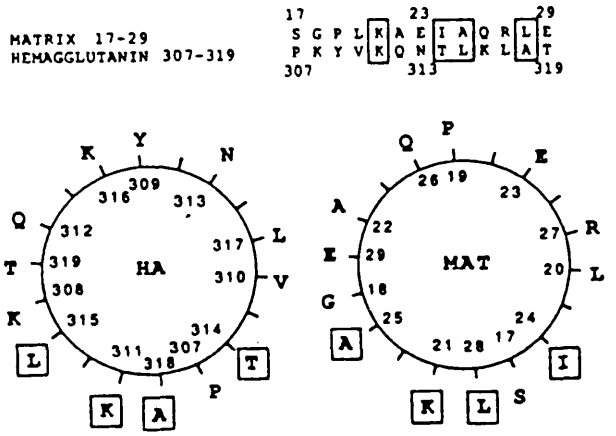


Figure taken from Rothbard et al., 1988.

b.

Alignment of Three Peptides Based on Structural Homology

	307	308	309	310	311	312	313	314	315	316	317	318	319	
Ha 307-319	P	K	Y	V	K	Q	N	T	L	K	L	A	T	
	17	18	19	20	21	22	23	24	25	26	27	28	29	
Mat 17-29	S	G	P	L	K	A	E	I	A	Q	R	L	E	
			3	4	5	6	7	8	9	10	11	12	13	14
Tub 3-14			R	V	K	R	G	L	T	V	A	V	A	G

Residues with structural homology are outlined
Based on Figure from Lamb et al., 1988.

and Mat 17-29 sequences were aligned based on the contacts they made with HLA-DR1Dw1. The two alignments were different both in terms of the relative alignment of the two peptide sequences and the residues predicted to contact the MHC protein (Figure 5.4a and Table 5.V). This illustrates quite clearly the problems of trying to determine the interactions between peptide antigen and MHC proteins using simply empirical analysis and even quite elegant functional assays. To determine the role of structural features within a peptide in binding to the MHC requires a direct assay of the interaction between them.

The T cell determinant Tub 3-14 was originally predicted based on the pattern of amino acids used by Rothbard et al. (1988) to align Ha 307-319 and Mat 17-29 (Lamb et al., 1988) (Fig 5.4b). The alignment of the Ha 307-319 and Tub 3-14 sequence based on the contacts they make with HLA-DR1Dw1 (Table 5.V.) was different from the empirical alignment shown in Figure 5.4b. This implies that the structural homology observed in the empirical alignment was either fortuitous or has some as yet undefined functional significance.

Alignment of Ha 307-319, Tub 3-14, and Mat 17-29 peptides based on a similar orientation and pattern of contacts made with HLA-DR1Dw1 (Table 5.V) revealed three homologous residues contacting the MHC protein. A large hydrophobic residue at i - 1, a small residue at i + 4, and a hydrocarbon side chain at position i + 7. The structural requirements at position i - 1 for all three peptides to bind to HLA-DR1Dw1 demonstrated the functional significance of the homology at this position and strongly suggested that this residue in each peptide may be interacting with a specific hydrophobic pocket within the MHC binding site. The importance of this residue in binding to the MHC is consistent with the experiments of Jardetzky et al. (1990) who were able to replace all residues within Ha 307-319 with Ala, apart from Lys at position 316 and Tyr at 309, and the peptide retained its affinity for HLA-DR1Dw1.

The structural significance of the small residue at position i + 4 has not been established for all three peptides. However, substitution of Ha 307-319 with a Tyr at this position has little effect on the capacity of the peptide to bind HLA-DR1Dw1 (Rothbard et al., 1989; Jardetzky et al., 1990) suggesting that there is not a specific requirement for a small residue at this residue for the peptide to bind. Similarly despite the presence of a branched hydrocarbon side chain in all three peptides at position i + 7, charged residues at this position in Ha 307-319 do not strongly affect its ability to bind (Rothbard et al., 1989). Therefore, the role of the two residues at positions i + 4 and i + 7 in the formation of a complex with HLA-DR1Dw1 is unclear, although the homology at this position suggests a functional role. One possibility is that these two residues may be important in the formation of the common conformation adopted by these three peptides.

5.3.2. Comparison of the structural features of five different peptides which bind to HLA-DR1Dw1

In Chapter 4 LCB lysine analogues of Myo 68-80 and Myo 110-121 were used to determine their contact residues with HLA-DR1Dw1. If the patterns of contacts made by these two peptides with the MHC protein were used to align them in a parallel fashion relative to the first three peptides then two positions of structural homology were apparent in all five peptides at positions which contacted the MHC protein (Table 5.V.). A large hydrophobic residue at relative position i -1 and a small residue at position i + 4. The two myoglobin peptides do not have an amino acid with a hydrocarbon side chain at position i + 7. Interestingly the lack of homology at this carboxyl terminal residue correlates with the different conformations which these two peptides adopt at their carboxyl termini compared to the first three peptides. This is consistent with the three positions of homology in the Ha 307-319, Mat 17-29 and Tub 3-14 peptides being important in their adopting a similar conformation over the central core. Similarly the large hydrophobic residue at i - 1 and small residue at i + 4 in all

five peptides may be important for them each to adopt a comparable conformation at the amino terminus. The effect of LCB lysine at i - 1 on binding of Myo 68-80 and Myo 110-121 indicated that this position was contacting HLA-DR1Dw1. The structural requirements at this position for the myoglobin peptides to bind was not established and therefore whether these peptides were binding in the same location in the DR binding site as the Ha 307-319, Mat 17-29, and Tub 3-14 peptides will require further investigation. However, the similarities in the conformations at the amino terminus along with the two positions of homology raises the possibility that they were also interacting with the same hydrophobic pocket within HLA-DR1Dw1.

5.3.3. Structural features of Lep 418-427

LCB lysine analogues of Lep 418-427 established a unique pattern of fluorescence for this peptide, compared to the other five peptides, when bound to cells expressing HLA-DR1Dw1 (Figure 4.12). There were two peaks of fluorescence within the pattern towards the carboxyl terminus and at relative positions i and i + 3. If the sequence was aligned with the other five in a parallel fashion with these two peaks aligned with relative positions i and i + 3 of the other peptides then there were no positions of homology between this sequence and the others. Since there were only two peaks of fluorescence these could also be aligned with relative positions i + 3 and i + 6 of the other peptides. In this alignment again none of the positions shared homology with all the other peptides. The lack of homology of the contact residues and the pattern of different contacts this peptide was making with HLA-DR1Dw1 both indicated that it was interacting with the MHC protein in a different fashion to the other peptides.

The sequence of this peptide does contain a large hydrophobic residue at position 418 and a small residue at position 423 five amino acids away. Despite the presence of the same structural features, with the same relative spacing (i -1 to i + 4), within the sequence, this peptide was unable to bind HLA-DR1Dw1 with the same

amino terminal conformation as the other peptides. The homology at i - 1 and i + 4 within the sequences of the first five peptides suggests the importance of these residues in the interactions of these peptides with HLA-DR1Dw1. However, while they may be necessary they are clearly not sufficient to result in the formation of a particular conformation at the amino terminal region of the peptides bound to HLA-DR1Dw1. The functional significance of the large hydrophobic residue at position 418 pf :e[418-427 is also unclear. LCB lysine at position 418 still leads to a low fluorescent signal indicating that this residue is contacting the MHC protein. However, LCB lysine at any of the amino terminal residues also leads to a low fluorescent signal. Whether residue 418 in this peptide requires a large hydrophobic amino acid for successful binding will require further testing.

The pattern of contacts made by Lep 418-427 with HLA-DR2Dw2A, which were very similar to the contacts of Ha 307-319, Mat 17-29 and Tub 3-14 with HLA-DR1Dw1, could be used to align the sequence of Lep 418-427 with the other peptides. Interestingly, if this was done in a parallel orientation then the large hydrophobic at position 418 aligned with the other hydrophobic residues at i -1 and the small residue at 423 aligned with the other small residues at i + 4 (Table 5.V). Although the structural significance of these two residues in binding HLA-DR2Dw2A was not shown, LCB lysine substitutions at these positions indicated that they were both contacting the MHC protein. The similarity in the conformations of Lep 418-427 bound to HLA-DR2Dw2A and Ha 307-319, Mat 17-29 and Tub 3-14 bound to HLA-DR1Dw1, combined with the structural homology at i - 1 and i + 4 in all four peptides, raised the possibility that residue i - 1 in Lep 418-427 was interacting with a specific hydrophobic pocket in HLA-DR2Dw2A. However, direct support for this hypothesis will require further experiments using analogues of Lep 418-427 substituted at position 418.

5.3.4. Analysis of the sequences of a large number of T cell determinants

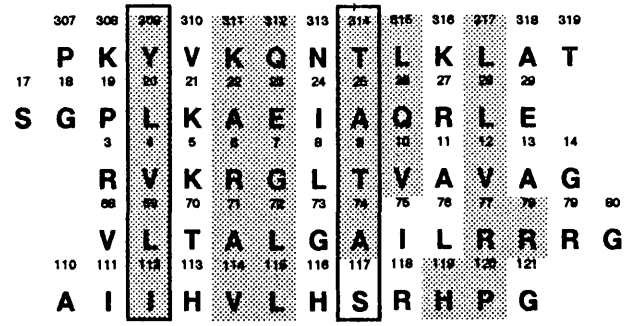
To extend the results outlined above, a large number of known T cell determinants were analysed for the presence of a large hydrophobic residue with a small amino acid 5 residues away (Table 5.VI). The list of determinants was not comprehensive, however, it did contain epitopes recognised in the context of both class I and class II, as well as mouse and human, MHC proteins, and was randomly chosen from the known T cell determinants. The probability of a large hydrophobic residue and a small residue 5 amino acids away within a random 15 mer is 0.46. The actual occurrence within the determinants shown was 56 out of 74 (76%) which a simple chi square test showed to be statistically significant. The statistical significance of these two structural features within these determinants suggests a functional significance. One possible hypothesis is that the residue at position i - 1 in each of these peptides is making an interaction with a hydrophobic pocket within the MHC binding site of all these different alleles, similar to the interaction of i - 1 within Ha 307-319, Mat 17-29 and Tub 3-14 binding to HLA-DR1Dw1. Further, the hydrophobic pocket may be a conserved pocket that appears in the same part of the binding site of each allele, suggesting that each peptide is binding in similar location in the MHC binding site.

5.3.5. Conserved pockets in the MHC binding site

As a preliminary step to examine the above hypothesis the binding site of different MHC proteins was examined for the possible presence of a conserved hydrophobic area. In Figure 5.5 a model of the HLA-DR1Dw1 binding site is shown, which was derived in Chapter 1 (Figure 1.6). All hydrophobic residues and small residues, which might combine to form a pocket able to accommodate a large hydrophobic amino acid, are shaded. There are two main areas which seem to contain suitable residues. The first consists of a cluster of four α chain residues 22, 24, 58,

Table 5.VI. Alignment of many T cell determinants

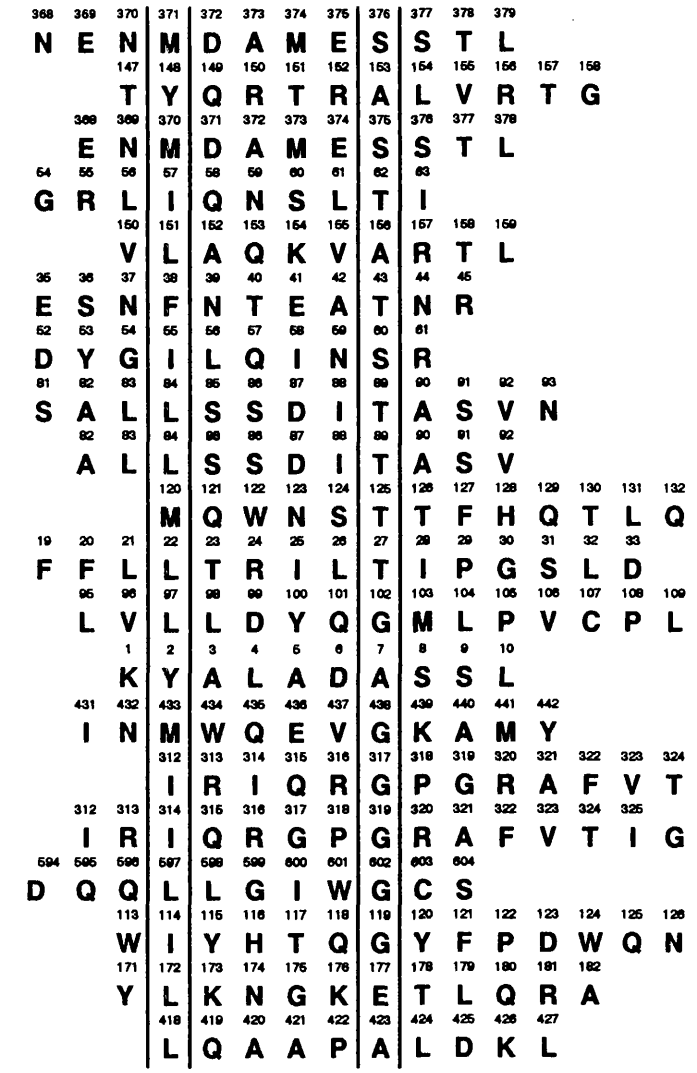
A.



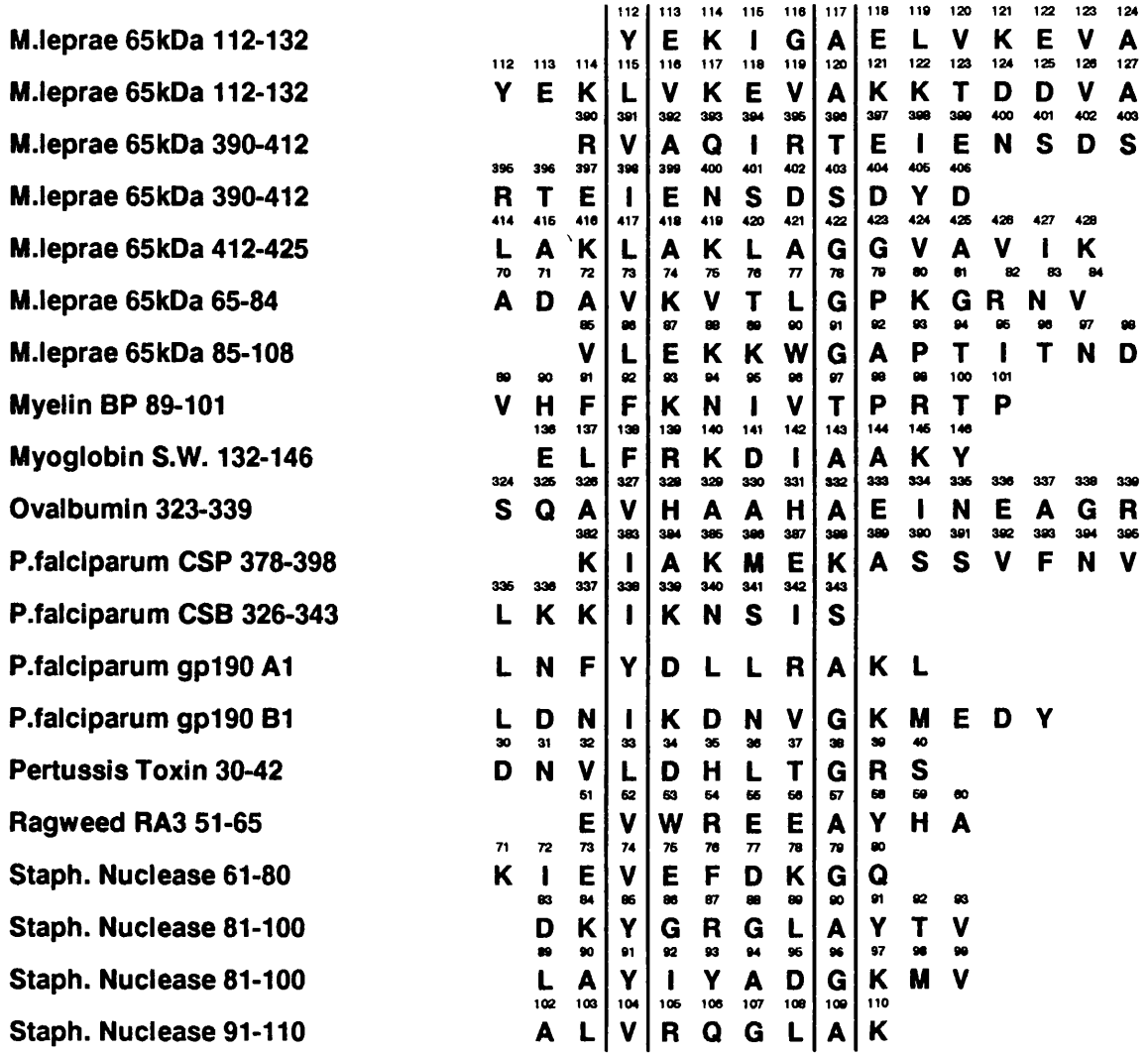
	Restriction*	Reference
Haemagglutinin 307-319	DR1Dw1	Lamb et al., 1983
Matrix 17-29	DR1Dw1	Rothbard et al., 1988
Tuberculosis 19 kDa 3-14	DR1Dw1	Lamb et al., 1988
Myoglobin 68-80	I-Ek	Livingstone et al., 1987
Myoglobin 110-121	I-Ed	Livingstone et al., 1987

B.

	Restriction*	Reference
A2 170-185	Kd	Maryanski et al., 1986b
Cytochrome horse 45-58	I-Ab,k	Suzuki and Schwartz, 1986
Cytochrome moth 89-103	I-Eb,k	Hedrick et al., 1982
Cytochrome pigeon 45-58	I-Ab,k	Suzuki and Schwartz, 1986
EBV LMP 43-53	A1	Thorley-Lawrenson, 1987
Flu HA 130-142	I-Ad	Hackett et al., 1985
Flu Matrix 57-68	A2	Gotch et al., 1987
Flu NP 383-396	DR	Hickling et al., 1990
Flu NP (34) 366-379	Db	Townsend et al., 1986
Flu NP (34/68) 335-349	B37	Townsend et al., 1986



Flu NP (68) 366-379	Db	Townsend et al., 1986
Flu NP 147-158	Kd	Taylor et al., 1987
Flu NP 366-379 (68)	Db	Townsend et al., 1986
Flu NP 50-63	Kk	Bastin et al., 1987
Foot and Mouth VP1 145-160	Guinea Pig	Francis et al., 1985
Hen Egg Lysozyme 34-45	I-Ak	Allen et al., 1984
Hen Egg Lysozyme 46-61	I-Ak	Babbitt et al., 1986
Hen Egg Lysozyme 78-93	I-Ab	Manca et al., 1984
Hen Egg Lysozyme 81-96	I-Ek,I-Ab	Shastri et al., 1985
Hepatitis B pre S 120-132	H-2k,q,s	Milich et al., 1986
Hepatitis B SAg 19-33	DPw4	Celis et al., 1988
Hepatitis B SAg 95-109	H-2q	Milich et al., 1985
Herpes Glyco D 1-23	I-Ab	Heber-Katz et al., 1986
HIV gp120 428-443	H2 k,s,d	Cease et al., 1987
HIV gp120 Env. 312-327	Dd	Takahashi, 1989
HIV gp120 Env. 312-327	Dd	Takahashi, 1989
HIV gp120 Env. 584-604	HLA Cl II	Schrier et al., 1988
HIV nef 113-128	HLA B17.37	Culmann et al., 1989
HLA CW3 171-182	Kd	Maryanski et al., 1986b
M.leprae 65kDa 418-427	DR2a	Anderson et al., 1988


M.leprae 65kDa 112-132 Y E K L V K E V A DR Lamb et al., 1987
M.leprae 65kDa 112-132 Y E K L V K E V A K K T D D V A DR Lamb et al., 1987
M.leprae 65kDa 390-412 R V A Q I R T E I E N S D S DR5 Lamb et al., 1987
M.leprae 65kDa 390-412 R T E I E N S D S D Y D DR5 Lamb et al., 1987
M.leprae 65kDa 412-425 L A K L A K L A G G V A V I K DR1 Van Schooten et al., 1989
M.leprae 65kDa 65-84 A D A V K V T L G P K G R N V DR1 Lamb et al., 1987
M.leprae 65kDa 85-108 V L E K K W G A P T I T N D DR5 Thole et al., 1988
Myelin BP 89-101 V H F F K N I V T P R T P I-As Sakai et al., 1988
Myoglobin S.W. 132-146 E L F R K D I A A K Y I-Ed Berkower et al., 1986
Ovalbumin 323-339 S Q A V H A A H A E I N E A G R I-Ad Shimonkevitz, 1984
P.falciparum CSP 378-398 K I A K M E K A S S V F N V DR Sinigaglia et al., 1988
P.falciparum CSB 326-343 L K K I K N S I S I-Ak Good et al., 1987
P.falciparum gp190 A1 L N F Y D L L R A K L DQ Crisanti et al., 1988
P.falciparum gp190 B1 L D N I K D N V G K M E D Y DR6 Crisanti et al., 1988
Pertussis Toxin 30-42 D N V L D H L T G R S DR1 De Magistris., 1989
Ragweed RA3 51-65 E V W R E E A Y H A H-2d,k,s,q Kurisaki et al., 1986
Staph. Nuclease 61-80 K I E V E F D K G Q I-Ek Finnegan et al., 1986
Staph. Nuclease 81-100 D K Y G R G L A Y T V I-Ek Finnegan et al., 1986
Staph. Nuclease 81-100 L A Y I Y A D G K M V I-Ek Finnegan et al., 1986
Staph. Nuclease 91-110 A L V R Q G L A K I-Ab Finnegan et al., 1986

Tetanus toxoid 947-969	948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 G N F T V S F W L R V P K V S 830 831 832 833 834 835 836 837 838 839 840 841	DR	Demotz et al., 1989a
Tetanus toxoid 830-843	Y I K A N S K F I G I T	DR	Demotz et al., 1989a

Table 5.VIII.C.

Cytochrome bovine 13-25	13 14 15 16 17 18 19 20 21 22 23 24 25 K C A Q C H T V E K G G K 111 112 113 114 115 116 117 118 119 120	I-Ad	Corradin et al., 1983
Flu HA 111-120	F E R F E I F P K E 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129	I-Ad	Hackett et al., 1983
Flu HA 114-131	A S S G T L E F I N E G F N W T 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63	Human	Lamb and Green., 1983
Flu HA 48-66	I C N N P H R I L D G I D C T 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154	H-2k	Mills et al., 1986
Hepatitis B SAg 140-154	T K P S D G N C T C I P I P S 263 264 265 266 267 268 269 270 271 272 273 274	H-2k	Milich et al., 1985
HIV gag p24 263-274	K R W I I L G L N K I V 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433	B27	Nixon et al., 1988
HIV gag p24 418-433	K E G H Q M K D C T E R Q A N F 112 113 114 115 116 117 118 119 120 121 122 123 124	A2	Claverie et al., 1988
HIV gp 120 112-124	H E D I I S L W N Q S L K 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424	H-2s	Cease et al., 1987
HIV gp 120 410-429	G S D T I T L P C R I K Q F I 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128	DR4	Siliciano et al., 1988
HIV nef 113-128	W I Y H T Q G Y F P D W Q N Y T 170 171 172 173 174 175 176 177 178 179 180 181 182	HLA B17.37	Culmann et al., 1989
HLA A24	R Y L E N G K E T L Q R A 171 172 173 174 175 176 177 178 179 180 181 182	Kd	Maryanski et al., 1986b
HLA CW3 171-182	Y L K N G K E T L Q R A 5 6 7 8 9 10 11 12 13 14 15	Kd	Maryanski et al., 1986b
Insulin B Chain bovine 5-15	H L C G S H L V E A L 12 13 14 15 16 17 18 19 20 21 22 23 24	I-Ad	Thomas et al., 1981
Lambda repressor 12-26	L E D A R R L K A I Y E K 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17	I-Ad	Guillet et al., 1986
M.leprae 65kDa 2-12	P G R D G E T Q P A S C G R P S 439 440 441 442 443 444 445 446 447 448 449	DR3	Van Schooten et al., 1989

M.leprae 65kDa 439-448	**H R I E D I V R N A K** (32 33 34 35 36 37 38 39 40 41 42 43 44)	DR1	**Van Schooten et al., 1989**
Rabies Glycoprot. 32-44	**D E G C T N L S G F S Y M** (1274 1275 1276 1277 1278 1279 1280 1281 1282 1283 1284)	H-2d	**McFarlane et al., 1984**
Tetanus toxoid 1272-1284	**Q I G N D P N R D I L**	DR	**Demotz et al., 1989b**

* The "Restriction" is defined as the MHC molecule by which the T cells originally used to define the epitope were restricted.

Shaded residues indicate those contacting the MHC protein.

Outlined residues are either a large hydrophobic at i -1 or a small residue at i + 4.

For convenience not all residues within every determinant are shown.

Large hydrophobics: I, L, V, Y, F, M. These are amino acids either present in peptides in Table VIII.A. at i -1 or amino acids which can be substituted at position 309 of Ha 307-319 without affecting binding.

Small residues: S, T, A. These are residues present at position i + 4 of the peptides in Table VIII.A.

A. Peptide sequences aligned based on contacts with HLA-DR1Dw1

B. Peptide sequences aligned based on the presence of a large hydrophobic residue at relative position i - 1 and a small residue at i + 4

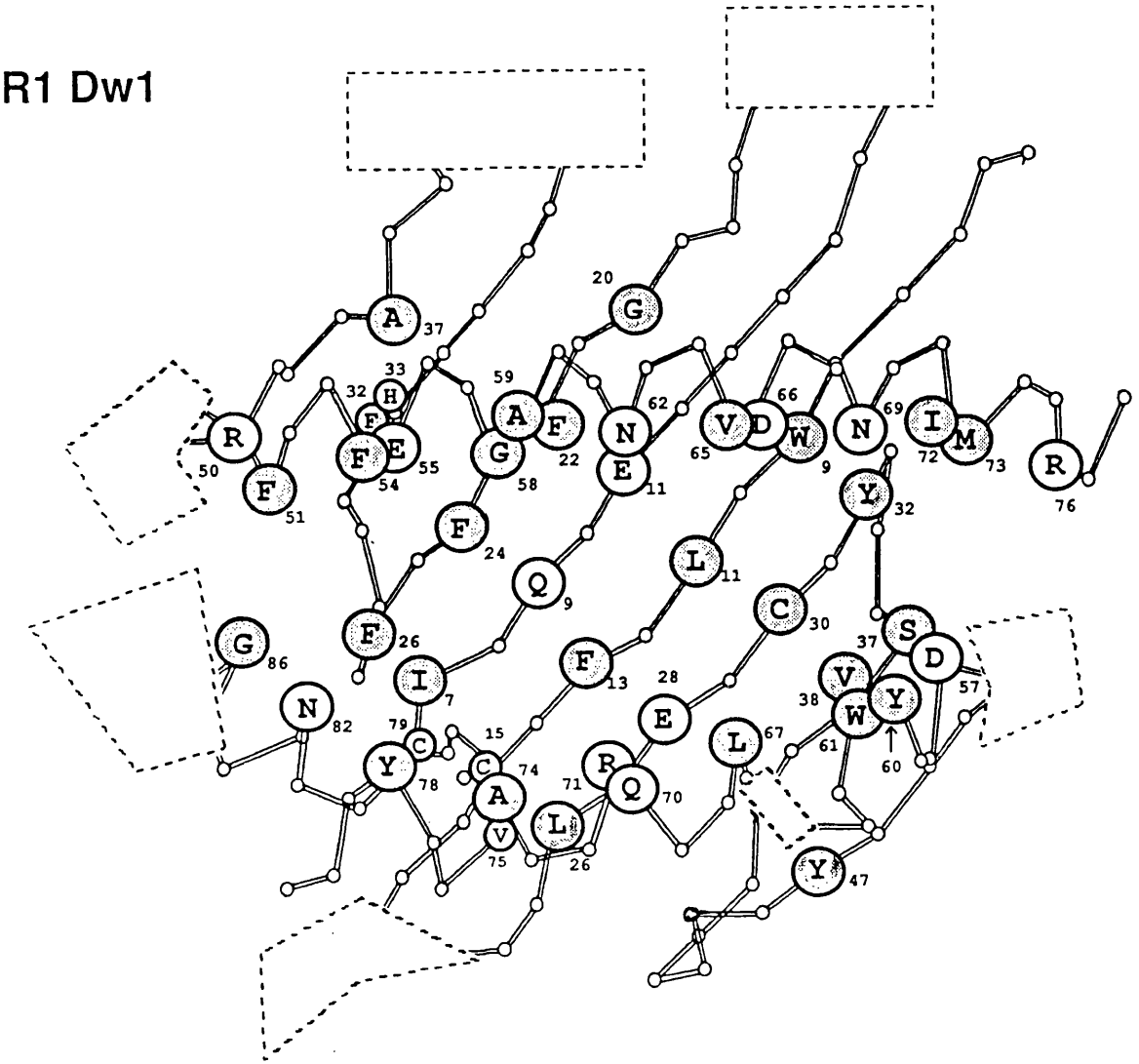
C. Peptide sequences which did not contain the pattern of a small and a large residue.

Figure 5.5.

Antigen binding site of HLA-DR1Dw1

Figure is essentially the same as Figure 1.6. except that shaded residues signify either small residues or large hydrophobic residues which might combine to make up a pocket able to accommodate a large hydrophobic residue.

HLA-DR1 Dw1


A 37
G 20
H 33
F 32
A 59
F 22
N 62
V 65
D 66
W 9
N 69
I 72
M 73
R 76
R 50
F 51
F 54
E 55
G 58
E 11
Y 32
F 24
Q 9
L 11
C 30
S 37
D 57
F 26
I 7
F 13
28
V 38
W 61
Y 60
G 86
N 82
79
C 15
E
L 67
Y 78
C 74
A
R 71
Q 70
V 75
L 26
Y 47

and 59. The second region consists of a cluster of four β chain residues 38, 47, 61, and 67. The first region residues are conserved in all DR molecules, however, residues at this position in the MHC binding site are predicted to be highly polymorphic amongst other class I and class II alleles, containing charged and polar as well as hydrophobic amino acids (Brown et al., 1988). The residues of the second region are also polymorphic, however, unlike the α chain, the residues at positions equivalent to 38, 47, and 61 in the HLA-DR1Dw1 MHC binding site are predicted to be hydrophobic within many class I and class II proteins (β 38 is Ala, Val, or Leu; β 47 is Phe, Trp, or Tyr; β 61 is Trp or Tyr) (Brown et al., 1988). Residue 67 is predicted to be hydrophobic in all class II molecules (Ile, Val, Phe, or Leu) but is charged in about 30% of class I molecules and hydrophobic in the rest. An additional factor is the location of this hydrophobic area. The short β chain helix which starts at about residue 50 and finishes at about residue 67 raises this end of the longer β chain helix up such that a "pocket" can be formed between the helical regions of the β chain and the strands of the β pleated sheet of the β chain. Hence the location of these conserved β chain residues is consistent with them forming a conserved specificity pocket in many different MHC protein binding sites. The location of a conserved hydrophobic pocket in many different MHC binding sites and the structural homology found between the different peptides in Tables V and VI is consistent with many peptides binding in a similar location in the MHC binding site.

Although the hypothesis outlined above is highly speculative it can also be easily tested. One prediction is that each of the peptides binding to different MHC proteins should share a structural requirement for a large hydrophobic residue at position i - 1. Similarly one would expect that the same peptide, if it was able to bind to a number of different MHC alleles, should have a structural requirement for a large hydrophobic amino acid at a position five amino acids away from a small residue in order to bind to each MHC protein. Busch et al. (1991) have recently analysed the ability of Ha 307-319, and an analogue with Ser at 309 (i - 1 in Table 5.V), to compete

for binding of Ha LCB N to a range of cell surface HLA-DR molecules. Many alleles had a structural requirement for the natural Tyr at position 309 for Ha 307-319 to bind, however,a number of alleles were able to bind the analogue with Ser at 309 as well as the natural sequence suggesting that they did not have a requirement for a large hydrophobic amino acid at this position. The structural requirement for a Tyr at position 309 correlated largely with the presence of a Gly at position 86 of the DR β chain. In the MHC alleles which bound both the natural sequence and the analogue with Ser at 309, position 86 was a Val. By using LCB lysine analogues of Ha 307-319 the amino acid change at position 86 was shown to correlate with a change in the entire conformation of the bound peptide. The differences in binding of Ha 307-319 to DR molecules with a Gly at 86, and those with a Val at 86, were not consistent with a local change in the structure of the DR molecule and suggested that the structure of the MHC binding site had been changed at several positions. The data indicated that the importance of a large hydrophobic residue at position i - 1, with a small residue at position i + 4, will depend on the MHC allele with which the peptide interacts. Hence although the hypothesis outlined above may be a valid mechanism for the formation of many peptide MHC protein complexes it is clearly not a universal mechanism of peptide binding and will require further investigation to test its validity.

5.3.6. Conclusions

Analysis of the structural features present in three peptides which bound to HLA-DR1Dw1 with similar conformation revealed structural homologies at three equivalent positions shown to be important for binding to the MHC molecule. A structural requirement for a large hydrophobic residue at one of these positions indicated that all three peptides were binding in a preferred location and orientation within the MHC protein binding site. This large hydrophobic amino acid at one position and a small amino acid at a position 5 residues away could be aligned with a similar pair of residues in a large number of T cell determinants and in 5 out of 6

peptide MHC protein complexes analysed, both these positions were making important contacts with the MHC molecule. The presence of these common structural features within many different determinants suggested a common location and orientation for the binding of diverse peptides to different MHC binding sites. Consistent with this was the identification of a potential hydrophobic interaction "pocket" within the HLA-DR1Dw1 binding site, which was highly conserved in all class I and class II proteins.

Chapter 6
General Discussion

6.1. Immunology of Human Papillomavirus

6.1.1. Introduction

The accumulating evidence for the causative role of human papillomaviruses in the development of epithelial malignancies has provided an exciting opportunity for cancer immunology. Several studies have indicated that HPV infection, and potentially therefore the associated lesions, could be modulated by immunotherapies (Chapter 3.1.3). However, the precise roles of the cellular and humoral immune response in regulating HPV infection are still poorly defined (Davies et al., 1991; Chapter 3.1.3). One of the principle reasons for this is the absence of an in vitro culture system to produce experimentally useful quantities of virus. Therefore, a large amount of effort is currently being focused on producing reagents to examine the different aspects of potential immune responses to HPV, including humoral, cytotoxic, and T helper responses.

An additional complication of HPV immunology is the range of over 60 sequenced HPV types (de Villiers, 1989). The high degree of antigen specificity of B and T cell responses results in the need for synthetic reagents to cover each HPV type in order to examine immune responses. For example, the B cell epitopes of the intact L1 proteins of HPV type 6 and 16 have been localised to distinct parts of each molecule and human sera is not crossreactive with the two types (Galloway and Jenison, 1990). In Chapter 3 of this thesis, experiments were described, which demonstrated that mouse T cells specific for peptides from L1 of HPV type 16 were not cross reactive with equivalent peptides from other HPV types (Table 3.V). Although this data was in contrast to studies in the human system (Strang et al., 1990; Chapter 3.3) the results

from both sets of studies suggested that L1 specific class II restricted T cell responses were unlikely to be generally cross reactive between different HPV types.

The diversity of different HPV types means that synthesis of antigens to cover all of them is impractical and therefore research efforts must be focused in some way. The range of lesions which are associated with the different HPV types is also diverse and one way to focus effort is to consider only prevalent HPV types which have a strong association with a highly pathogenic condition. For example, HPV type 16 has a strong association with cervical cancer which is a major form of cancer amongst women in the developed world (Cuzick and Boyle, 1988; zur Hausen, 1989). Once a particular virus or subset of viruses has been chosen from the available epidemiological data there are a number of approaches which can be used to analyse potential immune responses.

6.1.2. Approaches to examine immune responses to HPV

Due to the absence of available intact virions, studies have focussed on examining immune responses to individual proteins within HPV types (reviewed by Davies et al., 1991). The approach used will depend to some extent on the reagents available. For example recombinant proteins produced in bacteria have been used to examine humoral responses to HPV type 16 and 6 antigens in human patients (Cubie et al., 1989; Galloway and McDougall, 1990) and to determine potential B cell epitopes within the HPV type 16 L1 protein in mice (Cason et al., 1989). Recombinant proteins have also been used to examine proliferative T cell responses to HPV type 16 antigens in human patients (Cubie et al., 1989) and animal models (Davies et al., 1990).

To examine murine cytotoxic T cell responses to whole HPV antigens two approaches have been used. Recently a vaccinia virus recombinant vector expressing the HPV type 16 L1 ORF has been used to immunise mice and hence generate murine CTLs specific for L1 expressing targets (Jian Zhou et al., 1991). An alternative strategy is based on the work of Maryanski et al. (1986) who immunised P815 cells,

transfected with the genes for human HLA-Cw3, into Balb/c mice and hence generated murine CTLs specific for HLA. Similarly, P815 cells transfected with the E7 ORF of HPV type 16 have recently been used to generate E7 specific CTLs in Balb/c mice (H.Davies and A.McIndoe, personal communication).

Synthetic peptide antigen has also be used to analyse the immune responses to HPV antigens. In Chapter 3 a predictive algorithm was used to identify potential T cell determinants within three ORFs of HPV type 16. Thirteen peptides containing the putative epitopes, and covering 24% of the protein sequence analysed, were synthesised and assayed directly for immunogenicity in mice. Five of the determinants were immunostimulatory in the strains of mice tested (Table 3.VII) and three of the determinants were shown to generate class II restricted T cell responses. As well as being used for establishing proliferative responses to antigen, such predicted peptides can also be used in in vitro priming experiments to determine potential CTL responses to an antigen (Carbone et al., 1987; H.Davies, personal communication). However, there are two disadvantages to using a limited number of predicted determinants from a protein to prime T cells in vivo or in vitro. Firstly, the existence of other epitopes within the protein is not established and secondly, the immunodominance of the immunostimulatory epitopes is not determined.

Synthesis of overlapping peptides spanning an entire protein, followed by in vivo or in vitro priming, provides a comprehensive way of establishing the total number of different T cell epitopes within a protein (Van der Zee et al., 1989), as long as the overlap is sufficient to identify epitopes on the boundary between peptides. However, this still does not establish the immunodominance of the peptide epitopes defined. One way to determine the immunodominance of peptide epitopes is to use intact antigen to stimulate peptide specific T cells in vitro (Gammon et al., 1987). For example, T cells specific for three peptides from L1 (Table 3.VII) were shown to cross

react with intact L1 protein on solubilised nitrocellulose, indicating that these epitopes were immunodominant within the protein (Davies et al., 1990).

As well as using peptides to generate primary responses they can also be used to help analyse in detail the responses to whole antigen, either elicited by natural infection or through immunisation. For example, the predicted epitopes (Table 3.II) from HPV type 16 have been used to analyse HPV specific proliferative responses in asymptomatic human subjects (Strang et al., 1990). Predicted peptides, or more comprehensively overlapping sets of peptides, could be used to determine both proliferative and cytotoxic T cell epitopes in the whole antigens described in some of the studies above. More recently overlapping peptides have also been used to determine possible linear B cell epitopes within HPV antigens. For example, overlapping peptides spanning a number of L1 and L2 ORFs from different HPV types have been used in ELISA based assays to examine the sera from patients for potential antibody epitopes (Dillner et al., 1990).

The study of the immunobiology of HPV is clearly technically very demanding. However, the recombinant DNA and synthetic peptide techniques which are currently available should gradually lead to a clear analysis of the important epitopes within many HPV antigens.

6.1.3. Immunotherapy

Any immunotherapy must stimulate an immune response which will prevent infection by HPV and/or aid recovery from a current infection. There are two main approaches to developing immunotherapies for HPV. The first involves developing an animal model of disease in order to test possible therapies directly. For example, primary epithelial cells (Baby Mouse Kidney cells) co-transfected with HPV type 16 DNA and an activated *ras* gene have produced cells able to produce tumours in inbred mice (Matlashewski et al., 1987; N. Almond unpublished data). Irradiated tumour cells were able to protect mice against subsequent challenge with un-irradiated cells,

however, the antigen possible specificity of the protective response has not been examined in detail (N. Almond unpublished data). The model allows potentially therapeutic responses to different antigens to be assayed directly, either empirically or having established details of the responses through previous studies. For example, the HPV type 16 determinants identified in Chapter 3 have been used to immunise mice which were subsequently challenged with the HPV type 16 expressing tumour cells. No significant protection against tumour challenge was observed in the immunised mice compared to control animals (N.Almond C.M.Hill, unpublished data). The advantage of these kinds of studies is the ability to assay possible immunotherapies relatively easily, the disadvantage is that the therapies may not be relevant to human infection.

The second approach to establishing successful immunotherapies for HPV infection is to establish the nature of a successful immune response in human patients. The immune responses of individuals who have recovered from an HPV infection needs to be compared with those who are currently infected and those who have no history of disease. Since the role of the different branches of the immune system in modulating infection is so uncertain both cellular and humoral immune responses need to be assayed. For certain HPV types these studies are almost impossible. For example HPV type 16 infection is nearly always treated with surgery before the disease is allowed to progress and hence obtaining patients who have successfully and naturally recovered from disease becomes impractical. Patients who have recovered from more obvious skin and/or genital warts are easier to obtain and would probably form the basis for such studies.

Many of the studies using human subjects which were described in the previous section (6.1.2) attempted to provide patient and control groups within their analyses. However, the seemingly high incidence of infection amongst the "normal" population (zur Hausen, 1989) means that control groups have to be very carefully selected. An additional factor which requires more careful analysis is the HPV types involved in

patients being examined for potential immune responses. The synthetic antigens used must either be selected to reflect the HPV types present or there must be a sufficient range of antigens to cover all possible HPV types within a lesion. Also, the immunodominant antigens within a successful immune response need to be established as well as the immunodominant epitopes within a protein. These studies will require a great deal of co-operative effort amongst clinicians, virologists, and immunologists for their success. However, the technology is now available to undertake the task of elucidating the role of the different branches of the immune system in regulating HPV infection. Having established the nature of a successful immune response, knowledge of the antigens and epitopes involved should allow the development of specific subunit vaccines and immunotherapies to regulate HPV infection and hence the epithelial malignancies associated with these viruses.

6.2. Peptide MHC Protein Interactions

6.2.1. Introduction

MHC proteins represent a unique family of cell surface ligand receptor molecules. For example, hormone receptors on the cell surface have extremely high affinities for their ligands (K_D 10^{-8} - 10^{-14}) and the interactions are exquisitely specific. Antibodies also can have great affinity for their ligands (K_D 10^{-5} - 10^{-10}) and the binding is in general very specific. In contrast MHC proteins rarely have such high overall affinities for antigenic peptides (K_D 10^{-5} - 10^{-8}) and are designed to bind their ligands with very limited specificity (Table 1.II) (Introduction, 1.4.5, 1.5.1). MHC proteins are also remarkable in that despite these comparatively low overall affinities the stability of the complexes formed is very high, with half lives of the order of days (Buus et al., 1986). The high stability of the complexes combined with the broad specificity of the interactions are essential properties of MHC proteins, which enable them to bind a wide range of different peptides and present them to the available repertoire of T cells. One possible explanation for the high stability of peptide MHC

protein complexes is the involvement of conformational changes in the protein upon binding of ligand (Introduction, **1**.7.2), leading to a physical entrapment of the peptide. The broad specificity of the MHC protein binding site could be explained by two possible extreme mechanisms of peptide binding (Chapter **4**.1). Firstly, different peptides could bind with many different conformations and in many different locations within the binding site. Alternatively, different peptides could bind with a preferred conformation and in a preferred location within the MHC binding site. If the latter were true then different peptides binding to the same MHC allele should share at least sufficient structural features to define the preferred conformation and location. The existence of such a common structural mechanism of peptide binding would simplify the systematic analysis of peptide MHC protein interactions, and subsequent elucidation of the molecular mechanisms of peptide binding. Understanding of these mechanisms would in turn allow the rational design of MHC binding peptides to act as both immune agonists (vaccine development) and immune antagonists (autoimmune disease therapy).

6.2.2. Conformation

To help determine whether there is a common mechanism of peptide binding to MHC proteins a number of groups have attempted to examine the conformations of peptides which interact with MHC molecules (Introduction, **1**.8.2; Chapter **4**.1). There were two main problems with the majority of these studies. Firstly, some of the analyses considered the theoretical propensity of peptides to form a particular conformation, or the propensity to form a particular conformation in solution. These techniques did not analyse the peptides bound to the MHC protein. Secondly, the studies which analysed the conformations of bound peptides used analogues monosubstituted with natural amino acids to try and define residues contacting the T cell receptor, the MHC protein, or both. The MHC binding site is designed to tolerate a large number of different peptide sequences and hence these studies failed to identify

clearly all the residues within a peptide which were contacting the MHC protein and were unable to easily infer a conformation.

The strategy of our group was to use the un-naturally large LCB lysine monosubstituted into the sequence of Ha 307-319 to determine its conformation when bound to cell surface HLA-DR1Dw1 (Rothbard et al., 1989b). In Chapter **4** LCB lysine analogues of a number of T cell determinants were used to determine their relative conformations and orientations when bound to cell surface HLA-DR1Dw1. The success of this strategy was dependant on two main factors. Firstly, the specificity of the interactions measured, and secondly, that the LCB lysine substitutions were only affecting the affinity of the peptide for the MHC protein.

Analysing the interactions with HLA-DR molecules on the surface of EBV B cell lines is complicated by the presence of structurally related molecules on the surface of the cell. Cells transfected with HLA-DR molecules could be used to help prove specificity however, cells expressing sufficiently high levels of HLA to use in the assay are rare and require repeated sorting and analysis to maintain the high levels of expression. In Chapter **4** anti-DR antibody inhibition studies were used to demonstrate that the LCB lysine analogues were interacting predominantly with cell surface HLA-DR1Dw1 (Table **4.III**). The disadvantage of such studies was the toxicity of the antibodies at high concentrations. The use of Fab fragments of such antibodies may provide a means to avoid such toxicity but requires further investigation.

The advantage of examining the interactions between peptides and cell surface, as opposed to purified, HLA-DR is that the protein is in its natural environment. Experiments using purified protein are easier to control for specificity, however, the conditions of the assay can effect the interactions measured. For example Roof et al. (1990) have shown that different lipids can effect the kinetics of the interactions between peptides and Ia molecules, and the different detergents used in binding assays with purified proteins may well also affect the interactions.

The ability of LCB lysine analogues to determine clearly the MHC protein contact residues within a peptide is also dependent on the assumption that the substitution influences solely the interaction between the amino acid substituted and the MHC protein. However, any structural change in the peptide (including substitutions with natural amino acids) may alter other properties, for example, the conformational propensity. Controls for such effects can be hard to perform. Analysis of the conformational propensity of different LCB lysine analogues of Ha 307-319 in solution indicated that the LCB lysine substitutions had little effect on this property of the peptide (Rothbard et al., 1989b; Busch, 1991). However, this may not have reflected possible changes in the overall conformation of the bound peptide. An alternative strategy is to compare the effects of different types of substitutions on peptide binding. For example, MHC protein contact residues within a peptide could be defined using both LCB lysine and another un-natural amino acid with distinctly different chemical and physical properties. If the residues defined are the same in both cases then this implies that both sets of substitutions have modified the possible conformations of the peptide in the same way. The simplest rationalisation of such a result is that neither substitution has had a significant effect on the overall conformation of the bound peptide. An analysis similar to this has been attempted with Ha 307-319, however, the second substitution was only with the natural amino acid lysine rather than a second unnatural amino acid. Lysine substituted peptides were assayed for their ability to compete for the binding of Ha LCB N to cell surface HLA-DR1Dw1 (Busch, 1991). In general where substitution with LCB lysine gave a low fluorescent signal, substitution with a lysine resulted in an inability to compete for binding of Ha LCB N. Two discrepancies between the two sets of data were at position 311 which is normally a Lys, and at position 312 which is normally a Gln. The correspondence between the two assays suggested that the LCB lysine was largely affecting local interactions between the peptide and the MHC protein rather than the overall conformation of the

peptide. The differences between the two assays can be explained by the normal ability of the MHC binding site to tolerate substitutions with natural amino acids.

The specificity controls in Chapter 4 established that the most likely interpretation of the variations in fluorescent signals along the length of the peptide was differences in the importance of individual residues in the formation of the peptide MHC protein complex (Figure **4.**5., Table **4.**III., and **4.**V.). The patterns of contacts between Ha 307-319, Mat 17-29, and Tub 3-14, and HLA-DR1Dw1, suggested that different peptides could bind with remarkably similar conformations to the same MHC protein (Figure **4.**14). However, the contacts made by Myo 68-80, Myo 110-121, and Lep 418-427 indicated that there were variations in the conformations that bound peptides could adopt (Figure **4.**16). Each of the peptides bound with a conformation which was consistent with at least a single turn of a helix within the length of the peptide, while other regions of the peptides showed conformational variation. These results may help to reconcile conflicting data from experiments which indicated that different peptides adopted similar conformations when bound to MHC proteins, for example the "helix swap" experiments, and experiments which were unable to find evidence for a regular conformation for peptides binding to MHC molecules (Introduction 1.8.2). Different bound peptides may share sufficient conformational features to allow the success of the "helix swap" experiments but these conformations may not require amino acids within the sequence which are characteristic of a particular regular conformation.

A particularly interesting result came from a comparison of the conformations of Ha 307-319, Mat 17-29, and Tub 3-14 bound to HLA-DR1Dw1 and the conformation of Lep 418-427 bound to HLA-DR2Dw2A. The similarities in the conformations of each of these bound peptides suggested that there may be a common conformation characteristic of immunogenic peptide MHC protein complexes. Clearly the data base

of four peptide MHC protein complexes is too small to make a strong statement but the data prompts further investigation.

The MHC binding site clearly does not require a single well defined conformation to enable a peptide to bind. However, three out of six peptides bound to HLA-DR1Dw1 adopted remarkably similar conformations and the conformational features shared by all six peptides suggest that there are a limited number of ways in which different peptides bind to a single MHC binding site. This suggests that there is not a unique well defined structural mechanism by which different peptides bind to an MHC protein but a limited number of closely related mechanisms by which different peptides bind.

6.2.3. Structural Features

The empirical studies of De Lisi and Berzofsky (1985) and Rothbard and Taylor (1988) established the existence of common structural features within T cell determinants and hence suggested that there may be a common structural mechanism by which different peptides bind to MHC proteins. However, such empirical studies failed to determine the structural significance of these features in peptide MHC protein interactions. A number of other studies have attempted to determine more precisely the residues within a peptide which contact the MHC protein using truncated and substituted peptides in both functional and direct binding assays (Introduction, 1.8.3). Unfortunately the ability of the MHC binding site to tolerate a large number of different peptides resulted in a failure to define clearly those residues which were contacting the MHC protein in different peptides. The structural significance of many of these studies in terms of peptide MHC protein interactions was therefore hard to interpret in detail. By using the unnaturally large LCB lysine as a substitution in a number of different T cell determinants allowed a clearer definition of the MHC protein contact residues within these peptides (Chapter 4). This enabled logical analysis of the structural

homologies and differences at these residue positions to determine their role in the formation of the peptide MHC protein complexes (Chapter 5).

Consistent with studies using other peptides substituted with natural amino acids, the results obtained with the LCB lysine substituted peptides indicated that the different determinants analysed made a limited number of contacts with the MHC protein. Three homologous residues at relative positions i - 1, i + 4, and i + 7 were identified in Ha 307-319, Mat 17-29, and Tub 3-14 peptides at positions contacting HLA-DR1Dw1 (Table 5.V). A large hydrophobic residue at relative position i - 1 defined an important shared structural requirement for Ha 307-319, Mat 17-29, and Tub 3-14 to bind to HLA-DR1Dw1 (Figure 5.3), indicating the functional significance of this homology and also suggesting that all three peptides were binding with a similar relative orientation and in a similar location within the MHC binding site. The homologies at the other two residues contacting the MHC protein (i + 4 and i + 7) in these three peptides suggested these positions may also be structurally important for interactions with the MHC protein. The functional significance of the homology at these positions has not been established although they may be involved in, for example, allowing the peptides to adopt a similar conformation (Chapter 5.3.1 / 2).

The structural similarities between these three peptides bound to HLA-DR1Dw1 suggests a common structural mechanism by which they bind to the MHC protein. Each peptide shares a similar structural requirement for a large hydrophobic residue at position i - 1 and in the case of Ha 307-319 this has been shown to be the only residue specifically required for a peptide to bind to HLA-DR1Dw1. However, the other residues in the peptide clearly can affect the ability of the peptides to bind. For example, even though Ha 307-319 and Mat 17-29 interact with HLA-DR1Dw1 in a similar fashion they have different affinities for HLA-DR1Dw1. Presumably certain residues in Mat 17-29 are making negative interactions with the MHC binding site, compared to the equivalent residues in Ha 307-319. To determine the structural basis

for this difference in affinity the contact residues within each peptide obviously should be compared. However, residues in the peptides other than those revealed by LCB lysine to be contacting the MHC protein have been shown to modulate the affinity of bound peptides. For example, Glu substituted for the natural Ala at position 318 of Ha 307-319 has been shown to decrease the affinity of the peptide, although several other substitutions have little effect (Busch, 1991). Interestingly the equivalent position in Mat 17-29 (residue 29) is also a Glu and this might explain part of the reason for the decreased affinity of Mat 17-29 relative to Ha 307-319. Although detailed studies have yet to be performed, alignment of the peptides based on their contacts with HLA-DR1Dw1 will allow rational systematic analysis of the differences in amino acids at equivalent positions in each peptide to determine the structural basis for the difference in affinity of the two peptides.

When the sequences of Myo 68-80 and Myo 110-121 were aligned with those of Ha 307-319, Mat 17-29, and Tub 3-14 based on similar patterns of contacts with HLA-DR1Dw1 then two positions of homology were revealed (Table 5.V). A large hydrophobic residue at position i - 1 and a small residue at position 1 + 4. The same pattern of a large hydrophobic and a small residue were also present at these relative positions when the Lep 418-427 peptide sequence was aligned with the other five peptides based on its contacts with HLA-DR2Dw2A. Although the functional significance of this homology has not been established in all these peptides, in five out of six both residues were shown to be contacting the MHC protein (Table 5.V). Further examination of a large number of T cell determinants also revealed this pattern of a large hydrophobic and a small residue in 76% of the sequences (Table 5.VI). The statistical significance of this result suggested a functional significance to the homology and one hypothesis was that the large hydrophobic residue within the determinants containing the pattern was interacting with a conserved hydrophobic interaction pocket within different MHC binding sites. Consistent with this was the identification of a potential hydrophobic pocket which is conserved in many different MHC molecules

(Figure 5.5; Chapter 5.3.5). Although this hypothesis was clearly not universal (Chapter 5.3.5) it is eminently testable and forms a model upon which to base further investigations of the interactions between these different determinants and their respective original restriction elements.

An extension of this hypothesis is that the same peptide could bind to different alleles with a similar orientation and in a similar location with the binding site, if it possesses a large hydrophobic residue and small residue and then has no additional negative interactions with other parts of the different binding sites. In Chapter 3 a number of determinants were identified within HPV type 16 which were able to form immunogenic complexes with several different MHC proteins (Table 3.VII). Examination of these sequences revealed the pattern of a large and a small amino acid in L1 40-63 (Val at 44 and Ser at 49) and twice in L1 279-294 (Leu at 286 and Gly at 291, Phe at 287 and Thr at 292) but not in L1 91-106. Therefore two of these peptides may be using this mechanism, at least in part, to bind to multiple MHC alleles, but clearly this will require more investigation.

6.3 Conclusions

The conformational and structural features defined using LCB lysine analogues of a number of different peptides are consistent with the existence of a limited number of related structural mechanisms by which diverse peptides interact with the binding site of MHC proteins. This should therefore allow a logical and rational approach to the elucidation of the details of these mechanisms. Techniques have now been established to uncover some of the structural details of these mechanisms using direct binding assays with both unnatural and natural amino acids as substitutions within different determinants. The minimal structural requirements for peptides to bind to particular MHC proteins can be defined and the effects of other residues within a peptide on both the conformation and the affinity of the peptides can be analysed. Establishing the important structural features of a peptide involved in binding to the MHC protein will

also help to identify important regions of the MHC binding site which are involved in peptide binding. These sites can then be further defined using mutations within the MHC binding site to compliment changes in the peptide and hence establish detailed models for peptide MHC protein complexes.

Establishing the details of peptide MHC interactions will be important in the rational design of peptides able to specifically modulate immune responsiveness. For example, by understanding the features of a peptide which will allow it to interact with different MHC proteins will help in the design of subunit vaccines suitable for use in the outbred human population. In contrast by understanding the structural features of a peptide important for interactions with specific MHC molecules will allow the rational design of peptides or peptidomimetics which are able to specifically bind a particular MHC protein and hence inhibit autoimmune responses restricted through it. Therefore, although the details of the mechanisms by which MHC binding sites are able to bind a diverse but limited range of peptides have not been fully elucidated, the existence of a limited number of closely related mechanisms and the means to elucidate these mechanisms has been established. The practical outcomes of these studies will have great impact on the rational design of new reagents to modulate immune responses and hence the outcome of many human diseases.

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Corrigenda

Appendix

Publications resulting from the work in this thesis

1. Hill, C.M., K.Howland, J. Hayball, A.D. Allison, R.Busch, J.B. Rothbard. (1991). Conformational and structural characteristics of peptides binding to HLA-DR molecules. J. Immunology. In Press.

2. Davies, D.H., Hill, C.M., Rothbard, J.B., and Chain, B.M. (1990). Definition of murine T helper cell determinants in the major capsid protein of human papillomavirus type 16. J. Gen. Virol. **71**: 2691-2698.

3. Rothbard, J.B., Busch, R., Hill, C.M., and Lamb, J. (1989). Structural analysis of a peptide-HLA class II complex. CSH Symp. Quant. Biol. **54**: 260-265.

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Corrigenda

2.7.3. Immunisation and harvesting lymph node cells

All immunisations were generously performed by Huw Davies of Univeresity College.