

**T cell Recognition of Endogenous and Exogenous Peptides
Presented by MHC Molecules**

by

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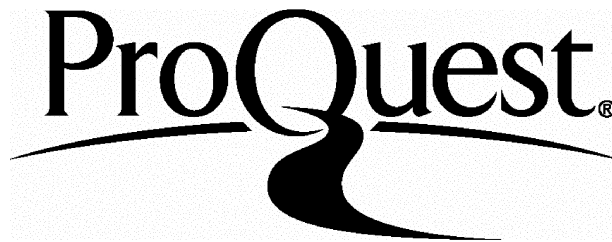
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ABSTRACT

This thesis presents data of analysing T cell responses to synthetic Ras peptides and to natural peptide epitopes derived from minor histocompatibility (mH) antigens.

Murine T helper and cytotoxic T lymphocyte (CTL) responses were induced by synthetic peptides corresponding to amino acids 1-23 of the normal or mutant p21Ras proteins containing a glycine to valine mutation at position 12. Specific T helper responses against mutant Ras peptide were observed in C3H/He, B10.BR but not C57BL/10 mice. These responses were mediated by CD4⁺ cells and restricted by MHC class II molecules. Moreover, mutant Ras peptide-specific T helper cells recognised the corresponding intact p21Ras protein. Ras peptides also induced CTL which were classical CD8⁺αβTCR⁺ T cells. None of the CTL could distinguish mutant and normal Ras peptides. Interestingly, these CTL lysed peptide pulsed murine cells of several different H-2 haplotypes, indicating that recognition was not MHC class I allele restricted. However, the real peptide epitopes recognised by these unconventional CTL were not identified since Ras peptides were not very pure and stable, and undefined minor components might be responsible for the CTL responses.

mH antigens cause rejection of MHC matched transplants. Bulk CTL responses were generated in H-2^b mice differing in one (H-1), two (H-1 and H-25) or multiple (>29) mH loci, and HPLC purification was used to analyse the complexity of CTL recognised peptides. Both anti-H-1 and anti-H-1/H-25 CTL recognised one immunodominant epitope which was encoded by the H-1 locus and presented by H-2K^b molecules. Anti-multiple mH antigen CTL recognised the same H-1 locus derived epitope and another genetically undefined epitope which was presented by H-2D^b molecules. Subfractionation of the CTL recognised HPLC fractions suggested that one mH locus encodes probably no more than one CTL epitope. Attempts were made to microsequence the CTL epitopes.

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LIST OF ABBREVIATIONS

2-ME	2-mercaptoethanol
⁵¹ Cr	⁵¹ chromium
ABC	ATP-binding cassette
APC	antigen presenting cells
β ₂ m	β ₂ microglobulin
CDR	complementarity-determining regions
CFA	complete freund adjuvant
Con-A	conconavelin A
cpm	counts per minute
CTL	cytotoxic T lymphocyte
DMSO	dimethyl sulphoxide
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GVHD	graft-versus-host disease
HLA	human leucocyte antigen
HPLC	high performance liquid chromatography
ICRF	Imperial Cancer Research Fund
Ig	immunoglobulins
i.p.	intra peritoneal
LCM	lymphocytic choriomeningitis
LMP	low molecular mass polypeptide
mAb	monoclonal antibody
MEM	modified Eagles medium
MHC	major histocompatibility complex
mH	minor histocompatibility
min	minute
MLR	mixed lymphocyte reaction
Mls	minor lymphocyte stimulating
MMTVs	mouse mammary tumour viruses
M-Ras	mutant Ras
MTF	maternally transmitted factor
Mtv	mammary tumour viruses
NP	nucleoprotein
NP40	Nonidet P40

N-Ras	normal Ras
PBS	phosphate buffered saline
SDS	sodium dodecyl sulphate
TAP	transport associated with antigen processing
TCR	T cell receptor
TFA	trifluoroacetic acid
T _H	T helper

CHAPTER 1. GENERAL INTRODUCTION

1.1. Genetics and molecular structure of the MHC

1.1.1. Discovery of the MHC.

The main function of the immune system is the discrimination of self from non-self, which leads to the destruction of non-self (e.g. pathogenic microorganisms, grafts and tumours) without causing damage to self. The first demonstration of self/non-self discrimination was that mice rejected the foreign grafts when tissues were transplanted between genetically distinct members of the same species. Later it was found that a set of closely linked genes were crucial in determining the fate of transplants. Because of its central role in tissue compatibility, this set of genes has been called the major histocompatibility complex (MHC, reviewed by Sachs, 1984).

The mouse MHC was named H-2 because it was the second antigen identified by Gorer in his early descriptions of the serology of histocompatibility antigens (Gorer, 1936). H-2 genes have been mapped to mouse chromosome 17. The human MHC was named human leukocyte antigens (HLA). These were first detected serologically by clinicians interested in blood typing who were analysing sera from multiply transfused patients (Dausset, 1958). HLA genes are located on human chromosome 6.

1.1.2. The gene organisation of the MHC.

MHC genes can be divided into three classes according to the molecules they encode, i.e., MHC class I, II and III. The genomic map of MHC is presented in Fig.1.1. In the mouse, class I genes are found in the H-2K, H-2D, H-2L, Qa, Tla and Hmt loci. H-2K, H-2D and H-2L genes encode classical transplantation antigens and are expressed by almost all nucleated cells. The Qa, Tla and Hmt loci are non-classical class I genes encoding molecules expressed in a tissue-specific manner. The function of the non-classical class I genes are not very clear. Similarly, the human class I region contains three well defined loci, called HLA-A, -B and -C which encode classical transplantation antigens. Also, some non-classical genes of

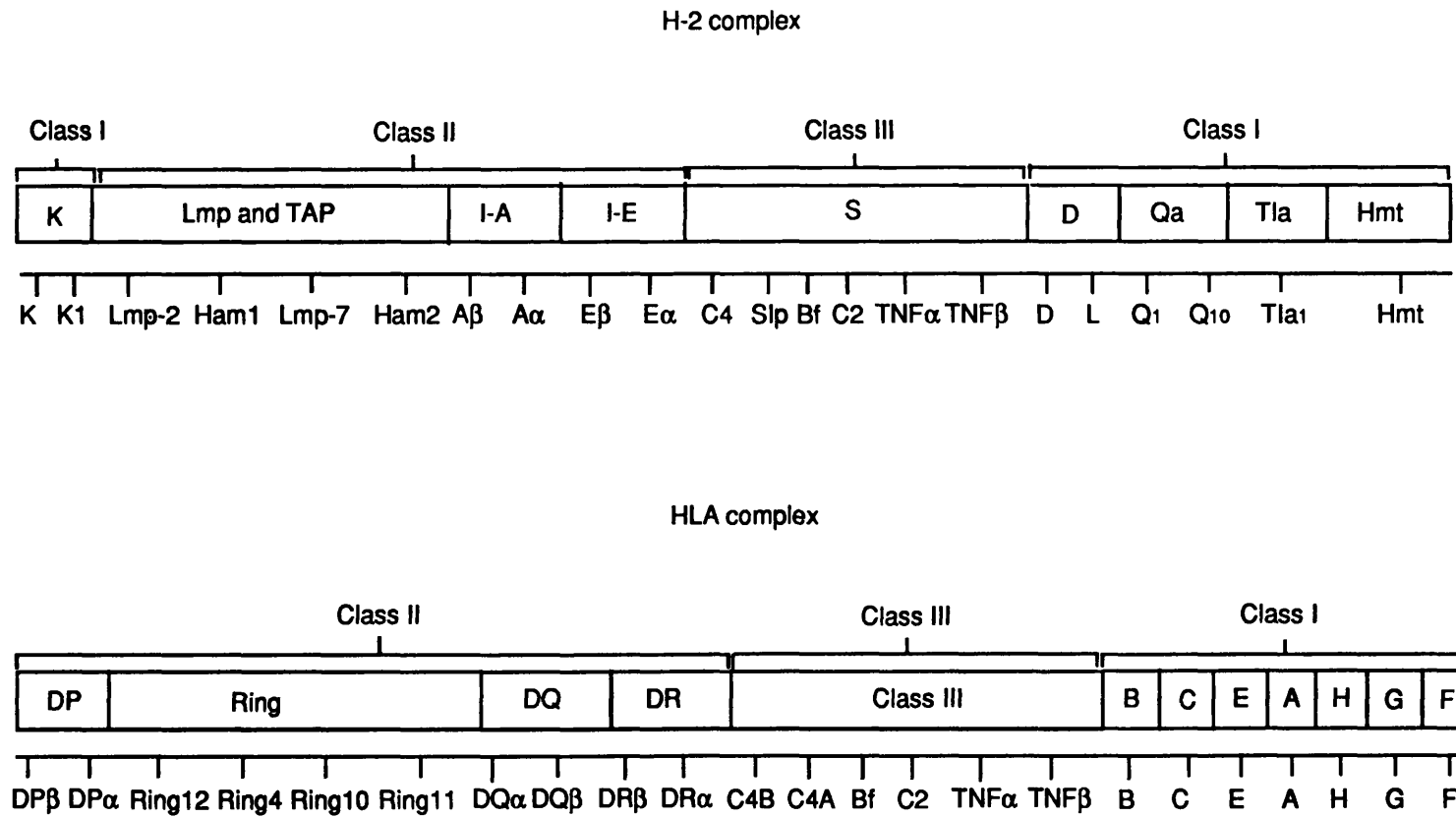


Fig. 1.1. Organization of murine and human MHC genes. The location and major loci of the class I, class II and class III regions are shown. Genes are represented by a vertical dash, and groups of genes are represented by an open box. Data are compiled from Male (Male, et al., 1991), Richards (Richards, et al., 1989) and Monaco (Monaco, 1992a).

unknown function have been mapped within the human class I region, e.g. HLA-E, -F, and -G (Male, et al., 1991; Murphy, 1992). Murine class II genes are located in the I-A and I-E regions of the MHC. Separate genes encode the α - and β -chains of murine class II molecules. There are two α -genes and six β -genes, but only four of them (A_α , E_α , A_β , E_β) appear to be expressed. Human class II genes are located in the HLA-D region which appears to be more complex than the corresponding murine region. There are six α -genes and ten β -genes forming three different gene families called DR, DQ and DP. Class II molecules are expressed predominantly on B lymphocytes, macrophages, monocytes, dendritic cells and some epithelial cells (Male, et al., 1991).

Recently the genes called low molecular mass polypeptide (LMP) complex and transport associated with antigen processing (TAP) have been mapped to the class II region. These genes are thought to participate in antigen processing and presentation (see section 1.4).

The MHC class III region encodes a heterogeneous mixture of genes including complement components, tumour necrosis factors and heat shock proteins, all of which may play important roles in immune functions (Male, et al., 1991).

1.1.3. Molecular structure of the MHC.

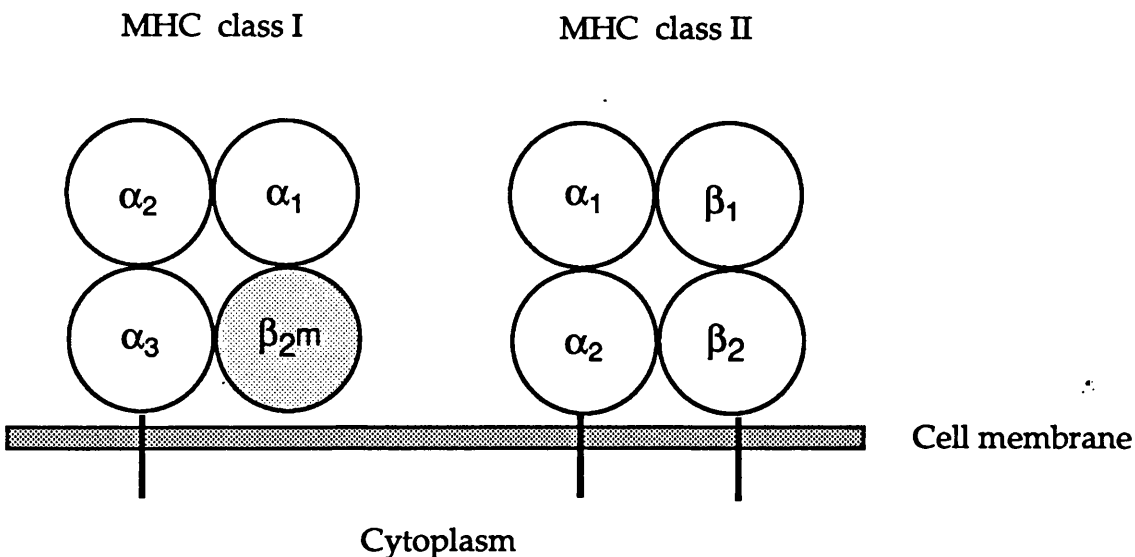


Fig.1.2. Schematic views of MHC molecules. The general features of MHC class I and class II molecules are illustrated.

A schematic representation of MHC class I and class II molecules is shown above. MHC class I molecules consist of a glycosylated polypeptide chain of 45 kD (heavy chain) and a 12 kD polypeptide, β_2 microglobulin (β_2m , light chain). The heavy chain, encoded by MHC class I genes, is divided into five regions: three extracellular domains designated α_1 , α_2 and α_3 , a hydrophobic transmembrane segment, and a hydrophilic cytoplasmic domain. The light chain, β_2m is encoded outside the MHC on human chromosome 15 and on mouse chromosome 2. Light chain and heavy chain are associated non-covalently (Male, et al., 1991).

MHC class II molecules are heterodimers comprising heavy (α) and light (β) glycoprotein chains. Each chain is divided into four major regions: two extracellular globular domains (α_1 , β_1 , α_2 , β_2), a short hydrophobic transmembrane region and a short hydrophilic cytoplasmic region. The α chain and β chain associate non-covalently (Male, et al., 1991).

The major feature of the MHC molecules is the high degree of polymorphism. Amino-acid sequence analysis shows that allelic variations are not random but occur clustered in particular hypervariable regions of the molecules. These hypervariable regions are localised in the α_1 and α_2 domains of class I molecules, α_1 and β_1 domains of class II molecules (Male, et al., 1991).

1.1.4. Crystallography of the MHC.

Crystallographic studies of human HLA-A2, HLA-Aw68, HLA-B27 and murine H-2K^b MHC class I molecules have elucidated the three-dimensional structure of the extracellular portion of class I molecules. The first crystallised molecule was HLA-A2 (Bjorkman, et al., 1987a, 1987b). It was found that the membrane-proximal α_3 and β_2m domains have tertiary structures resembling antibody domains and paired in a novel manner. The membrane-distal α_1 and α_2 domains form a platform composed of a single β -pleated sheet topped by α -helices with a long groove between the helices. The groove is about 25Å long, 10Å wide and 11Å deep, and was occupied by extra electron density which was postulated to be unknown antigenic peptides. Most of the polymorphic residues of the class I molecules are located in the bottom and sides of the groove. Substitutions of the amino acids sitting in the groove very often alter antigen-specific T cell recognition. Based on these structural features, it

was proposed that the prominent groove between the α -helices is a binding site for processed antigen (Bjorkman, et al., 1987a, 1987b).

The crystals of HLA-Aw68 showed similar structures to that of HLA-A2 (Garrett, et al., 1989). Subsites (pockets) of the putative peptide binding groove were identified. The groove contains a number of pockets which accommodate amino-acid side chains of antigenic peptides. The specific binding of a peptide is determined by a few side chains (anchor residues) of the peptide fitting into certain pockets of class I molecules. Therefore, by having various subsites, different class I alleles bind different sets of peptides. In certain circumstances, one class I allele accommodates peptides which do not have allele-specific anchor residues, possibly by using different sets of pockets (Garrett, et al., 1989).

X-ray crystallography of HLA-B27 revealed that the extra electron-dense material found in the peptide binding groove were nonameric peptides bound in an extended conformation (Madden, et al., 1991). The bound peptides shared a common main-chain structure with the N- and C-termini precisely positioned by numerous hydrogen bonds at both ends of the binding site (Madden, et al., 1992). HLA-Aw68 molecules showed the same atomic structures of the bound peptides (Silver, et al., 1992).

Recently, the structures of murine H-2K^b molecules were analysed in complex with two different viral peptides, VSV-8 [vesicular stomatitis virus nucleoprotein (52-59)] and SEV-9 [Sendai virus nucleoprotein (324-332)] (Fremont, et al., 1992; Matsumura, et al., 1992). The overall structure of H-2K^b molecule is similar to human HLA class I, although H-2K^b has slightly altered domain dispositions. The peptides bind to H-2K^b in extended conformations with most of their surface buried in the binding groove. Two deep but highly conserved pockets at each end of the peptide binding groove accommodate the N- and C-termini of peptides, whereas a deep polymorphic pocket in the middle of the groove interacts with the main anchor residue of the peptide and hence, determines allele-specific peptide binding.

Although no crystal structures of class II molecules are available yet, molecular modelling studies have been carried out, based on the comparison of conserved and polymorphic residues in class I and class II sequences. It was proposed in a hypothetical model that the α 1 and β 1 domains of class II molecules are paired in a similar fashion to the class I molecules to form an analogous peptide-binding groove (Brown, et al., 1988).

1.2. MHC restriction.

An important function of MHC molecules is presenting antigens to T lymphocytes. It has been demonstrated convincingly in many systems that T cell receptors (TCR) expressed on the T cell surface co-recognise antigens and MHC molecules. Antigens are recognised by T cells only when they are presented by MHC matched antigen presenting cells (APC). This process of dual recognition is referred to as MHC restriction.

The phenomenon of MHC restriction was revealed in the early 70's. First, Katz and colleagues demonstrated that the co-operation of T cells and B cells in mice was restricted by MHC (Katz, et al., 1973). They showed that only those T cells which had the same H-2 haplotype as B cells could provide help to B cells for antibody production. Subsequently, Rosenthal and Shevach investigated the role of MHC in T cell-macrophage interaction using cells from inbred strain 2 and 13 guinea pigs (Rosenthal and Shevach, 1973). They found that antigen-induced T cell proliferation could only be obtained when the antigens were presented by H-2 matched macrophages. The most convincing experiments demonstrating MHC restriction were done by Zinkernagel and Doherty in 1974 (Zinkernagel and Doherty, 1974). They showed that CTL generated from mice infected with live lymphocytic choriomeningitis (LCM) virus only killed LCM virus-infected target cells which shared the same H-2 haplotype as CTL. By then, it was clear that MHC restriction was a general property of all T cell antigen recognition. Later it was identified that MHC class I and class II molecules were the restriction elements for CTL and helper T cell recognition respectively (reviewed by Schwartz, 1984).

The mechanism of MHC restriction was a puzzle for long time. By analysing the recognition pattern of T cell clones, it was demonstrated that a single T cell receptor co-recognised antigen and H-2 molecules (Kappler, et al., 1981; Hunig and Bevan, 1982). The molecular basis of this recognition was revealed by the studies of antigen processing and presentation.

1.3. Antigen processing and presentation.

1.3.1. Nature of the antigen recognised by T cells.

Antibodies recognise native protein directly, while T cells tend to recognise degraded forms of antigen, i.e. antigen needs to be processed before it can be recognised by T cells. The concept of antigen processing was first proposed by Ziegler and Unanue. They found that there was a lag time between the binding of *Listeria monocytogenes* antigen to the macrophages and the detection of antigen recognition by MHC class II-restricted T cells. Fixation with paraformaldehyde before but not after the lag time rendered the macrophages incapable of presenting antigen. Therefore, it seemed that antigen presentation to T cells needed intracellular handling of the antigen molecule which was an energy-requiring and time-dependent process (Ziegler and Unanue, 1981). Moreover, this intracellular handling step was inhibited by lysosomal activity inhibitors such as ammonia and chloroquine (Ziegler and Unanue, 1982). These results suggested that antigens need to be processed intracellularly into smaller fragments in order to be recognised by T cells (Ziegler and Unanue, 1982). This hypothesis was verified with the demonstration that metabolically inactive APCs failed to present native antigen but could still present chemically or enzymatically degraded antigenic proteins to T helper cells (Shimonkevitz, et al., 1983).

The evidence that class I restricted T cells recognise processed antigen fragments mainly came from the studies of influenza virus (reviewed by Townsend and Bodmer, 1989a). First it was found that influenza-specific CTL could recognise internal viral proteins such as nucleoprotein (NP) which were not expressed on the cell surface (Townsend and Skehel, 1982). NP transfected L cells were recognised efficiently by class I restricted CTL, despite the lack of detectable NP at the cell surface using NP-specific antibody (Townsend, et al., 1984). These findings raised the question of how, and in what form the CTL epitopes were transported to the cell surface. Clearly the transport was not dependent on a signal sequence since CTL also recognised L cells transfected with segments of NP which did not contain signal-like sequences (Townsend, et al., 1985). Therefore, it was proposed that CTL recognised a degraded form, i.e. short peptides of NP at the cell surface. This hypothesis was verified by Townsend and colleagues who showed that synthetic NP peptides sensitised target cells for lysis by NP-specific CTL (Townsend, et al., 1986).

Based on these experiments, the concept that T cells usually recognise antigenic peptide fragments presented by MHC class I or class II

molecules was established. The identification of a putative peptide-binding groove in MHC molecules by crystallographic studies provided structural basis for this theory.

1.3.2. Two pathways of antigen processing and presentation.

There are two antigen processing and presentation pathways described by various names: MHC class I (cytosolic or endogenous) pathway, and MHC class II (endocytic or exogenous) pathway. Usually MHC class I molecules present peptides derived from endogenous proteins to CD8⁺ cytotoxic T cells, while MHC class II molecules present peptides derived from exogenous proteins to CD4⁺ helper T cells. Endogenous and exogenous antigens take different intracellular trafficking routes and bind to class I or class II molecules in different locations. Class I molecules bind to endogenous peptides in the endoplasmic reticulum (ER) whereas class II molecules associate with exogenous peptides in endocytic compartments (reviewed by Brodsky, 1991). The two pathways of antigen processing and presentation can be distinguished by their different sensitivities to a variety of inhibitors. The MHC class I pathway can be inhibited by two agents: 1) brefeldin A, which blocks the exit of newly synthesised molecules from the ER, was originally reported to inhibit class I restricted presentation specifically (Nuchtern, et al., 1989). However, more investigations showed that prolonged incubation of APC with brefeldin A also inhibited MHC class II restricted antigen presentation (Adorini, et al., 1990). A possible explanation for this is that brefeldin A not only affects glycoprotein transport out of the ER, but also the morphology of the trans-Golgi reticulum and endosomes, and the transport of molecules from late endosomes to lysosomes (Wood, et al., 1991; Lippincott-Schwartz, et al., 1991); 2) adenovirus E3/19K gene product, which specifically retains newly synthesised class I molecules in the ER (Cox, et al., 1990). MHC class II pathway is sensitive to two types of inhibitors: 1) lysosomotropic agents, such as chloroquine and ammonium chloride, which raise the pH of intracellular compartments (Ziegler and Unanue, 1982); 2) inhibitors of endosomal proteases (Takahashi, et al., 1989). The distinction of MHC class I and class II pathways is not absolute. There are some exceptions which will be discussed in the section below.

1.4. MHC class I antigen processing and presentation pathway.

1.4.1. Characteristics of peptides naturally bound to MHC class I molecules.

The features of naturally produced peptides presented by MHC class I molecules was revealed by two groups in 1990 (Rotzschke, et al., 1990a; Van Bleek and Nathenson, 1990). The experimental approaches that they took were similar. Rammensee's group extracted peptides from whole virally infected cells by using an acid elution method, and Nathenson's group isolated peptides from purified MHC class I molecules of virally infected cells. The low molecular weight peptides were then separated by reversed-phase high performance liquid chromatography (HPLC). The peptide fractions were analysed by antigen specific CTL in comparison with synthetic peptides. The results showed that naturally processed H-2K^d- and D^b-restricted peptides from influenza nucleoprotein were nine amino acids long and H-2K^b-presented vesicular stomatitis virus peptides were eight amino acids long (Rotzschke, et al., 1990a; Van Bleek and Nathenson, 1990).

Using the same procedure, Rammensee's group sequenced self-peptide blends eluted from MHC class I molecules H-2K^d, H-2K^b, H-2D^b and HLA-A2 and identified allele-specific peptide-binding motifs defined by the presence of conserved amino acids at certain positions (Falk, et al., 1991a). It was found that K^d-, D^b- and A2-presented peptides are nonamers, whereas K^b-presented peptides seem to be octamers. For each peptide motif, there are two anchor positions occupied by conserved residues that are present in the majority of the bound peptides, while other residues at other positions are heterogeneous.

Subsequently, Jardetzky and colleagues analysed the endogenous peptides pool bound to HLA-B27 (Jardetzky, et al., 1991). Eleven self peptides purified from HLA-B27 were sequenced. All of them were nonamers and shared a sequence motif. Most of these self peptides derived from abundant intracellular proteins.

Using a more sensitive technique, microcapillary HPLC-electrospray ionisation-tandem mass spectrometry, Hunt and colleagues characterised eight peptides bound to HLA-A2.1 molecules (Hunt, et al., 1992a). These peptides were nine residues long and shared a distinct structural motif.

Synthetic peptides had been used to map T cell epitopes by sensitising targets in CTL assays. However, features of such peptides can be very different from endogenously processed peptides (Fox, et al., 1988;

Bodmer, et al., 1989). By comparing the HPLC eluting profiles of synthetic peptides and natural processed peptides, it was found that natural influenza nucleoprotein peptides coeluted with some minor peptidic by-products present in synthetic peptide preparations which were originally described to contain the respective CTL epitope. These minor by-products were identified as nonapeptides and recognised by CTL much more efficiently than the longer peptides of the main product (Rotzschke, et al., 1990a; Falk, et al., 1991b). Moreover, in peptide binding assays, class I molecules of living cells selectively bound nonapeptides in a mixture of longer peptides even though the short peptides were a minor component (Schumacher, et al., 1991).

Summarising all these data, MHC class I molecules seem to bind preferentially to nonamer and octamer peptides which exhibit allele-specific structural motifs. The length stringency of class I bound peptides can be explained by the structural features of MHC class I molecules. The peptide binding groove of class I molecules has been found to be closed at both ends by highly conserved residues (Madden, et al., 1992; Matsumura, et al., 1992). This fixed dimension only accommodates 8-9 amino acid peptides. However, in certain circumstances, longer peptides can be bound as well. It has been reported that peptides eluted from HLA-Aw68 have heterogeneous lengths ranging from 9 to 11 amino acids (Guo, et al., 1992). Crystallographic studies revealed that these longer peptides bound to class I molecules by burying the residues at both ends in conserved pockets of the peptide binding groove and bulging out the middle of the peptides (Guo, et al., 1992).

1.4.2. Generation of endogenous peptides.

MHC class I molecules preferentially present peptides derived from endogenously synthesised proteins. The peptides are assumed to be generated in the cytosol during the normal turnover of cytoplasmic proteins. The proteases involved in the generation of these peptides are not defined yet. However, it has been suggested that proteasomes (multicatalytic proteinase complex) probably mediate the cytoplasmic proteins degradation. This proposal was based on the fact that proteasomes reside in the cytosol, possess proteolytic activity, and the interesting finding that proteasome-related genes, such as LMP genes are located in MHC class II region (Glynn, et al., 1991; Brown, et al., 1991; Martinez and

Monaco, 1991; Kelly, et al., 1991; Ortiz-Navarrete, et al., 1991). Another piece of supporting evidence is that like class I, class II and the peptide transporter molecules, the proteasome components can be up-regulated by γ -interferon (Glynne, et al., 1991; Ortiz-Navarrete, et al., 1991; Kelly, et al., 1991, Yang, et al., 1992). However, there is currently no functional evidence supporting the role of proteasomes in antigen processing for class I molecules.

Recently, two reports challenged the assumption that proteasomes function in the generation of MHC class I-binding peptides (Arnold, et al., 1992; Momburg, et al., 1992). It was found that the absence of two MHC-encoded proteasome subunit genes in mutant cell line 721.174 or T2 did not affect the stable class I molecules surface expression and normal antigen processing and presentation. These results leave several possibilities open: firstly the MHC encoded proteasomes may not play roles in antigen processing; secondly proteasomes are only one of several proteolytic enzymes participating the generation of MHC class I-binding peptides (Arnold, et al., 1992; Momburg, et al., 1992); thirdly proteasomes are only important in peptide presentation during immune stimulation.

1.4.3. Peptide transport into the ER.

Since MHC class I molecules are assembled in the ER, the peptides generated in the cytosol must be transported into the ER to load on class I molecules. It was speculated that some transporter-like proteins are responsible for pumping peptides into the ER. The search for peptide transporters for class I molecules was prompted by the isolation of a number of mutant cell lines with an antigen-processing defect. The mouse mutant cell line RMA-S was identified first. It was found that RMA-S had drastically reduced class I expression at the cell surface that could be restored by exposure to exogenous synthetic peptides. Also, RMA-S were unable to present intracellular viral antigens to CTL but could present defined epitopes in the form of synthetic peptides (Townsend, et al., 1989b). Subsequently, the human mutant cell lines, 721.134, 721.174 and its derivative T2 were found to share similar properties (Cerundolo, et al., 1990). The phenotype of these mutant cell lines suggested a deficiency in the provision of endogenously produced peptides to class I molecules. It was postulated that these cell lines have a defect in peptide transport from the cytoplasm into the ER.

The genetic defect of 721.174 and T2 cell lines was mapped to a large deletion in MHC class II region (Cerundolo, et al., 1990). It was speculated that this deletion area contains a gene(s) encoding a peptide transporter(s). Recently two genes have been isolated from MHC class II regions in different species. They were commonly named as TAP1 and TAP2 (Monaco, 1992a), corresponding to the original names Ham-1 and Ham-2 in mouse (Monaco, et al., 1990), mtp1 and mtp2 in rat (Deverson, et al., 1990), RING4, PSF1 or Y3, and RING10, PSF2 or Y1 in human (Trowsdale, et al., 1990; Spies, et al., 1990). TAP1 and TAP2 genes encode proteins homologous to members of ABC (ATP-binding cassette) superfamily of transmembrane transporter proteins which are capable of transporting a range of substrates, including peptides. The evidence implicating TAP1 and TAP2 genes in peptide transport came from gene transfer experiments. Transfection of TAP1 genes into the mutant cell line 721.134, and TAP2 into RMA-S resulted in the restoration of class I expression on the cell surface and the ability to process and present class I restricted antigens to CTL (Spies and DeMars, 1991; Powis, et al., 1991; Attaya, et al., 1992). However, no similar effect was observed in 721.174 mutant cells, which have a homozygous deletion in the class II region that includes both TAP1 and TAP2 (Spies and DeMars, 1991). It was proposed that TAP1 and TAP2 gene products associate as a heterodimer to be a functional transporter. This hypothesis was supported by two recent papers showing that the antigen processing defects of 721.174 and T2 were completely corrected by transfecting TAP1 and TAP2 genes together (Arnold, et al., 1992; Momburg, et al., 1992).

Comparing peptide elution profiles of cells expressing different forms of a rat transporter gene mtp2, Powis and colleague demonstrated that polymorphism of mtp2 determined the loading of class I molecules with distinct populations of peptides and resulted in the expression of different antigenic forms of rat class I molecules (Powis, et al., 1992). These results indicated a specific relationship between the transporter and the resulting peptides bound in the groove of class I molecules.

Using TAP1-deficient mice, Van Kaer et al provided more evidence supporting the functional role of TAP protein in peptide transportation (Van Kaer, et al., 1992). They found that the cells from TAP1-deficient mice closely resembled the phenotype of RMA-S. These cells were defective in the stable assembly and intracellular transport of class I molecules, lacked

class I cell surface expression and were unable to present cytosolic antigens to class I restricted CTL.

The report that ATP is required for class I molecules assembly but not for the translocation of peptide across the ER membrane argued against the function of the peptide transporters as peptide pump (Levy, et al., 1991). It was suggested that peptide transporters provide the ER lumen with some type of cofactor, other than the proposed peptide, needed for class I molecules assembly. To resolve the conflict, direct evidence is required to show whether the putative peptide transporters transport peptides.

1.4.4. MHC class I assembly.

After being transported into the ER, endogenous peptides assemble with newly synthesised class I molecules in the ER. Much has been learned about class I assembly from the studies of mutant RMA-S. As described above, RMA-S have low level of class I expression on the cell surface resulting from a deficient supply of intracellular peptides. It was found that the class I expression on RMA-S could be increased by either incubating with specific peptides or culturing cells at reduced temperature (19-33°C; Townsend, et al., 1989b; Ljunggren, et al., 1990). In the latter case, RMA-S produce empty class I molecules, suggesting that in certain circumstances class I heavy chains can assemble with $\beta 2m$ in the absence of peptides. However, these empty class I molecules are highly unstable at body temperature (Ljunggren, et al., 1990). Addition of specific peptides to either intact cells or detergent lysates could stabilise these empty class I molecules (Ljunggren, et al., 1990; Schumacher, et al., 1990; Townsend, et al., 1990). These results indicate that peptides play crucial roles in the formation and expression of stable class I molecules.

In vitro studies using cell lysates suggested that peptides have two co-operative functions in class I assembly, 1) to promote the assembly of class I heavy chain with $\beta 2m$ by inducing a conformational change in free class I heavy chain which may provide a high-affinity ligand for $\beta 2m$. This function seems only be performed by the peptides which have the correct size, i.e. nonamer peptides (Elliott, et al., 1991). 2) to stabilise the conformation of class I- $\beta 2m$ complexes. Both nonamer and longer peptides were shown to accomplish this function (Elliott, et al., 1991). Even though longer peptides can bind to class I- $\beta 2m$ complexes, it has been

shown that they have faster dissociation rate than optimal sized peptides (Cerundolo, et al., 1991). Therefore, only short peptides form the stable class I complexes which are expressed on cell surface.

Besides length dependency, peptide binding and stable class I assembly has structural requirements as well. First, the peptide needs to have a MHC-binding motif. It was shown that single substitutions of the main anchor residues of the peptides strongly affected the ability of the peptides to bind class I molecules (Elliott, et al., 1992; Jameson and Bevan, 1992; Shibata, et al., 1992). However, possession of good binding motifs does not guarantee that peptides will bind to MHC molecules, since some peptides bearing the correct anchor residues are not able to stabilise class I molecules (Jameson and Bevan, 1992; Deres, et al., 1992). The reason for this is that peptide induced class I assembly is an accumulated effect influenced by all facets of peptide sequence. The residues flanking the anchor positions may have positive or negative effects in the formation of stable peptide-class I complexes (Elliott, et al., 1992; Deres, et al., 1992, Jameson and Bevan, 1992). Moreover, the α amino and carboxyl groups at the N and C termini of the peptides were also shown to play an important role in both inducing the conformational change of free heavy chain and formation of a stable class I-peptide complex (Elliott, et al., 1992).

The mechanism by which peptide-MHC complexes are formed and then signalled for transit out of the ER is poorly understood. A chaperonin-like molecule, p88 was recently identified and proposed to play a role in MHC assembly (Degen and Williams, 1991). This protein could function to retain the nascent class I molecules in the ER until assembly with β 2m and peptide is completed.

What size are the peptides binding to newly synthesised class I molecules in the ER under physiological conditions? This question is not yet answered. Two possibilities exist. Firstly, a large peptide may bind to the class I molecule and then be trimmed by a protease to the optimal size (Falk, et al., 1990). Alternatively, octomer or nonamer peptides directly bind to class I molecules. These short peptides would be generated either by the specialised proteolytic machinery or would derive from long peptides which are trimmed by using peptide transporters as template (Townsend, 1992).

1.4.5. Alternative pathway of MHC class I antigen presentation.

Analysing the peptides presented by HLA-A2 class I molecules in the mutant cell line T2, two laboratories independently reported that these HLA-A2 molecules are occupied by a limited set of endogenous peptides which are derived from the signal peptide domains of normal cellular proteins (Henderson, et al., 1992, Wei and Cresswell, 1992). These signal peptides are longer than nine amino acids and can be isolated from HLA-A2 in normal cells as well. These results suggested a second pathway of antigen presentation, i.e. peptides capable of binding to class I molecules can enter the ER by the classical signal dependent mechanism, and they can be signal peptides themselves. Supporting this theory, a similar observation was made with T2 cells transfected with engineered minigenes. When an ER translocation signal sequence was coupled to the minigene encoding an antigenic influenza matrix protein-derived peptide M57-68, the transfected T2 were rendered susceptible to lysis by peptide-specific CTL; in contrast, a minigene encoding the matrix peptide alone was ineffective (Anderson, et al., 1991).

1.5. MHC class II antigen processing and presentation pathway.

1.5.1. Characteristics of MHC class II bound peptides.

Following the studies of class I peptides, the nature of class II peptides was revealed by using similar purification procedures. It was found that the two classes of peptides are very different. The extensive studies of sequencing natural processed peptides bound to class II molecules I-A^b, I-A^d, I-A^s, I-E^b and HLA-DR1 demonstrated that class II peptides have a wide range of lengths, 12 to 25 amino acids long (Rudensky, et al., 1991; Hunt, et al., 1992b; Chicz, et al., 1992; Rudensky, et al., 1992). In all these studies, allele-specific peptide sequence motifs were suggested in which the positions of anchor residues of class II peptides were not fixed, but varied among different peptides binding to the same class II allele. Truncation by a few residues at either N-terminus or C-terminus of peptides did not affect their capacity to bind MHC class II molecules and to be recognised by T cells (Hunt, et al., 1992b; Chicz, et al., 1992; Rudensky, et al., 1992).

It has been proposed that MHC class I and class II molecules have similar structures and analogous peptide-binding grooves. Why do they preferentially bind different sets of peptides? This has been interpreted on

the structural differences between the two types of MHC molecules. As discussed above, the peptide binding groove of class I molecules is closed at the both ends. In contrast, both ends of the peptide binding groove of class II molecules are open, and therefore peptides can extend in either direction of the groove from a central core region. As a result, MHC class II molecules can accommodate a set of peptides having heterogeneous size (Rudensky, et al., 1991; Rudensky, et al., 1992).

1.5.2. Intracellular trafficking of exogenous antigens.

MHC class II molecules preferentially bind to peptides derived from exogenous proteins which travel in a different pathway (endocytic pathway) from endogenous peptides. Exogenous antigenic proteins are internalised by APC via endocytosis. The endocytosed antigens go through early endosomal, late endosomal and lysosomal compartments of the endocytic pathway. These compartments are increasingly acidic (pH 6.5-4.5) and contain abundant proteases such as cathepsin B and cathepsin D, which can proteolyse antigenic proteins (Reviewed by Schwartz, 1990). It seems that antigenic peptides can be generated in early as well as late stages of the endocytic pathway. However, the proteases in the later endocytic compartments seem to be more efficient for cleavage of antigenic proteins into peptides due to the low pH (Guagliardi, et al., 1990; Harding, et al., 1991; Brodsky, 1992). It is not known exactly where in the endocytic pathway class II molecules bind exogenous peptides and probably different cell types have different binding locations (reviewed by Neefjes and Ploegh, 1992). Also, the mechanism by which MHC class II-peptide complexes are transported from the endocytic compartments to the cell surface remains as a puzzle.

There is no clear evidence to show whether exogenous peptides, like endogenous peptides, need transporters to be transported from their site of cleavage to the compartment where class II-peptide assembly happens. A peptide-binding protein 72/74 kDa (PBP72/74), a member of the heat shock protein 70 family, has been postulated to play a role in transporting processed peptides in the endocytic pathway and also in facilitating the binding of peptides to MHC class II molecules (DeNagel and Pierce, 1992).

There is evidence that class II molecules can be internalised and recycled after their expression on the cell surface (Reid and Watts, 1990;

Brodsky, 1992). The functional role of these recycled class II molecules in antigen processing and presentation is not very clear. A recent report suggested that the physiologically processed antigenic peptides bind to newly synthesised rather than recycled class II molecules whereas exogenously added peptides preferentially bind to the pre-existing cell surface class II molecules (Davidson et al., 1991).

1.5.3. Interaction of peptides and MHC class II molecules.

As is the case with class I molecules, peptides have been demonstrated to be important in determining class II MHC structure (Sadegh-Nasseri and Germain, 1991; Germain and Hendrix, 1991). Purified class II molecules show three distinct forms on electrophoresis in SDS-polyacrylamide gels: compact dimers with molecular mass about 57 kD, floppy dimers (64 kD) which are partially denatured compact dimers, and disassembled α and β chains (Dornmair, et al., 1989). It was found that interactions of peptides and purified class II molecules promoted the generation of compact dimers, suggesting that binding of peptides induced a conformational change of free class II molecules leading to a compact, stable structure (Sadegh-Nasseri and Germain, 1991). Moreover, incubating spleen cells with specific peptides increased the generation of stable class II dimers and the overall surface expression of class II molecules. Conversion of class II dimers to the compact, stable structure is a chloroquine-sensitive process that only occurs with molecules free of intact invariant chain and only involves newly synthesised class II molecules. A significant proportion of the class II molecules on spleen cell surface was found to be in the unstable state and were proposed to be functionally empty. These empty class II molecules have a shorter half-life than peptide occupied class II molecules (Germain and Hendrix, 1991). They may represent a functional pool of class II molecules that could bind antigenic peptides at the cell surface or following endocytosis and recycling. However, a recent report from Germain's laboratory argued against the unstable class II dimers detected in SDS-polyacrylamide gels being empty (Germain, BSI autumn meeting, 1992).

1.5.4. The functional role of invariant chain.

Both class I and class II MHC molecules are assembled in the ER. Why do class I but not class II MHC molecules preferentially bind to endogenous peptides in the ER? The invariant chain seems to account for this segregation. In the ER, MHC class II α and β chains are associated with each other and coassemble with a third subunit, invariant chain which is a type II transmembrane protein, i.e. the amino terminus is intracellular (Kvist, et al., 1982; Cresswell, 1992). Invariant chain mainly has two functions in antigen processing: 1) to prevent endogenous peptides from binding to class II molecules (Teyton, et al., 1990; Roche and Cresswell, 1990). The mechanism is not very clear. One possibility is that binding of invariant chain sterically hinders access of peptides to the binding groove of class II molecules. Alternatively, invariant chain binding may cause a conformational change in the class II molecules such that the peptide-binding groove is functionally impaired (Teyton, et al., 1990; Roche and Cresswell, 1990). 2) to target class II molecules to the endosomal/lysosomal compartments. It has been found that the cytoplasmic tail of invariant chain has a targeting signal which directs the class II-invariant chain complexes to be transported through Golgi apparatus into the endosomal compartment (Lotteau, et al., 1990). During this transit the invariant chain is degraded by endosomal proteases and free class II molecules can bind peptides generated in the endocytic pathway (Blum and Cresswell, 1988).

1.5.5. Presentation of endogenous antigen by class II molecules.

Many observations have suggested that MHC class II molecules do not present only exogenous peptides but also certain endogenous peptides. There are studies showing that some viral proteins produced in the cytosol, such as proteins derived from hepatitis virus (Jin, et al., 1988), measles virus (Jacobsen, et al., 1989) and influenza virus (Nuchtern, et al., 1990), can be presented by MHC class II molecules. Weiss and Bogen demonstrated that intracellular immunoglobulin light chain ($\lambda 2^{315}$) were processed possibly in the ER and then presented to T cells in association with class II molecules (Weiss and Bogen, 1991). Some self-MHC molecules were shown to be presented by class II molecules as well (Chen, et al., 1990; Benichou, et al., 1990).

The processing pathways for the presentation of endogenous antigen by MHC class II molecules are not very clear. In a recent report, Malnati and colleagues demonstrated that there are several mechanisms

accounting for the generation of CD4⁺ T cell epitopes from processing of cytosolic proteins (Malnati, et al., 1992). In their study, a cytosolic form of the H3 haemagglutinin (cytoH3) or a short cytosolic H3 peptide (miniH3) was inserted into a recombinant vaccinia virus and then used to infect TAP1-deficient mutant 721.134. They found that DR1-restricted T cells only recognised 721.134 cells expressing cytoH3 but not miniH3 and transfection of 721.134 with TAP1 cDNA restored the recognition of miniH3. Therefore, endogenous antigen can be processed by two distinct class II pathways, TAP-dependent and TAP-independent. TAP-dependent pathway is probably similar to the class I pathway using TAP to transport short cytosolic peptides. TAP-independent pathway may not involve short peptides, but rather the delivery of larger cytosolic molecules to an endosomal/lysosomal compartment for processing (Malnati, et al., 1992).

1.6. TCR recognition of peptide/MHC complexes.

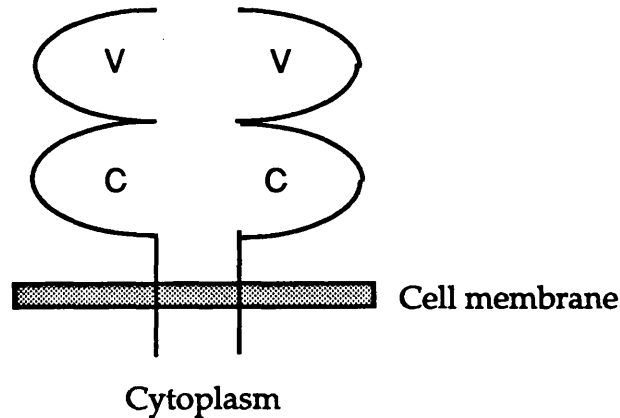
1.6.1. TCR structures.

T cells recognise antigenic peptides presented by MHC molecules via the antigen receptors expressed on the cell surface. T cell receptor (TCR) is a heterodimer made of two different polymorphic chains, either α and β chains forming $\alpha\beta$ TCR or γ and δ chains forming $\gamma\delta$ TCR. Most of the T cells express $\alpha\beta$ TCR. The $\gamma\delta$ TCR is found on a minor population of T cells, which are often associated with epithelial tissues. Both $\alpha\beta$ TCR and $\gamma\delta$ TCR are associated with CD3 polypeptide complex.

The chromosomal locations of TCR genes have been mapped both in mouse and man. Similar to the immunoglobulins (Igs), TCR loci contain V, J and C gene segments (some loci also contain D gene segment). During T cell development in the thymus, gene rearrangement occurs to generate receptor diversity which allows T cells to recognise an extensive collection of antigens (Male, et al., 1991).

As shown in Fig.1.3, a TCR polypeptide chain consists of five domains: V, Ig-like variable domain; C, Ig-like constant domain; CP, connecting peptide; TM, transmembrane domain; CYT, cytoplasmic tail. The overall structures of $\alpha\beta$ TCR and $\gamma\delta$ TCR are similar. There is no direct TCR structural data available yet. However, modelling studies were carried out based on the structural information of Igs, since TCR shares high degree of amino acid sequence identity with Igs. It has been proposed

Fig.1.3. Schematic view of TCR. The main domains of TCR molecules are shown. V, Ig-like variable region; C, Ig-like constant region. $\alpha\beta$ and $\gamma\delta$ TCRs have similar structures.



that TCR folds like Igs and the antigen-binding sites of these two molecules are comparable (Davis and Bjorkman, 1988). The antigen-binding sites of the Igs are formed by three complementarity-determining regions (CDR1, 2, and 3) present in the variable domains of heavy and light chains (Amit, et al., 1986). Corresponding CDR loops have been suggested for TCR. The putative CDR3 loops are encoded by the V(D)J junctions and are most diverse. The CDR1 and CDR2 loops are encoded within the germline sequences of the V gene segment and are less variable.

1.6.2. The mechanism of T cell recognition.

During early development in the thymus, immature T cells undergo a program of proliferation, T cell receptor (TCR) gene rearrangement and differentiation. T cell repertoire is shaped by positively selecting the T cells bearing TCR which recognise self MHC molecules and deleting the potentially autoreactive T cells bearing TCR which recognise self peptide/MHC complexes (reviewed by Blackman, et al., 1990). As a consequence, mature T cells in the periphery can only be activated by foreign antigenic peptides presented by self MHC molecules.

It is not well known how TCR interacts with a peptide/MHC complex. A model has been proposed in which CDR3 loops of $\alpha\beta$ TCR are involved in contacts with the peptide fragments lying in the MHC groove, while the flanking CDR1 and 2 loops contact the α helices of MHC which make up the walls of the peptide-binding cleft (Davis and Bjorkman, 1988). Two recent reports provided supporting evidence for this model. Using variant peptide to immunise TCR single-chain transgenic mice, Jorgensen and colleagues have mapped peptide binding sites to CDR3 equivalents of

TCR (Jorgensen, et al., 1992). In an alloreactive system, Janeway's laboratory made the similar finding that CDR3 equivalents of both α and β TCR chains were required for antigen-MHC recognition. Moreover, they mapped an MHC interaction site to the amino terminal portion of the TCR α chain containing the putative CDR1 and CDR2 regions (Hong, et al., 1992).

The number of MHC-peptide complexes required to activate T cells has been estimated both for MHC class I and class II restricted TCR recognition. Two studies showed that T cells were activated by APC that expressed about 60-300 specific peptide/class II complexes (Harding and Unanue, 1990; Demotz, et al., 1990). One report demonstrated that target cells were sensitised for lysis by effector CTL when as few as 200 specific peptide/class I complexes were present per cell (Christinck, et al., 1991).

A series of accessory molecules expressed on cell surface of T cells have been identified that participate in TCR recognition of antigen. Among them, CD8 and CD4 molecules have been shown to interact with MHC class I and class II antigens, respectively, and function as co-receptors to give efficient antigen recognition. Possible mechanisms by which CD4 and CD8 molecules modulates T cell activation are that they augment the avidity of T cell-target cell interactions and transmit intracellular signals. CD4 or CD8 function is especially important when T cell stimulation by antigen is suboptimal, i.e. when the affinity of the TCR for antigen is low or when antigen is limiting in quantity (reviewed by Bierer, et al., 1989).

The function and antigen specificity of $\gamma\delta$ T cells is not very clear. Emerging data suggests that these T cells perform different functions from $\alpha\beta$ T cells. It has been shown that $\gamma\delta$ T cells recognise antigens either in the complexes of MHC molecules or in a MHC unrestricted manner (Allison and Havran, 1991; Bluestone, et al., 1991). Non-classical MHC molecules may preferentially present peptides to $\gamma\delta$ T cells (Murphy, 1992).

1.7. Superantigens.

1.7.1. Nature of superantigens.

The term "superantigens" has been proposed by White and colleagues to describe a group of molecules that stimulate potent T cell immune responses by a novel mechanism (White, et al., 1989). So far two types of superantigens have been described: self-superantigens exemplified

by the murine minor lymphocyte stimulating (Mls) determinants and foreign superantigens such as bacterial toxins.

Originally described by Festenstein, Mls determinants stimulated a strong primary mixed lymphocyte reaction (MLR) between cells from Mls-disparate, MHC identical mice (Festenstein, 1973). The striking phenomenon was that a high frequency of naive T cells responded to Mls determinants. The molecular nature of Mls determinants remained a mystery for a long time. Recently, several groups have demonstrated that Mls determinants and Mls-like molecules are genetically tightly linked to mouse mammary tumour viruses (MMTVs), suggesting that murine self-superantigens are gene products of MMTVs (Marrack, et al., 1991; Franke, et al., 1991; Woodland, et al., 1991; Dyson, et al., 1991). Furthermore, transfection and transgenic mice studies showed that one of the self-superantigens was encoded in the open reading frame of the long terminal repeat of a MMTV (Choi, et al., 1991; Acha-Orbea, et al., 1991). A more recent report further characterised this self-superantigen biochemically. It was demonstrated that the *Mtv-7* encoded Mls determinant is synthesised as a 45 kd transmembrane glycoprotein precursor which undergoes proteolytic cleavage to yield a mature, functional form 18.5 kd surface protein (Winslow, et al., 1992).

A number of bacterial toxins have been found to share similar properties with self-superantigens, e.g. staphylococcal enterotoxins, toxic shock syndrome toxin, streptococcal pyrogenic exotoxins (Herman, et al., 1991). These foreign superantigens are well characterised and provide a easy way to examine superantigens-mediated immune responses.

1.7.2. Characteristics of superantigens.

Superantigens differ from conventional peptide antigens in several aspects. Firstly, superantigens stimulate a large number of naive T cells bearing particular V β elements. As described above, the specific interaction of TCR and peptide antigens involves all the variable elements of both TCR α and β chains (V α , J α , V β , D β , J β). There are potentially millions of possible combinations of these variable elements, and so the frequency of responding T cells to a given peptide antigen is usually very low (10^{-4} to 10^{-6}). In contrast, superantigens have been shown to interact almost exclusively with the V β elements of the TCR which have restricted numbers of variations (Kappler, et al., 1988; MacDonald, et al., 1988;

Pullen, et al., 1988). Therefore, virtually all T cells bearing these particular V β gene products can be stimulated. As a consequence, a large number of T cells (1/20-1/3) respond to particular superantigens (Herman, et al., 1991). When self-superantigens are expressed in the developing mouse, they cause a massive clonal deletion of self-reactive T cells (Kappler, et al., 1988; MacDonald, et al., 1988). Foreign superantigens also have been shown to induce V β -specific clonal deletion when they are injected neonatally (White, et al., 1989).

Secondly, the TCR interaction site for superantigens has been mapped to a β -pleated sheet region of V β which lies away from the predicted site of TCR interaction with peptide-MHC complexes (Choi, et al., 1990; Pullen, et al., 1990; Cazenave, et al., 1990).

Thirdly, superantigens differ from peptide antigens in that the TCR recognition of superantigens are not restricted by the allele of the presenting MHC class II molecules. It has been shown that presentation of superantigens requires MHC class II molecules on APC (Mollick, et al., 1989; White, et al., 1989). Unconventionally, superantigens can be presented by many different class II molecules to a single T cell clone, even though there is a hierarchy, i.e., one particular superantigen is presented by some class II alleles better than others (Kappler, et al., 1988; Mollick, et al., 1989; White, et al., 1989). Complicating the case, some V β 17⁺ T cells have been recently shown to recognise the Mtv-9 encoded superantigen, vSAG9 in a MHC class II restricted fashion (Blackman, et al., 1992). It has been postulated that superantigen recognition is generally not MHC restricted because most of the superantigens have strong interaction with either class II molecules or TCRV β elements, rendering the interaction between TCR and class II irrelevant. However, when superantigens have lower affinity for class II molecules or TCRV β elements, the interactions between TCR and class II molecules become necessary, resulting in MHC biased patterns of recognition (Blackman, et al., 1992).

Fourth, superantigens are presented by MHC class II molecules in a different manner from peptide antigens. Using a series of mutant I-A^k molecules, Dellabonna et al demonstrated that the mutations in peptide binding groove of I-A^k molecules strongly affected the presentation of a conventional peptide antigen to T cells but not of bacterial toxins (Dellabonna, et al., 1990). Thus it was proposed that superantigens interact with MHC class II molecules outside of peptide binding groove. By measuring the binding of two bacterial superantigens, staphylococcal

enterotoxins A and E to a panel of chimeric class II molecules expressed on transfected cells, Karp and Long identified a binding site of the superantigens lying on the outer face of the β chain α helix, pointing away from the peptide binding groove (Karp and Long, 1992).

Fifth, unlike conventional protein antigens, superantigens do not require intracellular processing prior to presentation by MHC class II molecules. It has been shown that metabolically inactivated APCs are still capable of presenting foreign superantigens to T cells, in contrast to their inability to present other intact protein molecules to T cells (Fleischer and Schrezenmeier, 1988; Yagi, et al., 1990; Carlsson, et al., 1988).

1.8. Minor histocompatibility antigens.

1.8.1. Definition of minor histocompatibility antigens.

The definition of mH antigens was originally based on graft rejection responses. For a successful transplantation, MHC matching is the most important. When tissues are exchanged between MHC unmatched individuals grafts are rejected rapidly (within 1-2 weeks). However, when MHC antigens are matched grafts are still rejected, but at a slower speed (weeks to months; Graff and Bailey, 1973). These non-MHC transplantation antigens were named minor histocompatibility (mH) antigens or non-MHC histocompatibility antigens. In fact the immunogenic strengths of mH antigens are variable. Some strong mH antigens can cause graft rejections almost as rapid as MHC antigens (Schultz, et al., 1976, Graff, 1978). The graft rejection responses stimulated by mH antigens can be life threatening. mH antigens appear "neither weak nor minor" and play important roles in transplantation (reviewed by Loveland and Simpson, 1986).

1.8.2. Genetics of mH antigens.

In mouse, the traditional method to identify mH loci was histogenetic analysis of congenic strains of mice. A congenic strain is created by placing a histocompatibility gene from various genetic sources onto a standard inbred-strain background by a regimen of crossing and selection (Bailey, 1975). The most well established system consists of a large set of congenic lines (the B6.C series) in which BALB/c mH genes were

transferred to C57BL/6 genetic background and selected by skin graft rejection. By identifying the differences between the congenic mice and background strain mice more than 50 mH loci have been mapped in the mouse genome (Graff and Bailey, 1973; Bailey, 1975; Lyon and Searle, 1990). The total number of mH loci is not known, but theoretical estimates based on histogenetic studies in mice suggest that there may be several hundred. These mH loci are scattered throughout the mouse genome (Loveland and Simpson, 1986).

mH loci have been further analysed by using T cell clones and lines as probes in classical backcross segregation and F1 complementation studies. The mouse H-3 mH locus was found to be comprised of at least three distinct genes: B2m and Cd-1 encode mH antigens recognised by specific CTL while Hd-1 encodes mH antigens recognised by specific T helper (T_H) cells (Roopenian and Davis, 1989). The mouse H-4 mH locus was found to contain two genes, H-46 and H-47 whose products stimulate T helper cells and CTL respectively (Davis and Roopenian, 1990). Similarly, there is evidence suggesting that the H-Y antigen recognised by T cells in association with MHC class I and class II molecules may be products of more than one gene (Scott, et al., 1991). Based on these data, Roopenian proposed that mH loci can be subdivided by the ability of their products to stimulate different T cell subsets. The traditional view that one mH locus is a single gene was revised to a model that one locus is a gene complex including T_H cell-defined genes and CTL-defined genes, together forming the functional unit detected by histogenetic analysis (Roopenian, 1992).

mH genes seem to be less polymorphic than MHC genes. Simpson et al found there was no allelism of H-Y, the mH antigen expressed by the male but not the female, in wild mouse strains (Simpson, et al., 1979). Rammensee and Klein showed that H-1, H-3 and H-4 had a low degree of polymorphism in wild mouse strains (Rammensee and Klein, 1983). Limited allelic variation for some human mH loci has also been suggested (Van Els, et al., 1992).

In man, the presence of mH antigens is indicated by the development of graft-versus-host disease (GVHD) and graft rejection after exchange tissue between HLA-matched siblings (Goulmy, 1988). However, there are no genetic mapping data for human mH genes, besides the H-Y gene being mapped to the long arm or centromeric region of the human Y chromosome (Simpson, et al., 1987a). Presently it is impossible to identify

individual mH differences in humans. To improve the survival of transplants immunosuppression is usually needed.

1.8.3. mH antigens mediated immune responses.

mH antigens are only recognised by T cells but not antibodies. Like most of the conventional antigens, the recognition of mH antigens by T cells is MHC restricted. This was first demonstrated by Bevan and Gordon early in 1975 in anti-multiple mH antigens and anti-H-Y CTL responses respectively (Bevan, 1975; Gordon, et al., 1975). The in vivo immune responses against mH antigens are reflected by acute or chronic transplant rejection, by GVHD and delayed type hypersensitivity responses. The detectable in vitro immune responses to mH antigens usually require in vivo immunisation followed by in vitro restimulation, probably because of the low frequency of anti-mH antigen T cell precursors (Loveland and Simpson, 1986). However, there have been two reports demonstrating that both murine and human anti-mH antigens CTL could be generated in vitro without prior in vivo priming. In one report, murine anti-multiple mH antigens CTL were generated by the primary mixed leukocyte culture receiving interleukin 2 on day 3 (Ando, et al., 1988). The human study showed that mH antigen-specific CTL were generated by stimulation with HLA-identical bone marrow mononuclear cells in stead of peripheral blood lymphocytes (Marijt, et al., 1991). As mentioned above, mH gene products include T_H- and CTL-determinants. Even though under certain conditions CD4⁺ T_H cells and CD8⁺ CTL can cause mH antigen-specific allograft rejection independently, usually the co-operation between the two T cell subsets is needed for an efficient anti-mH antigen response (Roopenian, 1992). The immune response to mH antigens is regulated by both MHC and non-MHC immune response genes. For example, it was found that genes inside and outside the H-2 complex control CTL responses to H-Y antigen (Fierz, et al., 1982).

When the immune system is challenged with multiple mH antigens only a small number of immunodominant mH antigens induce immune responses. Johnson and co-workers observed that H-Y-incompatible skin grafts inhibited the rejection of concurrently applied H-1-, H-34-, and H-36-incompatible skin graft (Johnson, et al., 1980). Wettstein and Bailey demonstrated that C57BL/6 anti-BALB.B CTL response is predominantly specific for two dominant mH antigens among

more than 40 mH antigens (Wettstein and Bailey, 1982; Wettstein, 1986a). The same phenomenon was also found in the BALB.B anti-C57BL/6 and B10.D2 anti-DBA/2 systems (Wettstein, 1986a). In another report Wettstein showed that when mice were immunised with H-Y antigen paired with H-4.2 or H-7.1 or H-3.1 mH antigens, no CTL were generated against H-Y antigen. CTL responses to H-Y antigen was also dominated by anti-multiple mH antigens (Wettstein, 1986b). Similarly Lai reported that CTL responses against H-25.3 mH antigen was dominated by the responses to multiple mH antigens on BALB.B cells (Lai, 1985).

1.8.4. Molecular nature of mH antigens.

The lack of antibodies against mH antigens has handicapped their structural identification. According to the features of immune responses to mH antigens, it has been postulated that they are presented as peptides by MHC molecules. Up to now, at least some of the CTL-determinants encoded by mH genes have been identified as peptides. As yet there are no similar reports regarding T_H-determinants.

Wallny and Rammensee demonstrated that natural peptides, produced by enzymatic digestion of antigenic cells and separated by reversed-phase HPLC, were recognised by CTL lines and clones raised in the minor histoincompatible mouse strain combination, C57BL/6 against BALB.B (Wallny and Rammensee, 1990). The second report from Rammensee's laboratory identified H-4 and H-Y mH antigens as naturally produced peptides derived from cellular proteins by showing that H-4 and H-Y specific CTL lines lysed the target cells pulsed with HPLC purified peptides from whole cell lysates (Rotzschke, et al., 1990b). Both reports showed that 1) the mH antigens were of peptidic nature, since they were destroyed by protease; 2) the CTL recognition of mH antigenic peptides is MHC class I restricted. Recently, using the same procedure, human mH peptides were also isolated. These peptides were recognised by a human mH antigen specific, HLA-B35 restricted CTL clone which was derived from a patient transplanted with HLA identical kidneys (Sekimata, et al., 1992).

Maternally transmitted factor (MTF) is an unusual murine mH antigen, which is maternally inherited and encoded by a mitochondrial gene. The CTL recognition of MTF is restricted by non-classical MHC class I Hmt molecules. Fischer Lindahl's group identified MTF as a hydrophobic

peptide derived from a mitochondrially encoded protein, ND1. Substitutions at a single residue created two allelic forms of MTF which can be recognised by MTF specific CTL in a Hmt restricted fashion. They suggested that mH antigens can arise by mutation in any protein, regardless of its function or cellular location (Loveland, et al., 1990). This idea was supported by a study in which allelic nuclear myxovirus-resistance protein (Mx) functioned as a mH antigen, induced class I restricted CTL responses and skin graft rejection (Speiser, et al., 1990). This illustrates that intracellular antigens exhibiting allelic differences may function as MHC class I-presented mH antigens.

$\beta 2m$ has been considered as an exceptional mH antigen which is encoded in H-3 locus. Using $\beta 2m^b$ mice to immunise $\beta 2m^a$ mice, specific CTL were induced to recognise endogenous as well as exogenous $\beta 2m^b$ proteins in the context of class I molecules (Rammensee, et al., 1986). It is unlikely that CTL recognise the amino acid difference of $\beta 2m$ directly. Possibly polymorphic $\beta 2m$ associate with MHC class I molecules in different conformations which are recognised by CTL. Another possibility was suggested by a report showing that different allelic forms of $\beta 2m$ proteins caused CTL stimulation by influencing the assortment of peptides that bind the antigen groove of the MHC molecules (Perarnau, et al., 1990). There is no data yet to show whether $\beta 2m$ incompatibility can lead to graft rejection.

It was demonstrated that retrovirus sequences were tightly linked to mH antigen genes (Rossomando and Meruelo, 1986, Wettstein and Melvold, 1986c). Either loss or gain of certain retrovirus sequences associated with the expression of new mH antigens detectable in skin transplant experiments and CTL assays (Wettstein and Melvold, 1986c, Colombo, et al, 1987). It was also reported that the DNA virus SV40 encoded T antigen could act as mH antigen in transgenic mice (Juretic and Knowles, 1989). These data suggested the possibility that at least some mH antigens are encoded by endogenous viral sequences, while other mH antigens may be encoded by cellular sequences whose expression is controlled by closely linked viral sequences (Simpson, 1987b).

1.9. Aim of the project.

Despite the rapid advances in understanding peptide presentation by MHC molecules and recognition by T cells it has remained difficult to predict the immunogenicity of protein antigens and to identify T cell

recognised epitopes. These two aspects of antigenicity and epitope identification were studied in this thesis. It was investigated whether synthetic peptides can be used to induce T cell responses against a protein of unknown immunogenicity. Specifically, synthetic peptides corresponding to sequences of normal and mutant p21 Ras protein were used to stimulate T_H and CTL responses. It was determined whether peptide induced T cells could recognise naturally processed Ras protein and whether specificity for the Ras mutation could be observed. Ras was chosen as a model antigen because of its possible use as a target for cancer immunotherapy. The feasibility of analysing peptide epitopes recognised by uncloned T cell populations generated against unknown proteins was also investigated. For these studies T cells were generated against genetically defined mH antigens and biochemical peptide purification including HPLC was used to determine the complexity of target peptide epitopes. Further refinement of this procedure may allow microsequencing of purified T cell epitopes.

CHAPTER 2. GENERAL MATERIALS AND METHODS.

2.1. Animals.

Mice:

B6.C-H-1^b, B6.C-H-25^c and C57BL/6BY were gifts from E. Simpson and supplied by the Clinical Research Centre, Northwick Park, Harrow; C3H/He, B10.BR, BALB/c, C57BL and C57BL/10 were from ICRF breeding colony. BALB.B, B10.A(4R) and B10.A(5R) were supplied by Harlan Olac Ltd., Shaw's Farm, Blackthorn, Becester, Oxon OX6 OTP. Mice were used at between 6 and 12 weeks old.

Rats:

Sprague Dawley outbred rats from University College London breeding colony were used at between 2 and 3 months old.

2.2. Reagents.

2.2.1. Media and sera.

RPMI 1640 (with L-glutamine and HEPES) was supplied by the ICRF Media Department. Dulbecco's modified MEM was from Gibco. Foetal calf serum (FCS) was from Gibco. Mouse serum was prepared by clotting blood of normal mice and then dialysing the serum against PBSA in the presence of 5×10^{-5} M 2-mercaptoethanol (2-ME, Sigma) for 48 hours. Complete T cell growth medium was made of Iscove's modification of Dulbecco's medium (Gibco) supplemented with 10% FCS, 2×10^{-5} M 2-ME, 100 U/ml penicillin (Gibco), 100 U/ml streptomycin (Gibco), 1mM sodium pyrovalate (Gibco), 5 μ g/ml transferrin (Sigma).

2.2.2. Monoclonal antibodies (mAb).

Purified mAb GK1.5 (anti-CD4) and YTS169V (anti-CD8) were gifts from R. Zamoyska, MRC, Mill Hill, London. Purified mAb TIB120 (anti-I-

A^b) and H40-481.3 (anti-I-A^k) were gifts from B. Chain, University College London.

Anti-mouse CD8 FITC (fluorescein isothiocyanate), anti-mouse CD4 PE, goat anti-mouse Ig-FITC and goat anti-rat Ig-FITC were purchased from Becton Dickinson. Anti-mouse CD8-biotin was purchased from PharMingen.

Anti-TCR mAb were used as cell culture supernatant, gifts from E. Simpson. KT11 (anti-TCRV β 11), KT50 (anti-TCRV α 8), KJ16 (anti-TCRV β 8.1.2) and 44-22.1 (anti-TCRV β 6) were rat Ig; F23.1 (anti-TCRV β 8.1.2.3), F23.2 (anti-TCRV β 8.2) and KJ23 (anti-TCRV β 17) were mouse Ig.

2.3. Cell culture and assays.

2.3.1. Cell lines and their maintenance.

RMA-S was derived from the Rauscher virus-induced H-2^b lymphoma RBL-5 by exposure to the mutagen ethyl methane sulphonate and repeated rounds of treatment with antisera against class I molecules and complement. RMA was derived from RBL-5 treated with mutagen ethyl methane sulphonate but not selected with antibodies (Ljunggren and Karre, 1985; Karre, et al., 1986). BW5147 is AKR derived thymoma (ATCC). P1HTR is a subline of P815 mastocytoma of DBA/2 origin (Van Pel, et al., 1985). YAC is an A/Sn mouse derived lymphoma, A-H-2⁻ is negative variant of YAC, they were gifts from K. Karre, Karolinska Institute, Stockholm. A-20 is BALB/c derived B cell lymphoma (ATCC). T2 is a human lymphoid cell line with a deletion in the MHC class II region resulting in loss of TAP-1 and TAP-2 transporter genes (Spies and DeMars, 1991). C1R is a human B lymphoma (Storkus, et al., 1987). Y-3 hybridoma, T2 transfected with H-2K^b and D^b, C1R transfected with K^b were generous gifts from A. Townsend, John Radcliffe Hospital, Oxford. C8166, a human T lymphoblastoid line and WMPT, an EBV-transformed B lymphoblastoid line were gifts from P.L.C. Beverley.

All the cell lines were kept in T-25 flask (Falcon) in RPMI 1640 medium plus 7% FCS (inactivated complement at 56°C in water bath for 30 min). When not required for experiments, cell lines were cryopreserved in medium with 20% FCS and 10% dimethylsulphoxide (DMSO, Aldrich).

2.3.2. Preparation of Con-A-stimulated rat spleen cell culture supernatant.

Spleens of normal rats were removed and teased into single cells which were pelleted in 50 ml tubes (Becton Dickenson) by centrifugation at 250g for 10 min and then washed once with MEM. Washed cells were counted and resuspended at concentration of 5×10^6 nucleated cells/ml in T-175 flask (Falcon) in complete T cell growth medium containing $3\mu\text{g/ml}$ Conconavelin A (Con-A, Sigma). After culture at 37°C , 5% CO_2 for 48 hours, cell suspension was centrifuged at 822g for 20 min, supernatant was harvested, filtered with $0.45\mu\text{M}$ filter (Nalgene) and stored in -20°C freezer before used for cell culture.

2.3.3. Lysis of red blood cells of spleens.

Solution: GEYS is made up with 20% solution A, 5% solution B, 5% solution C and 70% dH_2O .

Solution A

NH_4Cl	35g
Na_2HPO_4	0.56g
KCl	1.85g
KH_2PO_4	0.12g
Glucose	5g
dH_2O	1000ml

Solution B

$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	2.1g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.7g
or MgSO_4	0.35g
CaCl_2	1.7g
dH_2O	500ml

Solution C

NaHCO_3	11.25g
dH_2O	500 ml

Single cell suspension was prepared by teasing spleens through a mesh. Cells were pelleted in an universal (Sterilin) by centrifugation at 250g for 5 min. Red blood cells were lysed by resuspending cell pellet in 5 ml GEYS. One minute later 15 ml MEM were added to dilute GEYS followed by spinning at 250g for 5 min. Supernatant was discarded, cells were resuspended in 20ml MEM and spun again. This procedure was repeated twice. Finally the cells were resuspended in the medium required by experiment.

2.3.4. Preparation of Con-A blasts as CTL targets.

Spleens of normal mice were removed and teased into single cells. Red blood cells were lysed as described above. Viable cells were counted and then resuspended at concentration of 5×10^6 /ml in complete T cell growth medium containing 3 μ g/ml Con-A. 10 ml culture was set up in T-25 flask at 37°C, 5% CO₂. Three days later, viable cells were counted, harvested by spinning at 250g for 5 min and used for ⁵¹chromium (⁵¹Cr) labelling.

2.3.5. Cloning by limiting dilution.

T cell clones were established from either 5 days bulk culture or some short term cell lines by limiting dilution of T cells. Viable T cells were plated out at 100 cells/well, 50 cells/well, 10 cells/well and 1 cell/well with 48 replicate wells in 96 flat-bottomed plates (Falcon). Cloning was carried out in total volume of 200 μ l /well containing 10^6 irradiated syngeneic feeder cells (Co⁶⁰ 3000R or X-ray 2000R), 10^3 irradiated stimulator cells for anti-mH antigen CTL or 2 μ g/ml Ras peptides for Ras peptide-specific T cells and 15% rat Con-A supernatant. After 7-10 days culture at 37°C, 5% CO₂, positive clones were selected from the T cell dilution group which had <30% positive wells. They were transferred into 24 well plates (Falcon) and expanded by weekly restimulation.

2.3.6. Cytotoxicity assays.

Effector T cells were harvested either on day 5 of bulk culture or on day 6 after restimulation of CTL lines or clones. Different number of T cells were added into 96 U-bottomed plates (Falcon) according to the E:T

ratio. $1-2 \times 10^6$ target cells, Con-A induced lymphoblasts or tumour cell lines, were resuspended in 50 μ l RPMI+5% FCS and labelled with 10 μ l ^{51}Cr (1mCi/ml, Amersham, England) in a 96 U-bottomed plate for 1 hour at 37°C, 5% CO_2 . Then the labelled cells were washed 4 times, resuspended in culture medium and added at 5×10^3 per well in 96-well microtiter plates containing effector CTL. The plates were centrifuged for 3 min at 247g, incubated at 37°C, 5% CO_2 . After 4 hours culture, 100 μ l out of 200 μ l medium per well were harvested and radioactivity was counted in a gamma counter (LKB). The specific killing was calculated by the equation: (experimental release-spontaneous release/maximum release-spontaneous release) \times 100%. Spontaneous release was usually <20% of maximal (1% SDS) release for Con-A blasts, and <10% for tumour cell lines.

2.3.7. Immunofluorescence staining and FACS analysis.

Staining buffer

Phosphate-buffered saline (PBS) A

0.1% FCS

Three staining methods were used.

1) Direct immunofluorescence staining: Cells were distributed into 96 U-bottomed plate at $1-2 \times 10^5$ cells/well and incubated with FITC or PE conjugated antibodies in a volume of 50 μ l per well on ice for 30 min. Control samples were incubated with staining buffer only. Cells were washed 4 times with staining buffer by spinning the plate for 3 min at 247g, and finally were resuspended in 200 μ l staining buffer and analysed with FACScan flow cytometer (Becton Dickinson).

2) Indirect immunofluorescence staining: For unconjugated antibodies, cells were incubated with a saturating concentration of mAb for 30 min on ice in a 96 U-bottomed plate, followed by washing three times with staining buffer. Then FITC goat anti-mouse or goat anti-rat antibodies (Jackson Immuno Research) were added as second layer. The cells were incubated for 30 min on ice, washed three times with staining buffer, and resuspended in 200 μ l staining buffer and analysed with FACScan flow cytometer.

3) Triple staining for TCR expression: T cells were added in 96 U-bottomed plate at 10^6 /well, followed with 5 incubation steps: 1) primary mAb 100 μ l/well for 30 min, wash three times; 2) goat anti-rat or goat anti-mouse FITC 50 μ l/well for 30 min, wash three times; 3) normal rat serum (Dakopatts, Glostrup, Denmark) 1/20 in staining buffer for 10 min, spin down and discard the supernatant; 4) mix of anti-CD4-PE (Becton Dickinson) and anti-CD8-biotin (PharMingen) 100 μ l/well for 30 min, wash three times; 5) Streptavidin-cychrome (PharMingen) 50 μ l/well for 30 min, wash three times. Cells were resuspended in 200 μ l staining buffer and analysed with FACScan flow cytometer. Results are expressed as mean fluorescence intensity.

2.3.8. HPLC separation of peptides.

Peptides purification was carried on a reverse-phase HPLC column (SuperPac Pep-S, particle size 5 μ m, pore size 100 Å, Pharmacia LKB). The HPLC column was calibrated by running solution A (0.1% trifluoroacetic acid (TFA) in H₂O) at 1 ml/min for 30 min before peptide samples were loaded on the column. Elution was performed on a Pharmacia LKB system: HPLC-pump model 2248; low pressure mixer model 2248; variable wavelength monitor model 2141; fraction collector model Frac 100. Eluents: solution A, 0.1% TFA in H₂O; solution B, acetonitrile with 0.1% TFA. 0-5 min, 100% solution A; 5-40 min, linear increase to 60% solution B; 40-45 min, 60% solution B; 45-50 min, linear decrease to 0% solution B. flow rate, 1ml/min; fraction size, 1 ml. Fifty individual fractions were collected in eppendorf tubes.

CHAPTER 3. T HELPER RESPONSES AGAINST SYNTHETIC RAS PEPTIDES.

3.1. SUMMARY.

Point mutations of p21Ras proteins correlate with many human malignancies. To determine whether the mutations of Ras proteins generate immunogenic determinants which can be recognised by T cells and possibly serve as targets for immunotherapy, we studied the murine T_H responses against synthetic Ras peptides corresponding to amino acids 1-23 of normal or mutant Ras proteins. When C3H/He and B10.BR mice were used for immunisation, class II MHC restricted T_H responses were elicited specifically against mutant Ras peptide containing valine mutation at position 12. In contrast, C57BL/10 mice generated T_H responses against both mutant Ras and normal Ras peptides and the in vitro responses were not exclusively specific for the immunising peptide. The corresponding intact mutant Ras proteins were recognised by mutant Ras peptide-specific T cells.

3.2. INTRODUCTION.

A major challenge for tumour immunologists is the identification of tumour specific antigens. It has been found that structural mutations in oncogenes are associated with the formation of a variety of animal and human malignancies. These mutated oncogenes encode mutant proteins with transforming activities. Consequently, tumour cells express mutant proteins which are not present in normal cells (reviewed by Urban and Schreiber, 1992). p21 Ras proteins are a group of the well defined cellular proteins which are encoded by the Ras proto-oncogenes and play an important role in signal transduction across the cell membrane and in the cell cycle. Mutant Ras proteins acquire transforming properties by single amino acid substitutions, usually at residues 12 or 13 or 61. One of the most frequent activating Ras mutations is changing residue 12 from glycine of the normal Ras protein to valine (Barbacid, 1987; Urban and Schreiber, 1992). Ras oncogenes are necessary not only for initiation, but also for maintenance of the transformed phenotype. Therefore, mutant Ras proteins are tumour-specific markers. The activating mutations of p21 Ras have been detected in a substantial proportion of many types of human cancers and the incidence can reach 90% in some types of human neoplasm (Barbacid, 1987; Bos, 1989).

An important question is whether tumour-specific proteins such as mutant Ras proteins can induce immune responses and be recognised specifically by the immune system. Tumour growth may indicate the failure of immune surveillance. However, that does not rule out the possibility of mutant Ras proteins acting as tumour-specific antigens. It is possible that mutant Ras proteins mediated immune responses in vivo are not efficient to destroy the cancer but can be amplified artificially, e.g. by immunisation with synthetic T cell determinants. In that case, mutant Ras proteins would be excellent targets for cancer immunotherapy, as they are widely expressed by various tumours and the mutations are so limited in number that the same exact mutation is shared by many tumours (Urban and Schreiber, 1992).

Animal studies show that T cells play a relatively larger role in tumour rejection than B cells. Furthermore, Ras proteins are inner membrane-bound proteins and therefore are not accessible to antibody-mediated immunity in living cells (Urban and Schreiber, 1992). T cells usually recognise intact protein derived peptide fragments associated with

MHC molecules (Townsend and Bodmer, 1989a; Brodsky, 1991). Intracellular or extracellular proteins are processed by APC into peptide fragments, bind to MHC molecules and are presented on the cell surface, where the peptide/MHC complexes are recognised by antigen receptors of T cells. The recognition of antigenic peptides by TCR is exquisitely specific that one amino acid difference in antigenic peptides can be discriminated by TCR (Lurquin, et al., 1989; Loveland, et al., 1990). Therefore, potentially host T cells can detect the activating amino acid substitutions within p21 Ras proteins if these mutations are presented by MHC molecules. Host T cells should not be tolerant to such mutant Ras peptides, since the mutations arise somatically after thymic education has occurred.

To test the possibility whether tumour-specific mutant Ras proteins can serve as antigenic determinants which might be used to direct immunotherapy, synthetic Ras peptides corresponding to amino acids 1-23 of normal or mutant Ras proteins were used to immunise mice in vivo and T_H responses were analysed in vitro.

3.3. MATERIALS AND METHODS.

3.3.1. Synthetic Ras peptides and recombinant proteins.

Ras peptides were synthesised using conventional solid phase Fmoc chemistry by the peptide laboratory of ICRF. The mutant and normal Ras peptides have the amino acid sequences:

MTEYKLVVVGAVGVGKSALTIQL,

MTEYKLVVVGAGGVGKSALTIQL,

respectively. As the peptides were highly hydrophobic, they were only partially dissolved in 100% DMSO (Aldrich). For *in vivo* immunisation, peptides dissolved in PBS were used. For *in vitro* restimulation, peptides dissolved in 100% DMSO were used.

Normal and mutant human Kirsten Ras proteins were provided by the Wellcome Research Laboratories. Both proteins were produced as recombinant proteins in *E.coli*. Mutant Ras protein had valine at codon 12 instead of glycine of normal Ras protein.

3.3.2. Immunisation protocols.

Ras peptides were dissolved in PBS and mixed with complete Freund's adjuvant (Difco) in 1:1 (v/v) ratio. The mixture was emulsified by sonication (Branson Sonifier). Mice were immunised by injection in two hind footpads and tail base with 200 µg Ras peptides emulsified with adjuvant. Control mice were immunised with adjuvant and PBS. Two weeks later, the mice were either sacrificed or boosted by injection in hind footpads and tail base with same amount of Ras peptides as the first immunisation. One week after boosting, popliteal and inguinal lymph nodes were removed from 2-5 immunised mice and pooled.

3.3.3. Generation of helper T cell lines and clones against Ras peptides.

Single cell suspensions were prepared from lymph nodes of immunised mice in complete T cell growth medium and cultured in 24 well plates at 10^7 cells per well in the presence of 2 µg/ml Ras peptide. One week later, T cells were harvested and cloned by limiting dilution as described in general materials and methods. The T cell lines and clones were restimulated weekly in 2ml cultures in 24 well plates containing

5×10^5 T cells, 3×10^6 irradiated syngeneic feeder cells, 10% rat Con-A supernatant and 2 $\mu\text{g}/\text{ml}$ Ras peptide.

3.3.4. Proliferation assays.

The lymph node cells from immunised mice were resuspended in complete T cell growth medium replacing the 10% FCS with 1% mouse serum, and cultured in 96-U-bottomed plates at 4×10^5 cells per well in a total volume of 200 μl in the presence or absence of Ras peptide or protein. For measuring proliferative responses of helper T cell clones, cloned T cells were cultured at 10^5 per well with 3×10^5 irradiated syngeneic feeder cells in the presence or absence of Ras peptide. After 3 days incubation at 37°C , 5% CO_2 , the plates were pulsed with 1 μCi [^3H]thymidine (Amersham) per well. On day 4, the cells were harvested onto glassfibre paper and the thymidine incorporation was measured by liquid scintillation counting. Results are given as the mean of triplicate determinations.

3.4. RESULTS.

3.4.1. Ras mutation was recognised specifically by helper T cells when the Ras peptide was associated with H-2^k but not H-2^b molecules.

C3H/He (H-2^k) mice were immunised subcutaneously with synthetic Ras peptides corresponding to amino acids 1-23 of normal p21 Ras protein or mutant Ras changing glycine to valine at position 12. The primed T cells from draining lymph nodes were restimulated *in vitro* with different concentrations of Ras peptides and proliferative responses were measured. Immunisation with mutant Ras but not normal Ras peptides stimulated T_H responses. T cells of C3H/He mice immunised with mutant Ras peptide specifically recognised mutant Ras but not normal Ras peptides (Fig. 3.1A).

As a comparison, the T_H responses to Ras peptides were analysed in C57BL/10 (H-2^b) mice as well. These mice showed a different response pattern to the Ras peptides. Immunisation with either mutant or normal Ras peptides induced T_H responses. Responses induced by mutant Ras peptide were consistently stronger than responses induced by normal Ras peptide. The T_H responses induced by mutant Ras peptide were not exclusively specific for the immunising peptide, but showed cross recognition of normal Ras peptide (Fig. 3.1B).

The different response patterns of C3H/He and C57BL/10 mice might be caused by the differences in either MHC genes or non-MHC genes. To address this question B10.BR mice, which have the same H-2^k haplotype as C3H/He and the same non-MHC genes as C57BL/10, were used for immunisation. It was found that B10.BR mice showed the same response pattern as C3H/He mice (Fig. 3.1C). Therefore, the specificity of T_H responses against Ras peptides was determined by H-2 genes and not by non-H-2 background genes.

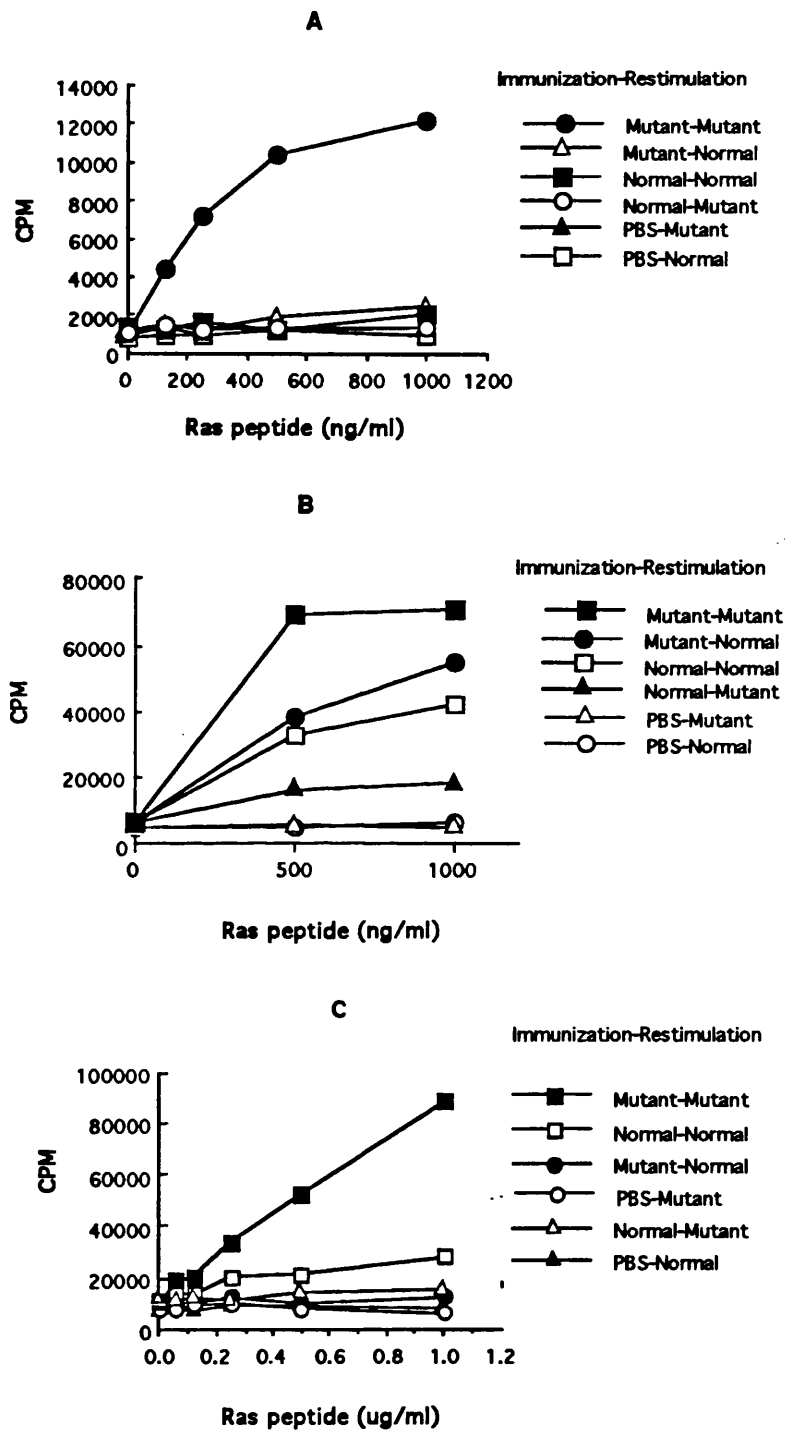


Fig. 3.1. T helper responses of C3H/He, C57BL/10, B10.BR mice against mutant and normal Ras peptides. C3H/He (Fig.A) or C57BL/10 (Fig.B) or B10.BR (Fig.C) mice were immunised subcutaneously with 200 μ g mutant or normal Ras peptides in complete Freund's adjuvant. Control mice were immunised with adjuvant and PBS. After 2 weeks, the draining lymph nodes cells were restimulated *in vitro* with the indicated concentrations of Ras peptides. The proliferative responses were measured by the incorporation of [3 H]thymidine. The data are the mean of triplicates.

3.4.2. Ras peptide-specific T_H cells were CD4⁺ and MHC class II restricted.

The phenotype of the Ras peptide-specific T cells was analysed by conventional antibody blocking assays. It was found that monoclonal antibodies directed against CD4 or MHC class II molecules blocked the peptide specific proliferative responses in either H-2^k mice or H-2^b mice (Fig. 3.2A and B). Furthermore, Ras peptide-specific T cells only recognised Ras peptide presented by syngeneic, but not class II-disparate APC. Fig. 3.3 showed that a representative Ras-specific CD4⁺ CD8⁻ clone from C57BL/10 mice responded to Ras peptide presented by C57BL/10 APC, but not DBA/2 (H-2^d) APC or C3H/He APC. Together these data demonstrated that Ras peptide-specific T_H responses were mediated by CD4⁺ T cells and restricted by MHC class II molecules.

3.4.3. Mutant Ras peptide-specific T_H cells recognised the corresponding intact Ras protein.

Although mutant Ras peptide can be recognised specifically by T cells from C3H/He mice, there is no guarantee that the corresponding Ras protein harbouring the mutation will be recognised. To be recognised by the peptide-specific T cells, intact Ras protein needs to be processed in a way that epitopes similar to the synthetic Ras peptides are generated and presented by MHC molecules. It was important to know whether such T cell epitopes would be generated during natural antigen processing of intact Ras protein. Thus, mutant Ras peptide primed T cells were stimulated by APC pulsed with normal or mutant Ras proteins. Fig. 3.4 shows that T cells from C3H/He mice immunised with mutant Ras peptide recognised mutant Ras protein containing the same mutation at position 12 as the immunising peptide, but not normal Ras protein.

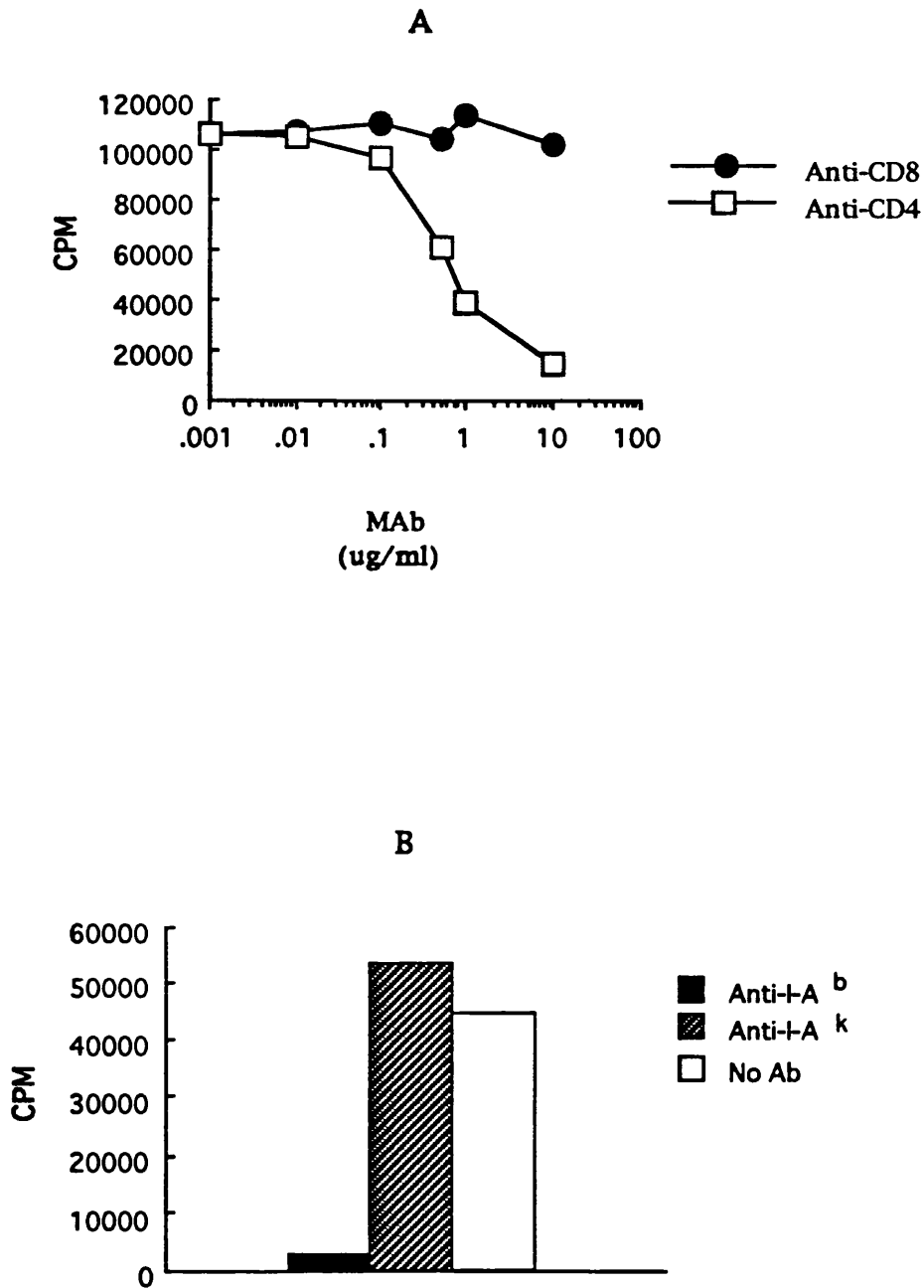


Fig. 3.2. Phenotype of Ras peptide-specific T_H cells. B10.BR (Fig.A) or C57BL/10 (Fig.B) mice were immunised subcutaneously with 200 µg mutant Ras peptide. The draining lymph node cells were restimulated with 1 µg/ml mutant Ras peptide *in vitro* in the absence or in the presence of the indicated monoclonal antibodies (mAb). The concentrations of anti-CD4 (GK1.5) or anti-CD8 (YTS169V) mAb varied as indicated (Fig.A). Anti-I-A^b mAb (TIB120) or anti-I-A^k mAb (H40-481.3) were used at 1 µg/ml. [³H]thymidine incorporation was measured on day 4.

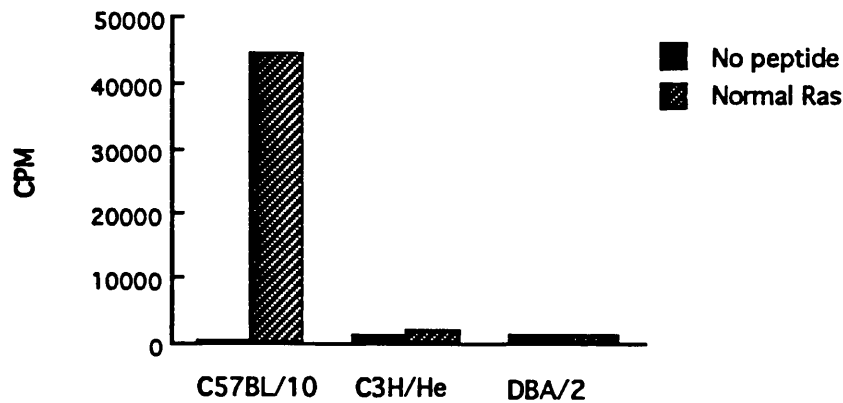


Fig.3.3. The presentation of Ras peptide to a CD4⁺ clone by different MHC class II molecules. A CD4⁺ CD8⁻ T cell clone was established from C57BL/10 mice immunised with normal Ras peptide. Cloned T cells were cultured with medium alone or 1 µg/ml normal Ras peptide in the presence of irradiated C57BL/10 or C3H/He or DBA/2 spleen cells. [³H] Thymidine incorporation was measured on day 4.

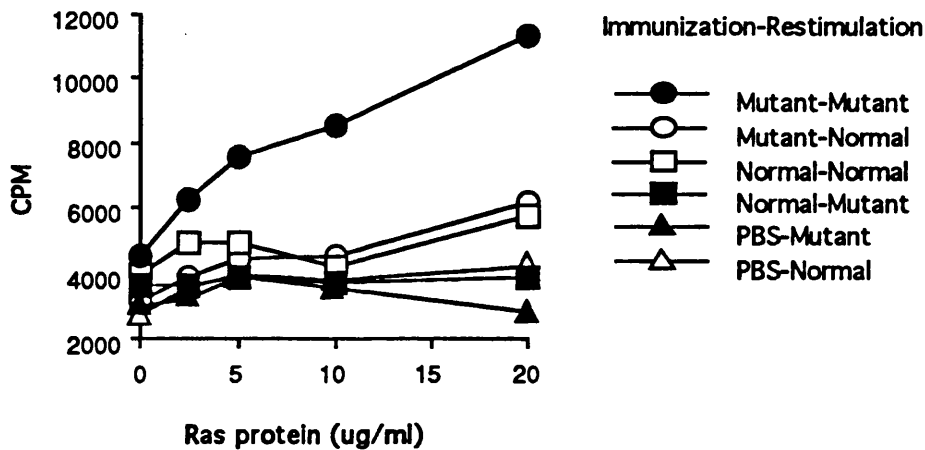


Fig.3.4. The recognition of intact p21Ras protein by Ras peptide-specific T_H cells. C3H/He mice were immunized subcutaneously with 200 µg mutant or normal Ras peptide or PBS. The draining lymph node cells were restimulated with either normal or mutant intact p21 Ras protein at the indicated concentrations. [³H]thymidine incorporation was measured on day 4.

3.5. DISCUSSION.

The valine-for-glycine substitution at position 12 is one of the most commonly found p21Ras mutations in human tumours. This study demonstrated that T_H responses specific to this oncogenic mutation of Ras were elicited in C3H/He and B10.BR mice (H-2^k) but not C57BL/10 mice (H-2^b) by immunisation with corresponding synthetic mutant Ras peptide. Processing of exogenous mutant Ras protein by APC produced the epitope recognised by mutant Ras peptide-specific CD4⁺ T cells. These results indicate that under some circumstances mutant Ras proteins can produce immunogenic determinants stimulating mutation-specific T_H responses.

Similar studies were reported recently by several groups. Jung and Schluesener established T cell lines from normal human peripheral blood lymphocytes by *in vitro* stimulation with a synthetic peptide, representing amino acids 5-16 of mutant Ras protein which has a valine mutation at position 12. These T cell lines recognised mutant but not normal Ras peptide in a MHC class II restricted fashion (Jung and Schluesener, 1991). Using a similar approach, another group raised several CD4⁺ T cell clones from a healthy donor with specificity for Ras peptides carrying different amino acid substitutions at residues 12 or 13 (Gedde-Dahl, et al., 1992a). Moreover, CD4⁺ memory T cells from a patient with a follicular thyroid carcinoma were found to recognise specifically a synthetic Ras peptide containing mutation at position 61 (Gedde-Dahl, et al., 1992b). In a murine system, Peace and colleagues immunised C57BL/6 or BALB/c mice with synthetic Ras peptides containing various transforming substitutions of Ras proteins either at residue 12 or 61. They found that some (including valine at residue 12), but not all, of transforming substitutions of Ras proteins could induce mutation-specific T_H responses in individual strains of mice. Also the peptide-specific T cells recognised corresponding mutant Ras protein (Peace, et al., 1991). All these data suggest that tumour-specific Ras mutations may be used to direct immunotherapy.

C57BL/10 mice seem not to be tolerant to the normal Ras peptide used in this study, since immunisation with normal Ras peptide stimulated T_H responses. According to the thymic negative selection theory, T cells bearing TCR which recognise self peptide/MHC complexes should be clonally deleted in the thymus (Blackman, et al., 1990). There are

several explanations of the intolerance of C57BL/10 mice to the synthetic normal Ras peptide. One possibility is that normally this peptide is not presented, or presented at a level too low to cause clonal deletion in C57BL/10 mice. Another possibility is that the length of the peptide used in this study was different from the physiologically produced peptide. It has been found that natural peptides bound to MHC class II molecules were heterogeneous in size, ranging from 13 to 25 amino acids (Rudensky, et al., 1991; Hunt, et al., 1992b; Chicz, et al., 1992; Rudensky, et al., 1992). We do not know the exact peptide length of naturally processed Ras peptides under physiological conditions. Possibly the 23 mer Ras peptide that we used is similar to the natural form of C3H/He and B10.BR mice but not C57BL/10 mice. Therefore, it was seen as foreign by the T cells from C57BL/10 mice. Peace et al found that synthetic Ras peptide having valine mutation at position 12 mounted specific T_H responses in both C57BL/6 and BALB/c mice while normal Ras peptide were not immunogenic (Peace, et al., 1991). There are two possible reasons accounting for the discrepancy between our results and theirs. Firstly, they used shorter peptides, 13 amino acids long corresponding to residues 5-16 of Ras proteins. Secondly, C57BL/6 and C57BL/10 may have different T cell repertoires.

The T_H responses of C57BL/10 mice induced by either normal or mutant Ras peptides were not specific to the immunising peptide. The crossreaction between the two peptides suggested that residue 12 of the peptides was not critical for specific T cell recognition. Using panels of single amino acid substituted analogues of antigenic peptides, it has been shown that some but not all residues within the peptides are crucial for T cell activation (Herman, et al., 1991).

The observation that different strains of mice had different reaction patterns to the immunising Ras peptide implied that this 23 mer mutant Ras peptide could not be used universally for inducing specific immune responses against mutant Ras protein. The principle of selecting peptide vaccine is that the peptide should be able to induce specific immune responses against the target without causing autoreaction. It is known that different MHC class II molecules present different naturally processed peptides (Rudensky, et al., 1991). Thus individuals expressing different MHC haplotypes may have different peptide pools to which the host T cells are tolerant. Therefore, we need to be very cautious when synthetic peptides are considered to be used for immunotherapy.

Ras proteins are intracellular proteins (Barbacid, 1987). Peptides derived from intracellular proteins are usually presented by MHC class I molecules and recognised by CD8⁺ CTL (Townsend and Bodmer, 1989a). However, in many situations, it has been demonstrated that MHC class II molecules also can present peptides derived from intracellular proteins (Unanue, 1992 and see section 1.5.5. of general introduction for detail). Furthermore, the detection of mutated Ras protein in the extracellular tumour environment (LaVecchio, et al., 1990) raised the possibility that these proteins produced by tumour cells might be taken up, processed and presented by host MHC class II⁺ APC. The hosts bearing Ras induced tumours may benefit from mutant Ras-specific T_H responses in three aspects, I) MHC class II restricted CD4⁺ cells were shown to confer tumour-specific immunity (Van Waes, et al., 1986; Nagarkatti, et al., 1990) and mediate eradication of established tumours independent of the CD8⁺ cells (Greenberg, et al., 1985; Fujiwara, et al., 1984); II) T_H responses may facilitate CTL responses against tumour cells by supplying co-stimulators like interleukin 2; III) activated T_H cells could release cytokines stimulating NK cells, macrophages and neutrophils which also play roles in anti-tumour immune responses. Recent experiments in our laboratory suggest that mutant Ras proteins not only contain T_H epitopes, but also determinants which are recognised by cytotoxic T cells (Skipper and Stauss, 1993). This raises the possibility of exploiting these determinants for immunotherapy of Ras induced cancers.

CHAPTER 4. CTL RESPONSES AGAINST SYNTHETIC RAS PEPTIDES.

4.1. SUMMARY.

Ras peptide-specific CTL responses were induced by immunising mice with synthetic Ras peptides corresponding to amino acids 1-23 of the normal or mutant Ras proteins. Both normal and mutant Ras peptides could induce CTL and none of the CTL lines and clones distinguished the two peptides in vitro. Although the CTL were CD8⁺ and expressed $\alpha\beta$ TCR, their specificity was unconventional. They were able to lyse peptide pulsed cells of many different H-2 haplotypes, but not MHC class I negative cells. Peptide pulsed human HLA expressing cells were not lysed but transfection with mouse class I molecules rendered them susceptible to peptide-specific lysis. Short term CTL lines showed biased TCR usage of V β 8. However, synthetic Ras peptides were very unstable and even HPLC purified material contained many minor peptide components. Furthermore, independently synthesised peptide batches contained different amounts of minor peptide components. These unusual features of Ras peptides caused difficulties in determining which peptide component was responsible for stimulating the MHC unrestricted CTL responses.

4.2. INTRODUCTION.

In chapter 3 it was shown that under certain circumstances mutant p21Ras proteins can create novel immunogenic determinants to elicit mutant peptide-specific CD4⁺ T_H responses. Since p21Ras is a cytoplasmic protein, Ras derived peptides are most probably presented by MHC class I molecules rather than class II molecules and recognised by CD8⁺ T cells. Moreover, MHC class I molecules are expressed by almost all nucleated cells while class II molecules are only present in limited types of cells such as dendritic cells, B lymphocytes, macrophages, monocytes and some epithelial cells (Male, et al., 1991). Tumours expressing mutant Ras proteins, therefore, are likely to express class I molecules and present Ras peptides to CD8⁺ T cells. The natural presentation of mutant Ras peptides to CD4⁺ T cells, if it occurs, is limited to those few types of MHC class II positive cells mentioned above. CTL, therefore, are more likely to recognise tumour cells expressing endogenous mutant Ras and destroy them.

The role of CTL in anti-tumour immune responses has been indicated by the detection of CTL reactive with autologous tumours in the blood, tumour, and tumour-draining lymph nodes of patients with various types of cancer (Anichini, et al., 1987). Adoptive transfer of tumour-specific CD8⁺ T cells in mouse has been shown to mediate tumour eradication under certain circumstances (Greenberg, 1991). In most of the cases, the nature of the antigens recognised by these CTL remain elusive. Therefore, it is difficult to develop a specific targeted cancer immunotherapy. Recently, Boon's group made a significant progress in identification of tumour antigens recognised by CTL. Using CTL clones as probes, they identified two groups of tumour antigens: 1) antigens derived from structurally abnormal proteins; 2) antigens resulting from the abnormal expression of a normal protein. The first group of antigens were represented by artificial tum⁻ antigens which were generated by *in vitro* mutagen treatment of mouse P815 mastocytoma cells. The gene conferring expression of tum⁻ antigen P91A has been cloned and found to contain a point mutation. The mutated exon encodes a antigenic peptide recognised by CTL (Lurquin, et al., 1989). The second group of antigens include the products of two genes, a mouse gene P1A encoding a major tumour rejection antigen of mastocytoma P815 (Van den Eynde, et al., 1991), and a human gene encoding a tumour antigen

(MZ2-E) expressed in a melanoma cell line derived from a patient (Van der Bruggen, et al., 1991). The DNA sequences of both genes are identical to that of the equivalent genes found in normal cells. Both of the genes appear to be silent on most normal tissues and are activated in the tumours.

Mutant Ras proteins are similar to tum^r antigen of P815, resulting from point mutations of normal genes. To test whether mutant Ras proteins contain CTL epitopes, synthetic peptides corresponding to amino acids 1-23 of normal Ras protein or mutant Ras protein were used to induce CTL responses in mice. Ras peptide-specific CTL lines and clones were isolated and analysed whether they could specifically recognise the mutation at position 12 of Ras protein.

4.3. MATERIALS AND METHODS.

4.3.1. Synthetic Ras peptides.

Ras peptides were synthesised using conventional solid phase Fmoc chemistry by the peptide laboratory of ICRF. For details see chapter 3.

4.3.2. Generation of CTL lines and clones against Ras peptides.

Ras peptides were emulsified with complete Freund's adjuvant as described in materials and methods of chapter 3. Mice were immunised i.p. with 200 µg Ras peptides emulsified with adjuvant. Two weeks later, the mice were boosted by injection either i.p. or in hind footpads and tail base with the same amount of peptides with adjuvant. One week after boosting spleens or popliteal and inguinal lymph nodes from immunised mice were removed. Single cell suspensions were prepared in complete T cell growth medium. Bulk cultures were set up either in T25 flasks containing 5×10^7 spleen cells with 2 µg/ml Ras peptides in a total volume of 10 ml culture medium or in 24 well plates containing 5×10^6 lymph node cells per well with 2 µg/ml Ras peptides in a total volume of 2 ml culture medium. Five days later, T cells were harvested and restimulated in 24 well plates at 5×10^5 cells per well with 3×10^6 irradiated syngeneic feeder cells, 15% rat Con-A supernatant and 2 µg/ml Ras peptides in a total volume of 2 ml culture medium. CTL activities of the cell lines were tested on day 6 after restimulation against target cells preincubated with peptides for 0.5 hour. CTL clones were established by limiting dilution from CTL lines which recognised Ras peptides specifically. The CTL lines and clones were restimulated weekly in 24 well plates.

4.4. RESULTS.

4.4.1. Cytotoxic T cells could not distinguish mutant and normal Ras peptides.

In parallel with the studies of T_H responses against Ras peptides, C3H/He, B10.BR and C57BL/10 mice were used for immunisation to generate CTL lines and clones. The mice were immunised i.p. or subcutaneously with synthetic Ras peptides, either normal Ras corresponding to amino acids 1-23 of normal p21 Ras protein or mutant Ras changing glycine to valine at position 12. The CTL lines were established by restimulating the spleen cells or lymph node cells from immunised mice with Ras peptides *in vitro*. In all three mouse strains, both mutant and normal Ras peptides stimulated CTL responses after two restimulations, although normal Ras peptide seemed to have lower efficiency to stimulate CTL responses. None of the CTL lines generated could distinguish mutant and normal Ras peptides. CTL immunised with mutant Ras peptide lysed target cells pulsed with either mutant or normal Ras peptide equally well (Fig.4.1A). The same results were obtained with normal Ras peptide-induced CTL (Fig.4.1B). The lysis was Ras peptide-specific, since the target cells pulsed with irrelevant peptide (H-2K^b143-167) were not lysed by Ras peptide-specific CTL (not shown).

Furthermore, Ras peptide-specific CTL clones were established from corresponding cell lines. Seven CTL clones tested recognised both mutant and normal Ras peptides. A representative example is shown in Fig.4.1C. These results ruled out the possibility that individual T cell clones within a whole population had distinct specificities for the two peptides.

4.4.2. Ras peptides-specific CTL were conventional CD8⁺ CD4⁻ $\alpha\beta$ TCR⁺ cells.

The phenotype of Ras peptide-specific CTL was determined by the conventional antibody blocking assays and cytofluorimetry. The addition of anti-CD8 but not anti-CD4 mAb in CTL assays inhibited lysis mediated by Ras peptide-specific CTL (Fig.4.2). Several Ras peptide-specific CTL lines and clones were analysed by cytofluorimetry. The FACS staining profile showed that these CTL were CD8⁺ CD4⁻ $\alpha\beta$ TCR⁺ cells (not shown).

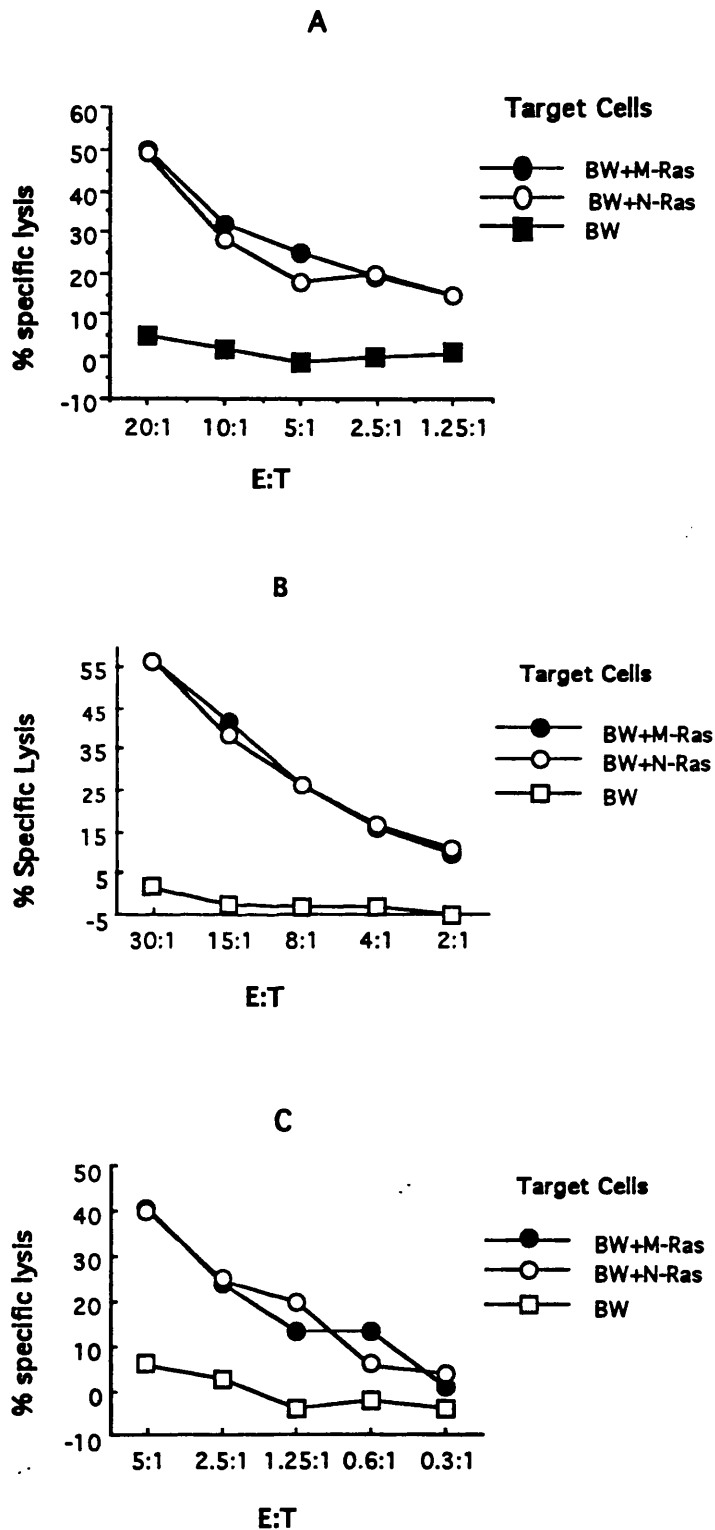


Fig.4.1. CTL recognition of Ras peptides by CTL lines and clones. C3H/He mice were immunised and boosted i.p. with 200 µg mutant (Fig.A) or normal (Fig.B) Ras peptides in complete Freund's adjuvant. One week after boosting, the spleen cells were restimulated *in vitro* with 2 µg/ml immunising peptides. The CTL lines were restimulated once a week. A CTL clone was established from B10.BR mice immunised with mutant Ras peptide (Fig.C). CTL activities were tested against BW targets pulsed with either mutant or normal Ras peptides in 4 hour chromium release assays.

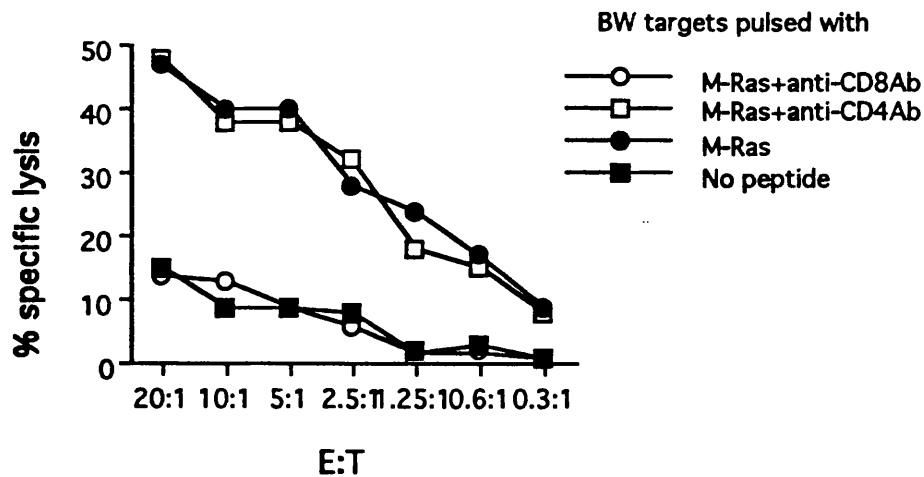


Fig.4.2. Phenotype of the Ras peptide-specific CTL. The CTL activity of a C3H/He derived, mutant Ras peptide-induced CTL line was tested against BW cells without pulsing or pulsed with mutant Ras peptide in the absence of antibodies or in the presence of 5 µg/ml anti-CD4 or anti-CD8 mAb.

4.4.3. The CTL recognition of Ras peptides was not MHC restricted but MHC dependent.

Experiments to map restricting MHC class I molecules gave surprising results. Ras peptide induced CTL lines lysed peptide pulsed target cells of H-2^{a,b,d,k} haplotypes. A representative example was shown in Fig.4.3A. A C3H/He derived CTL line lysed Ras peptide pulsed targets BW(H-2^k), RMA(H-2^b), A20(H-2^d) and YAC(H-2^a) equally well. Cross-recognition of allogeneic class I molecules did not account for this H-2 unrestricted lysis, since peptide unpulsed allogeneic cells were not lysed. Furthermore, CTL clones also lysed H-2 disparate target cells, demonstrating that the unrestricted recognition was not limited to bulk T cell lines (Table 4.1).

To extend the analysis of H-2 haplotypes Con-A stimulated spleen cells of H-2^{s, p} and ^u haplotype were used as target cells for C3H/He derived CTL lines. Although Con-A blasts were found to be inefficient targets for peptide-specific CTL, similar levels of lysis were observed against peptide pulsed syngeneic H-2^k and allogeneic H-2^{s,p,u} target cells (not shown).

To determine whether MHC class I molecules were required for CTL recognition of Ras peptide the lysis against class I expressing YAC cells

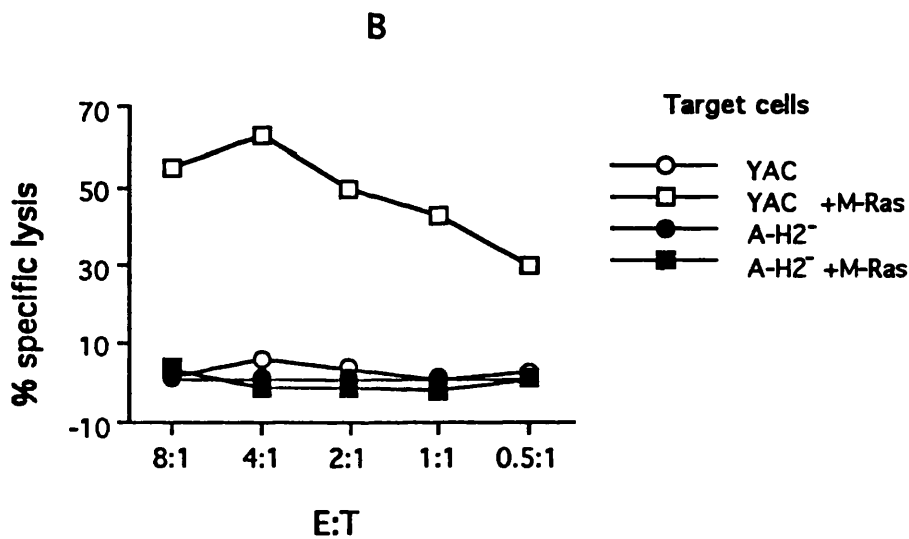
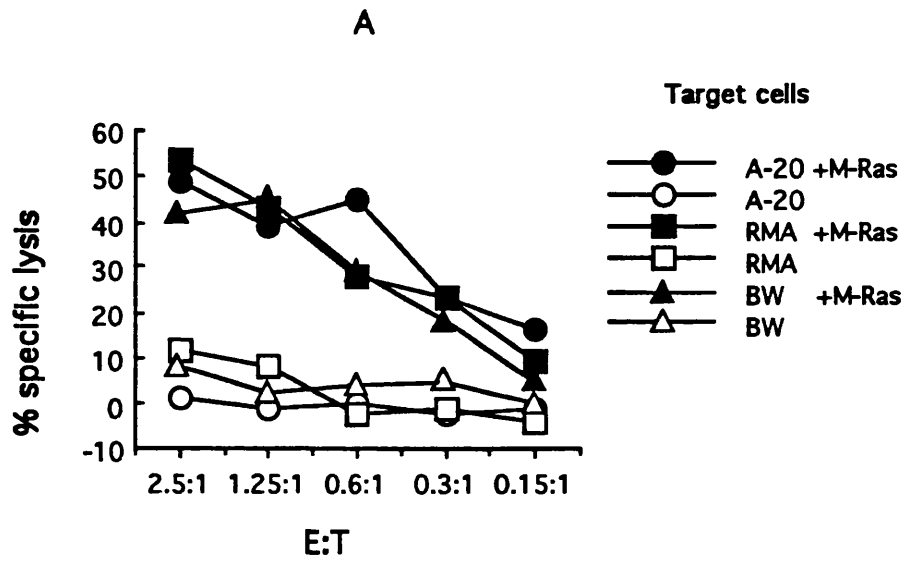


Fig.4.3. CTL recognition of mutant Ras peptide presented by murine cells expressing different H-2 haplotypes. The CTL lines were derived from C3H/He mice immunised with mutant Ras peptide. The CTL activities of the CTL lines were tested against different target cells without pulsing or pulsed with 10 µg/ml mutant Ras peptide.

Table 4.1. Mutant Ras peptide induced CTL lyse target cells expressing different H-2 class I molecules.

CTL*	Origin	H-2 Haplotype of Target Cells			
		H-2 ^b	H-2 ^k	H-2 ^d	H-2 ^a
		% Specific Lysis**			
Line 17	C3H	65	50	22	n.d.
Line 20	C3H	66	53	58	36
Line 21	C3H	77	27	64	49
Line 22	C3H	65	42	26	44
Clone 12	B10.BR	25	47	45	n.d.
Clone 16	B10.BR	35	66	55	n.d.
Clone 18	B10.BR	25	36	47	n.d.

* CTL lines and clones were established from mice after in vivo immunisation and in vitro restimulation with mutant Ras peptide.

** The lysis of target cells (H-2^b=RMA-S; H-2^k=BW; H-2^d=A 20; H-2^a=YAC) that were pulsed with mutant Ras peptide is shown. Lysis by CTL clones and lines was determined in independent experiments using E:T ratios from 1:1 to 10:1. In all experiments the lysis of unpulsed target cells was <10%.

and against a class I negative variant of YAC (A-H2⁻) was compared. Fig.4.3B showed that only peptide pulsed YAC cells were lysed but not the A-H2⁻ cells.

4.4.4 CTL lysis required peptide presentation by murine MHC class I molecules.

After observing of the Ras peptide presentation by many different murine class I molecules we tested whether human MHC class I molecules could present this Ras peptide to murine CTL lines. Three lymphoid human cell lines T2, C8166 and WMPT were used as CTL targets. None of them were lysed by CTL lines and clones of either H-2^k or H-2^b origin (Fig.4.4). However human cells were not inherently resistant to Ras specific murine CTL since transfection of a single mouse class I

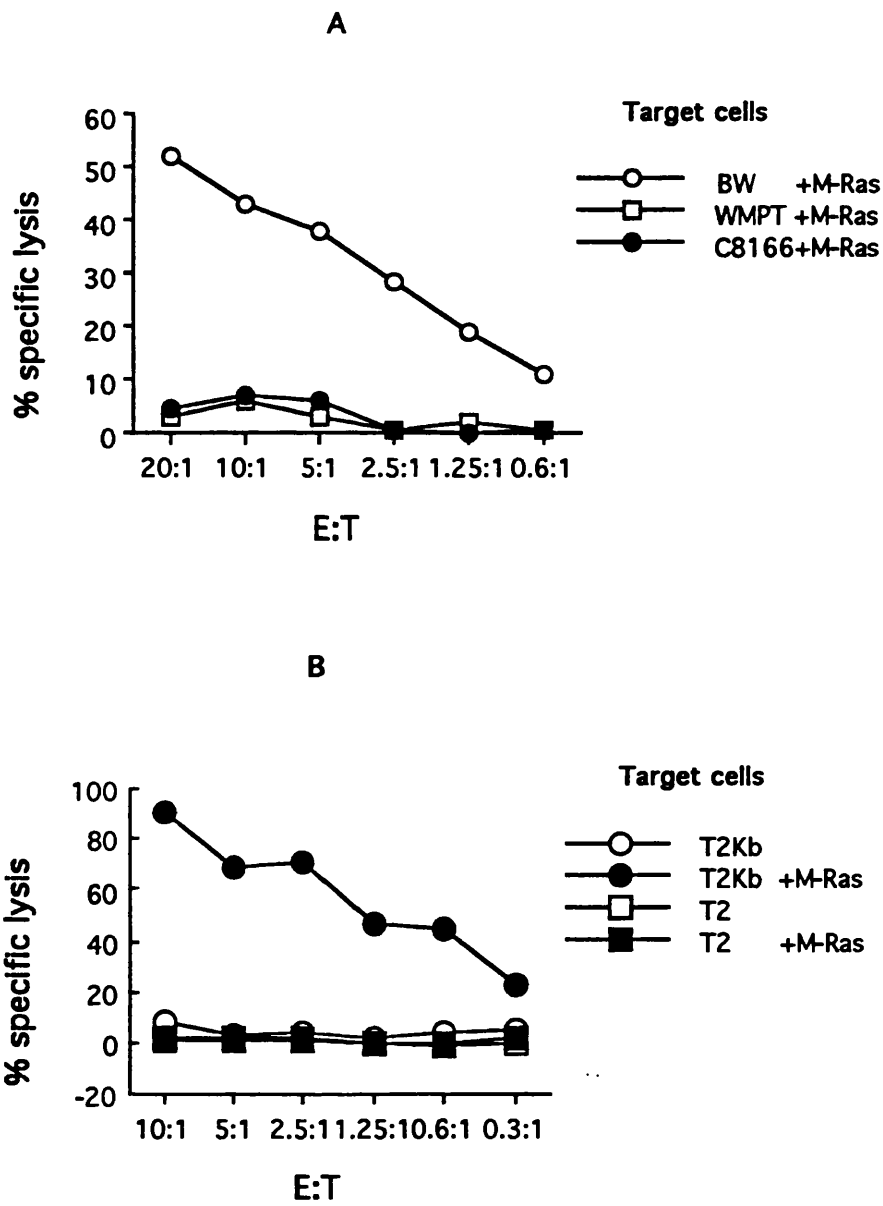


Fig.4.4. CTL recognition of mutant Ras peptide presented by murine and human cells. Indicated murine or human cell lines were labeled with ^{51}Cr , pulsed with $10\ \mu\text{g}/\text{ml}$ mutant Ras peptide for half hour and then lysed by CTL lines derived from C57BL/10 mice (Fig.A) or C3H/He mice (Fig.B) primed with mutant Ras peptide.

allele led to CTL lysis. Ras peptide pulsed K^b transfected T2 cells were lysed while untransfected T2 cells were not (Fig.4.4B).

4.4.5. Biased usage of V β TCR.

It was interesting to know whether these MHC class I unrestricted, Ras peptide-specific CTL preferentially use particular V β elements of TCR like T_H cells recognising MHC class II-dependent superantigens. Four independently derived CTL lines were analysed 2 weeks after *in vitro* passage with a set of mAb directed to different components of TCR. FACS analysis showed that these CTL mainly expressed V β 8 TCR. The triple staining data demonstrated that 42-88% of the CD8⁺ T cells were V β 8.1.2.3 positive (Table 4.2).

Table 4.2. TCR usage of Ras-specific CTL lines

CTL*	% of positive cells**								
	V α 8	V β 6	V β 8.1.2	V β 8.1.2.3	V β 8.2	V β 11	V β 17	CD8 ⁺ /4 ⁺	V β 8.1.2.3 ⁺ of CD8 ⁺
Line 20	2	6	48	49	14	3	1	91/1	54
Line 21	3	7	64	72	41	6	3	82/0	88
Line 22	4	4	nd	62	56	4	5	82/0.5	67
Line 23	2	5	29	36	27	2	2	66/5	42

* CTL lines were established from C3H/He mice after *in vivo* immunisation and *in vitro* restimulation with mutant Ras peptide.

** Triple staining for TCR expression were carried out as described in general material and methods. Briefly, 10⁶ T cells were incubated with indicated mAb for 30 min followed by incubation with goat anti-rat or goat anti-mouse FITC, normal rat serum, mix of L3T4-PE and Lyt-2-biotin and streptavidin-cychrome.

4.4.6. The size of the CTL recognised Ras peptide.

It has been demonstrated that optimal peptide size for recognition by class I restricted CTL was 8 or 9 amino acids (Rotzschke, et al., 1990). In order to find out whether a similar size core sequence can be identified in the 23 mer Ras peptide, two shorter peptides were synthesised. 5 residues were removed from either the N or C-terminus of the 23 mer long peptide and neither of these 18 mer peptides was able to sensitise efficiently target cells for CTL lysis (Fig.4.5).

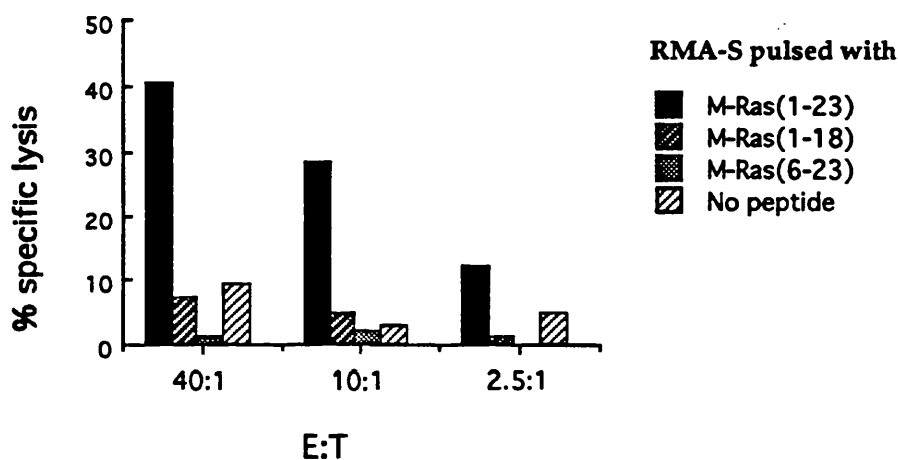


Fig. 4.5. CTL recognition of truncated Ras peptides. RMA-S targets were pulsed with either the full length mutant Ras peptide (1-23) or peptides shortened by 5 residues at the N terminus (6-23) or the C terminus (1-18). The CTL line was derived from C3H/He mice immunised with mutant Ras peptide.

Afterwards, we asked the question what was the minimum length of the Ras peptide epitope. A set of peptides truncated by 1-5 amino acids at either N or C-terminus were synthesised and used to pulse target cells. Unexpectedly, this batch of peptides (including 23 mer Ras peptide) were not recognised by the CTL raised against previous batch of 23 mer Ras peptide. Therefore, several CTL lines were generated against the two different batches of Ras peptide and it was found that many CTL lines could not cross recognise both batches of peptide (not shown).

The two batches of peptide were subjected to a reversed-phase HPLC column. The eluting profiles showed that both batches of peptide contained many minor peaks besides the major peaks and these minor peaks were different between the two batches of peptide (Fig.4.6),

indicating that the minor components of the two batches of peptide were different. Sequencing the peptides present in the major peaks revealed that the major components of the the two batches of peptide were identical 23 mer mutant Ras peptide.

Since the original synthetic Ras peptide did not appear very pure, we thought to use HPLC purified major component for our experiments. To test the stability of this peptide, we run the HPLC purified major component over the HPLC column after it had been kept either at 4°C or -20°C for short time. Surprisingly, several distinct peaks showed up in the elution profile (Fig.4.7). Therefore, it seemed that this 23 mer Ras peptide was very unstable and it was not feasible to use HPLC purified fractions for our studies.

Because of the impurity and instability of the Ras peptide, the epitope mapping became difficult. One possible approach was using freshly purified HPLC fractions of crude Ras peptide to pulse target cells and then analysing these targets with CTL raised against crude Ras peptide. In one experiment, we found that a minor component was recognised best by a short term CTL line (Fig.4.8). Other components might also stimulate CTL responses since there were many peptide fractions that were recognised weakly by this CTL line.

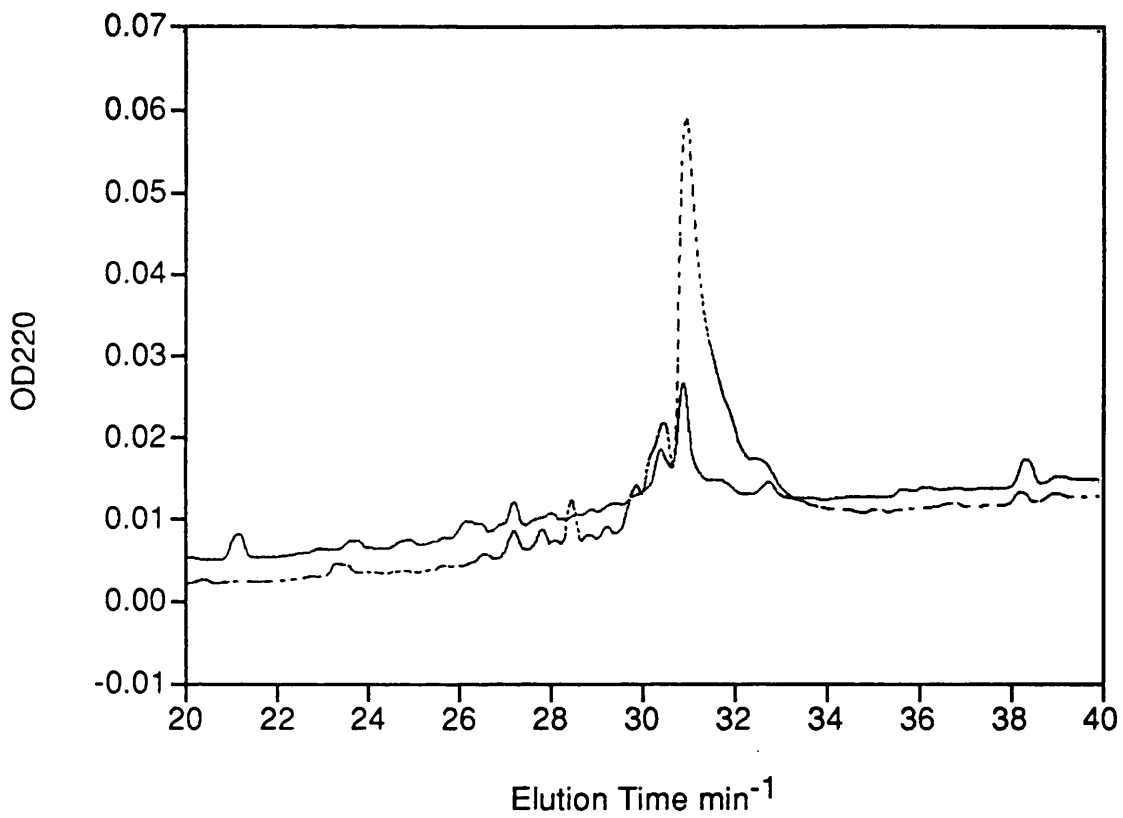


Fig.4.6. HPLC profiles of Ras peptides. Two batches of independently synthesised mutant Ras peptides were dissolved in 0.1% TFA at a final concentration of 1mg/ml. 30 μ l each of the solutions were subjected to HPLC purification.

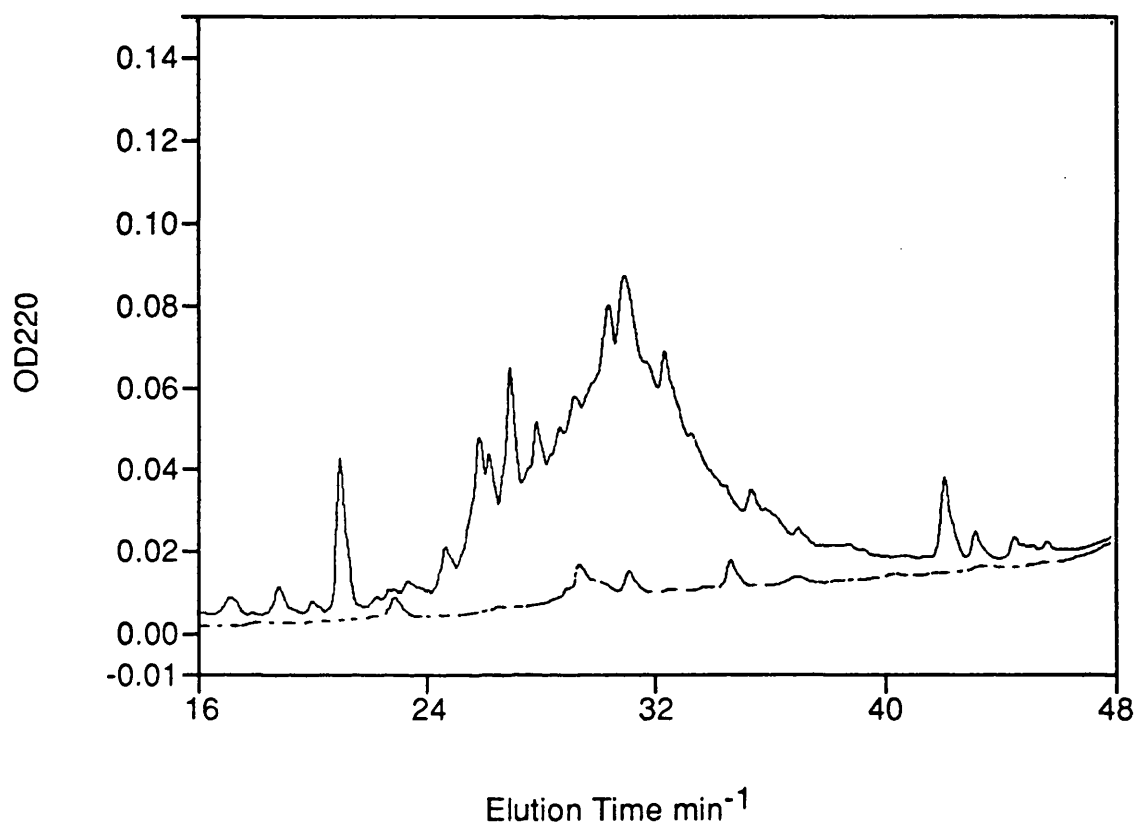


Fig.4.7. HPLC elution profiles of Ras peptides. 0.5 mg of mutant Ras peptide was dissolved in 1.5 ml of 0.1% TFA and purified by HPLC. The elution profile is shown as a solid line. 1 ml peptide fractions were collected and stored at 4 °C overnight. 100 μ l of HPLC fraction 32 was subjected to HPLC purification again. The elution profile is shown as a dashed line.

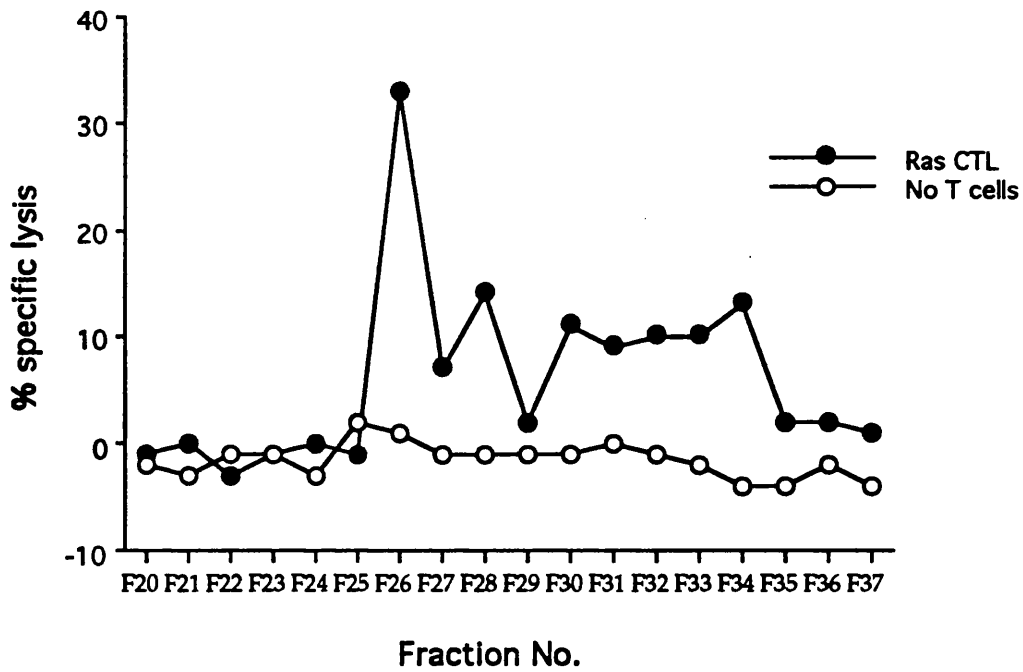


Fig. 4.8. CTL recognition of purified HPLC fractions of Ras peptide. 1 mg synthetic mutant Ras peptide was dissolved in 1.5 ml 0.1% TFA and subjected to HPLC purification. 1 ml fractions were collected and 10 μ l of each fraction was used to pulse ^{51}Cr labeled BW target for 30 min before T cells were added. The CTL line was derived from C3H/He mice immunised with mutant Ras peptide. E:T =6:1.

4.5. DISCUSSION.

The analysis of CTL responses induced by synthetic Ras peptides corresponding to amino acids 1-23 of normal Ras protein or containing a valine change at residue 12 gave us surprising, exciting and frustrating results. It was surprising that the CTL lines and clones raised against these peptides showed unconventional specificities. It was exciting that these Ras-specific CTL recognised peptides in a MHC class I-dependent but unrestricted fashion, which was similar to superantigen recognition by T_H cells. It was frustrating that these Ras peptides were very impure and unstable so that the epitope mapping was difficult.

CTL raised against Ras peptides showed different specificities from that of T_H cells induced against the same Ras peptides (see chapter 3). T_H cells in C3H/He and B10.BR mice recognised the mutation at position 12 of Ras peptide. However, none of the Ras peptide induced CTL lines and clones could distinguish normal and mutant Ras peptides. In fact, these CTL recognised Ras peptides in a very unconventional way. The most striking feature shown by the Ras peptide-specific CTL was that they recognised the Ras peptide presented by many different H-2 class I alleles. Peptide binding is usually MHC class I allele-specific (Falk, et al., 1991a), which is mediated by fitting the peptide anchor residues into the major pockets of peptide binding groove of class I molecules (Fremont, et al., 1992; Matsumura, et al., 1992). Although by using different sets of pockets one given peptide possibly can bind to different class I molecules (Matsumura, et al., 1992), the TCR of CTL usually recognises specifically one peptide/MHC combination. Several Ras-specific CTL clones were analysed in this study, and all of them showed recognition of Ras peptide in association with H-2^a, H-2^b, H-2^d and H-2^k class I molecules. Therefore, single TCR recognised the different Ras peptide/MHC combinations. Furthermore, we found that these CTL lines and clones expressed $\alpha\beta$ TCR rather than $\gamma\delta$ TCR which is commonly associated with MHC unrestricted killing (Allison and Havran, 1991).

MHC restriction is a fundamental property of T cell recognition. How should we explain this unconventional CTL recognition of the Ras peptides? One possibility is that the highly hydrophobic Ras peptides bound in the groove of class I molecules in an unusual way and the TCR of Ras peptide-specific CTL recognised peptide residues directly and co-recognition of the peptide presenting MHC molecules is not involved.

Alternatively, co-recognition of MHC may involve residues which are conserved among all murine class I molecules analysed in this study. Another possibility is that the Ras peptides bound to MHC class I molecules outside binding groove, like MHC class II-dependent superantigens.

In contrast to most of T cell recognised antigens, superantigens violate the rules of MHC allele restriction as both self-superantigens and foreign superantigens have been shown to be recognised by CD4⁺ cells in the context of many different class II alleles (reviewed by Herman, et al., 1991). Superantigens stimulate T cells in an unconventional way by engaging the outer face rather than peptide binding groove of class II molecules (Karp and Long, 1992) and the lateral surface of the TCR β chain (Choi, et al., 1990; Pullen et al., 1990; Cazenave, et al., 1990). The Ras peptides used in this study resembled the MHC class II-dependent superantigens in that they could be presented by many different MHC class I molecules. However, it is uncertain whether Ras peptides bridge class I molecules and TCR in the same way as superantigens.

Superantigens activate T cells bearing particular V β (Herman, et al., 1991). We analysed the TCRV β usage of a few Ras peptide induced short term CTL lines and found predominant expression of V β 8. Limited by the availability of anti-TCR mAb, we did not analyse many TCR elements. Therefore, we do not know how diverse Ras peptide-specific CTL repertoire is and whether Ras peptide-specific CTL responses are only determined by V β chains which probably reflects the specific interaction of Ras peptide with V β chains.

Although CTL recognised Ras peptides in the context of many different murine class I molecules, human class I molecules failed to present the peptides to murine CTL. The lack of recognition of human target cells may be caused by failure of Ras peptide binding to HLA molecules or by inability of CTL to recognise peptide/HLA combinations. We tried to measure Ras peptide binding to class I molecules by incubating the peptide with mutant RMA-S cells and then detecting the level of MHC class I expression (Townsend, et al., 1989b). No significant increase of surface expression of class I molecules was observed. Therefore, this peptide binding assay was not sensitive enough to detect the weak binding of Ras peptides to class I molecules. Thus we could not distinguish the two possibilities accounting for the lack of recognition of human target cells.

Our finding of MHC unrestricted CTL recognition is not unique. There are several other reports demonstrating similar results in different systems. Carbone et al reported that specific CTL clones recognised cyanogen bromide-cleaved ovalbumin on restricting elements encoded by the H-2^b, H-2^d and H-2^k haplotypes. However, the peptide responsible for target sensitisation was not identified in that study (Carbone, et al., 1988). Sponaas et al isolated tumour cells induced CTL lines which lysed a number of tumour cell lines of H-2^b, H-2^d, H-2^a, and H-2^k haplotypes (Sponaas, et al., 1988). Some CTL lines established from lymph nodes of patients with pancreatic adenocarcinoma were shown to recognise a tumour associated antigen, mucin in a MHC unrestricted manner and these CTL were identified as $\alpha\beta$ T cells (Barnd, et al., 1989). Sherman and Lara showed that murine CTL specific to hemin, the heme moiety of hemoglobin recognised this antigen presented by H-2^b, H-2^d, H-2^k, and even a human class I molecules (Sherman and Lara, 1989). Maccario et al obtained HSV-specific human CTL which lysed target cells mismatched for HLA loci (Maccario, et al., 1993). All these data together with our data suggest that T cell repertoire does contain some T cells bearing $\alpha\beta$ TCR which can recognise antigens presented by MHC class I molecules in a unconventional way. The biological significance of this type of T cells needs to be explored.

It has been very well documented that class I molecules predominantly present 8 or 9 mer peptides. Although longer peptides can be presented and recognised as well, they are less efficient to sensitise CTL lysis (Rotzschke, et al., 1990a; Van Bleek and Nathenson, 1990; Falk, et al., 1991a). Our data showed that truncation of the 23 mer Ras peptide by 5 residues at the N or C-terminus led to the loss of lysis mediated by MHC unrestricted CTL. However, since later experiments indicated that independently synthesised peptides contained different components and the minor components might be responsible for the CTL responses, it became impossible to interpretate the results with shorter peptide versions.

The real epitope recognised by the Ras peptide-specific CTL were not defined in this study. It was uncertain that the 23 mer Ras peptide was the CTL epitope, because 1) two batches of synthetic peptide identically contained this major component but could not be co-recognised by many CTL; 2) one CTL line was shown to recognise a minor component of the peptide preparation. However, we had no information about those minor

components appeared in the HPLC elution profile. They could be either degraded or modified forms of the 23 mer Ras peptide. Micro-sequencing of those minor components would be informative.

Synthetic peptides are widely used in the laboratories. Our experience told us that we have to be very cautious to use synthetic peptides for studies. Peptides like Ras are very unstable, got degraded or modified easily. Special attention needs to be paid to CTL epitope mapping for this kind of peptides.

CHAPTER 5. IDENTIFICATION OF PEPTIDES RECOGNISED BY MINOR : HISTOCOMPATIBILITY ANTIGEN-SPECIFIC CTL.

5.1. SUMMARY.

The peptide epitopes involved in CTL responses to single or multiple mH antigens were analysed. Using acid elution and HPLC separation techniques, mH antigenic peptides were purified from spleen cells of H-2^b mice. The HPLC peptide fractions were analysed with mH antigen-specific CTL lines which were generated in H-2^b mice differing in one (H-1), two (H-1 and H-25) or multiple (>29) mH loci. Anti-H-1 CTL recognised one out of 50 HPLC peptide fractions and the recognition was H-2K^b restricted. The same peptide fraction was also recognised by anti-H-1/H-25 CTL and no additional epitope was detected, indicating that the H-25 locus was dominated by H-1 locus. CTL generated to multiple mH loci (including H-1 and H-25) recognised two HPLC peptide fractions. One fraction was the same as that recognised by anti-H-1 and anti-H-1/H-25 CTL and it was shown to contain a K^b presented, H-1-derived peptide. The other fraction was presented by H-2D^b and could not be isolated from purified H-2K^b molecules. Peptide extraction from recombinant mice further confirmed that the two CTL epitopes were D^b- and K^b-restricted respectively. The CTL recognised peptide fractions were further subfractionated and the results suggested that probably a single peptide in one HPLC fraction was recognised by the bulk CTL populations. The D^b-restricted CTL epitope was attempted to be sequenced.

5.2. INTRODUCTION.

The expression of disparate mH antigens causes GVHD or the rejection of organ transplants which are matched for MHC (Loveland and Simpson, 1986). At present, the GVHD and graft rejection mediated by mH antigens can not be overcome completely because most of the mH antigens are poorly characterised. Generally mH antigens stimulate T cell immune responses but not antibody responses. The lack of mH antigen-specific antibodies makes the structural identification of mH antigens difficult. The only tools that can be used to identify mH antigens are the mH antigen-specific T cell lines and clones. It has been demonstrated recently both in mouse and man that mH antigens in general are peptides derived from cellular proteins and presented by MHC molecules (Wallny and Rammensee, 1990; Rotzschke, et al., 1990b; Loveland, et al., 1990; Sekimata, et al., 1992). However, attempts to identify peptide sequences and the protein origin of mH antigens have been unsuccessful. To date, the maternally transmitted factor (MTF) is the only molecularly identified mH antigen. The sequence of maternally transmitted factor was determined by sequencing the entire mitochondrial genomes from three mouse strains that differ in Mta (Loveland, et al., 1990). This approach is not practical for other mH antigens which are mostly encoded in the nuclear genome.

Recently, Rammensee's group developed biochemical techniques to purify peptides from cell extracts by HPLC. This biochemical approach allowed them to sequence the self-peptides mixtures eluted from MHC class I molecules (Falk, et al., 1991a). Using similar approaches, many laboratories have succeeded to obtain the sequence of certain abundant peptides associated with MHC molecules (Jardetzky, et al., 1991; Hunt, et al., 1992a; Rudensky, et al., 1991; Hunt, et al., 1992b; Chicz, et al., 1992; Rudensky, et al., 1992). Most encouragingly, Udaka and colleagues have purified and sequenced a natural peptide from mouse spleen that is recognised by an alloreactive CD8⁺ CTL clone in association with the MHC class I molecule H-2L^d (Udaka, et al., 1992). Up to now, this is the only report that a T cell epitope has been identified by using this painstaking biochemical approach.

This study tried to identify the murine mH loci encoded CTL epitopes by using acid extraction and HPLC separation techniques. More than 50 mH loci have been mapped in the mouse genome (Snell, 1981). It

was proposed that one mH locus contains at least two distinct genes which encode proteins that are processed and presented by MHC class I and class II molecules respectively (Roopenian, 1992). It is currently unknown how many peptide epitopes are encoded by one mH locus and how this relates to the strength of CTL responses. Taking the advantage of genetically defined congenic mice, we analysed the CTL responses against one, two or multiple mH loci differences. Attempts were made to obtain microsequence of one CTL recognised peptide epitope.

5.3. MATERIALS AND METHODS.

5.3.1. Generation of CTL lines and clones against mH antigens.

Mice were immunised by i.p. injection of 2×10^7 spleen cells from mH antigen disparate mice followed by an injection of the same cell number after two weeks. Two to four weeks after boosting, single cell suspensions were prepared from the spleens of the immunised mice. Red blood cells were lysed with GEYS solution. Mononuclear cells were restimulated *in vitro* with irradiated (Co^{60} 3000R, or X-ray 2000R) stimulator cells for 5 days. The restimulation was done in 24 well plates (Falcon) containing 5×10^6 responder cells and 5×10^6 stimulator cells in 2 ml complete T cell growth medium. On day 5, T cells were harvested from the plates and tested for CTL activities in a standard 4-hour ^{51}Cr chromium release assay. CTL lines were established by weekly restimulation in 24 well plates containing 5×10^5 responder cells, 4×10^6 irradiated syngeneic spleen cells, 5×10^5 stimulator cells and 15% rat Con-A supernatant in 2ml culture. CTL assays were usually done on day 6 after restimulation.

Anti-mH antigen CTL clones were established from 5 days bulk culture by limiting dilution of cytotoxic T cells as described in general materials and methods.

5.3.2. Preparation of mH antigenic peptides from whole cell lysates.

Protocol 1.

Six spleens were removed from naive mice. Single cell suspension was prepared and the red blood cells in spleens were lysed with GEYS solution. Cells were pelleted by centrifugation and the pellet was resuspended in 5 ml 0.1% TFA followed by adding 1 ml 1% TFA. The suspension was homogenised with a Dounce homogeniser followed by ultrasonication (20 bursts, Branson sonifier) on ice. After stirring for 30 min on ice, the suspension was centrifuged at 188,000g, 18°C for 30 min (Beckman ultracentrifuge). Supernatant was lyophilised overnight, resuspended in 1 ml 0.1% TFA and then was homogenised and transferred into an eppendorf tube. After spinning in an eppendorf centrifuge (Micro Centaur) at 4°C for 10 min, the supernatant was loaded onto a G25 Sephadex column (Pharmacia). Gelfiltration separation program was: 120 min run, flow rate 1 ml/min, absorbance 280 nm, elution buffer 0.1% TFA,

fraction size, 5 ml. Material of MW<5000 Da was collected and lyophilised again. Then the material was resuspended in 1 ml 0.1% TFA, homogenised and spun as before. The supernatant was used for HPLC separation.

Protocol 2.

Six to 12 spleens or thymi were removed from naive mice. Single cell suspensions were prepared and the red blood cells in spleens were lysed with GEYS solution. Cells were pelleted by centrifugation and the pellet was resuspended in 3 ml 0.7% TFA. The suspension was homogenised with a Dounce homogeniser followed by ultrasonication (20 bursts, Branson sonifier) on ice and vortexing for 3 min. The supernatant was collected after spinning the suspension in a microcentrifuge (Sigma 2K15) at 17,608g, 4°C for 30 min. The pellet was resuspended in 2 ml 0.1% TFA and homogenised, sonicated and vortexed as before. The 2 ml suspension plus previous 3 ml supernatant were transferred into polyallomer tubes and spun (Beckman ultracentrifuge) at 154,324g for 30 min. The supernatant was transferred to 10 kDa centricon filters (Amicon) and centrifuged at 5000g for 3 hours. The filtrate was used for HPLC separation.

5.3.3. Preparation of mH antigenic peptides from purified MHC class I molecules.

5.3.3.1. Production of Y-3 mAb from ascites.

Adult female BALB/c mice were primed by i.p. injection of 0.5 ml pristane (sigma). Two weeks later, $4-6 \times 10^6$ Y-3 cells were injected i.p. in a volume of 0.5 ml. The ascites fluid was collected within 1-2 weeks after injection and centrifuged at 822g for 20 min. The supernatant was removed and stored in -20°C.

5.3.3.2. Purification of Y-3 mAb using a protein A column.

Buffers: Tris buffer:	0.05 M Tris
	0.15 M NaCl
	pH=8.6
Glycine buffer:	0.05 M Glycine
	0.15 M NaCl

pH=2.3

Protein A column was washed with 10 volumes of Tris buffer first. Y-3 ascites fluid was precleared by centrifugation at 13,000g for 30 min at 4°C and the pH of the supernatant was adjusted to 8 by adding 1 M Tris (pH=8). The supernatant was loaded on the protein A column with a flow rate of about 30 ml/hour and recirculated overnight with a flow rate of about 15 ml/hour by using a 2132 Microperpex Peristaltic pump. The following day, the column was washed with 10 volumes of Tris buffer. Y-3 mAb was eluted from the column by running glycine buffer and collected under the supervision of UV monitor (LKB Bromma). The protein concentration was identified by absorbance at 280 nm with a spectrometer (ULTROSPEC II, LKB Biochrom).

5.3.3.3. Coupling of Y-3 mAb to CNBr-activated sepharose 4B beads.

Buffer:

Coupling buffer: 0.1 M NaHCO₃
0.5 M NaCl

Acetate buffer: 0.1 M Na-acetate-trihydrate
0.5 M NaCl
pH=4

Tris buffer: 0.1 M Tris-HCl
0.5 M NaCl
pH=8

The purified Y-3 mAb was dialysed against coupling buffer overnight. Freeze dried CNBr-activated sepharose 4B beads powder (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) was washed with 1 mM HCl in a sintered glass filter. Dialysed Y-3 mAb was mixed with washed beads in a 10 ml tube. 5-10 mg mAb were used for coupling 1 ml beads. The tube was rotated end-over-end for 2 hours at room temperature. Coupled beads were washed 3 times with coupling buffer and resuspended in 0.1 M Tris-HCl (pH=8). After 2 hours rotating, beads were washed with 3 cycles of alternating pH; Acetate buffer first, Tris buffer afterwards. The beads were resuspended in Tris buffer and packed in a column.

5.3.3.4. Affinity purification of K^b molecules.

Naive spleen cells were treated with GEYS to deplete red blood cells, washed, and lysed at a concentration of $1-2 \times 10^8$ cells per ml in PBS containing 1 % Nonidet P40 (NP40), 5 mM Na-orthovanadate, 25 mM iodoacetamide, and 1 mM phenylmethanesulfonyl fluoride. The lysate was stirred for 30 min on ice and then cleared of nuclei and debris by centrifugation at 250g for 5 min at room temperature followed by 13,000g for 30 min at 4°C. The cleared lysates were loaded on a Y-3 affinity chromatography column and recirculated overnight with a flow rate of 2-4 ml/hour. The column was then washed with 15 column volumes of PBS containing 0.5 % NP40 and 0.1 % SDS, followed by 2 column volumes of PBS containing 1 % n-octyl-glucoside. The bound K^b molecules were eluted with 0.05 M diethylamine in 0.15 M NaCl containing 1 % n-octyl-glucoside (pH=10.5). The eluate was dialysed against 10 mM ammonium bicarbonate buffer for 24 hours at 4°C.

5.3.3.5. Separation of mH peptides from MHC class I molecules.

The dialysed eluate was transferred to 2 ml eppendorf tubes and concentrated on a speed vac evaporater (speed vac SC200, Savant) at medium drying rate to a final total volume about 2 ml. The sample was then transferred to a 10 kDa centricon filter and centrifuged at 5000g at 4°C for 120 min. The concentrated sample, retentate (MW>10,000) was denatured by incubating in 1 ml guanidine chloride buffer (6 M guanidine chloride, 20 mM Tris, 1 mM 2ME) for 30 min in a 37°C water bath. The denatured sample was transferred to a 10 kDa centricon filter and centrifuged at 5000g for 50 min at 4°C. The filtrate was used for HPLC separation.

5.3.4. HPLC separation of mH antigenic peptides.

The 1-2 ml peptide preparation derived from either whole cell lysates using Protocol 1 or purified K^b molecules was loaded onto a reverse-phase HPLC column. The 5 ml peptide preparation derived from whole cell lysates using Protocol 2 was loaded on the HPLC column in batches of up to 2 ml per injection. The HPLC separation procedures were described in general materials and methods. Individual fractions were

collected. Either the whole fractions or aliquots of the fractions were dried by vacuum centrifugation and dissolved in PBS. After 5 min sonication in a water bath, the samples were stored at -20°C before they were used in CTL assays. Rest of the aliquoted samples were stored at -20°C in the original eluting buffer before they were further subfractionated.

5.3.5. HPLC subfractionation of CTL recognised mH peptides.

CTL recognised fractions obtained from first HPLC separation were transferred to a 3 kDa centricon filter (Amicon) and spun in a Beckman centrifuge at 7,500 g at 4°C for 3 hours. The filtrate was loaded on a Applied Biosystem HPLC column (particle size 7 mm, pore size 300 Å) in batches of up to 2 ml per injection. The peptides were eluted using the following program: flow rate, 0.4 ml/min; 0-5 mins, 0% buffer B; 5-55 mins, linear increase to 50% buffer B; 55-60 mins, increase to 90% buffer B; 60-65 mins, decrease to 0% buffer B. 0.4 ml fractions were collected. 30 µl per fraction was aliquoted, dried by vacuum centrifugation, dissolved in 30 µl PBS and sonicated in water bath. These samples were used for CTL assays. Rest of the samples were stored at -20°C in the original eluting buffer before they were used for rechromatography.

5.3.6. Rechromatography of subfractionated CTL recognised mH peptides.

Fractions containing activity were rechromatographed by HPLC system (HEWLETT-PACKARD 1090, ABI) with diode array detection. Micropore columns 1.0 x 100 mm, aquopore OD300 (C18) packing were employed to further purify and concentrate active peptides prior to sequence analysis. Buffer A: 30 mM sodium acetate, pH 5.5; Buffer B: 35 mM sodium acetate, pH 5.5; 80% acetonitrile. For elution a linear gradient of 0-40% over 40 mins was employed and flow rate was set at 50 µl/min. 25 µl fractions were collected automatically. Real time monitoring of the fraction collector and U.V. detector was recorded on a flatbed chart recorder (at 214 nm).

5.3.7. CTL assays.

General procedures of CTL assays were described in general materials and methods. For detecting CTL activities of HPLC fractions,

usually 10 μ l peptide solution from each fraction was added into 96 U-bottomed plates followed by adding ^{51}Cr -labeled targets 5×10^3 per well in a volume of 50 μ l. The plates were incubated for 60 min at 37°C, 5% CO_2 . Then CTL were added to give a total volume of 200 μ l per well followed by culture for 5 hours before the supernatants were harvested. For peptide pulsing, RMA-S were temperature induced by preculturing at 25°C overnight. No special treatment for other cell lines.

5.4. RESULTS.

5.4.1. mH antigen-specific CTL responses *in vitro*.

The study focused on four mouse strains, BALB.B, B6.C-H-1^b, B6.C-H-25^c and C57BL/6BY. All of them express H-2^b haplotype but differ at mH genes (Table 5.1).

Table 5.1. mH antigens of mouse strains used to generate CTL

RESPONDER STRAIN				STIMULATOR STRAIN				mH difference
Name	H-1	H-25	mH background	Name	H-1	H-25	mH background	
B6.C-H-1 ^b	b	b	B6	C57BL/6BY	c	b	B6	H-1
B6.C-H-1 ^b	b	b	B6	B6.C.-H.25 ^c	c	c	B6	H-1+H-25
BALB.B	b	c	BALB	C57BL/6BY	c	b	B6	H-1+H-25+ multiple H

mH antigen-specific CTL were induced against one (H-1), two (H-1+H-25) or multiple mH antigenic differences by immunising mice and restimulating the immunised spleen cells *in vitro* with antigenic cells. CTL activities were analysed on day 5 after restimulation. The three CTL bulk lines, anti-H-1, anti-H-1/H-25 and anti-multiple mH antigen CTL, recognised Con-A blast targets derived from mice bearing the mH antigen used for immunisation but not the syngeneic spleen cells. These CTL lines also lysed RMA cells which are of H-2^b origin, indicating these cells express the related mH antigens. However, none of the CTL lines lysed the mutant RMA-S cells (Fig.5.1). More than twenty mH antigen-specific CTL clones were established from bulk anti-H-1 and anti-H-1/H-25 CTL lines. These clones had the same antigen recognition pattern as the bulk lines. A representative experiment is shown in Fig.5.1C.

Antibody blocking assays showed that anti-CD8 but not anti-CD4 antibodies inhibited the lysis mediated by mH antigen-specific CTL in a concentration dependent fashion (Fig.5.2). Cytofluorimetric analysis revealed that mH antigen-specific CTL lines and clones were CD8⁺ CD4⁻ (not shown).

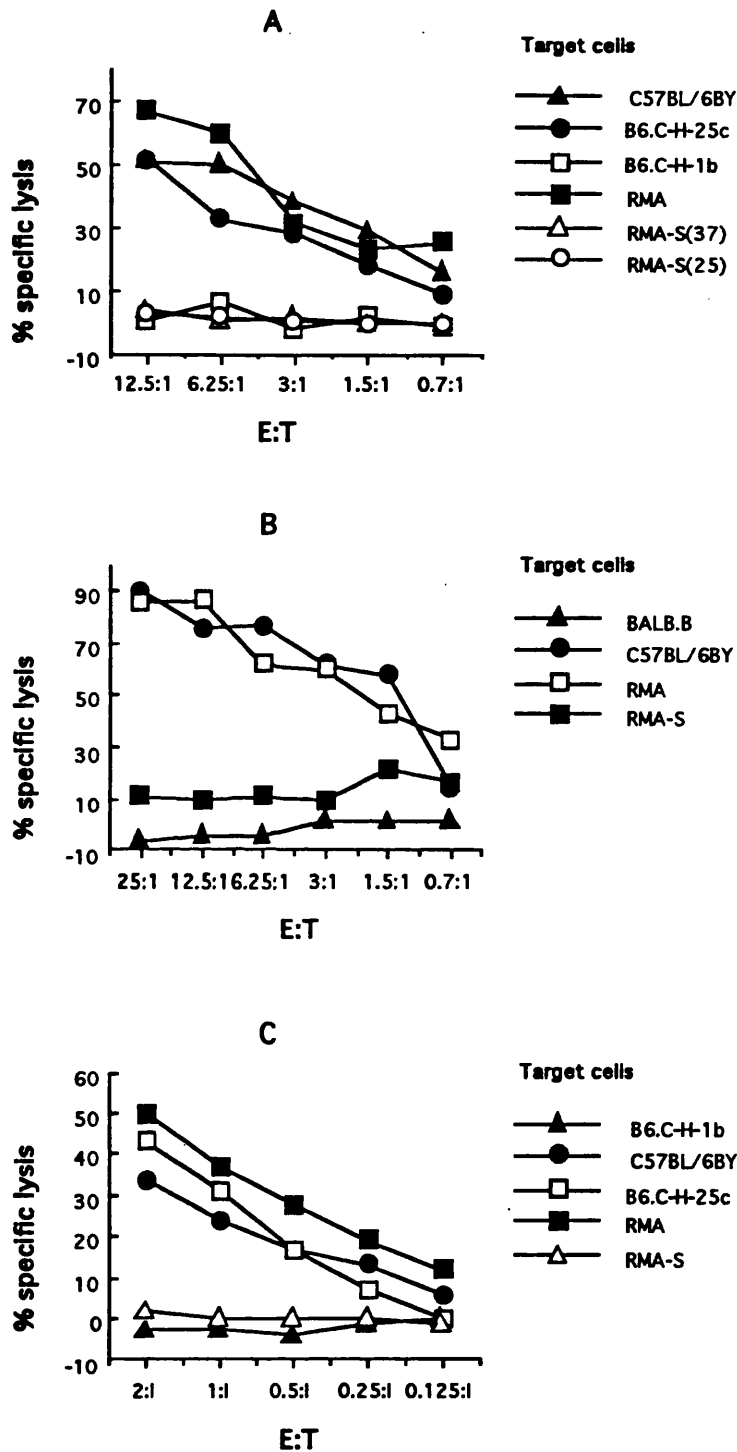


Fig.5.1. Specificity of anti-mH antigen CTL bulk lines and clones. Anti-H-1/H-25 CTL (Fig.A) and anti-multiple mH antigen CTL (Fig.B) were assayed on day 5 after first *in vitro* restimulation. The CTL clone was established from an anti-H-1/H-25 CTL bulk line and assayed on day 6 after restimulation (Fig.C). Target cells B6.C-H-1^b, B6.C-H-25^c, BALB.B and C57BL/6BY were splenic Con-A blasts. RMA and RMA-S(37) were cultured at 37°C before labeling. RMA-S(25) were shifted to 25°C one day prior to the CTL assay. Anti-H-1 CTL showed the same specificities as anti-H-1/H-25 CTL lines (not shown).

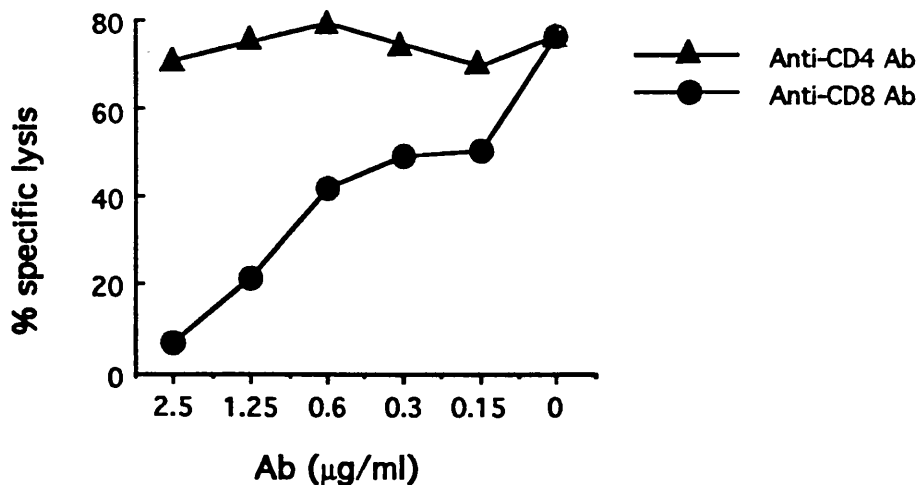


Fig.5.2. Phenotype of anti-H-1/25 CTL. The activity of an anti-H-1/H-25 CTL line was tested in a standard 4 hour ^{51}Cr release assay using RMA as target cells in the absence or presence of indicated concentrations of anti-CD8 (YTS169V) mAb or anti-CD4 (GK1.5) mAb. E:T=20:1.

It is important that none of the mH antigen-specific CTL lines or clones lysed RMA-S and even the low temperature induced RMA-S which have increased expression of MHC class I molecules were not lysed (Fig.5.1). Since RMA-S were shown to present efficiently exogenously added peptides (Townsend, et al., 1989b), this allowed us to use them for CTL analysis of HPLC separated peptide material.

5.4.2. Recognition of HPLC fractions by mH antigen-specific CTL.

Using the TFA extraction technique natural peptides were isolated from whole spleen cells. Low molecular mass material was separated by gel filtration or centricon filters and then separated further by reversed-phase HPLC. A typical peptide elution profile is shown in Fig.5.3A. 50 individual fractions were collected from HPLC separation and analysed with mH antigen-specific CTL by pulsing RMA-S targets in CTL assays. To avoid outgrowth of dominant CTL clones, short term bulk CTL lines (after one or two times restimulation *in vitro*) were used for these experiments. When peptides extracted from C57BL/6BY were tested, it was found that anti-multiple mH antigen CTL recognised the HPLC fraction 20 and fraction 27 among 50 fractions (Fig.5.3B). Anti-H-1 or anti-H-1/H-25 CTL only recognised peptides present in fraction 27 (Fig. 5.4A).

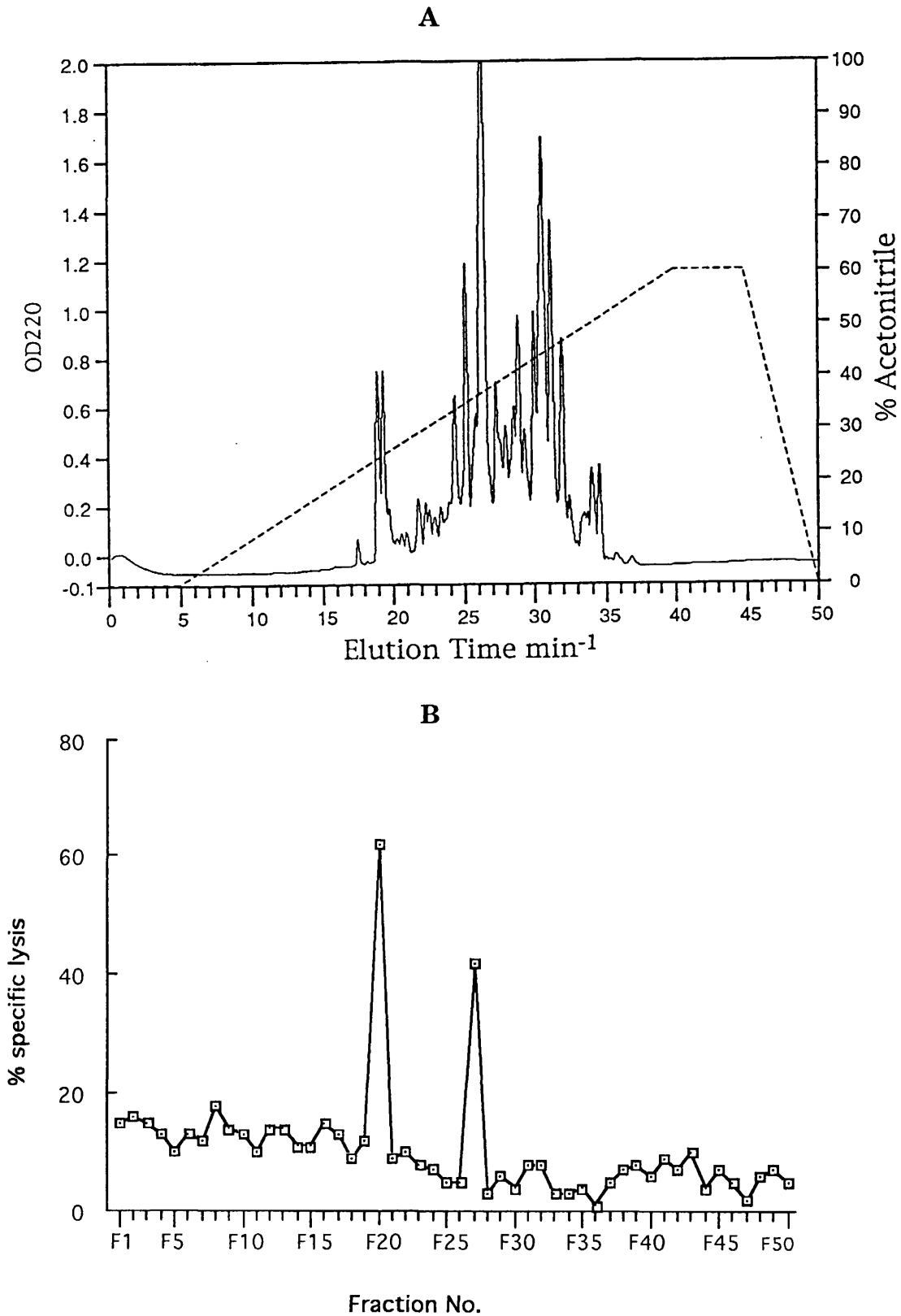


Fig.5.3. HPLC peptide elution profile and CTL recognition of peptides isolated from C57BL/6BY spleen cells. Peptides were isolated from C57BL/6BY spleen cells by acid extraction, separated by HPLC and 50 fractions were collected. The elution profile is shown in Fig.A. HPLC fractions were dried down and resuspended in 300 μ l PBS and 10 μ l was used to pulse temperature induced, 51 Cr-labeled RMA-S for 1 hour. Peptides pulsed target cells were cultured with anti-multiple mH antigen CTL for 5 hours at E:T ratio of 5:1 (Fig.B).

To test the influence of MHC and mH genes on cellular peptide composition, peptides were extracted from BALB.B (H-2^b) and B10.BR (H-2^k) spleen cells. BALB.B has the same H-2 genes as C57BL/6BY but different mH genes; in contrast, B10.BR has the same mH genes as C57BL/6BY but different H-2 genes. The results showed that neither BALB.B nor B10.BR cells contained the peptides recognised by anti-H-1 or anti-H-1/H-25 or anti-multiple mH antigen CTL (Fig.5.4). This indicated that the presence of mH antigen peptides were dependent on both H-2 and mH genes.

5.4.3. MHC class I restricted mH peptide presentation.

To determine the MHC class I restriction element, P1HTR (H-2^d) and P1HTRK^b, P1HTRD^b transfectant cells were used as APC to present C57BL/6BY derived peptides to mH antigen-specific CTL lines. As expected, MHC mismatched P1HTR did not present the positive peptide to anti-H-1 (not shown) or anti-H-1/H-25 or anti-multiple mH antigen CTL lines (Fig.5.5). P1HTRK^b but not P1HTRD^b were sensitised to lysis by the peptide present in HPLC fraction 26/27, indicating that CTL recognition of the peptides in this fraction was K^b restricted (Fig.5.5). Furthermore, peptides were extracted from K^b molecules which were purified from C57BL/6BY spleen cells by an immunoaffinity column. The CTL recognition of peptide fractions derived from whole cell lysates and immunoaffinity purified class I molecules were compared. Only CTL epitope present in fraction 27 but not fraction 20 of whole cell lysates were detected in peptide preparation of purified K^b molecules (Fig.5.6), further confirming that peptides in fraction 27 were K^b restricted.

The peptide present in HPLC fraction 20 could not be presented by P1HTRK^b, thus this epitope was probably D^b restricted. However, using P1HTRD^b to map the restriction element was not successful since this cell line was lysed by anti-multiple mH antigen CTL in the absence of mH peptides (Fig.5.7). The interpretation would be that P1HTR contain the relevant mH genes encoding mH antigens and transfection of D^b molecules led to the expression of D^b restricted mH peptides.

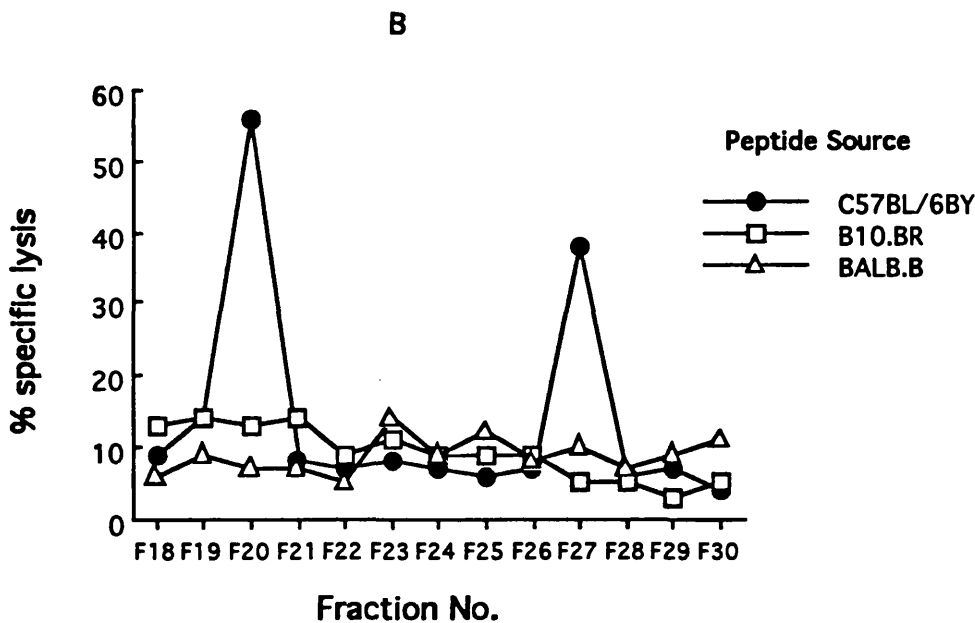
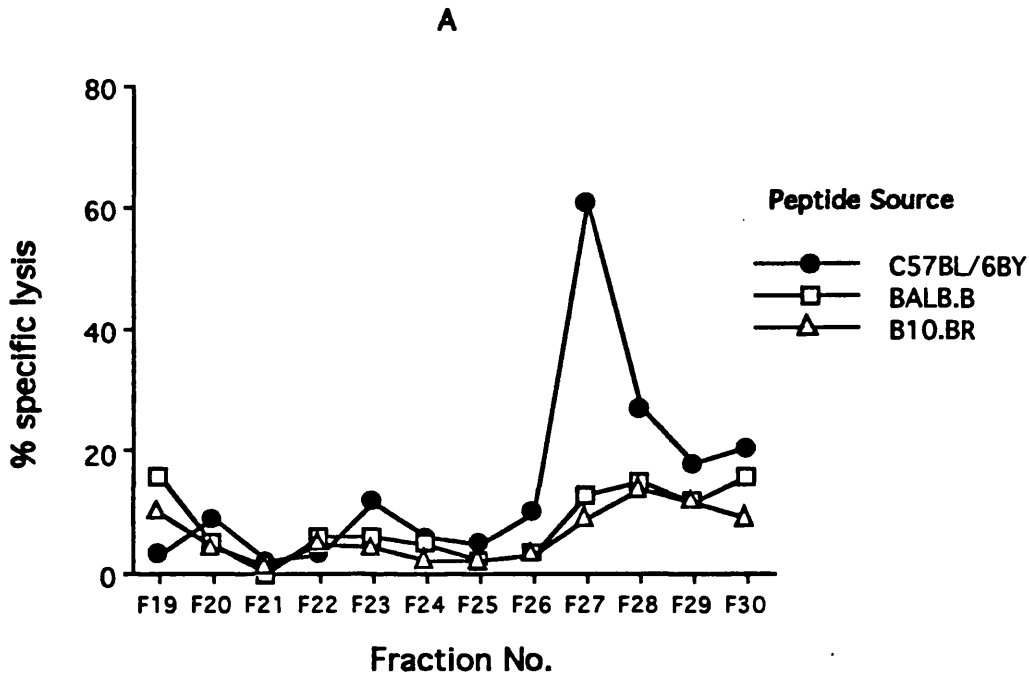


Fig.5.4. CTL recognition of peptides isolated from C57BL/6BY, B10.BR and BALB.B spleen cells. Peptides were isolated from C57BL/6BY, B10.BR and BALB.B spleen cells by acid extraction, separated by HPLC. Peptide fractions were resuspended in 300 μ l PBS and 10 μ l was used to pulse temperature induced, chromium labeled RMA-S for 1 hour. Peptides pulsed target cells were cultured with anti-H-1/H-25 CTL (Fig. A) or anti-multiple mH CTL (Fig. B) for 5 hours at an E:T ratios of 10:1 (A) and 4:1 (B). Fractions 1-19 and 30-50 did not contain any CTL recognised peptides (not shown). Anti-H1 CTL recognised the same fraction as anti-H-1/H-25 CTL (Fig. A).

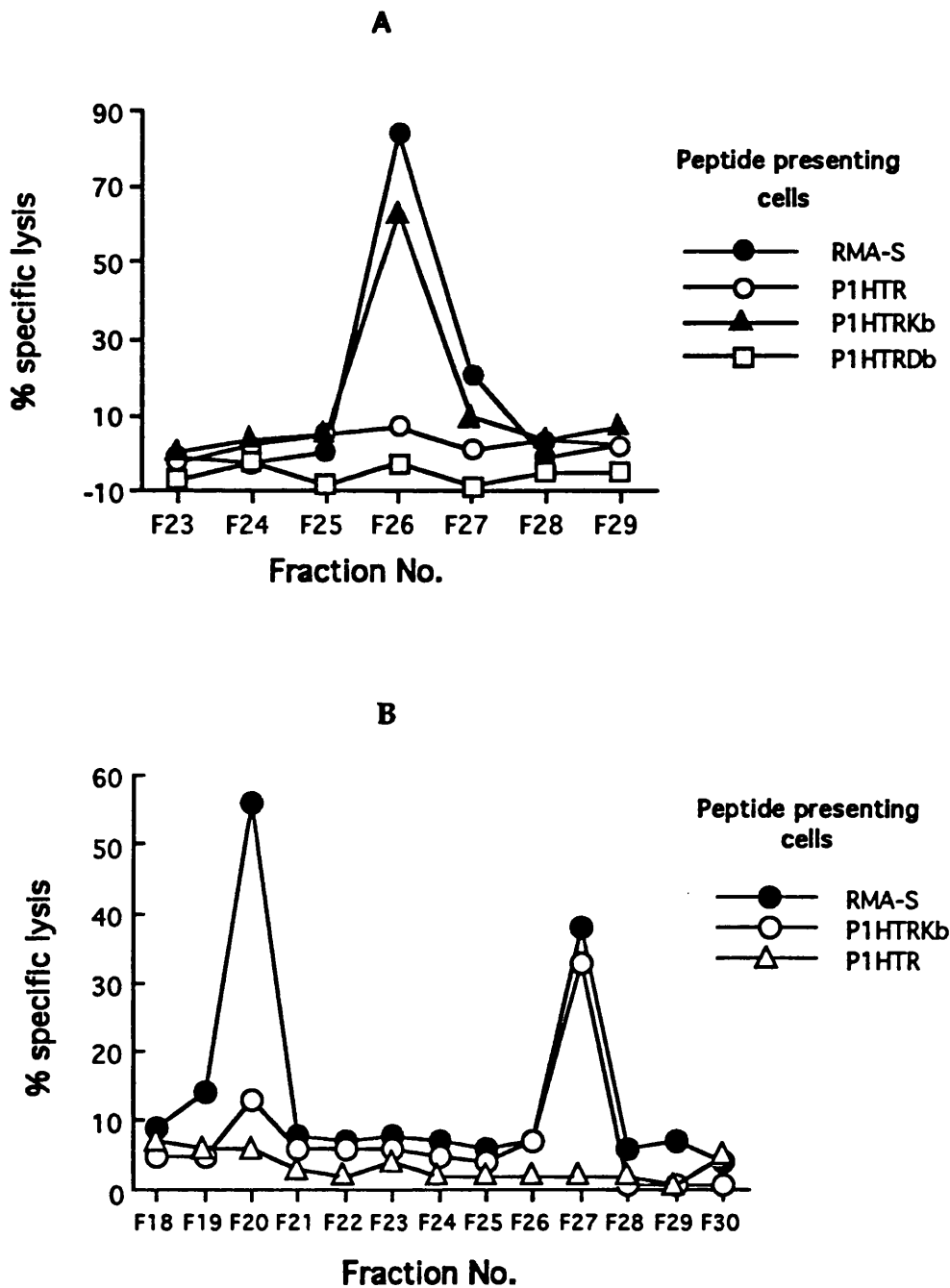


Fig.5.5. H-2 K^b and D^b restricted CTL recognition of HPLC purified peptide fractions. Peptide fractions purified from C57BL/6BY spleen cells were used to pulse indicated ⁵¹Cr labeled target cells for 1 hour. RMA-S were temperature induced. Peptides pulsed target cells were cultured with anti-H-1/H-25 CTL (Fig.A) or anti-multiple mH antigen CTL (Fig.B) for 5 hours at E:T ratios of 5:1 and 4:1 respectively. In Fig.A the positive peptide were eluted in fraction 26, resulted from experimental deviation.

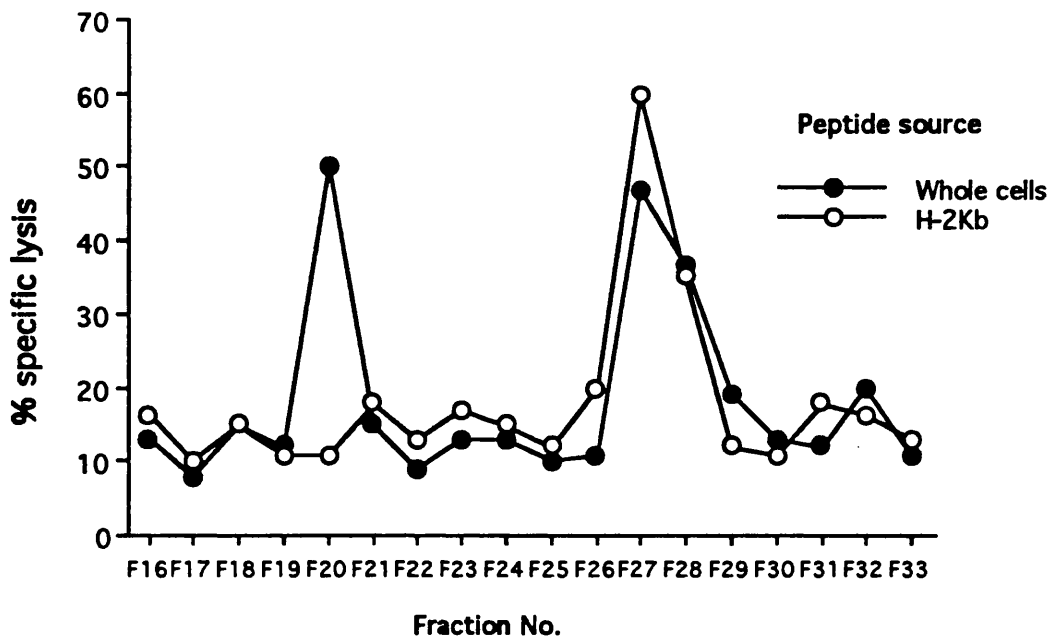


Fig.5.6. CTL recognition of mH peptides extracted from whole cells and H-2K^b class I molecules. Peptides were isolated from either whole spleen cells of C57BL/6BY or H-2K^b class I molecules purified from C57BL/6BY spleens. RMA-S pulsed with indicated peptide fractions were cultured with anti-multiple mH antigen CTL for 5 hours at E:T ratio of 16:1.

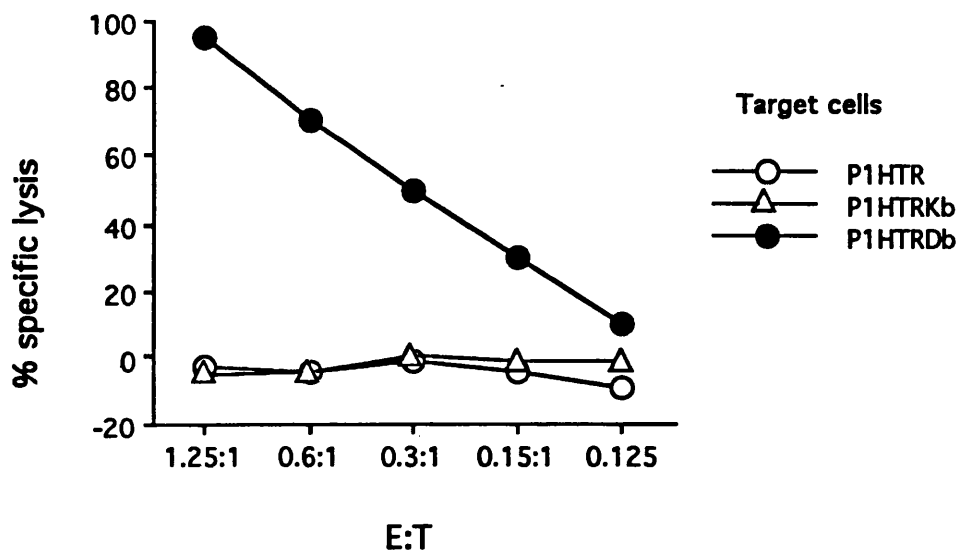


Fig.5.7. CTL recognition of P1HTR and its transfectants. Indicated target cells were lysed by anti-multiple mH antigen CTL in the absence of peptides in a standard CTL assay.

Trying to demonstrate that peptides in fraction 20 were presented by D^b molecules, we purified peptides from spleen cells of recombinant mice: B10.A(4R) mice which express K^k , D^b and B10.A(5R) mice which express K^b , D^d and L^d (Lyon and Searle, 1990). The CTL recognition showed that the D^b expressing mice contained CTL epitope present in fraction 20 while K^b expressing mice contained CTL epitope present in fraction 26/27 (Fig.5.8). Therefore, the peptides present in fraction 20 and 26/27 were restricted by D^b and K^b molecules respectively.

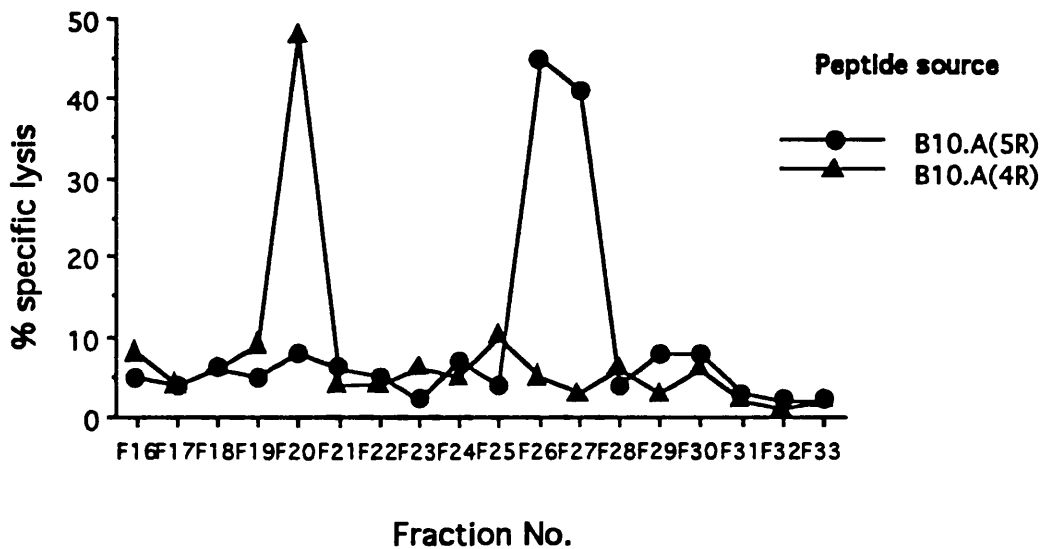


Fig.5.8. CTL recognition of mH peptides extracted from recombinant mice. Peptides were isolated from spleen cells of B10.A(4R) or B10.A(5R) mice. HPLC separated peptide fractions were used to pulse temperature induced RMA-S target. The target cells were lysed with anti-multiple mH antigen CTL at E:T ratio of 4:1.

5.4.4. H-1 locus encoded peptide epitope.

Since anti-H-1, anti-H-1/H-25 and anti-multiple mH antigen CTL recognised the same HPLC fraction 27, it was possible that H-1 derived peptide accounted for the CTL recognition by the three different lines. To test this possibility, peptides were extracted from H-1 mismatched mice B6.C-H-1^b (Table 5.1) and used to pulse target cells. As shown in Fig.5.9, B6.C-H-1^b mice did not contain the positive peptide which is present in HPLC fraction 26 of C57BL/6BY derived peptide preparation. Anti-H-1 or anti-H-1/H-25 or anti-multiple mH antigen CTL lysed target cells pulsed with fraction 26 peptide extracted from C57BL/6BY but not from B6.C-H-

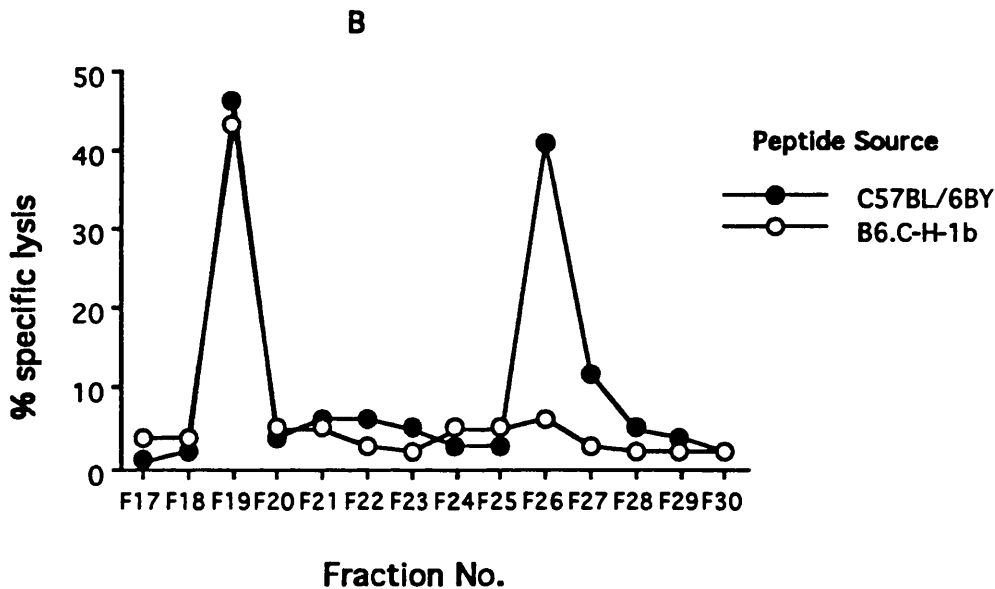
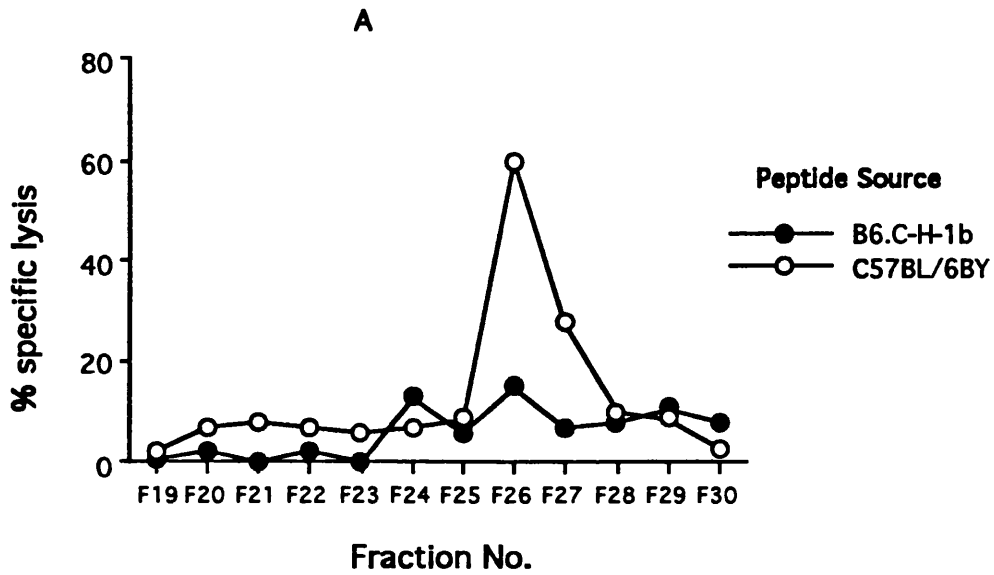


Fig.5.9. H-1 mismatched cells (B6.C-H-1^b) do not provide the peptides recognised by anti-H-1/H-25 and anti-multiple mH antigen CTL. Peptides isolated from B6.C-H-1^b or C57BL/6BY spleen cells were used to pulse ⁵¹Cr labelled RMA-S cells and recognised by anti-H-1/H-25 (Fig.A) or anti-multiple mH antigen CTL (Fig.B). CTL recognised peptides of fraction 26 are missing in preparations from B6.C-H-1b cells while peptides eluting in fraction 19 are present (Fig.B).

1^b. Therefore, all the three CTL lines recognised the same peptide, i.e. H-1 encoded epitope. As a control, B6.C-H-1^b still contained the positive peptide present in HPLC fraction 19, demonstrating that this peptide was not derived from the H-1 locus.

5.4.5. Subfractionation of a positive peptide fraction.

Our ambition was to sequence one of the mH antigenic peptides recognised by mH antigen-specific CTL. To achieve microsequence, peptides need to be highly pure and highly enriched in quantity (minimum 3-5 picomolar peptide was needed to get sequence by Edman degradation). To accumulate sufficient amounts of peptides, a modified purification procedure was developed (protocol 2, see materials and methods in this section). The advantages of this procedure are: 1) less time consuming, can be finished within one day; 2) less loss of peptide activity, since there is no drying down step such as lyophilization. Using this procedure, peptides were purified from total 216 mice. Both spleens and thymi were used for peptide purification, since the peptides extracted from spleens and thymi had the same CTL recognition pattern (not shown). Also, four strains of mice were used, C57BL, C57BL/10, C57BL/6BY and B6.C-H-25^c and all of them contained the active peptides being studied (the reason was that we could not get enough supply of a single mouse strain).

To avoid HPLC overloading, peptides were prepared in batches maximum using 12 mice. Every peptide preparation was screened by CTL assays. Positive fractions representing the epitope in fractions 19/20 were pooled together from 4 batches and then passed through centricon 3 to remove peptides larger than 3kDa. The filtrates were subjected to another HPLC column which had higher resolving capacity. Fig.5.10A shows that the original HPLC fraction resolved into 6 or 7 distinct peptide peaks. The CTL assays showed that CTL recognised mH peptides were present in one HPLC subfraction, suggesting that few (possibly one) peptides were recognised by bulk CTL populations (Fig.5.10B). Using similar approach, HPLC fractions 26/27 were also subfractionated and tested. Only one subfraction was found to be recognised by CTL (not shown).

The subfractionated HPLC fractions containing the CTL recognised mH peptide epitopes were rechromatographed by a more sensitive liquid chromatograph system (see materials and methods). So far, we have tried

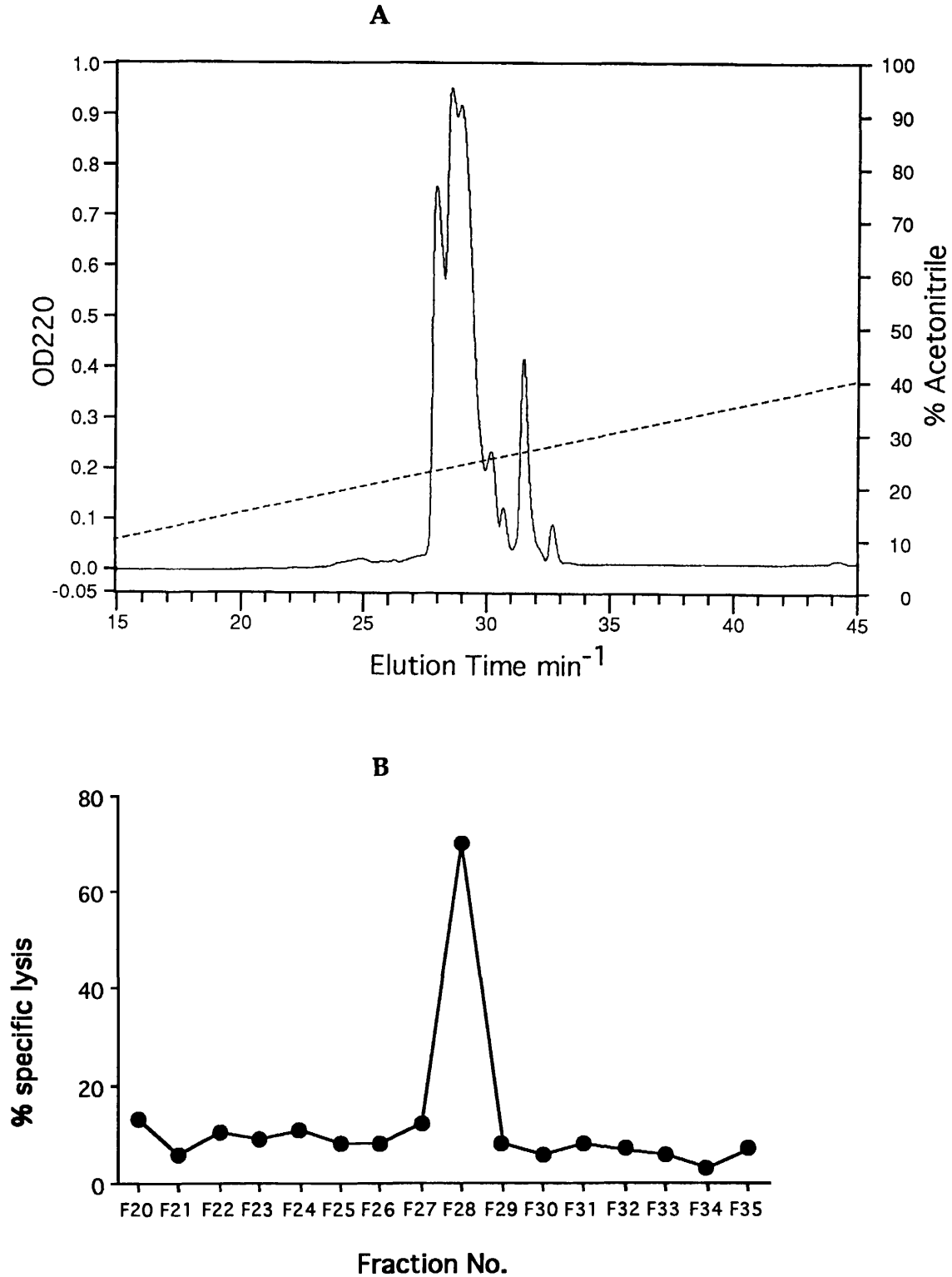


Fig.5.10. Subfractionation of peptides and CTL recognition of collected subfractions. Fractions 19/20 of HPLC separated C57BL/6BY peptides, which contained CTL recognised peptides, were subfractionated as described in Materials and Methods and the peptide elution profile is shown in Fig.A. Collected subfractions were used to pulse temperature induced RMA-S and recognised by anti-multiple mH antigen CTL (Fig.B).

to rechromatograph some portions of the positive HPLC subfractions for several times. However, none of these rechromatographed peptide fractions could sensitise target lysis by mH antigen-specific CTL (not shown). Presently, we are trying to pool all the positive subfractions together and then rechromatograph them. Hopefully we can still trace the CTL epitope and then sequence it by Edman degradation.

5.5. DISCUSSION.

The results presented here are in agreement with several reports demonstrating that mH antigens are peptides derived from cellular proteins and presented by MHC molecules (Wallny and Rammensee, 1990; Rotzschke, et al., 1990b; Loveland, et al., 1990; Sekimata, et al., 1992). This study identified two immunodominant peptide epitopes recognised by mH antigen-specific CTL, which were present in HPLC fraction 20 and 26/27 respectively. Using genetically defined mice, one peptide epitope was mapped to the H-1 locus while the genetic origin of the second peptide epitope was unknown.

We found that the expression of the mH antigenic peptides was dependent on the presence of appropriate H-2 and mH genes, since they were only extracted from the cells expressing both genes. Analysing the expression pattern of H-4^b, H-Y and an unmapped BALB.B mH antigen, called mapki, Rammensee's group had similar finding that the expression of mH antigenic peptides but not mH antigenic proteins was dependent on coexpression of the restricting MHC class I molecules (Falk, et al., 1990; Griem, et al., 1991). These results indicate that MHC molecules are directly involved in the processing of mH antigens and the binding of mH antigenic peptides to class I molecules is allele-specific. In Falk et al's report, H-4^b mH antigen was found to also contain another MHC-independent peptide which was isolated from several strains of mice expressing different H-2. It was speculated that this MHC-independent peptide was a larger precursor molecule from which the smaller, MHC-dependent, peptide was derived upon interaction with MHC molecules (Falk, et al., 1990).

Cellular studies have shown that one mH locus includes at least two genes which encode CTL and T_H epitopes respectively (Roopenian, 1992). Now we have brought the analysis of mH loci encoded products to the peptide level. Using sequential HPLC separation of both H-1 derived peptide and an undefined mH locus derived peptide it was found that one mH locus probably encodes no more than one CTL epitope. This is in agreement with the molecular analysis of the mH antigen Mta, which was shown to be a single peptide derived from the N terminus of a mitochondrial protein (Loveland, et al., 1990). Other mH loci, e.g. H-Y and mapki, which have been analysed by using HPLC separation appeared to encode single peptide epitopes as well (Rotzschke, et al., 1990b). However,

since these studies have used long term CTL lines or CTL clones rather than short term bulk CTL populations the number of potential peptide epitopes derived from one mH locus remained unclear. In the case of H-4, it was found that two distinct peptide fractions, one was MHC-dependent and another was MHC-independent, could sensitise for CTL lysis. However, as described above, they might represent one common peptide epitope (Falk, et al., 1990). Further analysis is needed to determine the number of CTL epitope encoded by each mH locus.

The phenomenon of immunodominance in anti-mH antigen CTL responses has been very well documented for a long time (see general introduction). We found that a H-1 locus derived peptide epitope was immunodominant in H-2^b mice. In anti-H-1/H-25 CTL responses, the H-1 derived peptide was the only detectable antigen. The antigenicity of H-25 locus was uncovered in the absence of dominant H-1 locus since we obtained anti-H-25 CTL when this locus was the only difference between responder and stimulator cells (not shown). This indicates that H-25 derived peptide epitopes do exist and that the T cell repertoire does contain antigen receptors to recognise them. In the CTL responses of BALB.B mice to potentially at least 29 mH differences (Bailey and Mobraaten, 1969) of C57BL/6 stimulator cells, only two dominant peptide epitopes were detected and one was H-1 locus derived, another was genetically undefined. This correlates with Wettstein's report that a maximum of only two antigens could be detected preferentially in anti-multiple mH antigen CTL responses (Wettstein, 1986a).

In accordance with our results that H-1^c was immunodominant mH antigen, it has been shown that H-1^c was most efficient in mediating tissue transplant rejection among 10 distinct mH antigens (Graff, et al., 1966). Also, *in vitro* CTL studies showed that H-1^c dominated over H-Y, H-25^c and H-7^a in H-2^b mice (Loveland and Simpson, 1986).

Immunodominance is a general property of T cell responses and is more pronounced in CTL responses than T_H responses (Berzofsky, 1991; Yewdell and Bennink, 1990). There are many possible mechanisms accounting for immunodominance. What are possible mechanisms for the immunodominance observed in this study? One mechanism could be competition of antigen presentation. Probably the two dominant peptide epitopes have higher affinity to bind MHC class I molecules and thus result in a higher epitope density in comparison to other dominated mH locus derived peptides. Subsequently these peptides with higher epitope

density stimulated dominant T cell responses. Epitope density may also depend on the expression levels of mH genes which are presently unknown. Antigen competition accounting for immunodominance has been suggested by Wettstein who showed that immunodominance could only be observed when the dominant and dominated mH antigens were presented on the same stimulating cells (Wettstein, 1986a). The second mechanism probably is that the T cell repertoire of H-2^b mice may recognise the two dominant peptide epitopes more efficiently than other dominated peptides, resulting in preferential stimulation of CTL against dominant peptides.

It is interesting that the two immunodominant CTL epitopes were rationally allocated, one was restricted by H-2K^b and another by H-2D^b molecules. This suggests that firstly one MHC class I allele predominantly presents one immunogenic peptide which has highest binding affinity; secondly T cells focus their attention to only one epitope presented by one MHC class I allele.

We have not yet reached the final goal--sequencing one CTL epitope encoded by a mH gene. Sequencing T cell epitopes of mH antigens by using acid elution and HPLC separation techniques has been proved extremely difficult (Rotzschke, personal communication; de Bueger, et al., 1993). There are two possible reasons accounting for the difficulty. Firstly, most of the mH antigenic peptides are produced and presented by MHC molecules in a very low level which might be just enough to stimulate CTL responses. Although this can be overcome by using a large amount of starting material, that means more irrelevant material is included and more purification steps are needed. Secondly, this biochemical procedure is not ideal for the purification of mH antigenic peptides. We could not detect any positive peptides after rechromatographing portions of the HPLC subfractions. The loss of activities might be caused by physical loss of peptides or modification of peptides such as oxidation during HPLC purification (Hsuan, personal communication). The purer the peptides were, the more significant these physical and chemical losses became. Using larger amount of materials for rechromatography probably can keep the peptides at certain concentration that enable us to get the peptide sequence.

CHAPTER 6. GENERAL DISCUSSION AND FUTURE PERSPECTIVE

This thesis explored the possibility of using synthetic Ras peptides to stimulate specific T_H and CTL responses against an oncogenic mutation at position 12 of p21 Ras proteins (Chapter 3 and 4). Clearly in some mouse strains mutant Ras peptide could induce T_H cells which specifically recognised the mutant peptide and protein. These results suggested that under certain circumstances tumour related a mutation in p21Ras protein could be immunogenic and probably mutant Ras peptides could be used to boost mutation-specific immune responses. Recently several research groups have reported similar findings (Jung and Schluesener, 1991; Peace, et al., 1991; Gedde-Dahl, et al., 1992a; Gedde-Dahl, et al., 1992b). One distinct point we made in this study was that mutant Ras peptide induced T_H cells in H-2^b mice also recognised normal Ras peptide. Since we did not analyse many mouse strains, it is difficult to tell how frequently T_H cells cross react to mutant and normal Ras peptides. However, it is a reasonable thinking that peptide vaccine should be designed according to each MHC haplotype.

The analysis of CTL responses against synthetic Ras peptides brought us into a different field from our initial interest which was to find a target for tumour immunotherapy. In three mouse strains analysed we did not get CTL which specifically recognised glycine to valine mutation at position 12 of Ras peptides. However, Ras peptides induced CTL showed very interesting features in that they recognised Ras peptides in a MHC unrestricted fashion. Unfortunately we have not managed to identify the real epitope recognised by these CTL. It will be very interesting to know what the size of this special CTL epitope is. It is possible that these MHC unrestricted CTL recognised 8 or 9 mer peptides like most of classical MHC restricted CTL do. Alternatively they might recognise longer peptides as we showed in one experiment that 18 mer Ras peptide could not sensitise CTL lysis. Presently we are trying to identify the nature of the minor components responsible for MHC unrestricted CTL responses. Identification of the peptide sequence would help us to understand the mechanism of MHC unrestricted CTL recognition.

Once the Ras CTL epitope has been identified, it would be interesting to determine: 1) do MHC class I molecules bind the Ras peptides in a similar way as classical peptide antigens, i.e. binding the peptides in the groove? This can possibly be addressed by peptide competition assays. We had some primary data showing that the CTL

recognition of Ras peptides could not be inhibited by a T cell epitope with a high affinity for binding to class I molecules. II) which domain of class I molecules is critical for binding Ras peptides? This can be answered by using cells transfected with chimeric class I molecules as APC to present Ras peptides. III) which part of TCR is involved in the CTL recognition of Ras peptides? More anti-TCR mAb should be used to analyse extensively the TCR usage of long term Ras peptide-specific CTL lines and clones. Also, anti-TCR mAb can be applied to block the CTL recognition in CTL assays.

Using synthetic peptides, especially longer peptides, for T cell epitope mapping can be very misleading. The lesson we learned was that to be sure with the T cell epitope being analysed, HPLC separated fractions of the synthetic peptides should be tested. Attention should also be paid to peptide synthesis. As shown in our study independent synthesised peptides can be biologically different.

There are a lot of questions which need to be answered about mH antigens mediated graft rejection and GVHD. T cell recognition of HPLC purified cellular peptides is a very useful tool which can help us to understand the molecular biology of mH antigens. The number of immunodominant mH peptides can be revealed by using this technique. We did not analyse the peptide recognition by mH antigen-specific T_H cells in this study. According to Roopenian's proposal one mH locus consists of at least two genes encoding T_H and CTL epitopes independently (Roopenian, 1992). Most likely the two different epitopes could be separated by a HPLC column with high resolution capacity. It will be interesting to compare the peptide recognition patterns of mH antigen-specific T_H cells and CTL.

Using HPLC purification technique to achieve microsequence of mH peptides definitely is not an easy job. Although we used more than 200 mice, we have not yet succeeded to obtain the microsequence. To reach this goal, we probably need to use better equipments which can minimise the loss of material and maximise the sensitivities of detection.

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