Recognition of antigen and superantigen by cytotoxic T lymphocytes

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A thesis submitted to the University of Oxford in partial fulfilment of the requirements for the degree of Doctor of Philosophy



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Trinity term 1993

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Abbreviations

APS	Ammonium persulphate
AS	Ankylosing Spondylitis
BCL	Human B lymphoblastoid cell line
bp	Base pair
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	Complementary DNA
CDR	Complementarity determining region
CDTa	*Clostridium difficile* toxin a
Ci	Curie
CPET	*Clostridium perfringens* enterotoxin
CTL	Cytotoxic T Lymphocyte
DNA	Deoxyribonucleic acid
EAE	Experimental Allergic Encephalomyelitis
EBV	Epstein Barr Virus
ERA	Enteropathic Reactive Arthritis
FACS	Fluorescence activated cell sorter
FCS	Fetal bovine serum
5-HT	5-Hydroxytryptamine
H-2	Murine histocompatibility system
HIV	Human Immunodeficiency Virus 1
HLA	Human Leukocyte Antigen
HPLC	High Pressure Liquid Chromatography
Ig	Immunogloblin
IL, IL-2R	Interleukin, Interleukin 2 Receptor
kb	Kilobase
kD	Kilo Dalton
LCMV	Lymphocytic Choriomeningitis Virus
M, mM, nM	Molar, millimolar, nanomalor
mAb	Monoclonal Antibody
MBP	Myelin Basic Protein
MCC	Moth Cytochrome C
MHC	Major Histocompatibility Complex
MIs	Minor lymphocyte stimulant
mmtv	Mouse Mammary Tumour Virus
mRNA	messenger RNA
NP	(Influenza A) Nucleoprotein

PAGE	Polyacrilamide Gel Electrophoresis
PBL	Peripheral blood leukocyte
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutinin
PPD	Purifid Protein Derivative
RBL	Rat Basophil Leukemia cell line 2H3
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
SA	Superantigen
SARA	Sexually Acquired Reactive Arthritis
SDS	Sodium Dodecyl Sulphate
SE (A-E)	Staphyloccal Enterotoxin (A-E)
ß2M	(Human) beta 2 Microglobulin
TCR	T Cell Receptor
TSST	(Staphyloccal) Toxic Shock Syndrome Toxin 1

Abbreviations of amino acids

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
I	Isoleucine
H	Histidine
L	Leucine
K	Lysine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

Recognition of antigen and superantigen by cytotoxic T lymphocytes

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DPhil thesis abstract Trinity term 1993

Human cytotoxic T lymphocytes (CTL) recognize antigen in the form of short peptide fragments presented by Human Leukocyte Antigen (HLA) class 1 molecules. The HLA B27-restricted CTL response to Influenza A virus is directed against a single nine amino acid epitope within the viral nucleoprotein NP383-391.

Influenza A-specific CTL lines and clones, generated from the peripheral blood of three unrelated individuals, exhibited remarkable fine specificity for NP383-391, failing to recognize synthetic variants containing single amino acid substitutions at positions 1, 4, 7 or 8. The sequences of rearranged T cell receptor (TCR) alpha and beta chain genes showed remarkable conservation of variable and joining gene segment usage, and also of non germline-encoded ß chain residues proposed to interact with peptide, even between clones from unrelated individuals. Correlation of fine specificity for peptide with TCR alpha chain usage suggested a possible orientation for the TCR/HLA/peptide ternary complex in this immune response. The finding of restricted TCR usage in the HLA B27-restricted CTL response to influenza A virus has implications for the study and potential therapy of HLA B27 associated autoimmune disease.

The TCR heterodimer from one clone was expressed in a rat basophil cell line and shown to confer specificity for HLA B27 + NP383-391.

Influenza A-specific CTL were also shown to recognize bacterial superantigens in an MHC class II dependent but unrestricted manner. A rapid screening assay for candidate superantigens was developed and used to identify a novel superantigen produced by *Clostridium perfringens*. This superantigen was found to have a unique specificity for human TCRß chains.

Acknowledgements

I would first and foremost like to thank my supervisor, Professor Andrew McMichael, for his constant support, enthusiasm and advice throughout the duration of this research. Professor John Bell has also contributed much interest and positive encouragement. I acknowledge the MRC for generously funding my training fellowship.

Particular thanks are due to Paul Moss in John Bell's laboratory for patiently teaching me anchor PCR and all the attendant molecular biological skills, and to Sarah Rowland-Jones for passing on her CTL expertise. Others who have provided practical help and advice include Marion Brown (MRC Immunochemistry Unit, Oxford) with Western blotting of RBL transfectants, Lynda Tussey, Awen Gallimore, Stephen McAdam, and Frances Gotch.

I would very much like to thank Hugh Reyburn, Ann Hill, Enzo Cerundolo, Tim Elliot and Ita Askonas for many lively immunological discussions and helpful suggestions, and for critical review of my manuscripts, thesis chapters and science in general!

Others who must be mentioned are Penny Catlin and Anne-Marie Moody for providing wonderfully competent E. Coli at critical moments, Ming Gao for help with peptide synthesis, Lazlo Pazmany for neighbourly advice, and Philip Thornton-Evison for cheerful Mycoplasma testing.

I very much enjoyed collaborating with and working alongside Tom Ottenhof in Richard Klausner's laboratory at the National Institutes of Health, Bethesda, USA.

Lastly a big thank you to my wife Gwithian for uprooting herself to Oxford and spending hours commuting on my account, and to our daughter Bryher (who has already displayed a remarkable aptitude for digesting turgid papers) for 6 months of joyous sleep deprivation — providing excellent preparation for thesis writing!

CHAPTER 1

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1.1. The specific immune system

The immune system protects an individual against infection and sometimes malignancy by recognition and elimination of non-self or altered self. The adaptive or specific immune system of higher animals is capable of recognizing with remarkable specificity and memory a huge variety of foreign substances (antigens). Specificity can be conferred by antibody (the humoral immune response) or by lymphocytes that traffic in the blood, lymph and associated organs (Gowans and Knight 1964). Two distinct lymphocyte lineages are known. B cells, so named because they undergo differentiation in the bursa of Fabricius in birds, are the precursors of antibody-forming plasma cells. T cells develop in the thymus (Miller 1961), and are responsible for the cell-mediated immune response. There are two major types of T cell — helper T lymohocytes and cytotoxic T lymphocytes (CTL). B lymphocytes, carrying on their cell surface an

immunoglobulin receptor for antigen can, after appropriate stimulation, divide and differentiate into plasma cells, which produce antibody of closely related structure and identical specificity. The specificity of the T cell receptor for antigen (TCR) is mediated by an immunoglobulin-like heterodimer of either $\alpha\beta$ or $\gamma\delta$ chains, but exhibits two major functional differences. The TCR is not secreted (with possible exceptions), and recognizes foreign antigen in the form of small peptide fragments bound to major histocompatibility (MHC) molecules. MHC moleules are highly polymorphic, and different MHC molecules bind different peptides (see below). Zingernagel and Doherty (1974) first demonstrated MHC restriction of murine T cells from mice infected with lymphocytic choriomeningitis virus (LCMV), which could only recognize and kill LCMV infected cells of the same MHC (H-2) haplotype. The same phenomenon was subsequently been widely reported in other species including man (Goulmy *et al.* 1977, McMichael 1978). However it is now clear that there are certain exceptions to the rule of MHC-restricted T cell recognition. A recently described class of bacterial- and retrovirus-derived proteins, the superantigens, appear to be recognized in a manner that is MHC-dependant without being MHC restricted (see below). Specific recognition by T cells bearing aß or $\gamma\delta$ TCR can also occur in the context of non-classical and indeed non-MHC molecules (Porcelli *et al.* 1989, Fisch *et al.* 1990), and it is also likely that the natural killer (NK) population of lymphocytes, which bear neither the $\alpha\beta$ nor the $\gamma\delta$ TCR, are able to recognize HLA gene products (Pantaleo *et al.* 1988, Colonna *et al.* 1992, Moretta *et al.* 1992).

Most circulating T lymphocytes carry either the CD4 (cluster determinant 4 — originally defined in the human by the OKT4 antibody (Reinherz *et al.* 1979)), or the CD8 cell surface glycoprotein (Reinherz *et al.* 1980). CD4 positive lymphocytes recognize antigen presented by class 2 MHC molecules (see below), providing "help" for humoral immune responses and mediating "delayed type" hypersensitivity reactions, principally through lymphokine secretion. Some CD4+ cells also exhibit

cytotoxicity restricted by class II antigens (Fleischer 1984). Possession of the CD8 molecule characterizes a population of cytotoxic T Lymphocytes (CTL) that recognize antigen in the context of class 1 MHC molecules. CD8+ lymphocytes can also mediate both delayed type hypersensitivity and suppressor functions although these phenotypes are less well defined. CTL were first identified as causing lysis of allogeneic cells *in vitro* (Cerottini and Brunner 1974), and are known to play a major role in the control of viral infection (Gardner *et al.* 1974, Askonas *et al.* 1982). CTL may be important in the control of some malignancies such as malignant melanoma, reviewed by McMichael (1992), but are also the cause of immune-mediated pathology in LCMV infection (Buchmeier *et al.* 1980).

This thesis examines the molecular basis for the antigenic specificity of human HLA class 1-restricted cytotoxic T lymphocytes. Much of the work concerns a model immune response, the HLA B27-restricted CTL response to Influenza A. An HLA B27-restricted response was studied because HLA B27 is strongly associated with a number of autoimmune rheumatic diseases, particularly ankylosing spondylitis (AS) and the sexually acquired (SARA) and enteropathic (ERA) reactive arthritides (Brewerton *et al.* 1973, Brewerton *et al.* 1974). Whilst a number of explanations for this association have been put forward (see section 1.6), application of Okkam's razor would suggest that understanding the immune function of HLA B27 might also explain its autoimmune function. Thus it is possible that these diseases are mediated by autoimmune HLA B27-restricted CTL specific for as yet unidentified peptide(s). Recently Hermann *et al.* (1993) have demonstrated that *Yersinia* and *Salmonella*-specific HLA B27-restricted CTL specific are present in the joints of patients with reactive arthritis. At the start of this study the only HLA B27-restricted responses for which the peptide epitopes were known were directed against the nucleoprotein (NP) of Influenza A virus and the gag protein of the human immunodeficiency virus HIV1, and so these responses were studied. A detailed analysis of the TCR usage of CTL

recognizing these antigens together with HLA B27 was undertaken for two reasons. Firstly it was hoped that an understanding of TCR usage in HLA B27-restricted responses would give further information about the immune function of HLA B27 and its possible role in autoimmunity. Secondly since the peptide and presenting HLA molecule had been clearly defined at the molecular level, characterisation of the TCR would give perhaps the most comprehensive picture of a ternary complex thus far obtained, and would thus serve as a model of TCR recognition of antigen.

This thesis also examines the recognition of superantigens (SA) by CTL. Although SA had been shown to require MHC class 2 products for presentation to T cells, at the start of this work a single report existed suggesting that CTL might interact with SA (Fleischer and Schrezenmeier 1988). Furthermore since one group of enteric human pathogens, the *Staphylococci*, had been shown to produce SA, a possible mechanism by which enteric bacteria such as *Salmonella* might induce ERA could be by SA production. Such a SA might, for example, have affinity for the TCRs of HLA B27-restricted autoreactive CTL, which could thus be activated and tolerance circumvented. Although this possibility was neither confirmed nor disproved, CTL of known specificity (for Influenza) and TCR usage proved a useful tool for screening and analysis of SA.

The remainder of this introduction describes the Influenza model system studied, and the function of HLA B27 and other class 1 molecules, before detailing current knowledge of T cell receptor biology and immune recognition of antigen and superantigen.

1.2. The cytotoxic T cell response to Influenza A virus infection

Influenza A virus is an orthomyxovirus with a segmented genome comprising 8 single stranded RNA fragments coding for 10 gene products: the polymerases PB2, PB1, and PA, haemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), Matrix 1 and 2, and

nonstructural proteins 1 and 2. The external surface of the Influenza virion is made up of a host cell-derived lipid bilayer into which are inserted the HA and NA glycoproteins. Matrix (M1) protein lines the inner membrane surface. The remaining structural proteins PB1, PB2 and PA, and NP in association with viral RNA, comprise the virion core. Although Influenza A viruses are highly variable, most variation occurs in the HA and NA proteins, where changes in the prevalent subtype, referred to as antigenic shift, have caused Influenza pandemics. The nucleoprotein is less variable, and indeed the two main subtypes differ at only 29 of 498 amino acids, none of which lie within the epitope NP383-391, described below, to which HLA B27-restricted CTL have been found to respond (Huet *et al.* 1990).

Whilst neutralizing antibodies are largely directed against the HA protein, the principle targets of the CTL responses to Influenza A viruses are the NP (Townsend *et al.* 1984, Yewdell *et al.* 1985) and matrix proteins, although other internal proteins may also be targets (Bastin *et al.* 1987, Gotch *et al.* 1987). Yap and Ada (1978) first showed that adoptive transfer of murine spleen culture cells depleted of T helper cells, but not CTL, reduced lung virus titres and protected against lethal infection. Lin and Askonas (1981) showed similar protection with a CTL clone, and McMichael *et al.* (1983) found that human volunteers with demonstrable CTL responses cleared Influenza A virus more effectively following experimental inoculation. Recovery parallels viral clearance (Mackenzie *et al.* 1989), brought about by cytolysis of infected cells, and probably aided by cytokine release (Morris *et al.* 1982). CTL responses are generally only detectable following a secondary *in vitro* stimulation with infective virus or cognate peptide. The small size of the Influenza genome enabled the first definition of CTL epitopes for any infection, initially using NP deletion mutants (Townsend *et al.* 1985, McMichael *et al.* 1986). More recently recombinant Vaccinia viruses containing Influenza genes or gene fragments have been used together with overlapping peptide sets to define epitopes including the HLA B27-restricted NP epitope studied in

this thesis (Huet *et al.* 1990). This study identified three equally potent synthetic peptides, recognized in the context of HLA B27, NP380-393, 384-398, and the octomer 384-391. Subsequent work by S. Rowland-Jones showed that the nonamer NP383-391 (SRYWAIRTR in single letter amino acid code) was in fact more potent, and exactly fits the sequence motif of Jardetzky *et al.* (1991), see below. Thus NP383-391 is almost certainly the epitope presented to CTL *in vivo*. It is interesting to note that a number of other epitopes have been defined, for different restriction elements, that lie close to or even overlap the HLA B27 epitope; these include the murine D^b-restricted epitope (366-374), and the human HLA B8 (380-388) and HLA B*2702 (381-389) epitopes (Sutton *et al.* 1993, S. Rowland-Jones unpublished data). This suggests that factors other than primary amino acid sequence affect epitope selection *in vivo.*

Although CTL responses have been described in "fresh" PBL from individuals recently infected with Influenza A (Ennis *et al.* 1982), almost all studies have stimulated CTL lines *in vitro* using autologous B cells pulsed with cognate peptide (Carbeno *et al.* 1992). In this thesis the more physiological approach of Gotch *et al.* (1987) is adopted, in which PBL are initially stimulated by infection (of only a proportion of cells) with small amounts of virus. Although the protocol used may not entirely reflect the *in vivo* CTL response, it is important to note that the peptide specificity of, for example, the HLA B27-restricted response was first defined without any *in vitro* peptide stimulation, and that the CTL lines and clones studied kill influenza A-infected target cells.

1.3. Antigen presentation by MHC class 1 molecules

MHC class 1 and 2

The human leukocyte antigen (HLA) molecules are members of the immunoglobulin gene superfamily (Williams and Barclay 1988), and are amongst the most polymorphic proteins known. The two principle classes of HLA molecule are class 1 and class 2. The principle HLA cass 1 loci are HLA A, B and, to a lesser extent C, with together over 120 alleles expressed. The principle murine class 1 loci are H-2 K, D, and L. HLA class 1 molecules are composed of two subunits, a 44kD highly polymorphic heavy chain, and a non-polymorphic 12kD light chain, beta 2 microglobulin (ß2M). The heavy chain comprises 338 amino acids and has three extracellular domains, α1, α2, and α3. The α1 and α2 domains contain most of the polymorphic residues and together make up the peptide-binding groove (see below), the α3 domain interacts with ß2M, and is involved in interaction with CD8 on the CTL cell surface (Norment *et al.* 1988). Class 2 molecules, HLA DR, DQ and DP, and I-E and I-A in the mouse, are also heterodimers, comprising a 34kD alpha chain and a 28kD beta chain. Both chains have two extracellular domains, with the membrane distal of these showing similarities with the α1 and α2 domains of class 1 molecules, and the membrane proximal with ß2M and the α3 domain of class 1 molecules. The HLA genes (but not ß2M) are located on the short arm of chromosome 6 (6p21 band), in a region spanning over 3Mb that has been extensively, but by no means completely, mapped (Campbell and Trowsdale 1993). The HLA class 2 genes lie most centromeric, with DP, DQ and DR α and ß loci. These are followed by a region known as the MHC class 3 region, which includes genes for complement factors, heat shock proteins and tumour necrosis factors α and ß. The HLA class 1 region includes HLA B, C, X, E, A, H, G and F loci.

The HLA system was first defined following observations that the serum from multiply transfused or pregnant individuals contained antibodies that agglutinated lymphocytes from some individuals but not others (e.g. van Rood and van Leeuwen 1958).

Following the demonstration of MHC restriction by Rosenthal and Shevach (1973) and Zingernagel and Doherty (1974), the role of MHC in presentation of antigen to T cells has received considerable attention. Townsend and Skehel (1982) first showed that murine CTL did not recognize cell-surface Influenza glycoproteins. It was then shown that CTL recognized a product of the nucleoprotein gene, which could be introduced into cells by transfection or by infection with recombinant vaccinia virus, although the NP protein could not be detected on the cell surface (Braciale *et al.* 1984, Townsend *et al.* 1984). It was subsequently found that fragments of the NP gene, or even short synthetic peptides, were sufficient for CTL recognition (Townsend *et al.* 1985, Townsend *et al.* 1986). The CTL response to different internal influenza proteins was restricted by different MHC class 1 alleles (Bennink *et al.* 1986), which have subsequently been shown to bind peptides with particular amino acid residues at key "anchor" residues (see below). This work is reviewed by Townsend and Bodmer (1989).

Further understanding of the role of MHC class 1 molecules in antigen presentation has come from the study of presentation-defective cell lines. Both murine (Ljunggren and Karre 1985) and human (DeMars *et al.* 1984) cell lines expressing little or no surface class 1 were generated by chemical or radiation mutagenesis. These cells failed to present virus-derived epitopes after infection, but could present exogenously added peptides, which also restored cell surface MHC expression (Townsend *et al.* 1989). The assembly of class 1 heavy chains and ß2 microglobulin could also be induced by peptide in cell lysates, implying that peptide has a critical role in the folding and final structure of class 1 molecules (Townsend *et al.* 1990). Chromosomal deletions in these mutant cell lines were localized to the MHC class 2 region, where a number of new genes were identified (Monaco *et al.* 1990, Spies *et al.* 1990, Trowsdale *et al.* 1990). These include the transport associated proteins TAP1 and 2, putative peptide transporters of the ATP binding casette gene transporter family, one of which can restore the defect in

the mutant cell line LBL721.134 (Spies and DeMars 1991, Spies *et al.* 1992). To date polymorphism of these proteins has only been shown to affect the nature of presented peptides in the rat, where the cim effect on CTL allorecognition of the RT1a class 1 molecule has been shown to be due to a TAP 2 polymorphism (Powis *et al.* 1992). Two low molecular weight members of the proteosome complex, LMP2 and LMP7, were also identified within the MHC region. LMP2 and LMP7 appear to modify protein breakdown by the proteosome favouring the generation of peptides with hydrophobic, basic or asparagine residues at their C termini e.g.(Driscoll *et al.* 1993).

Structure of Class 1 MHC molecules

Functional evidence suggesting the physical association of MHC and peptide was strongly supported by the crystal structure of the HLA A2 molecule, which contained a "groove" 2.5nm long and 1nm wide on its membrane-distal surface (Bjorkman *et al.* 1987, Bjorkman *et al.* 1987). This groove, bordered by the alpha 1 and alpha 2 helices and with a floor of 8 beta pleated strands, was seen to contain electron density compatible with bound peptide. The crystal structure of HLA B27 subsequently revealed the presence of nonamer peptides in the groove, bound in an extended but kinked conformation (Madden *et al.* 1991). The structure of HLA B27, shown in figure 1.1. and described in detail below, revealed a number of features of peptide binding to class 1 molecules. Thus the side chains of certain residues were accommodated in pockets, named B to E, under the alpha helices, and the N and C termini were tightly bound by a web of hydrogen bonds in the A and F pockets respectively (Madden *et al.* 1992). Different MHC molecules have pockets with different physical characteristics (Parham *et al.* 1988), and would be expected to accommodate different side chain residues. Recently crystal structures have been obtained for single peptides bound

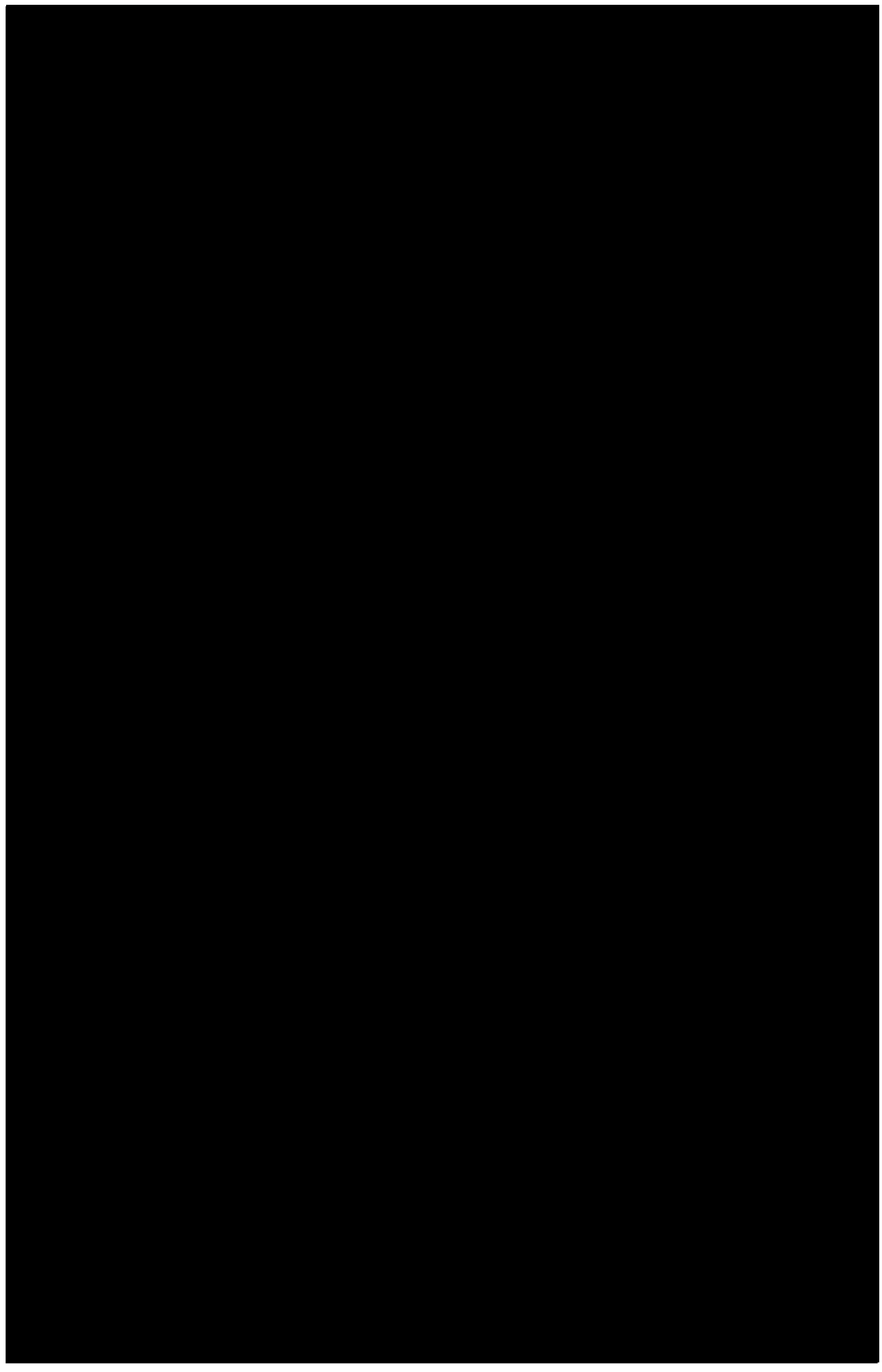


Figure 1.1. The peptide-binding cleft of HLA B27. Top view, from Madden *et al* (1992). A) The peptide-binding cleft is shown in grey. Peptide side chains are shown contacting model peptide, RRIKAITLK, in red. Residues conserved in most class 1 MHC molecules, shown in yellow, are clustered around the peptide termini. Polymorphic contact side chain are shown in blue. B) 12 water molecules, shown as blue spheres, are bound in the centre of the peptide-binding cleft between HLA B27 and peptide model ARA7, shown in yellow.

to a number of MHC class 1 molecules (Fremont *et al.* 1992, Silver *et al.* 1992). It is clear that the peptide backbone is relatively fixed towards both ends, whereas the central portion of the peptide is less constrained and indeed peptides of different lengths can be accommodated by "bulging out" in this region (Guo *et al.* 1992). Small changes in the conformation of the H-2K^b structure were seen to occur on binding peptide (Fremont *et al.* 1992).

Peptide selection by class 1 molecules

Naturally processed peptides were first isolated from whole cellular extracts by acid extraction, gel filtration and high pressure liquid chromatography (Rotzschke *et al.* 1990). Peptide elution from purified class 1 molecules was used to identify a naturally processed epitope derived from Vesicular Stomatitis virus (Van-Bleek and Nathenson 1990), although this technique has subsequently been more effective in identifying self peptides bound to particular MHC molecules. Peptides, usually octomers or nonamers, eluted from H-2K^d, K^b, K^k, K^{km1}, D^b, HLA-A*0201 and *0205, HLA B*2705, HLA B53, HLA B35 and HLA B37, contain invariant "anchor" residues at one or two positions (usually the second, fifth or C-terminal residues) with aliphatic or charged residues at the C terminus (Falk *et al.* 1991, Rotzschke and Falk 1991, Hill *et al.* 1992, Falk *et al.* 1993), and reviewed by (Rammensee *et al.* 1993). The nature of these anchor residues correlates with the physical characteristics of the pockets of different MHC molecules, and it thus appears that the polymorphic pockets of MHC molecules select peptide epitopes with particular amino acids at certain key positions. This gives specificity and at the same time promiscuity by permitting a variety of residues at other positions. Using this knowledge of anchor residues the strategy of "reverse immunogenetics" has recently been employed to identify Listerial and malarial epitopes e.g. (Hill *et al.* 1992). However it must be stressed that epitopes probably cannot be predicted from database searches as attempted by, for example, Ohno (1992), without confirmation of processing, MHC binding, and immunogenicity *in vivo*.

Non-classical MHC class 1-like molecules, such as Qa-2 and related molecules, also selectively bind peptides, including formylated peptides, and so are probably ligands for TCR recognition (Fischer-Lindhal *et al.* 1989, Rotzschke *et al.* 1993).

Thus some general rules for peptide binding to MHC class 1 molecules are now becoming clear. Peptides of about 9 amino acids in length are tightly bound in the class 1 groove, with many main chain atoms buried. Peptides are almost certainly always bound in the same orientation, with the N terminus in the A pocket, and C terminus in the F pocket. Binding occurs in the endoplasmic reticulum, and the MHC molecule folds around the peptide, acquiring a stable conformation. Certain "anchor" residues are critical for MHC binding, others appear to be TCR contact residues. Not all potential epitopes are in fact presented to T cells, and gene products involved in antigen processing, such as the TAP and LMP products, undoubtedly limit the pool of peptides available for MHC binding.

The crystal structure of the HLA class 2 molecule DR1 shows considerable similarity to that of class 1 (Brown *et al.* 1993), as predicted previously (Brown *et al.* 1988). The principle difference is that the ends of the peptide-binding groove are open, allowing longer peptides to bind. This finding is in agreement with those involving peptide elution, suggesting that peptide lengths of 13-17 amino acids or more are common (Rudensky *et al.* 1991). The striking similarity of MHC class 1 and 2 molecules, despite quite substantial differences in primary amino acid structure, could perhaps have been predicted from the fact that similar or identical TCR elements can be involved in recognizing both structures.

HLA B27 structure and peptide binding

The structure of the HLA B27 has been determined by X-ray crystallography (Madden *et al.* 1991, Madden *et al.* 1992). A ribbon diagram of the alpha helices and peptide-binding groove, together with bound peptide and water molecules, is shown in fig. 1.1. Although HLA B27 from a human B cell line was studied, containing a mixture of bound peptides, clear electron density was seen for the main chain peptide backbone. Peptide was bound in a largely extended conformation, although there was a kink at amino acids three and four, such that the side chain of position 3 pointed forward along along the direction of the peptide, and under the side chain of residue 5. The main chain atoms of the central portion of bound peptide were seen to arch up from the floor of the groove. The N and C termini are tightly bound in the A and F pockets, with the side chains of the second, third, seventh and ninth residues accommodated in the B, D, E, and C pockets respectively. The most distinctive structural feature of HLA B27 molecules is the B or "45" pocket, which is lined with residues, particularly a glutamic acid (position 45) at its base, permitting the accommodation of an arginine side chain at the second position of bound peptide. The elution of self peptides isolated from an HLA B*2705 positive BCL by (Jardetzky *et al.* 1991), showed that an arginine at the second position was indeed the anchor for HLA B27. The amino acid sequences of the eluted self-peptides are shown in table 1.1, together with the known HLA B27-specific viral epitopes. It can be seen that, in addition to the arginine (R) anchor residue at position 2, there appears to be a preference for aromatic or aliphatic residues at position three, and for a positive charge at position 9. These preferences were, to a large extent, predicted by comparison of the minimal peptides from HIV gag and Influenza A nucleoprotein (NP) also shown in table 1.1 (Huet *et al.* 1990). They are also consistent with the D and C pockets probable amino acid binding specificity. Thus the D pocket contains both non-polar residues such as L156, and also aromatic amino

EPITOPE	PEPTIDE SEQUENCE
[redacted]	

Table 1.1. Amino acid sequences (single letter code) of viral epitopes and self peptides presented by HLA B27. Residues shown in bold denote anchor (position 2) or frequent residues. References: Eluted peptide from Jardetzky *et al* (1991). Viral epitopes from * Nixon *et al* (1988). ** Huet *et al* (1990). *** Brookes *et al* (1993).

acids (Y99 and Y156). The C pocket, which accommodates the side chain of the C terminal peptide residue (with the carboxy terminus itself bound in the F pocket) contains both non-polar amino acids and two basal negative charges, D77 and D116. It could thus readily accommodate the C-terminal arginine residue of the influenza NP383-391 (SRYWAIRTR) peptide studied in this thesis. At 2.1A definition (Madden *et al.* 1992) it was possible to accurately determine the hydrogen bond network binding the N and C termini of the peptide, and also the positions of 12 water molecules inherent in the overall structure, shown in figure 1.1. However the exact positions of the side chains of the central

residues could not be determined, implying that a variety of residues could be accommodated in different orientations in this region. Unfortunately HLA B27 has not yet been successfully crystallized with individual peptides, and so the exact structure of the HLA B27/NP383-391 complex studied in this thesis is not known.

Six naturally occuring subtypes of HLA B27 have been identified, which can be distinguished by isoelectric focussing (Choo *et al.* 1986), and which all share the common 45 pocket structure. The most frequent subtype found in Caucasians is HLA B*2705, and in this thesis use of the abbreviation HLA B27 denotes HLA B*2705. HLA B*2702, the next most frequent in Caucasians, is also associated with autoimmune arthritis, as are B*2704 and B*2706 (see below). HLA B*2702 differs at only three amino acid residues from HLA B*2705, 77, 80, and 81, where the changes are D to N, T to I, and L to A respectively (2705 residues shown first). Brookes *et al.* (1993) have recently demonstrated that HLA B*2702 and B*2705 can both present the same nonamer epitope from Epstein-Barr virus (EBV) nuclear antigen, EBNA 3C 258-266, shown in table 1.1.

1.4. The T Cell Receptor for antigen

Structure and genomic organisation

The T lymphocyte receptor for antigen comprises a heterodimer of two highly polymorphic glycoprotein molecules, non-covalently linked with five other invariant chains which make up the CD3 complex (Samelson *et al.* 1985). A simplified cartoon of the cell-surface arrangement of these proteins is shown in fig. 1.2. The term T Cell Receptor (TCR) is used to describe both the entire complex and also the idiotypic heterodimer (strictly referred to as ti) which confers antigenic specificity (see below). For simplicity the term TCR will be used in this thesis to describe the αß heterodimer that

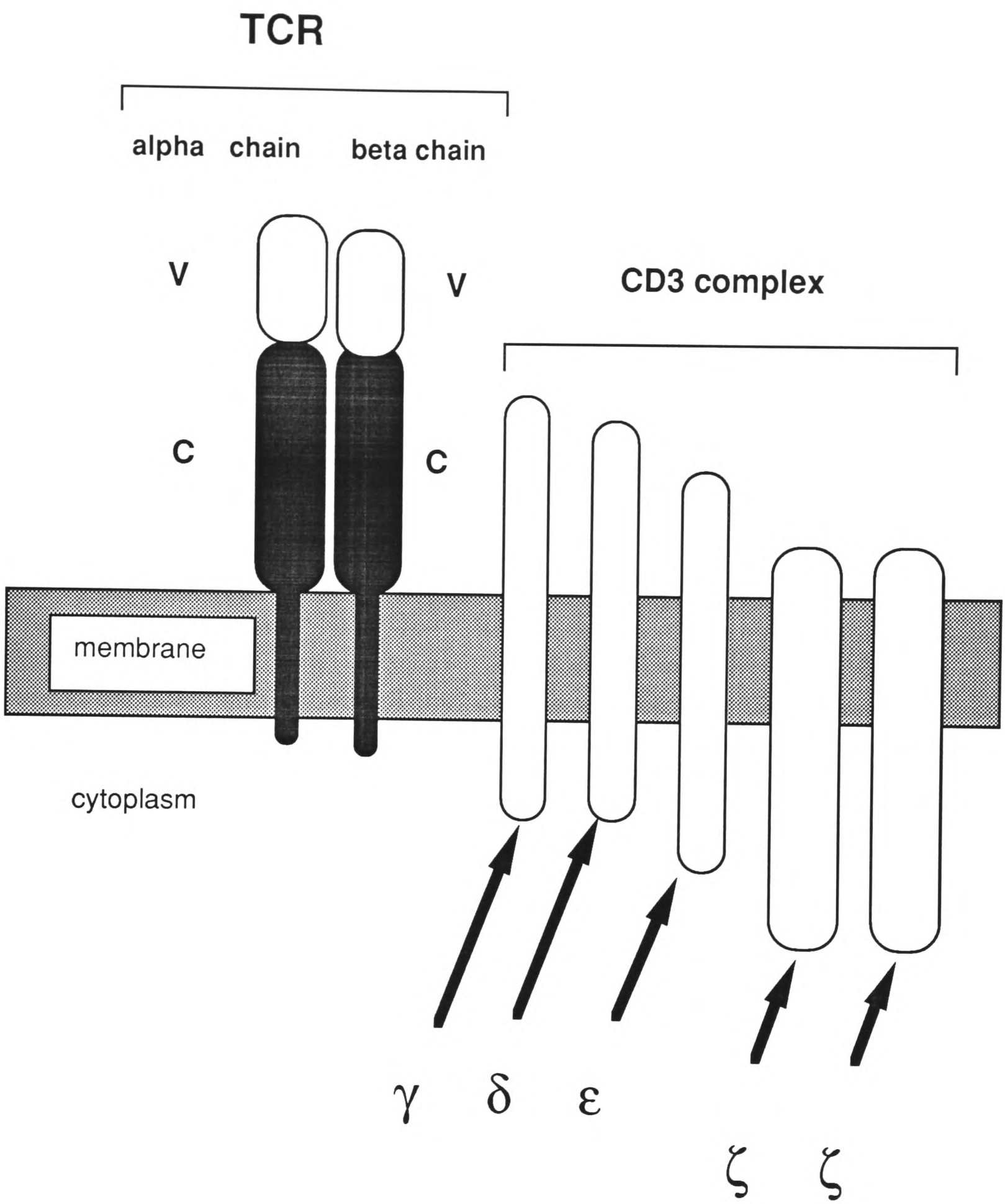


Figure 1.2. Schematic representation of the αß T cell receptor polypeptides. The ζ homodimer may be replaced with a ζη heterodimer in some cells.

is found on 95% of circulating mature T lymphocytes (Brenner *et al.* 1987). A minority of T cells express a γδ heterodimer. The role of γδ cells, reviewed by Raulet (1989) and by Bluestone *et al.* (1991), remains largely unknown.

TCR were first identified on murine and human T cells with clonotypic antibodies, and shown to be disulphide-linked heterodimers of combined MW 80-90kDa (Haskins *et al,* 1983). The murine TCR ß chain cDNA sequence was obtained in 1984 by Hedrick *et al.* (1984), using the technique of differential nucleic acid hybridisation. Yanagi and colleagues (1984) identified the human counterpart. Further T-cell specific cDNAs were identified encoding gamma —initially thought to be the partner of the beta chain— (Saito *et al.* 1984), alpha (Chien *et al.* 1984, Saito *et al.* 1984), and lastly delta chains (Chien *et al.* 1987). All these molecules are closely related members of the immunoglobulin superfamily (Hannum *et al.* 1984). The TCR beta gene complex was subsequently mapped to chromosome seven in the human (Caccia *et al.* 1984) where it spans approximately 600Kb (Lai *et al.* 1988), and TCR alpha complex to chromosome 14 (Caccia *et al.* 1985). Much is now understood about the organisation and rearrangement of the TCR gene segments, which closely parallels that previously described for immunoglobulins (Tonegawa 1983, Wood and Tonegawa 1983), and appears relatively conserved between mice and humans (Lai *et al.* 1988).

One of the central immunological mysteries has been the mechanism of generation of Ig and TCR molecules with almost infinite different specificities, on which clonal selection can act (Burnet 1959). It is now known that the enormous diversity of peripheral lymphocyte TCR (and Ig) molecules is generated by a unique mechanism involving the combinatorial association of variable (V), joining (J) and constant (C) gene segments to form rearranged genes. The ß chain has additional diversity (D) segments, as do immunoglobulin heavy chain genes. During T cell

development in the thymus, Vα-Jα or Vß-Dß-Jß gene segments are brought together by recombination events under control of the Recombinase Activating genes RAG1 and 2, reviewed by (Alt *et al.* 1992). Recombination usually occurs by looping-out and deletion of the intervening chromosomal DNA (Okazaki *et al.* 1987). A simplified schema of this process, which occurs in the thymus, is illustrated in fig. 1.3.

There are now thought to be approximately 50 Vα and 70 Jα segments (Kimura *et al.* 1986, Roman-Roman *et al.* 1991) These are shown in appendices 1 and 2. In this thesis the Jα nomenclature of (Moss *et al.* 1993) is used, although during writing a new WHO nomenclature was introduced (WHO Bulletin 1993). 57 Vß segments have been identified, and are grouped into 24 families (Kimura *et al.* 1986, Kimura *et al.* 1987, Ferradini *et al.* 1991), as shown in appendix 3. There are 13 Jß, two Dß and two Cß gene segments (Toyonaga *et al.* 1985). The two Cß segments, separated by 8Kb, are each associated with an upstream set of Jß segments and a Dß segment (Toyonaga *et al.* 1985). Splicing of a Vß region to a Jß segment commits the transcript to using the downstream Cß segment, although rearrangement to a Jß2 segment after a failed Jß1 rearrangement appears possible. The human gamma chain locus on chromosome 7 comprises 8 functional Vγ segments and two sets of Jγ segments, each with a constant region complex. The human delta chain gene complex lies within the alpha (Chien *et al.* 1987). Whilst there are only three Vδ segments (one of which, Vδ3, is 3' of Cδ and in an inverse orientation) considerable junctional combinatorial diversity is possible because up to three Dδ segments can be used in a single transcript (Takihara *et al.* 1989). The potential diversity generated by combinatorial association of V, (D), and J segments is further augmented by imprecision of joining and by N region addition of non germline-encoded nucleotides at V-(D)-J junctions, catalysed by the enzyme terminal

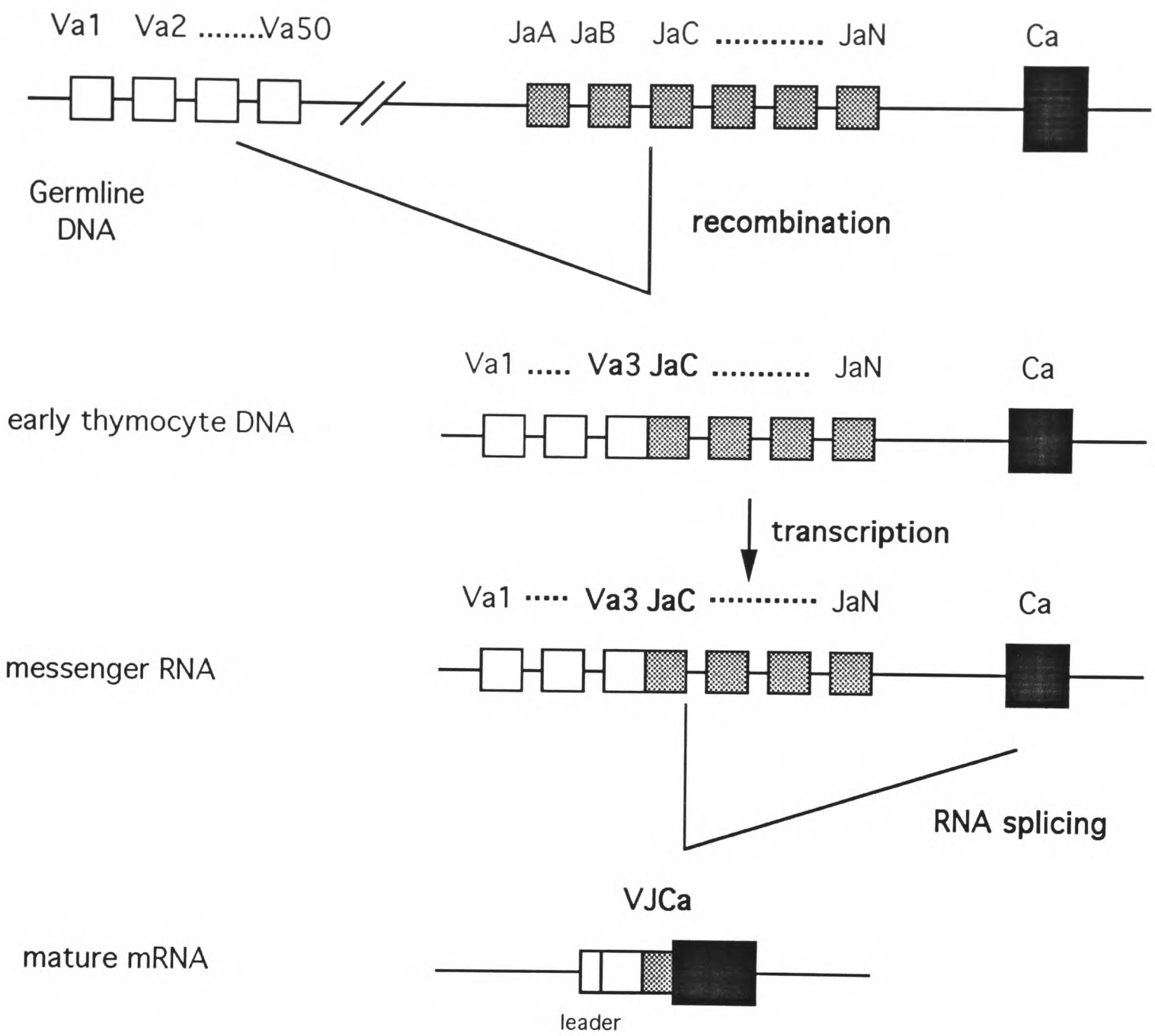


Figure 1.3. Schematic representation of TCR alpha gene complex recombination. The delta complex, lying between the V alpha and J alpha segments, is not shown.

deoxynucleotidyl transferase or TdT (Basu *et al.* 1983). TCRs have an even greater theoretical junctional repertoire than immunoglobulins, estimated at 10^{15} combinations (Davis 1990), although in contrast to immunoglobulins, alternative splicing and somatic hypermutation do not appear to occur {reviewed in (Davis 1990)}. The available evidence suggests that, as with immunoglobulin expression on B cells (Coleclough *et al.* 1981), allelic exclusion operates to prevent rearrangement of both TCR ß alleles. Study of TCRα transgenic mice and of murine T cell clones suggests that alpha chain allelic exclusion is at best leaky and may not occur at all (Malissen *et al.* 1988, Casanova *et al.* 1991). Only one report exists of a T cell clone expressing two beta chains (Matis *et al.* 1988). Thus whilst any one T cell and its progeny express only one (or perhaps two) TCRs, the repertoire of any individual is vast, and is probably limited, subject to thymic selection, to the total number of T lymphocytes in the body — of the order of 10^{11} in humans.

Whilst TCR V gene segments share only approximately 25% sequence homology with immunoglobulin domains, their amino acid diversity is similarly clustered in three regions, equivalent to the immunoglobulin complementarity determining regions CDR1, 2, and 3 (Wu and Kabat 1970). The CDR3-equivalent is the most diverse, with V, N (DN) and J region contributions. This observation led several groups to propose that the TCR had a similar tertiary (and quaternary) structure to immunoglobulin, with the CDR3 regions contacting MHC-bound peptide and the (V region-encoded) CDR1 and 2 regions contacting MHC (Davis *et al.* 1986, Chothia *et al.* 1988, Claverie *et al.* 1989). Figure 1.4 shows the structure of an immunoglobulin domain. Chothia, Boswell and Lesk (1988) further identified framework residues conserved between Ig and TCR, allowing numbering of amino acid residues and comparison of CDR length. A modified cartoon of this model is shown in fig. 1.5. This model also predicted the

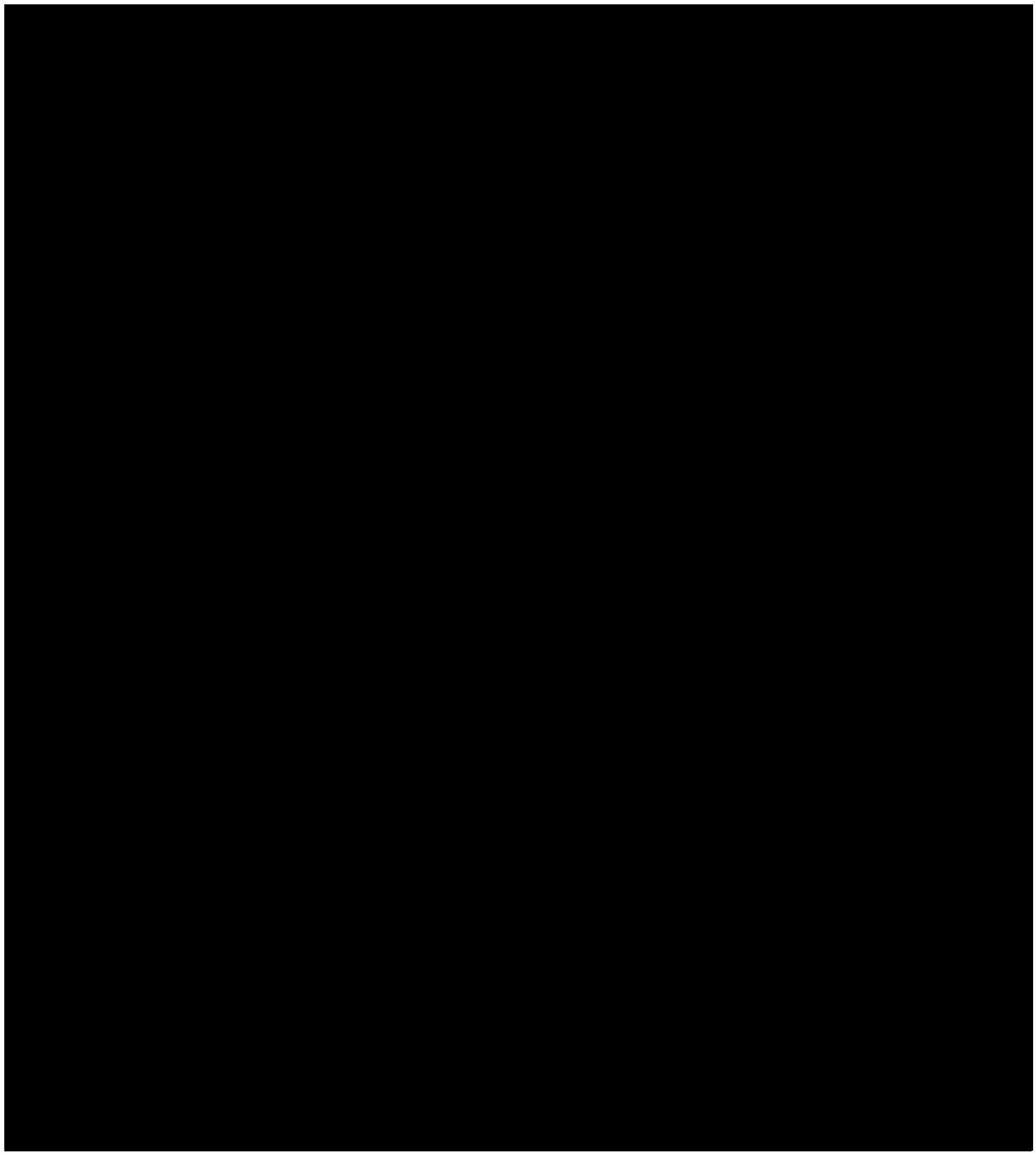


Figure 1.4. Schematic representation of a single Ig V domain. Ribbon diagram, side view, from Patten *et al* (1993). CDR1, 2, and 3 loops are composed of loops between a series of nine antiparallel ß strands. The A, B, E, and D strands are predicted to be exposed to solvent. The DE loop contributes to the so called CDR4 loop.



Figure 1.5. Model of TCR CDR interaction with peptide and MHC alpha helices. After Chothia *et al* (1988), scale approximate.

CDR3 residues likely to interact with MHC-bound peptide, and is supported by current experimental data, including mutagenesis of CDR3ß (Engel and Hedrick 1988), CDR1ß (Pullen *et al.* 1990, White *et al.* 1993) and of alpha chain CDRs (Nafelski *et al.* 1990, 1992).

Development of the TCR Repertoire

In this thesis the concept of a TCR repertoire is used to describe a collection of functional TCR specificities for foreign or self antigen, although it is also widely but less precisely used to describe the usage of a particular gene segment (e.g. Vß repertoire). TCR repertoire is studied in this thesis by analysis of the fine specificity of individual T cell clones, and by determining the rearranged TCR gene sequence of both PBL and antigen-specific CTL. It is assumed that an individual's available repertoire of TCR specificities limits and defines their response to antigens. There is evidence that this is true for some immune responses (Nanda *et al.* 1991), but not others (Ruberti *et al.* 1991).

The development of the TCR repertoire is not random and is subject to both genetic and environmental influence. Genetic factors include TCR deletions and polymorphisms e.g. (Li *et al.* 1990), however the major genetic determinants of the TCR repertoire are MHC genes (and perhaps others such as peptide transporters) that act during thymic maturation. Only a small proportion of T cells entering the thymus survive, interaction of the TCR with MHC molecules may result in positive selection, i.e. proliferation and survival, or negative selection, i.e. death by apoptosis (Kappler *et al.* 1987, Teh *et al.* 1988, Fink *et al.* 1990). In the absence of any interaction T lymphocytes probably also die. It is probable, but not proven, that selection is peptide driven e.g. (Berg *et al.* 1990). Recent support for this proposition comes from the study of TAP 1 deficient mice, which have a greatly reduced CD8+ population, that can be "rescued" by thymic peptide extracts (Ashton-Rickardt *et al.* 1993). The

effects of MHC on TCR repertoire selection have been more difficult to ascertain in (outbred) human populations but have now been repeatedly demonstrated (Loveridge *et al.* 1991, Akolkar *et al.* 1993). Some workers have suggested that negative selection occurs at an early stage in T cell maturation, at or before the CD4+ CD8+ double positive stage (Kisielow *et al.* 1988), whereas others suggest that commitment to either the CD4+ or CD8+ lineage is required (Guidos *et al.* 1990). Positive and negative selection can probably occur simultaneously, although they might or might not occur on different cell types (Marrack *et al.* 1988). In addition to the MHC, certain minor histocompatibility antigens also shape the TCR repertoire. Minor histocompatibility antigens are generally polymorphic self proteins that can be presented to T cells (Wallny and Rammensee 1990). It is now clear that another potent modifier of the TCR repertoire in mice, the minor lymphocytic stimulating (Mls) determinants first described by Festenstein (1973), are superantigens encoded by endogenous retroviruses (see below). Endogenous SA do not appear to modify TCR repertoire in rats (Baccala *et al.* 1991, Smith *et al.* 1992), and may in fact be confined to mice.

Study of TCR repertoire

Several techniques are now available with which to study the TCR repertoire. Monoclonal antibodies (Mab) to TCR V regions are simple to use, assess cell membrane expression, and can give accurate tissue localization. To date Mab have been raised to the following human V regions: Vα2, 12 and 24, and Vß2, 3, 5, 6.7a, 8, 12, 13.1, 13.2, 17, 19 (Bigler *et al.* 1983, Acuto *et al.* 1985, Bigler *et al.* 1985, Boylston *et al.* 1986, Posnett *et al.* 1986, Lipoldova *et al.* 1989, Choi *et al.* 1991, Devaux *et al.* 1991, Friedman *et al.* 1991, Diu *et al.* 1993). Thus less than one third of the human Vß repertoire and even less of the Vα repertoire can be studied. Moreover, with the exception of certain clonotypic antibodies, such reagents do not give information on D and junctional sequence usage, and hence do not give any indication of the functional TCR repertoire.

Recombinant DNA techniques have been used to study either TCR gene rearrangement, or cellular TCR RNA transcripts. Southern blot analysis together with restriction fragment length polymorphism (RFLP) can be used to study both germline variation between individuals (Robinson 1986), and to look for TCR region rearrangements in mature lymphocytes and hence give an indication of clonality. Most recombinant techniques study TCR RNA transcripts. RNase protection assays have employed the ability of family-specific oligonucleotide probes to selectively bind RNA transcripts from different Vß families (Smith *et al.* 1992). Polymerase chain reaction (PCR) amplification of cDNA reverse transcribed from TCR mRNA is now widely used. TCR PCR has proved difficult because V region variability generally precludes the use of a 5' consensus primer, although relatively degenerate primers have now been employed successfully in the rat. V region family-specific primers have been widely used, and have the advantages of speed and ease of use, but are difficult to quantify because each V region is amplified in a separate reaction. Indeed as more Mabs have become available family-specific PCR has been shown to be unreliable (Diu *et al.* 1993). Furthermore this technique cannot identify new V gene segments and is not often used to obtain information about the frame, (ND)N nucleotide sequence or J segment usage of TCR transcripts. The problem of 5' variability can be overcome by circularizing the transcript - 'inverse' PCR (Ochman *et al.* 1988), or by adding a tail or "anchor" sequence to all cDNA transcripts, to which a consensus primer can then be annealed - "anPCR" (Loh *et al.* 1989). AnPCR has been used to estimate the TCR repertoire of human peripheral blood lymphocytes (PBL)(Rosenberg *et al.* 1991, Moss *et al.* 1993). From this work it is clear that certain families, for example Vß4, 6, and 12, are common, whilst others, such as Vß14-24, are less frequently used. AnPCR is used in this thesis to study the TCR usage of PBL, CTL lines and CTL clones from HLA B27-positive individuals, and is discussed further in chapter 4.

TCR gene usage in specific immune responses

Much work has gone into attempting to define the relative contributions and orientation of the TCRα and ß chains in the recognition of antigen and MHC, i.e. the trimolecular complex. Most studies have shown that a simple dual receptor model, in which antigen and MHC are recognized separately, is unlikely to be correct. Thus Kappler *et al.* (1981) showed that fusion of T cells with unrelated specificities did not lead to reassortment of MHC and antigen specificities. Confirmation that the TCR alpha and beta chains together conferred specificity for antigen and MHC was obtained by cell fusion experiments (Yague *et al.* 1985), and ultimately by gene transfer (Dembic *et al.* 1986, Saito and Germain 1987). Exceptionally, individual chains have been shown to confer specificity for antigen, e.g. the hapten p-azobenzenearsonate (Tan *et al.* 1988), or MHC (Saito and Germain 1987). Study of TCR usage of murine antigen specific T cell clones and hybridomas provided further evidence of the importance of both chains, by demonstrating conservation of primary TCRα and/or ß sequences. Thus both chains were conserved in the I-$E^{k/s/b}$ restricted response to moth cytochrome c (Fink *et al.* 1986, Winoto *et al.* 1986). Specificity for MHC could be correlated with Vß usage, and the presence of alloreactivity with Jα usage (Sorger *et al.* 1987). Subsequent studies have shown conservation of various elements in a variety of immune responses, although with no clear pattern correlation suggesting a common topology for TCR interaction with peptide and MHC. Notably most studies involved MHC class 2-restricted responses to artificial antigens in inbred mouse strains, e.g.Hedrick *et al.* (1988), Danska *et al.* (1990), Ruberti *et al.* (1991). A study of beta chain usage in the H-$2K^b$ restricted response to the hapten TNP found evidence of V, D and Jß conservation (Hochgeschwender *et al.* 1987). Another study, of only four AEB specific CTL clones using two different restriction elements, found little TCR homology between clones, although two clones did use the same Jα (and Jß) elements (Iwamoto *et al.* 1987). Recently studies of rather more physiological responses, the D^d-restricted responses to LCMV infection, and the human

HLA A2-restricted response to Influenza A matrix protein, have found conservation of both TCR alpha and beta chains (Aebischer *et al.* 1990, Yanagi *et al.* 1990, Brandle *et al.* 1991, Moss *et al.* 1991).

Taken together these studies suggest that different elements of the TCR are conserved in different immune responses. Interestingly similar observations were made for immunoglobulin gene usage, with, for example, conservation of V_H, J_H and J_K in the murine response to phosphorylcholine (Perlmutter *et al.* 1984)), but of V_K (V_K21C) in the response to influenza haemagglutinin (Clarke *et al.* 1985). Subsequently a number of antibody-antigen complexes have been crystallized, and it is clear that a variety of different configurations are possible, from "lock and key" to relatively planar, reviewed by (Davis *et al.* 1990). Furthermore whilst either the heavy or light chain CDR3 usually play an important role in recognition, not all CDRs contact antigen in every complex.

Pending crystallographic data on TCR structure, a number of other approaches can be undertaken to analyse TCR recognition. These include *in vitro* expression of αß pairs in which one chain is kept constant and single amino acid residues, regions or indeed different chains introduced in the partner chain (Engel and Hedrick 1988). Similar experiments have been performed *in vivo* by studying the immune responses of mice made "hemitransgenic" for either the α or ß TCR chains of particular T cell clones. Jorgensen *et al.* (1992) found that mice, hemitransgenic for the TCR α chain of an I-E^k-restricted moth cytochrome c-specific clone 5C.C7, responded poorly to a peptide with a charge change at residue 99 of the MCC In contrast ß chain hemi-transgenic mice responded well, indicating that the (no longer fixed) TCRα chain recognized this position of the peptide. Sequencing of the non-transgenic TCRα chains revealed a reciprocal charge change at position 93 of the TCRα CDR3 in T cell hybridomas responding to the mutated peptide. Substitutions of T102 of the peptide induced amino acid changes

at position 97 of the beta chain CDR3, confirming the importance of both CDR3 regions in peptide contact and also establishing the orientation of the TCR in this immune response. Notably changes at position 102 also had a dramatic effect on TCR Vß usage, showing the importance of elements other than the CDR3s in establishing peptide contact. Recently Romero *et al.* (1993a,b) have reported chemical cross-linking of the TCR using photoaffinity-labelled peptide. This promises to be a very powerful technique, but may be limited to murine models since chemical modification of natural epitopes frequntly abolishes TCR recognition.

Finally it is important to note that, despite the striking conservation of TCR usage observed in some immune responses, the immune system is also capable of remarkable plasticity. Thus identical V segments can be used by both MHC class-1 and class 2-restricted T cells (Rupp *et al.* 1985). Even when a specific immune response preferentially uses one (Vß) gene segment, mice lacking or prevented from using this element may be able to mount an equally vigorous immune response using alternative elements (Ruberti *et al.* 1991).

TCR usage and autoimmunity

Restricted TCR usage of pathogenic T cell clones has been clearly demonstrated in the murine autoimmune disease experimental allergic encephalomyelitis (EAE), a model for multiple sclerosis. T cell clones specific for the N-terminal 11 amino acids of myelin basic protein (MBP1-11) are capable of transferring EAE in PLJ mice (Acha-Orbea *et al.* 1988). These clones almost invariably use Vß8, and antibodies to Vß8 can be used to prevent disease induced by MBP1-11 or to ameliorate that induced by whole MBP (Urban *et al.* 1988).

Restricted TCR usage has been reported in a variety of human autoimmune disease, including multiple sclerosis, where a predominance of Vα12- and Vß5.2-containing transcripts in involved plaques has been reported (Oksenberg *et al.* 1990,

Oksenberg *et al.* 1993). Current knowledge of TCR repertoire in HLA B27-associated and other autoimmune rheumatic diseases is reviewed by Bowness and Bell (1992). Notably study of synovial membrane and fluid from patients with rheumatoid arthritis (RA) has produced conflicting results (Howell *et al.* 1991, Paliard *et al.* 1991).

TCR expression

Many attempts have been made to express TCRs for both functional and structural study, because of the relatively low levels of TCR on the surface of lymphocytes — approximately 20-40,000 molecules (Samelson *et al.* 1983) — and the paucity of immortal T cell lines of known antigenic specificity. Expression of single human beta chains in murine T-hybridoma cells lacking an endogenous ß chain has been used to raise mAbs to Vß elements (Choi *et al.* 1991). T cell heterodimers have been expressed in phosphotidyl inositol (PI)-glycan linked form, permitting cleavage from the cell surface and solubilisation (Lin *et al.* 1990). However cell surface expression appears relatively inefficient, largely because correct pairing of the αß heterodimer and association with the CD3 complex appear to be required for cell surface expression. Procaryotic expression has been attempted, including using "linker" segments to join Vα and Vß elements, but it is not clear if the resulting protein is correctly folded. Engel *et al.* (1992) have recently expressed TCR heterodimers as fusion proteins with the transmembrane and cytoplasmic domains of the CD3 ζ chain, linked by a thrombin cleavage site to allow solubilization. The ζ chains facilitate dimerization and efficient cell surface expression, and also signal in the rat basophil leukaemia cell line used (RBL-2H3), confirming the functional integrity of the transfected TCR. The above approach to TCR expression is adopted in this thesis.

1.5. T cell recognition of superantigens

The term superantigen (SA) has been applied to a group of bacterial and retroviral gene products that stimulate large numbers of T-lymphocytes in a manner distinct from that of

classical antigens (Kappler *et al.* 1989), reviewed by Marrack *et al.* (1990). Two distinct types of SA have been described which, whilst almost certainly having different structures and evolutionary origins, appear to interact with the T cell TCR in a similar manner. The *Staphylococcal* enterotoxins (SEA-SEE), the major causes of the symptoms of food poisoning, are the prototypic bacterial or "exogenous" SA. A variety of other bacterial toxins are SA, including the *Staphylococcal* toxic shock toxin 1 (TSST), and the *Streptococcal* exfoliative toxins. The second group of SA are the retroviral or "endogenous" SA, principally the products of the murine Mls loci.

SA have profound immunological effects *in vitro* (Choi *et al.* 1989) and when injected into mice (White *et al.* 1989). Many of these toxins cause important human disease, although it is not certain that this is mediated by their superantigenic action. Staphylococcal toxic shock syndrome toxin 1 (TSST) is implicated in toxic shock (Choi *et al.* 1990), and the staphylococcal enterotoxins (SE) are potent emetics, although it is not clear if their emetic action is a direct result of their superantigenic properties (Fleischer *et al.* 1989).

The "endogenous" SA, including the Mls, have recently been shown by several groups to be the products of retroviruses, principally the mouse mammary tumour viruses (MMTV), e.g. (Choi *et al.* 1991). Almost all mouse strains have several MMTV incorporated into their genome (hence the term "endogenous"), although some MMTV are transmitted from mother to offspring in milk (Marrack *et al.* 1991). Thymic expression of the product(s) of the MMTV 3' open reading frame, results in deletion of T cells expressing MMTV-responsive TCR Vß elements. It appears that endogenous SA, unlike exogenous, may require an element of processing and/or cell surface expression (Choi *et al.* 1992). Mice are protected from exogenous infection if they possess endogenous MMTV that delete reactive T cells in the thymus (Golovkina *et al.* 1992). It is important to note that there is currently no reliable evidence to suggest that retroviral

SA play a role in shaping the human TCR repertoire, although it has been suggested that the human immunodeficiency virus (HIV) may produce a SA (Imberti *et al.* 1991, Laurence *et al.* 1992).

Interaction of superantigens with lymphocyte T cell receptor

SA are thought to bind to the T cell receptor (TCR) Vß chain. Kaye and Hedrick (1988) first demonstrated that Mls specificity could be transferred by transfection of the TCRß chain. Stimulation with SEB and other SA was shown to enrich T cells bearing certain human (Kappler *et al.* 1989) and murine (White *et al.* 1989) Vß elements. The known TCR Vß specificities of the bacterial SA are shown in

[redacted]

Table 1.2. TCR Vß-specificity of bacterial superantigens. Dominant responding Vß elements are shown in bold type. Refs: Choi *et al*, (1989), Marrack P and Kappler J., (1990). *Fleischer B *et al.* (1991). () indicates proliferation but not killing. ** this thesis. # Friedman *et al*, (1991). Vß8 proliferation with SEB was not observed by Choi *et al* (1990), and SEB did not induce cytolysis by a Vß8 CTL clone (this thesis).

It has proved particularly difficult to demonstrate direct binding of SA to the TCR, although this has recently been demonstrated for a soluble TCRß chain molecule (Gascoigne and Ames 1991). Whilst the role of the TCRß chain has been unequivocally demonstrated, it is important to note that there is evidence for TCRα chain involvement, that is not simply explained by pairing bias (Vacchio *et al.* 1992), and that SA have also been shown to bind γδ cells (Fleischer and Schrezenmeier 1988), and possibly also immunoglobulins (Pascual and Capra 1991).

The exact nature of the interaction between the TCR Vß and the SA is unknown, although both Mls and bacterial SA are thought to bind to a solvent exposed site on the side of the Vß chain (Choi *et al.* 1990, Pullen *et al.* 1990). Thus site-directed mutagenesis of the TCRß chain residue 22 (N to D), or of residues 70 (E to K) together with 71 (N to K), conferred Mls-1a reactivity to a T cell hybridoma using the otherwise Mls-1a unreactive Vß8.2a TCR, probably by preventing N-linked glycosylation at these sites (Pullen *et al.* 1990, Pullen *et al.* 1991). Similarly transfer of residues 67-77 (encompassing most of the so called TCRß CDR4 region) of the human Vß13.2 segment conferred SEC2 reactivity on a murine TCRa negative hybridoma transfected with a human Vß13.1-containing ß chain. In contrast (Patten *et al.* 1993) were able to transfer SA reactivity by grafting of the CDR2 region, and to a lesser extent CDR1 (but not CDR3), although the significance of these findings is uncertain as very high SA concentrations were required for recognition. SEB has recently been crystallized, and its structure determined (Swaminathan *et al.* 1992). A shallow cavity was observed that is thought to be the TCR binding site. Notably the TCR binding site appears to be contributed to by residues from both its domains, explaining apparent discrepancies in previous mutagenic studies e.g. Buelow *et al.* (1992). In addition the TCR and MHC binding sites are on opposite faces of the SEB molecule, consistent with the model of binding shown in figure 1.6.

The interaction of SA with the MHC class 2 is even less well characterized. However it is clear that SA are not bound in the antigen-binding groove in the "classical" manner (Dellabona *et al.* 1990), and that different SA have different affinities for different class 2 molecules. Fraser (1989) demonstrated direct binding of SEA and SEB to the same site of HLA DR molecules. Herman *et al.* (1990, 1991) showed that different HLA DR alleles differed in their ability to present SA, implicating differences in the polymorphic DRß chain. In contrast Karp *et al.* (1990) reported that the HLA DR α1 domain was essential for TSST binding. It should be noted that binding to MHC class 2 molecules is not essential for T cell activation (Hewitt *et al.* 1992). Interestingly SA interaction with the class 2 molecules of B cells can results in B cell stimulation, which is probably T cell mediated and may be important *in vivo* (Mourad *et al.* 1989).

Whilst SA engage both CD4- and CD8-positive T cells (Herrmann *et al.* 1990), these populations appear to be affected differently in mice in *vivo* (Kawabe and Ochi 1991). It has also been shown that certain SA may induce proliferation but not cytolysis (Fleischer and Schrezenmeier 1988). This may simply reflect a quantitative difference in affinity of the SA/TCR interaction, consistent with the demonstration that the Vß specificities of different SA are relative only (Fleischer *et al.* 1991). Similarly under varying conditions SA appear to be able to induce either T cell proliferation, anergy (O'Hehir and Lamb 1990, Rellahan *et al.* 1990), or deletion, both *in vitro* (Jenkinson *et al.* 1990), and *in vivo* (White *et al.* 1989, Kawabe and Ochi 1991).

Although the SA produced by human pathogens, such as the *Staphylococci*, do interact with murine TCR, they stimulate a smaller proportion of the total (murine) TCR repertoire. Similarly the SA mitogen produced by *Mycoplasma arthritidis* (MAM), a pathogen of rats and mice, stimulates 20% of murine T cells but only 8% of human (Matthes *et al.* 1988), predominantly Vß17 (Friedman *et al.* 1991). It has been suggested that this

indicates the co-evolution of host TCR and parasite SA (Fleischer *et al.* 1991).

Thus SA are potent T cell stimulators that are recognized by the TCR in an entirely different manner to that of classical antigen. Although the exact stoichiometry of recognition is not known, it is thought that SA may form a "bridge" between TCR and MHC class 2 molecules, as shown in figure 1.6.

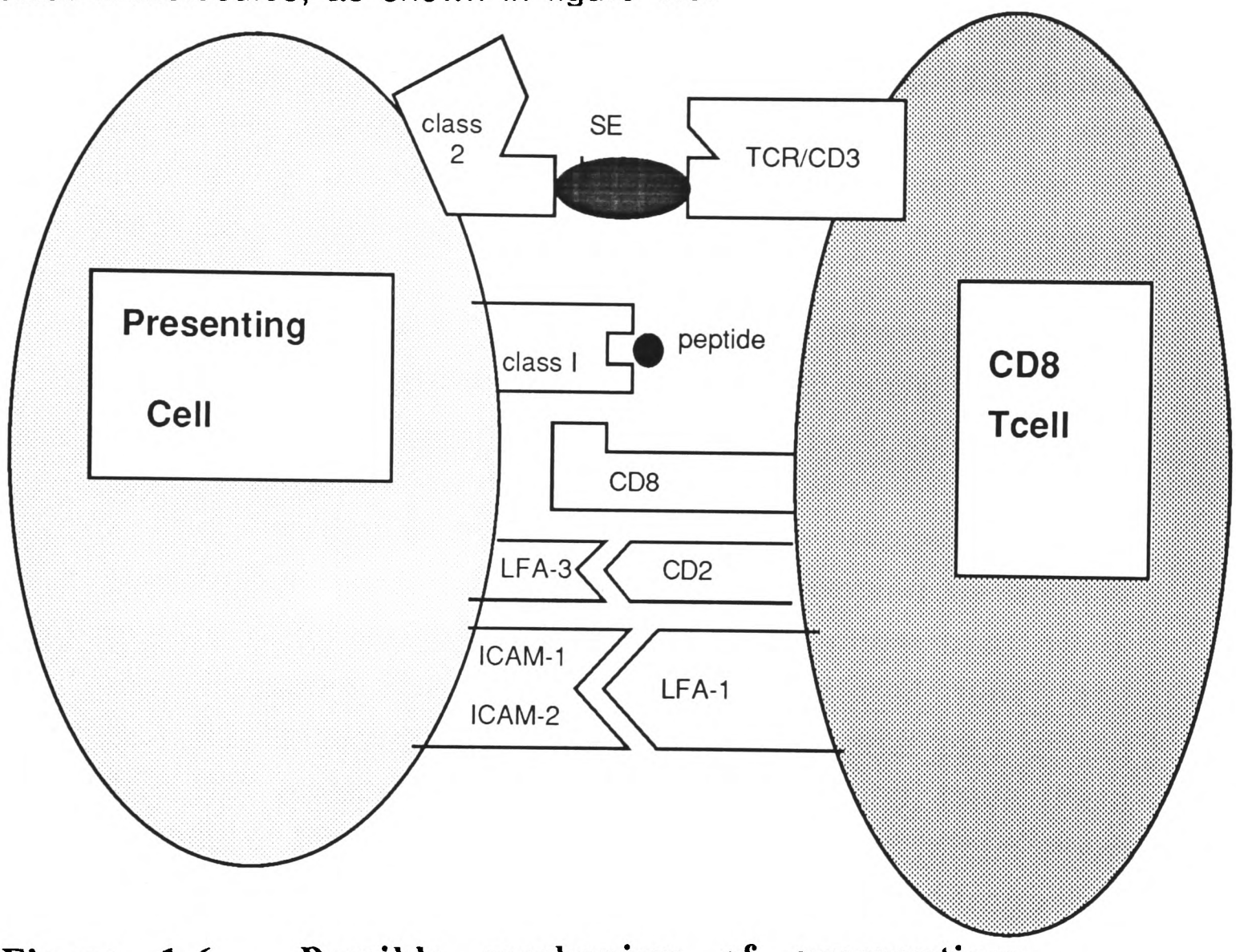


Figure 1.6. Possible mechanism of superantigen recognition by Cytotoxic T cells.

1.6. HLA B27 and susceptibility to autoimmune arthritis

The major reason why an HLA B27-restricted immune response was studied in this thesis was that possession of HLA B27 is very strongly associated with the development of autoimmune arthritides, the relative risk for developing ankylosing spondylitis (AS) in individuals with HLA B27 being over 300. It was hoped that molecular characterisation of the HLA B27-restricted

response to a defined antigen might be relevant to studies in these conditions. Although the mechanism by which HLA B27 confers susceptibility to autoimmune arthritis remains unknown, a number of theories have been propounded.

Theories in which the immune role of HLA B27 is incidental

- HLA B27 may be a marker for a "linked-gene", i.e. HLA B27 is not itself involved in pathogenesis. Whilst the incidence of AS is higher in relatives of affected individuals than in other HLA B27-positive individuals, implying a role for other genes, disease in HLA B27 transgenic rats (Hamer *et al.* 1990) strongly suggests a direct role for HLA B27.

- HLA B27 may be a cell surface receptor on the gut mucosa that facilitates dissemination of those organisms that induce arthritis.

- The "molecular mimicry" hypothesis suggests that a peptide sequence in the HLA B27 is mimicked by another one as in, for example, *Klebsiella sp.* (Ebringer *et al.* 1976, Van Bohemen *et al.* 1984, Schwimmbeck *et al.* 1987), and that AS is caused by auto-antibodies to this part of the HLA B27 molecule. Although widely espoused, there are a number of theoretical objections to this model. Thus whilst the motif QTDRED is present both in *Klebsiella* nitrogenase reductase (residues 188-193), and in HLA B*2705 (72-77), it is absent in the other B27 subtypes clearly associated with AS, HLA B*2702, 4, and 6 (Breur-Vriesendorp *et al.* 1987). These subtypes have N or S at position 77. The remaining motif (QTDRE) is commonly found in HLA molecules not associated with autoimmunity such as HLA A25, A32, and B8. Notably Lahesma *et al.* (1992) also identified a TDRE motif in the *Yersinia enterocolitica* YadA protein. Whilst 1/3 of patients with Yersiniosis were found to have antibodies to an 18-mer peptide covering this

region, these were in fact shown to recognize non-crossreactive flanking peptide residues.

• A controversial variant of the crossreactivity hypothesis suggests that *Klebsiella* sp. produce HLA B27 "modifying factors" that alter the HLA B27 of individuals with AS (Geczy *et al.* 1983). Other groups have not been able to confirm these findings.

Theories in which the immune role of HLA B27 is central

The remaining hypotheses directly invoke the immune role of HLA B27 in predisposing to autoimmunity.

• HLA B27 could select a repertoire of potentially arthritogenic T cells. One variation of this hypothesis, discussed above, would be that HLA B27-restricted autoreactive T cells may use particular Vß element(s) that permit stimulation by a particular "arthritogenic" superantigen.

• The apparent stability of empty HLA B27 molecules on the cell surface has lead to the suggestion that it may be particularly good at binding extracellular peptides (Benjamin and Parham 1991, Benjamin and Parham 1992).

• The "arthritogenic peptide" hypothesis suggests that certain peptide(s) derived from arthritogenic organisms and presented by HLA B27, closely or exactly resemble self peptides presented in a similar manner (Benjamin and Parham 1990). This model, favoured by the author, has recently received support from the work of Hermann *et al.* (1993), who have for the first time isolated *Salmonella*- and *Yersinia*-specific CTL clones from the joints of patients with reactive arthritis precipitated by these organisms.

• In the light of recent data suggesting that peptides derived from HLA molecules are frequently presented by both class 1 and class 2 molecules, it is also possible that

both class 1 and class 2 molecules, it is also possible that HLA B27 could itself provide an epitope, presented to crossreactive T cells by another HLA molecule or molecules, which would necessarily also be strongly associated with disease. Although such an association has not been described, studies to date have been of insufficient power to detect weaker or multiple associations of AS with HLA class 2 alleles. Interestingly CD4+ T cells are more readily isolated from joints of patients with ReA than CD8+ cells (Hermann *et al.* 1989, Hermann *et al.* 1990).

• The unique unpaired cysteine at position 67 might be involved in the pathogenesis of AS (Archer *et al.* 1990). The possibility that ligand binding to this residue may result in recognition by CTL of HLA B27 as "altered self" is reviewed by Benjamin and Parham (1990).

All models must attempt to explain the tissue-specificity of the spondylarthropathies, in which disease involvement is restricted to the entheses and fascia, sacroiliac and peripheral joints (usually lower limb), aortic arch, mucous membranes including conjunctivae, and iris. The arthritogenic peptide model can easily be modified to predict that any cross-reactive self peptide would be expressed only in these tissues. This would also explain why alloreactive HLA B27-specific CTL clones have not distinguished BCL from AS sufferers from those of unaffected HLA B27-positive individuals (Czitrom *et al.* 1986). It is notable that some groups have reported the prolonged sequestration of *Chlamydial* antigens in the joints of patients with SARA, reviewed by Kingsley and Sieper (1993). It is thus possible that these diseases are in fact immune, with T cell responses directed entirely against foreign peptides, rather than autoimmune.

1.7. Aims of the work undertaken

The aims of the work undertaken were firstly to characterize the molecular anatomy of cytotoxic T cell recognition of HLA B27

the TCR usage and fine specificity of peptide recognition of a number of HLA B27-restricted NP383-391-specific CTL clones were studied. This study was greatly aided by the publication of the crystal structure of the third element of the trimolecular complex, HLA B27. A preliminary study of TCR usage of HLA B27-restricted CTL lines recognizing different peptides was also undertaken to look for common TCR segment usage. It was envisaged that the results would give information about the molecular basis of immune recognition in general, and might also give an indication of the role of HLA B27 in the pathogenesis of autoimmune arthritides.

The recognition of bacterial superantigens by CTL was also studied, both to establish if a variety of superantigens could interact with the CTL TCR, and to see if (novel) superantigens produced by enteric bacteria might have the potential to induce autoimmune arthritis in this manner.

CHAPTER 2

MATERIALS AND METHODS

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2.1. Tissue culture

All tissue culture was carried out in a containment class 2 cabinet (Mat UK). Cell lines were incubated at 37°C in humidified 5.5% CO_2. Cells were cultured throughout in RPMI 1640 medium (Gibco laboratories, Grand Island NY) supplemented with penicillin and streptomycin (50 U/ml, Gibco), 2mM glutamine (Gibco), and 10% fetal calf serum (Gibco) — abbreviated to R10, except for rat basophil leukemia (RBL) cells, which were cultured in Dulbecco's Modified Eagle medium (DMEM) containing 4.5g/l glucose, together with the same supplements. CTL lines were grown in R10 with recombinant IL2 (a generous gift from Cetus Corp. Emeryville, USA) added at 5-10 U/ml. For CTL clones 10% lymphocult T (Biotest AG,

Dreieich, FRG) was added. Cells were pelleted by centrifugation at 1,000 rpm in a bench top centrifuge (Beckman U.K). For freezing, cell pellets were chilled on ice and resuspended in 1ml of ice cold freezing medium containing 90% FCS and 10% dimethylsulfoxide (DMSO). The cell suspension was immediately transferred to a prechilled freezing vial (Nunc UK) and placed in a -70°C freezer. Vials of cells were thawed at room temperature until their contents had just melted, then the cells were transferred to 20ml universal container, and 5ml R10 added dropwise. The cells were then pelleted and washed again in R10 before being resuspended in 5mls of appropriate medium.

Separation of peripheral blood leucocytes (PBL).

20-50ml venous blood was transferred to 1-2 50ml tubes (Falcon UK) containing approximately 100 units of preservative free heparin (Leo laboratories), and an equal volume of RPMI 1640 medium immediately added. 15ml aliquots of diluted blood were overlayed onto 5ml of lymphocyte separation medium (Flow laboratories Ltd., Scotland), and centrifuged for 30 minutes at room temperature at 1,200 rpm (with braking off). The buffy coat layer was removed from the interface of plasma and lymphocyte separation medium using a plastic pasteur pipette, and transferred to a sterile universal tube. The cells were washed once at 2,000rpm for 15' and once at 1,000rpm for 10' (to leave platelets unpelleted). PBL were resuspended in 5-10 mls supplemented medium (or RPMI without serum for subsequent infection with EBV or Influenza virus) and counted using a slide haemocytometer (Coulter UK).

HLA typing of fresh PBL was performed by the tissue-typing laboratory, Nuffield Dept. Surgery, Churchill hospital, Oxford. Table 2.1 shows the HLA types of donors of PBL, BCL and CTL used in this thesis.

DONOR	HLA TYPE				
	A	B	C	DR	DQ
GR	3, 31	*2705	w2	4, w53	
HF	2, 3	7, *2705	w1, 7		
SD	2,32	*2705,49	w2	4	8
JM	2	15, 51		4	
CM	1,2	7,8		3	
NW	3, 33	8, *2702		3, 14	
DN	24,29	51,60	3	13	1
PM	1,3	7,8	7	3	2

Table 2.1. HLA types of donors of PBL, BCL and CTL. All except HF were laboratory workers.

Establishment and maintenance of human B-lymphoblastoid cell lines (BCL).

Approximately 10^7 PBL were pelleted and resuspended in 1ml EBV-containing supernatant obtained from a marmoset cell line B95-8, productively infected with EBV, and stored in 1ml aliquots at 4°C. After incubation for 1 hour at 37°C, 5ml of R10 containing 1µg/ml cyclosporin A (Sandoz) was added and cells were transferred to 50ml tissue culture flasks (Nunc, UK). 1µg/ml cyclosporin selection was maintained for 1 month to suppress T lymphocyte growth. BCL were periodically tested for mycoplasma contamination by co-culture with a mycoplasma free indicator cell line and fluorescent staining using Hoescht 33258. EBV supernatant production and mycoplasma testing was kindly performed by P. Thornton-Evison.

In addition to BCL, the following cell lines were used as APC or CTL targets:

- C1RHMy2, a B cell/myeloma cell line in which HLA class 1 expression was "reduced" by mutagenesis (Edwards *et al.* 1982), transfected with HLA B*2705 or B*2702 (gifts from J. Lopez de Castro).

- P815, a murine mastocytoma cell line, transfected with HLA B*2705 or B*2702 (gifts from Elizabeth Weiss).
- T2, derived from a human EBV-transformed BCL with homozygous radiation-induced deletions reducing both MHC class 1 and 2 expression (DeMars *et al.* 1984), transfected with HLA B*2705 (a gift of P. Creswell, USA).

Establishment and culture of Influenza A-specific cytotoxic T cell lines and clones

Influenza A virus specific cytotoxic T cell (CTL) lines were generated as previously described (Gotch *et al.* 1987), from five naturally immune donors. Three were HLA B27 positive (GR, HF, and SD), one HLA A2 positive (JM), and one was HLA B8 positive (CM). $1.2x10^7$ PBL in 12 ml supplemented but serum-free RPMI 1640 were cultured for one hour with trace amounts (6µl) of Influenza A. The AX/31 strain, an H3N2 recombinant of A/Aichi/68 and A/PR8/34 (Lubeck *et al.* 1979), was grown in the allantoic cavity of 11-day old embryonated chicken eggs by Dr. F.M. Gotch (IMM, Oxford), and batches with haemagglutinin titres greater than 1 in 1,000 were stored at -70°C in 20-100µl aliquots. After 1 hour 1.2ml FCS was added to inactivate the virus and the cells were transferred to 50ml flasks. Cell were incubated for 5-7 days before testing for cytolytic activity. After 1 week R10 with IL2 at 10U/ml was added. CTL lines were obtained by stimulation with peptide-pulsed autologous B lymphoblastoid cells (BCL). A pellet of approximately $5x10^6$ BCL was incubated with peptide at approximately 50-100µM for one hour, and then irradiated for 5.6 minutes in a Compugamma irradiator (Atomic Energy Authority of Canada) receiving a dose of 3,000 rads (1 rad = 0.01 Gy). The BCL were washed twice in R10, resuspended at 10^6/ml in R10 with IL2 at 5-10U/ml, and 200-500µl added to 10^6 CTL in 24 well Costar plates. CTL were "restimulated" in this manner initially after 10-14 days, and weekly thereafter. HLA A2-restricted JM CTL were stimulated using the Influenza matrix peptide 57-68 or 58-66 (Gotch *et al.* 1987). Initially the nucleoprotein (NP) peptides 380-391, and 384-394 were used to stimulate HLA B27-restricted HF, GR and SD CTL (Huet *et al.* 1990). When the optimal nonamer 383-391 was defined by S. Rowland-Jones (Colbert *et al.* 1993), this was

used in preference. NP 380-391 was used to restimulate HLA B8-restricted CTL. CTL were grown in 24 well costar plates, and medium was replaced thrice weekly with R10 containing IL2 at 5-10 U/ml.

CTL clones were obtained by limiting dilution at 0.3-1 cells/well in 96 well flat bottom plates (Nunc) in the presence of 100µl "cloning mixture"/well. 10 mls of cloning mixture was made up containing 10^5 peptide-pulsed irradiated autologous BCL/ml, 10^6 irradiated allogeneic PBL/ml and PHA (Wellcome) at 5ug/ml, following a method described by Spits (Spits *et al.* 1982), and kindly brought to our attention by Dr. R O'Hehir (St. Mary's Hospital Medical School, London). After 4 and 7 days wells were fed with 50µl R10 medium with 10%-20% lymphocult T, and after 11 days positive wells were transferred to individual wells of 24 well Costar plates in the presence of 1ml cloning mixture/well. CTL clones could usually be tested in CTL assays at 10-16 days. Cloning efficiencies are shown in table 5.1.

CTL cytotoxicity assays

CTL lysis assays were performed as described by (Gotch *et al.* 1987). Target cells were pelleted and resuspended in 100µl of $NaCr^{51}O_4$ (Amersham, 0.3mCi/ml in sterile PBS). After 1 hour labelling at 37°C, cells were pelleted and the supernatant removed into a lead shielded container. The cells were washed twice in R10 and made up to 1-2x10^5/ml in warm R10. 50µl were aliquoted into 96 well U-bottomed plates giving either 5x10^3 or 10^4 targets/well, to which 100µl of washed CTL and 5µl of appropriately diluted peptide, superantigen or R10 were added. The final volume was thus 155µl. Each experimental value was performed in duplicate or triplicate. Total labelling was assessed by adding 105µl 5% Triton X detergent solution to 4 wells of BCL. Spontaneous release was assessed by adding 105µl R10 to 4 wells of targets. After 4-5 hour incubation at 37°C, 20µl supernatant was harvested onto filtermats (Pharmacia). These were dried for 10' at 80°C, placed in plastic sleeves, and 10ml scintillation fluid (Pharmacia) added, before counting in an LKB Pharmacia flat bed γß-plate counter. Results were calculated as percent specific lysis:

= (experimental cpm - spontaneous release cpm) x 100%
maximal release cpm - spontaneous release cpm

Background spontaneous chromium releases was calculated:

= spontaneous release x 100%
detergent control

and was less than 20% in all assays.

Proliferation assays

PBL from donor NW were cultured with autologous irradiated (5,000 rads) antigen presenting cells (APC) in 96 well plates at 10^5 cells/well. As APC PBMC were fixed with 0.025% glutaraldehyde for 90 seconds before adding 0.2 M lysine and washing three times in medium. APC were incubated for two hours with purified protein derivative (PPD) (100,000 u/ml, Evans Medical, Horsham) diluted 1/200 or 1/500, tetanus toxoid diluted 1/1,000, CPET at 3-300nM, SEB at 3nM or PHA at 5ug/ml before washing twice. After 3, 5 or 6 days 1uCi ^3H thymidine was added, cells were harvested after 6 hours and counted using the ß plate counter.

Cytofluorography

The following monoclonal antibodies (mAbs) were used to stain cells:

W6/32: A murine IgG2a mAb reacting with all known human HLA class 1 molecules when assembled with ß2M. For FACS staining ascites was diluted 1:1000 in PBS.

ME 1: A murine IgG1 mAb specific for ß2m assembled HLA B27, B7 Bw22 and Bw42 (Ellis *et al.* 1982). Neat supernatant or ascites diluted 1 in 500 with PBS was used for FACS staining.

ßF1: A murine IgG1 mAb specific for the Cß region of the TCR (Brenner *et al.* 1987). This does not stain the TCR of unfixed lymphocytes but stained RBL cells transfected with TCR CD3 zeta fusion heterodimers (see below).

Jovi 1: A generous gift from J. Viney/ M. Owen (ICRF London). A recently generated IgG2a mAb staining approximately 60% of CD3-positive PBL, TCR ß chain-specific.

42/1C2 31/1C2 MX6 Gifts from A. Boylston (Leeds University). mAbs specific for Vß5.2/3 and Vß8 respectively (Boylston *et al.* 1986, Clark *et al.* 1986).

Approximately 10^5 cells were pelleted in Falcon 2054 tubes and resuspended in 100µl ice-cold PBS or appropriate mAb diluted in PBS. After 30' on ice cells were washed with ice cold PBS containing 1% BSA and 0.06% Na azide. Cells were resuspended in 100µl of FITC-labelled goat anti mouse antibody (Sigma, diluted 1 in 40 in PBS/BSA/azide). After 30-45' on ice cells were washed, resuspended in 300µl PBS/BSA/azide and analysed immediately. Alternatively cells fixed in PBS/1%FCS/1% formaldehyde could be stored at 4°C in the dark for several days prior to analysis.

Cytofluorography was carried out using a Beckton-Dickinson FACSCAN instrument and the Consort 30 acquisition and analysis program. Cell sorting of TCR transfectants, stained under sterile conditions using filtered DMEM10 instead of PBS/BSA/azide, was carried out by Mr. R Boon (IMM) using a Beckton Dickinson FACSSTAR cell sorter and Lysis II software. Sorted cells (brightest 10% FL1) were washed twice in DMEM10 before transfer to 50 ml flasks.

Selection of activated lymphocytes for TCR analysis

PBL from donors WR, GR, PM, and PB were cultured for 72 hours at 10^6 cells/ml R10 in 24 well costar plates. 10^7 cells in 5 wells were cultured with each of CPET, TSST, SEB, CDT (all at 100ng/ml) or PHA at 5ug/ml. The cultured cells were then washed with cold PBS and incubated on ice for 45' with the monoclonal antibody 23A9.3 (a gift of C. Mawas, Marseille), specific for the IL2 receptor (IL2-R). Lymphocytes bearing the IL2 receptor were selected with magnetic beads coated with goat anti-mouse immunoglobulin (Dynal, Wirral, U.K). Fresh unselected PBMC or IL2-R selected PHA-stimulated PBMC were used as controls.

2.2. Peptide synthesis

Synthetic nonamer peptide analogues of Influenza A NP residues 383-391 were purchased from CRB, Cambridge UK, or synthesized on a SMPS 350 peptide synthesizer (Zinsser analytic, FRG). Standard Fmoc chemistry was used. After synthesis, the amino-terminal Fmoc group was deprotected using piperidine, and peptides were then cleaved from the resin overnight (and the peptide side

chains deprotected) using a cleavage solution composed of 92 parts trifluoracetic acid (TFA), 4 parts EDT, 2 parts anisol and 2 parts phenol. This step was prolonged because arginine side chains are difficult to deprotect. Peptides were then frozen in dry ice/methanol before freeze-drying in a Micro Modulyo freeze dryer (Edwards UK). 200μg (dry weight) of each peptide was dissolved in 1% TFA and analyzed by HPLC to assess purity and approximate concentration. All peptides tested gave one dominant peak comprising 42% (for 7D and 8F) to 91% (7E and 8A) of the total. Significant lesser peaks running at later time points (i.e. more hydrophobic) were observed in one batch of peptides, marked with an asterisk in table 6.1 and including 7D and 8F, which probably represent failed synthesis or failed deprotection of arginine residues (manufacturers comments). Whilst these peaks were not further analysed, it is unlikely that they could have significant biological activity as R2 is essential for binding to HLA B27, as is thought to be a positively charged side chain at position 9, and R7 is critical for TCR recognition (see below). Peptides were stored as powder at room temperature or as 1mM stock solutions in RPMI 1640 at -20°C. On the day of assay 10 fold dilutions were made in RPMI 1640, these were freeze-thawed only once before discarding.

2.3. Purification of enterotoxins

SEA, SEB, SEC2, SED, SEE and CPET were purified by H. Tranter (PHLS, Porton Down, Hampshire) from culture supernatants by dye ligand affinity chromatography as described (Reynolds *et al.* 1986, Brehm *et al.* 1990). TSST was a gift from Rosalind Brehm (PHLS, Wilts, UK). CDT, a gift from Dr. April Roberts (PHLS), was purified by anion exchange chromatography and isoelectric point precipitation in a separate facility to that used to purify CPET.

2.4. HLA B27 assembly assays

HLA B27 assembly was promoted *in vitro* by the addition of peptides to lysates of metabolically radiolabelled T2 cells transfected with HLA B27 (obtained from P. Cresswell USA), based on a method developed by (Elvin *et al.* 1991). Assembled HLA B27 (and other class 1 molecules) were then immunoprecipitated with W6/32 and identified and quantitated by one dimensional isoelectric focussing

(Neefjes *et al.* 1986). When this method was followed exactly it was found that a majority of the HLA B27 was in a W6/32 precipitable form, even in the absence of added peptide (data not shown). Consequently the method was modified to include heat denaturation, according to an unpublished protocol of B. Colbert (UNC USA). 10^7 log phase T2/B27 cells per condition were washed in PBS and resuspended in 2 ml methionine-free supplemented RPMI 1640 (Gibco UK). After 30' incubation at 37°C, 100µCi/condition of S^{35} methionine with a specific activity of 1000 Ci/mM (Amersham), was added for a further 1-2 hours. After washing and appropriate disposal of S^{35} waste, the cells were lysed into 0.5ml/condition lysis/wash solution. If 24 samples were being processed 40ml would be made up as follows:

40ml lysis buffer (0.5% NP40, 20mM Tris pH 8, 10mM EDTA, 100mM NaCL)
200mg Mega 9 (Sigma)
400µl IAA
400µl PMSF

From this point all work was carried out on ice or at 4°C although it is not clear if this was strictly necessary. After 30' on ice, the cells were transferred to eppendorf tubes and the nuclei spun down at 15,000rpm for 15'. At this point the supernatants were split into into 1 eppendorf/condition (e.g. 12 or 24 tubes), and peptides/PBS added. 250µl 10% *Staphylococcus* A cells (Pansorbin), thrice washed in lysis buffer, were then added to the eppendorfs, which were then heated to 60°C for 20 minutes, and precleared by rotation end-over-end overnight. The Pansorbin was sedimented and 4µl W6/32 ascites added to the supernatants. HC10, a mAb precipitating all class 1 heavy chains, was used as a control to measure total assembled and unassembled HLA B27. After 1 hour rotation 100µl washed 10% Protein A sepharose (Sigma) in lysis buffer was added for a further hour. Finally the complexes with Protein A sepharose beads were washed four times with lysis/wash buffer, and resuspended in 60µl sample buffer (9.5M urea, 2%NP40, 2% ampholines 3.5-10, 5% 2ME), and incubated at room temperature for 30-60'. The beads were sedimented by brief centrifugation, and 40µl supernatant loaded on the IEF gel. The pellet was saved for re-elution if required.

Isoelectric focussing gel was made up as follows:

16.5g Urea (final conc. 9M)
6.6ml dd water
6mls 10% NP40 (final conc. 2%)
4.5ml acrilamide stock (30% acrilamide/1.6% Bis acrilamide (Bio-rad)) (final conc. 4.5%)
1.2ml pH 5-7 ampholines (Pharmacia) (final conc. 4%)
0.3ml pH 3.5-10 ampholines (Pharmacia) (final conc. 1%)
60µl 10% w/v APS
30µl TEMED.

The upper buffer was 50mM NaOH, the lower buffer contained 20mM H_3PO_4. Two 16mm gels were run simultaneously in a Bio-rad Protean II gel apparatus at 880V, 12mA, and 8W for 13-16 hr. Gels were then fixed in 10% acetic acid and treated with Amplify (Amersham UK) for 30', dried and exposed to Kodak XAR film. HLA B*2705 bands were identified with reference to published data and to C1R B27 transfectant controls. Using a developed film as a template, the 1.2 x 0.6cm sections of the dried gel were cut out, stuck onto acetate, placed in a plastic bag with scintillant, and counted using the ß plate counter.

2.5. Anchor PCR analysis of TCR gene usage

Isolation of total cellular RNA

RNA was usually isolated from 5 x 10^6 activated lymphocytes, CTL lines or clones using the RNAzol kit (Biogenesis, Bournemouth, UK), although as few as 10^6 were successfully used. The cells were washed once, resuspended in 1ml PBS, transferred to a 1.5ml eppendorf and spun down at 4,000rpm for 4'. Cells were resuspended in 20µl of PBS, 1ml RNAzol solution (200µl/10^6 cells), containing guanidinium thiocyanate and phenol, was added. 100µl chloroform was then added and the tube shaken vigorously for 15". The eppendorf was then placed on ice for 5', before centrifugation at 15,000rpm for 30' at 4°C. The upper aqueous phase was transferred to a fresh labelled eppendorf and the RNA precipitated by adding an equal volume of isopropanol. Following incubation on ice for 45' and centrifugation at 15,000 rpm for 15', the pellet was washed once in 70% ethanol (all solutions were made using DEPC-

treated water), dried and resuspended in 100µl of water. The amount of RNA was estimated by spectrophotometry of 5µl diluted in 1ml of water at a wavelength of 260nm (1 O.D. unit = 40µg/ml RNA, total RNA content in µg = O.D. reading x 200 x 40), and protein contamination estimated by a reading at 280nm. RNA was stored at -70°C as an ethanol precipitate (adding 10µl 3M Na acetate and 275µl 100% ethanol).

cDNA synthesis

cDNA was synthesized from 5µg of total cellular RNA. The RNA, stored as an ethanol precipitate, was centrifuged, washed with 70% ethanol and resuspended in 16µl DEPC-treated water (aided by heating to 55°C for 3'). 4µl of oligo dT primer at 50U/ml (Collaborative Research Inc. Bedford Mass, USA) was added and the mixture heated to 70°C for 3' to denature any secondary nucleic acid structure. After 3' on ice 0.55µl RNAsin (40,000 U/ml Promega) was added, followed by 8µl 5mM dNTPs (Pharmacia Ultrapure), 4µl 0.1M DTT, 4µl RTase buffer, and finally 1.5µl Avian Myeloblastosis Virus reverse transcriptase (Life Sciences, Inc, Fl). The reverse transcription reaction was performed for 1 hour at 42°C. The reaction products were precipitated three times with an equal volume of ammonium acetate and three volumes of 100% ethanol, and finally washed with 70% ethanol, dried and resuspended in 11µl water. A poly G tail was added to the first strand cDNA as follows: 4µl manufacturers buffer (5x), 3µl CoCl (diluted 1/5 before use), 1µl 100µM dG, and 1µl (25U) TdT (Boehringer Mannheim) were mixed and incubated at 37°C for 15'. The reaction products were phenol chloroform extracted and stored at -70°C in a final volume of 200µl water.

Anchored PCR

AnPCR was performed following the method of (Loh *et al.* 1989), as modified for human TCR αß amplification by Rosenberg *et al.* (1991) and Moss *et al.* (1991). PCR was performed using Taq polymerase (Boehringer Mannheim) together with manufacturers buffer and dNTPs at 200nM. The oligonucleotide PCR primers, listed in table 2.2, were provided by P. Moss and P. Catlin. They had been synthesized on an Applied Biosystems 392 DNA/RNA synthesizer,

eluted with 1.5ml NH_4OH, ethanol precipitated, resuspended in water and used at 250nM. The 3' Bgl 2-containing primers were used for early amplifications of cDNA from CTL lines, the 3' Sal 1 primers were used subsequently. The following PCR conditions were used: 95°C for 4 minutes, 55° for 2 minutes, and 72° C for 10 minutes for 1 cycle, followed by 95°C for 1', 55° for 2', and 72° C for 10' repeated for 30 cycles.

Amplification products were digested with either Not 1 followed by Bgl 2, or with Not 1 together with Sal 1, using Promega enzymes and recommended buffer(s) in a final volume of 20µl. Digested products were size separated in a non-denaturing 4.5% polyacrilamide gel prepared as follows:

100ml acrilamide stock solution (75ml of Protogel 30%,
50ml 10x TBE and 375ml water)
100µl TMED
600µl 10% APS.

5µl Orange G loading buffer was added to the samples, which were loaded and run at 400V for 40' and then 300V or 2 hours, soaked in 100ml TBE containing 5µl ethidium bromide for 15', and the bands cut out minimizing exposure to (long) wavelength UV light. DNA was extracted using the "crush soak" technique of Maxam and Gilbert as detailed in (Sambrook *et al.* 1989). The purified insert was resuspended in 50µl water and ligated for 4-16 hours at 16°C at an approximately 1:1 molar ratio into an

For anPCR

anchor poly C 5'-GCATGCGC**GCGGCCGC**GGAGG-[C]$_{14}$-3' sense
Not1

Cα Bgl2 5'-GAT**AGATCT**TAGAGTCTCTCAGC antisense

Cß Bgl2 5'-CGCGAATTC**AGATCT**CTGCTTCTGATG antisense
Bgl2

3'α sal1 5'-TGACCGCA**GTCGAC**AGACTTGTCACTGGATT-3' antisense

3'β sal1 5'-CGTTTGTC**GTCGAC**CTCCTTCCCATTCACC-3' antisense
Sal1

For expression of clone GRb α and ß chains

TO#10: **Kozak and Vα14 leader**

5'-ccttccttcc/**CTC GAG**/CCGCCACC/ATG GCA TGC CCT GGC
Xho1 Kozak TTC CTG TGG GCA CT-3'sense

TO#3: -**Cα**

5'-ccttccttcc/**GGA TCC**/CC/ACA GGA ACT TTC TGG GCTG-3'
BamH1 Spacer Cys and Cα 393-377 antisense

TO#12: **Vß7.1 5'UTR**

5'-ccttccttcc/**GTC GAC**/GAG GCT AGC ATG GGC TGC-3'
Sal1 Vß 7.1 5'UTR 21-38 sense

TO#4: **Cß2**

5'-ccttccttcc/**GGA TCC**/CC/ACA GTC TGC TCT ACC CC-3'
Bam H1 Spacer Cys and Cß 393-377 antisense

Sequencing oligos

M13 -17 universal primer 5'-GTTTTCCCAGTCACGAC-3' antisense

Px3' SRα 3189-3209 5'-AACTCTACGTCTTTGTTTCG-3' sense

CαRT. Cα 5'-CAAAATCGGTGAATAGGC-3' antisense

CßRT. Cß 5'-AGAAGCCTGTGGCCAG-3' antisense

TO#6. Cα 2-21 5'-CAAATATCCAGAACCCTGAC-3' sense

TO#7. Cß 16-25 5'-GTGTTCCCACCCGAGGTCGC-3' sense

Detection oligos

Cß	5'-CGACTTCGGGTGGGAACAC-3'
Vß6.9	5'-TGGAGAAAGATCCCTTAG-3'
Vß22	5'-AGTGAAATTTGATCCATCAG-3'
Vß 24	5'-AAAGCAGAAAGAAGTGTT-3'

Table 2.2. Oligonucleotides used for PCR, DNA sequencing and Southern blot hybridisation.

appropriately cut modified M13mp18 vector, containing the pBluescript II KS+ polylinker (Rosenberg *et al.* 1991). When Bgl 2 was used the M13 was further modified to deleted an intrinsic Bgl 2 site, this was performed by W. Rosenberg in J. Bell's laboratory (Rosenberg *et al.* 1991). Competent JM101 or DH5 alpha E coli were prepared using either the method of Hanahan (Sambrook *et al.* 1989), or of Chung. *et al.* (1989). Aliquots of 210µl freshly prepared competent cells were incubated on ice for 40' with 3 µl of a 10µl ligation mix in Falcon 2059 14 ml tubes, the remainder being stored at -20°C for reuse as necessary. The cells were heat-shocked for 2' at 42°C, and then placed on ice for 2'. 3ml molten soft top 2YT medium (at 55°C), 300µl bacterial lawn, 40µl 100mM ITPG and 40µl XGAL solution (20mg/ml in dimethyl formamide) were added and the mixture poured onto pre-warmed 9cm 2XYT plates. After drying plates were inverted and incubated for 16 hours at 27°C. Clear plaques were picked into 1.5ml 2XYT medium, and after 6 hours at 37°C single stranded DNA was prepared by polyethylene glycol precipitation as described by Sambrook *et al.* (1989). Dideoxy sequencing was performed using the method of Sanger *et al.* (1977), using T7 DNA polymerase and Sequenase reagents (USB) and the universal M13 -17 primer (table 2.2).

Southern blot TCR Vß family hybridisation

This was used either to identify plaques containing TCR inserts (if the majority of cloned material was not TCR), or to quantify the TCR Vß family member expression of large numbers of cloned products. Plates were blotted onto nitrocellulose filters (HiBond C Amersham UK), baked at 80°C for 2 hours, and hybridized with P^{32} radiolabelled oligonucleotide probes for Cß (5'-CGACTTCGGGTGGGAACAC), Vß6.9 (5'-TGGAGAAAGATCCCTTAG), Vß22 (5'-AGTGAAATTTGATCCATCAG), or Vß 24 (5'-AAAGCAGAAAGAAGTGTT).

2.6. TCR Expression

Cloning of CTL clone GRb α and ß chains into the expression vector SRαζ.

Full length sequences of CTL clone GRb α and ß chains were obtained by sequencing M13 clones following anPCR. The Vß7 sequence of GRb corresponded to the published IGRb19 sequence (Ferradini *et al.* 1991). The Vα14 coding sequence was identical to that previously published (Kimura *et al* 1097).

PCR of cDNA prepared by the author was performed by Dr. Tom Ottenhof, N.I.H. Bethesda, USA. The cloning and transfection steps were performed together with Tom Ottenhof in Richard Klausner's laboratory. Screening and functional studies were performed in Oxford.

The expression vector used, "SRαζ", was derived by Isaac Engel in Richard Klausner's laboratory from the vector pcDL-SRα296 (Takabe *et al* 1988), and is illustrated in figure 2.1. The SRα vector contains an ampicillin resistance gene for bacterial selection, and uses an SV40 origin together with an HTLV1 promoter and enhancer for eucaryotic transcription. Briefly, the pcDL-SRα296 polylinker was excised at Xho1 sites 37 and 3244, and replaced with that from pxs (5 'Xho1, 3' Sal1). A thrombin cleavage site (GD**LVPRGS**SRLD) was introduced, together with residues 31-164 of the murine TCRζ chain, comprising the transmembrane and cytoplasmic portions. This procedure created a Bagl2 site at the 5' of the linker containing the thrombin cleavage site, which was used to clone the GRb TCR α and ß chains, using the oligonucleotide primer pairs TO#10 and TO#3, and TO#12 and TO#4, shown in table 2.2. A 5' Vß7 amplification oligonucleotide containing a Sal1 cloning site was used because the GRb ß chain contains an internal Xho1 site at the Jß-Cß junction (see appendix 6). Consequently, although compatible with the 5' Xho1 cloning site of SRαζ, cloning resulted in a loss of this 5' site. Similarly the 3' site was also lost because the GRb α and ß chains were amplified with 3' Bam1-containing primers. The oligonucleotide primers used for α and ß chain amplification,



Figure 2.1. Cartoon of the modified expression vector "SRαζ". Residues 3244-37 of the vector pcDL-SRα (Takabe *et al.* 1988) were replaced with the pxs polylinker at the sites marked with ●, into which was cloned the murine TCR ζ chain (residues 31-164) and a thrombin cleavage site. The GRb alpha and beta chains were inserted at the points marked ↙.

shown in table 2.2, were designed by T. Ottenhof and used to amplify suitable fragments for cloning from the same cDNA used for anchor PCR. Notably the M13 cloned fragments were not suitable because anPCR does not amplify enough of the TCR constant regions. Although the 5' untranslated region (5'UTR) of Vα14 was obtained, an oligonucleotide primer, TO#10, using a Kozak ribosome binding sequence, was successfully employed. The PCR conditions were as follows:
denaturation: 94°C 5' (then 92°C 2'), annealing: 55°C 2', extension: 72°C 1.5', for 20 cycles.

PCR products were digested with Sal1 (or Xho1) and Bam1, and cloned into Xho1/ Bgl2-cut SRαζ at a molar ratio of 2:1, using 30ng of 0.8kB insert and 70ng of 4Kb cut vector. The ligations were transformed into competent DH5α (BRL, Bethesda USA). 3 α and 3 ß colonies were picked, grown overnight in 250ml LB amp, and DNA prepared using Quiagen columns, exactly following manufacturers instructions. Aliquots of 16μg (4x4μg) of DNA were denatured for 5' at room temperature using:

DNA 4μg x 4
4μl Denaturing buffer (10x) 2M NaOH 25mM EDTA
water to make up 40μl

Samples were ethanol precipitated, washed with 70% ethanol and resuspended in (e.g. 4 x 6μl) water. 2μl sequencing primers were added at 10ng/ml, together with 2μl annealing buffer. The mixture was heated to 65°C for 15', allowed to cool to room temperature, and sequenced using Sequenase reagents as described previously. One GRb ß clone was found to contain errors, presumably resulting from the PCR.

Approximately 50μg of DNA was linearized as follows:

GRb α/SRαζ

DNA	50μl (50μg)
Promega buffer D	10μl
BSA	10μl
Sal1	12μl (100U)

water to a total volume 100μl

GRb ß/SRαζ

DNA	50μg
Promega buffer B	10μl
BSA	10μl
Hnd3	8μl (100U)

water to a total volume 100μl

Following incubation at 37°C for approximately 2 hours, an aliquot was run on a 1% agarose gel to confirm linearisation (Sal 1 cuts the vector twice), and the remaining DNA was phenol/chloroform

extracted, ethanol precipitated, and resuspended in 50µl sterile water. Linearized DNA could either be transfected immediately or stored at -20°C.

Transfection of RBL-2H3 cells

Rat basophil leukaemia RBL-2H3 cells were obtained from H. Metzger (N.I.H). RBL are adherent cells, and possess IgE Fc receptors facilitating IgE-mediated degranulation. The transfection protocol follows that described by Engel *et al.* (1992).

RBL cells were harvested by trypsinisation, washed with PBS and resuspended in DMEM10 at 4 x 10^7 cells/ml. 250µl cell suspension was added to a 0.4 cm gap electroporation cuvette (Biorad) together with 19-20µg linearized DNA (1-2µg pfNeo, 9µg GR$\alpha\zeta$ and 9µg GRßζ). After 10' at room temperature a charge of 250V and 500µfarads was applied using a Bio-rad gene pulser. After a 10' recovery period cells were put into 20ml DMEM with 15%FCS. Selection for neomycin resistance was started adding G418 (Geneticin, Gibco), 100µl of a 160mg/ml solution at 16-24 and 42-48 hours, to give a final concentration of 1.6mg/ml. This was maintained for approximately 2-3 weeks. The medium was replaced after 3-5 days and there were usually sufficient cells to FACS at 11-14 days.

Western blot analysis of TCR transfectants

3x10^6 RBL-2H3 cells transfected with GR$\alpha\zeta$, GRßζ and Pfneo, were washed, lysed in 100µl sample loading buffer, boiled for 5', iodoacetamide added, and run on a 10% SDS gel for 45' at 200V. The separating gel was prepared as follows:

3.33ml Acrilamide/Bisacrilamide (Protogel)
2.5ml 1.5M Tris-HCl pH 8.8
0.1ml 10%SDS

The human T cell line Jurkat 3x10^6 cells) was used as a positive control for CD3 ζ expression, and untransfected RBL as negative controls. The gel was transferred onto a Hybond nitrocellulose filter (Amersham) overnight at 4°C at 50V in transfer buffer (3.3g Tris, 14 glycine, 200ml methanol per litre). The blot was blocked with 5% Marvel milk (10g in 200ml PBS/azide) for 2 hours with rocking. This

was poured off and the first layer antibody, anti-ζ mAb TIA-2 (Coulter U.K), added at 1/25 in PBS/azide/1%BSA. After 2 hours rocking the blot was washed three times with 0.1% Tween 20 (Sigma), and the second layer antibody added — peroxidase-conjugated rabbit anti-mouse serum (Dako), diluted 1/2,000 in Marvel blotting solution. After a further 1 hour rocking, and 3 further washes with 0.1% Tween, the blot was visualized using the Enhanced Chemiluminescense reagents (Amersham, UK), following manufacturers instructions. 15" exposure to X-ray film gave satisfactory images.

2.7. RBL degranulation assays

The release of 5 hydroxytryptamine (5HT, serotonin) by RBL cells can be measured using tritium labelled 5-HT (5 hydroxytryptamine creatine sulphate Amersham UK), which is taken up by RBL cells. The method described is a simplified and modified version of that used by Engel *et al.* (1992).

On the day prior to the assay, RBL transfectants (and untransfected controls) were harvested, and 20-40μl tritiated 5-HT (1mCi/ml) added to $3x10^6$ cells in 10ml DMEM10 (for each 96 well plate). This was equivalent to $3x10^4$ cells/well and 0.2-0.4μCi 5-HT/well. Round and flat bottomed wells were found to be equally satisfactory. On the day of assay cells were washed three times with warm DMEM10, before adding 100μl antigen presenting cells (APC), see below, medium with or without added antibodies, PHA at 5ug/ml or triton X controls. In addition to PHA, the mAbs ßF1 or Jovi 1 were used as positive controls. Hybridoma supernatants were either used neat or diluted 1/10 in DMEM, together with 4μl of dynabeads coated with goat anti-mouse immunoglobulin (Dynal) to ensure cross-linking.

BCL, HMy2/C1R cells transfected with HLA B*2705 or HLA A2, or P815 cells transfected with HLA B27 were used as APC. APC were washed once in R10 and then incubated for 2-4 hours with peptides in R10. APC were usually, but not invariably, washed again before being made up to a final concentration of $1-4x10^6$ cells/ml. 100μl were added to each well of RBL in triplicate and the plates spun

down briefly at 400rpm in a benchtop centrifuge. After 45-60' incubation at 37°C in 5.5% CO_2 20µl supernatant was harvested onto ß plate filters and these were counted on an LKB ß-plate counter. Results were expressed as c.p.m. 3H 5-HT release or as specific 3H 5-HT release (deducting DMEM medium release) for comparison with untransfected RBL cells. Medium release was usually less than 5% of Triton X release and less than 20% of PHA release.

CHAPTER 3

SUPERANTIGEN RECOGNITION BY CYTOTOXIC T CELLS

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3.1. Introduction.

Superantigens (SA) may be defined as proteins that interact with lymphocyte TCRs in a Vß-selective, MHC unrestricted but class 2-dependent manner. SA do not require antigenic processing for T cell recognition and are active at nanomolar concentrations. This

purely operational definition, used here, allows SA to be distinguished from conventional antigens, which require processing and show MHC restriction, and mitogens, which are not TCR Vß-selective. This definition may however exclude certain endogenously presented SA and possible B cell SA (Pascual and Capra 1991), and will probably be superceded when the exact nature of the SA TCR interaction is more fully understood.

When the work reported in this thesis was commenced, preliminary experiments, performed by Fleischer and Schrezenmeier (1988), had suggested that the superantigens SEA and SEB could interact with human cytotoxic T cells (CTL), although Zehavi-Wilner and Berke (1986) had reported that this interaction could not induce cytolysis. Although CTL were known to recognize specific antigenic peptides in the context of MHC class 1 molecules, there did not appear to be any reason why they could not also recognize SA in the context of MHC class 2 molecules, because SA recognition had been shown to be independent of (classical) antigenic specificity. In order best to address this question it was planned to use CTL of known specificity (as a positive control for CTL function), and therefore influenza A-specific CTL were used. Since different SA had been shown to have different TCR Vß specificities, summarized in table 1. (Kappler *et al.* 1989), CTL from a number of donors were used. Preliminary evidence obtained by P. Moss in J. Bell's laboratory had shown that HLA A2-restricted influenza A matrix peptide M58-66-specific CTL lines used almost exclusively Vß17-containing TCR ß chains. It was considered likely that CTL with different specificities would utilize different predominant TCR Vß elements, and therefore influenza-specific CTL restricted by three different HLA class 1 alleles, HLA A2, B8 and B27, were used. Indeed it was hoped to use SA as probes to study TCR usage of these CTL.

The second reason for studying the interaction of CTL with SA was to investigate the possibility that the enteric bacteria associated with autoimmune rheumatic disease might activate (autoreactive) T cells by producing SA, as subsequently proposed by Friedman *et al.* (1991). Although this question remains unresolved, the CTL screening test developed to look for superantigenic activity was

used to identify two novel candidate SA produced by *Clostridium* species, one of which, *Clostridium perfringens* enterotoxin, was characterized in detail.

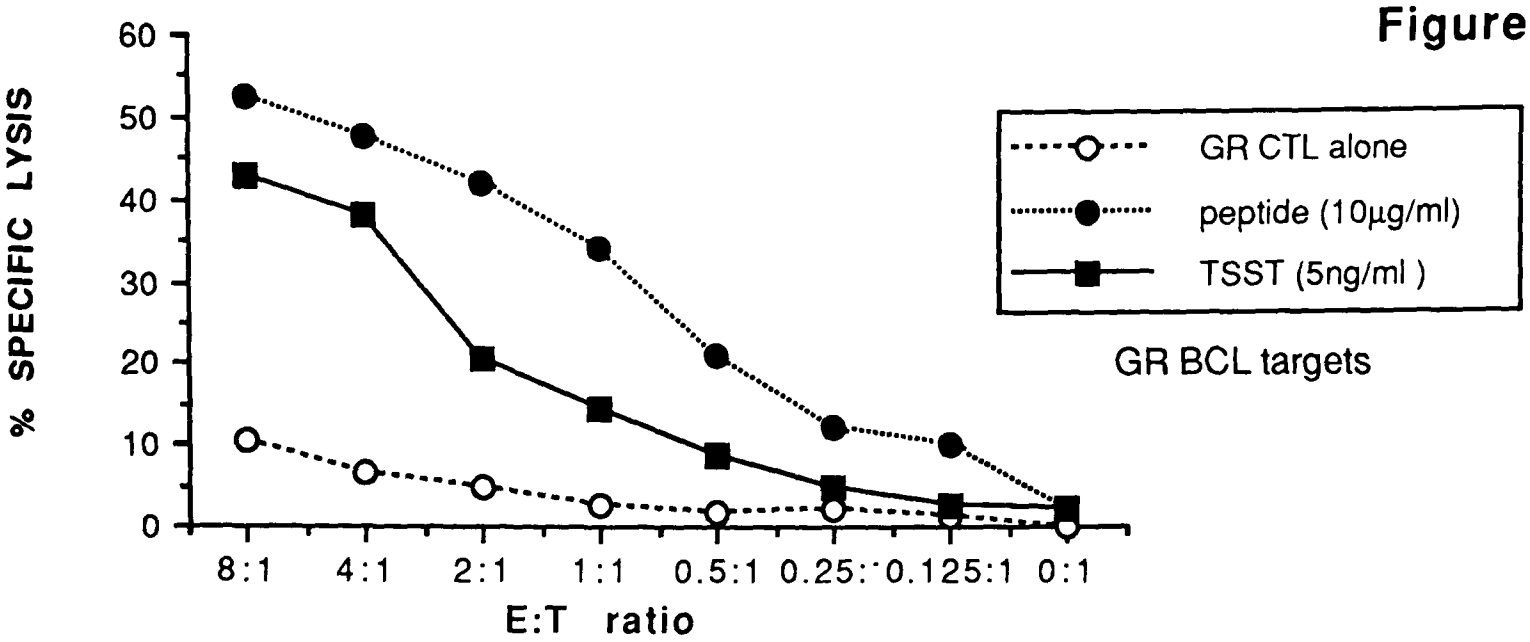
3.2. Interaction of superantigens with CTL.

The superantigen TSST induces cytolysis by HLA B27-restricted CTL from donor GR, but not by HLA A2-restricted CTL from donor JM

In preliminary experiments the SA Toxic Shock Syndrome Toxin 1 (TSST), produced by *Staphylococcus* aureus, was shown not to be toxic to chromium-labelled human BCL targets alone (data not shown), but induced cytolysis in the presence of an HLA B27-restricted influenza NP peptide 383-391-specific GR CTL line, as shown in figure 3.1. Approximately a 4-fold higher Effector:Target (E:T) ratio was needed to induce cytolysis comparable with that found for cognate peptide. In contrast, figure 3.2a shows that a JM CTL line, which was HLA A2-restricted and specific for the influenza A matrix peptide M58-66, did not kill autologous BCL targets in the presence of TSST. In the same experiment JM CTL did however recognize Staphylococcal enterotoxin B (SEB), as illustrated in figure 3.2b. The TCR Vß usage of these lines was studied by Vß family-specific PCR amplification and agarose gel electrophoresis of PCR products (J. Loveridge and P. Bowness, unpublished data). For GRCTL, the Vß7 family and Vß13.1 amplified strongly, and weaker bands were observed for Vß1, 5.1/2, 6 and 15. For JM CTL strong bands were seen for Vß13.1 and 17, and weaker bands for Vß 6 and 14.

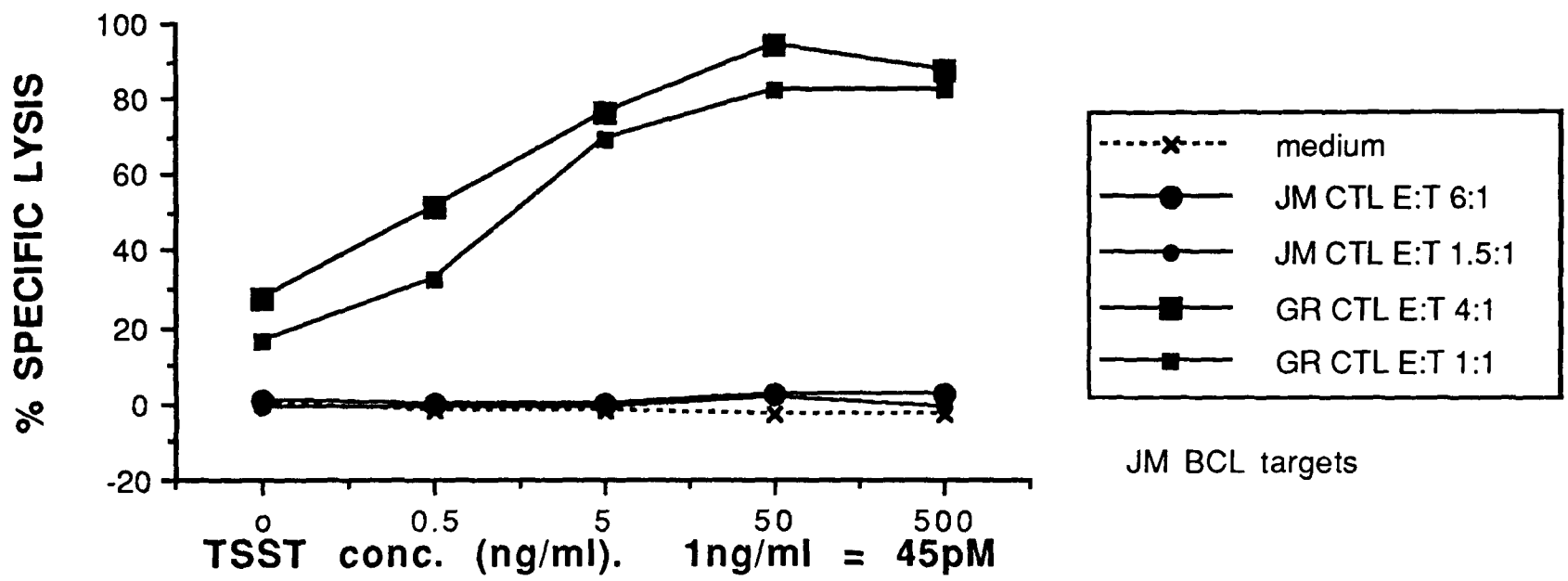
Killing by GR of autologous BCL targets with both NP382-395 and TSST

Figure 3.1



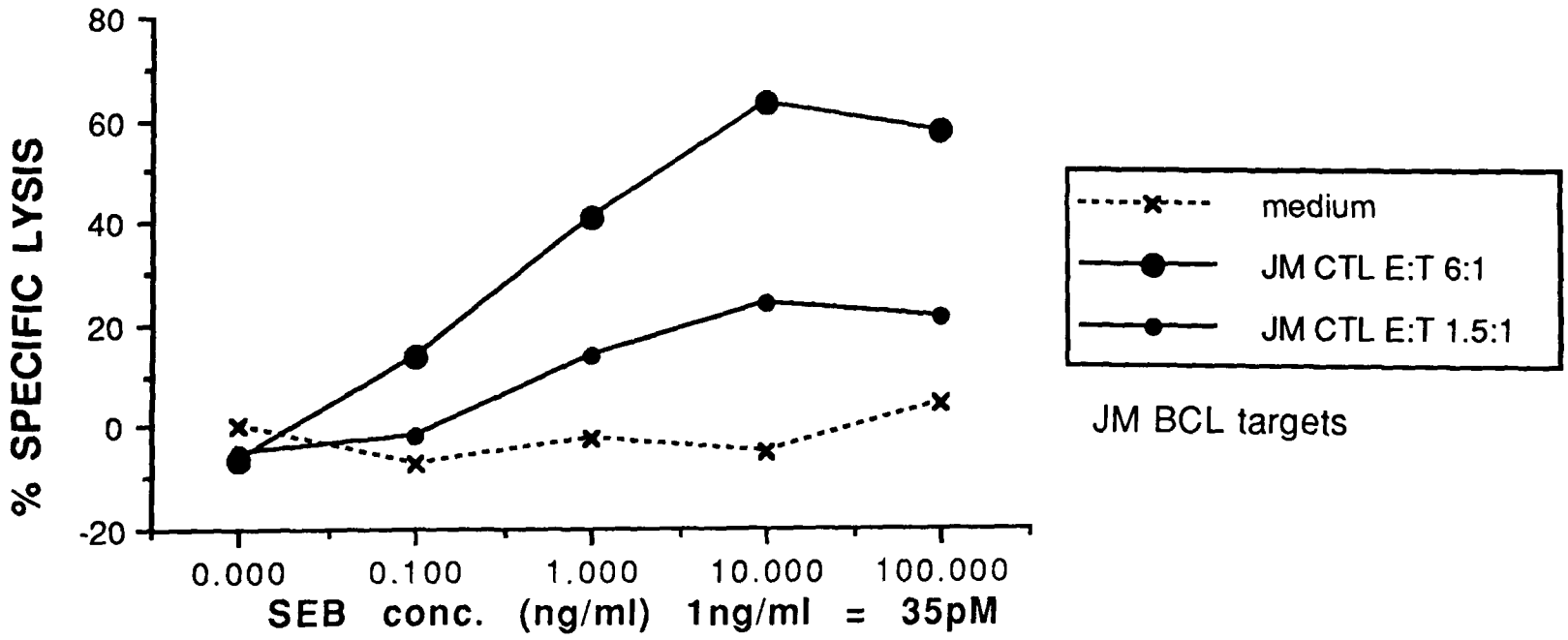
GR CTL but not JM CTL recognize TSST

Figure 3.2a



JM CTL kill autologous targets in the presence of SEB

Figure 3.2b



Figures 3.1 and 3.2. Superantigens induce target cytolysis by Cytotoxic T Lymphocytes.

Superantigen-induced cytolysis can be inhibited by antibodies to LFA3, and requires target cell MHC class 2 expression

In the experiment shown in figure 3.3a, the mAb W6/32 (anti-HLA class 1) did not inhibit SEB- or TSST-mediated cytolysis of autologous targets by GR CTL, at concentrations sufficient to abrogate recognition of cognate peptide*. Figure 3.3b shows that cytolysis was inhibited by the anti-DR mAb MHM31, and more profoundly by the anti LFA3 alpha chain antibody MHM23. Notably both these antibodies also blocked peptide-induced killing, and it is possible that they were toxic to the CTL themselves (which express HLA class 2). Furthermore in the experiment shown in figure 3.3b, MF8 and W6/32 were found to inhibit SA-induced cytolysis, possibly via a similar mechanism. Since conflicting results were obtained with these mAbs, cytolysis of HLA class 2 negative targets was studied. The mutant cell line T2, which expresses very low levels of HLA class 2 (and of HLA A2), was killed by the HLA A2-restricted JM CTL line after preincubation with its specific peptide epitope M58-66 (data not shown), but not by either JM CTL (data not shown) or GR CTL (figure 3.6b) in the presence of SEB.

Superantigen "repertoires" as indicators of TCR usage?

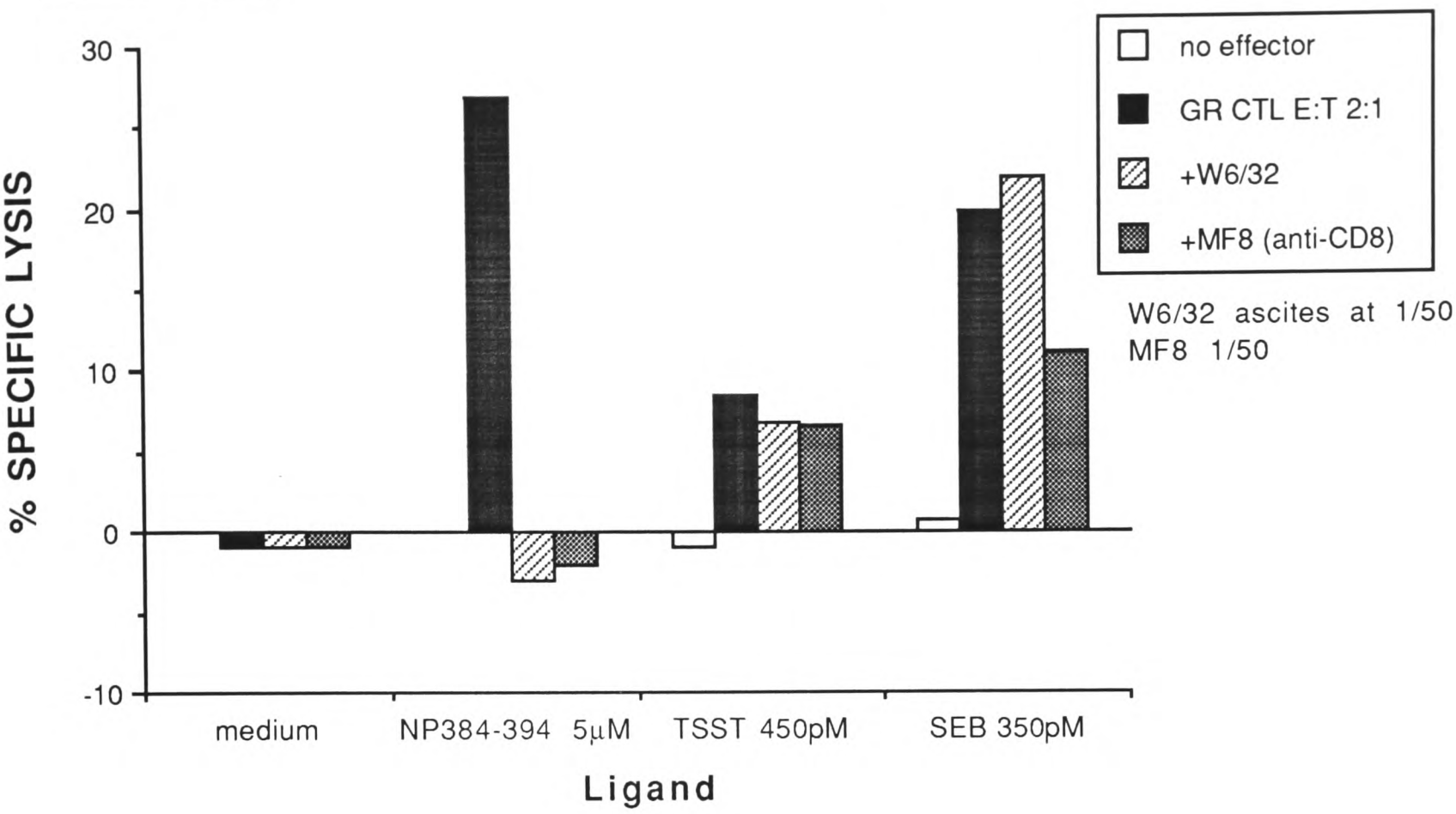
The HLA B8-restricted NP 380-393-specific CM CTL line, and also one HLA B27-restricted GR3 CTL, showed broad superantigen reactivity, killing autologous targets in the presence of SEA, SEB, SEC2, SEE and TSST (data not shown). In contrast, although different HLA A2-restricted JM CTL lines tested at different times appeared to have different SA reactivities, none ever recognized either SEE or TSST. One of the putative HLA B27-restricted NP-specific CTL "clones" described in chapter 5 was found to react with all SA tested (and was therefore discarded as being polyclonal), whereas clones GRb, SD10 and SD16, which used Vß7, recognized only SEA.

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*In this experiment the peptide NP384-394 was used at optimal concentration. Although the peptide NP383-391 was subsequently shown by S. Rowland -Jones (unpublished data) to be the most potent, and is assumed to be the naturally processed epitope, other peptides including 384-391 and 380-393 were used in early assays and were well recognized.

Superantigen-mediated cytolysis is not inhibited by W6/32

Figure 3.3a



Inhibition of superantigen-mediated cytolysis by anti-DR and anti-LFA3 mAbs

Figure 3.3b

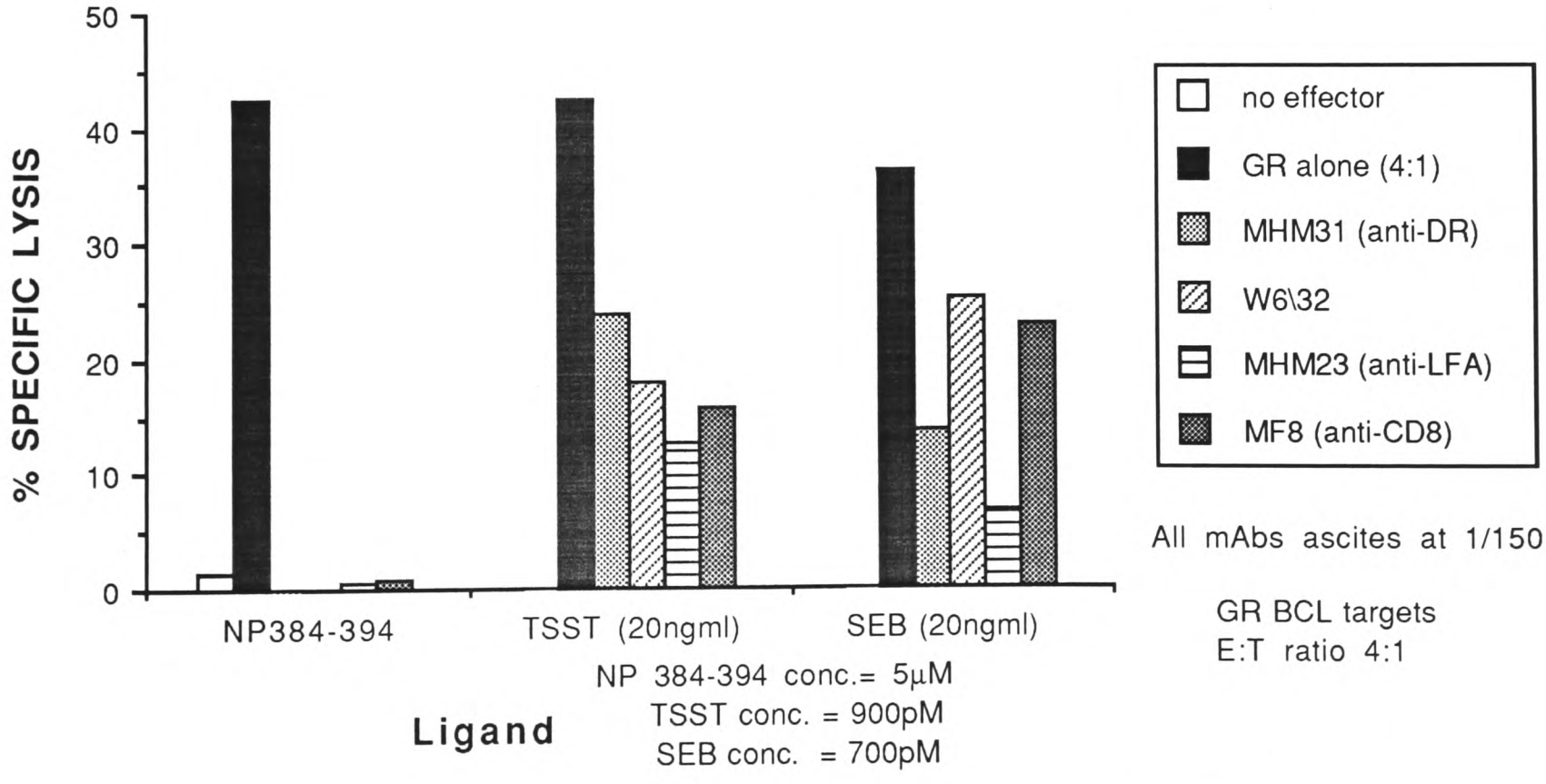


Figure 3.3. Inhibition of SA-mediated cytolysis by mAbs.

CTL recognition can be used to screen for bacterial SA production
CTL lines CM and GR3, with broad reactivity to known SA, were used to screen for SA production by a variety of enteric bacteria. Clinical isolates were grown in beef heart infusion (BHI) medium by Dr. I. Bowler (Bacteriology, John Radcliffe hospital, Oxford), and cell-free culture supernatants tested in CTL assays. Figure 3.4 shows the results of one screening assay using CM CTL. Cytolysis was induced by cognate peptide, by control SA preparations and also by culture supernatants from a group A *Streptococcus* isolated from blood, and by a laboratory reference strain of *Staphylococcus aureus*. The supernatants from a skin isolate of group A *Streptococcus* and also a *Listeria monocytogenes* isolate were toxic to the BCL targets used. Isolates from *Streptococcus bovis* and *Eschericia coli* type 0157 (associated with the haemolytic uraemic syndrome) gave equivocal results. The remaining isolates, including a *Salmonella enteritidis* isolate from a patient with reactive arthritis, were negative. Figure 3.5 shows that the supernatant from a clinical isolate of *Staphylococcus aureus*, grown in BHI broth, induced CTL-mediated lysis at a dilution of 1/640.

3.3. Identification and characterization of novel superantigens produced by *Clostridium perfringens* and *Clostridium difficile.*

In addition to the bacterial supernatants described, preparations of enterotoxins produced by two *Clostridium* species, *C. perfringens* and *C. difficile,* were obtained from the PHLS at Porton Down, Wilts, UK. The enterotoxin of *C. perfringens* (CPET) is a 34 KD polypeptide which has been shown to produce the diarrhoea and abdominal pain associated with *C. perfringens* food poisoning, reviewed by McClane *et al.* (1988). *C. perfringens* is a major cause of food poisoning in the USA and the UK, and is also an important cause of skin and soft tissue sepsis. *C. difficile* is the major causative agent of pseudomembranous colitis in humans and produces two toxins. Only one of these, the 308 KD polypeptide enterotoxin (CDTa), was studied. The CPET preparation was made by H. Tranter and the CDTa preparation by A. Roberts, as described in chapter 2.3.

Figure 3.4

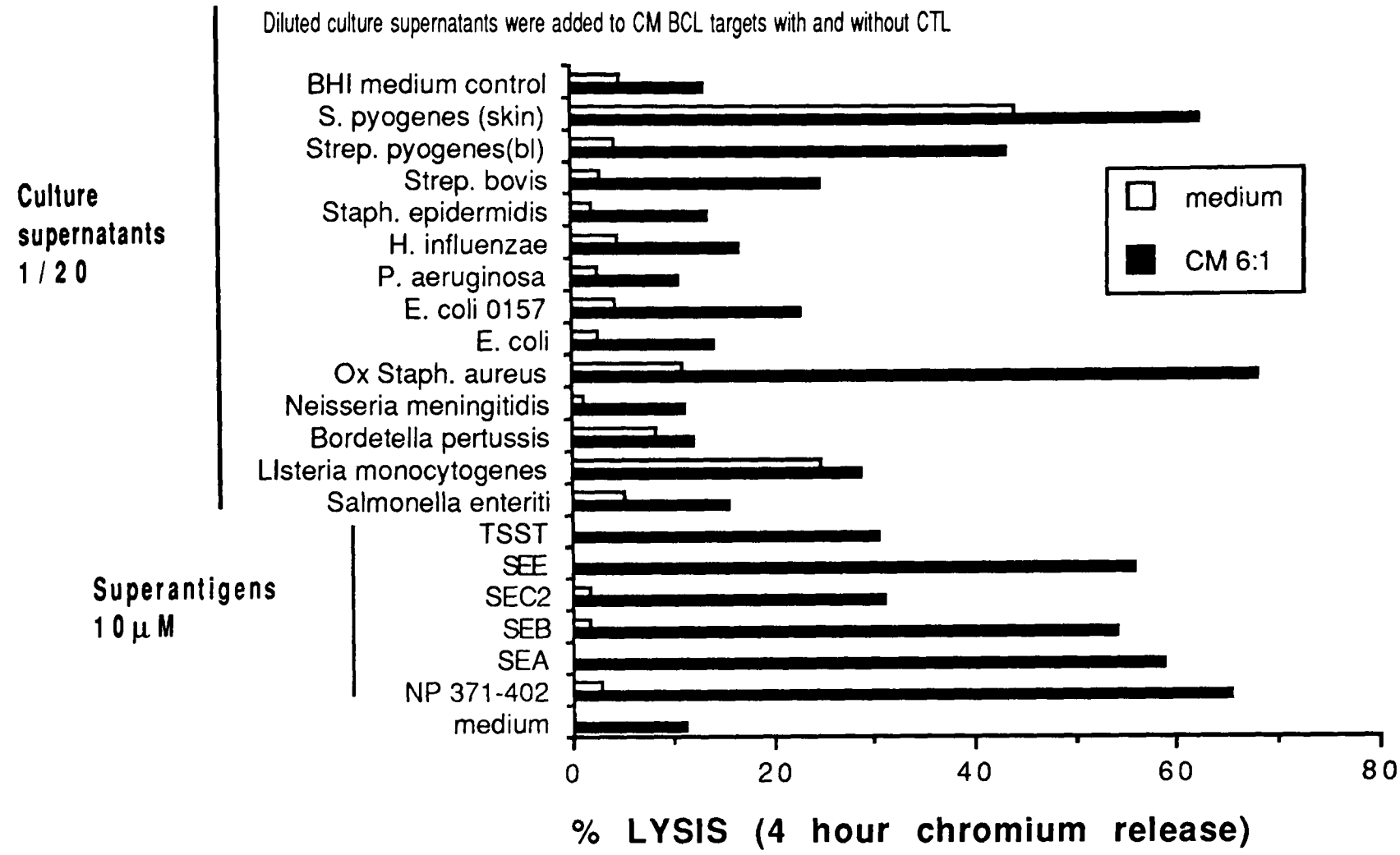
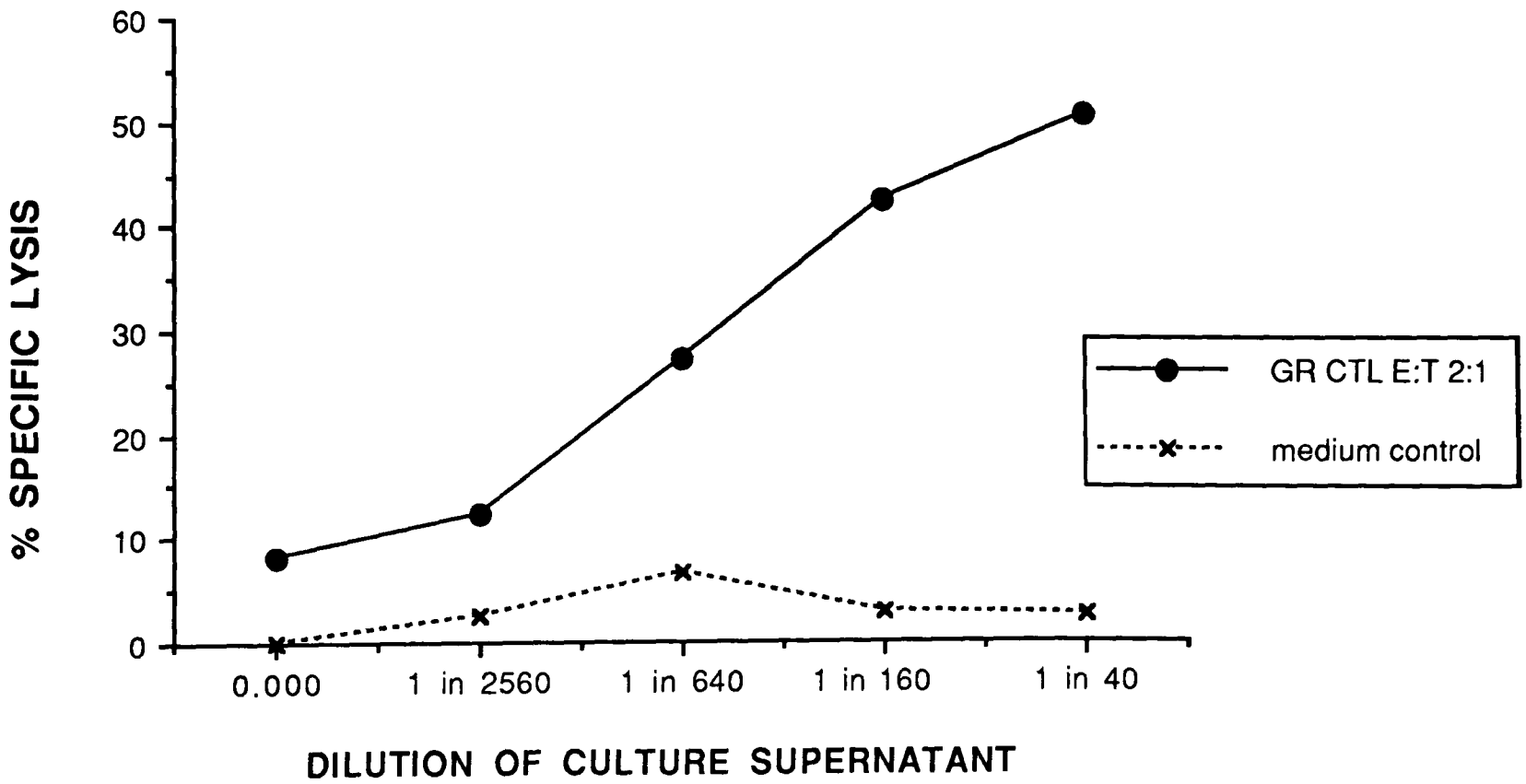


Figure 3.5

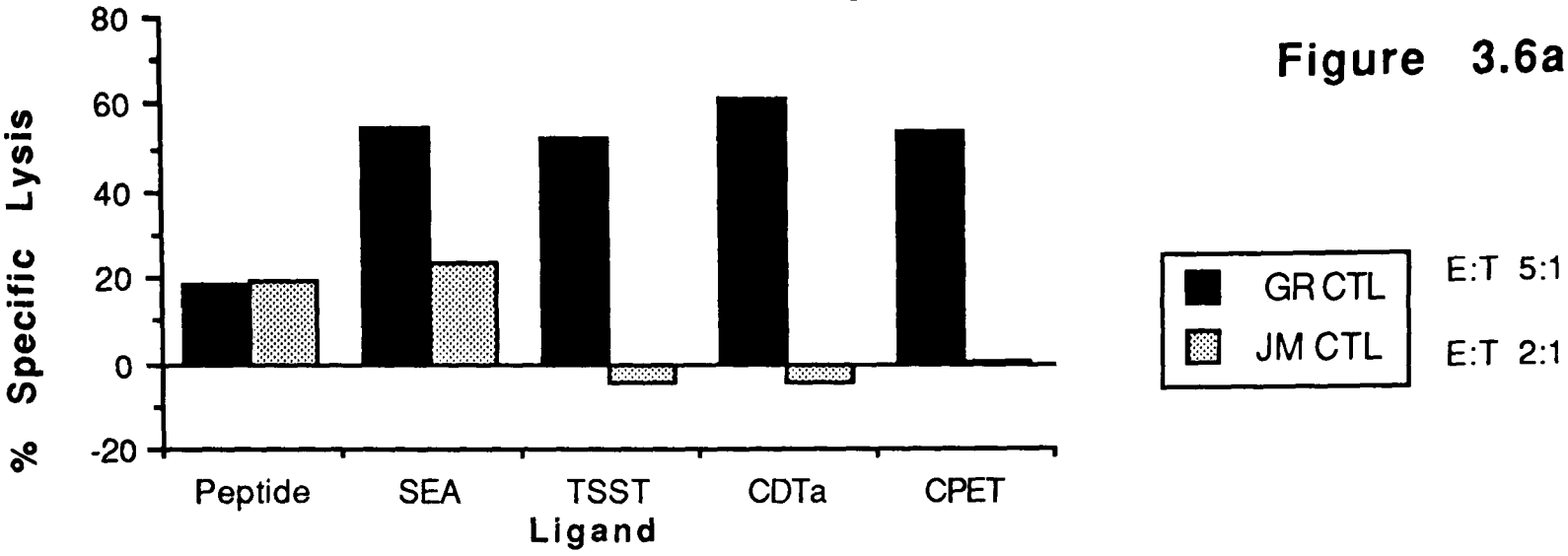


CPET and CDTa facilitate cytolysis by some but not all CTL.
Fig 3.6a shows that CPET and CDTa induced killing of autologous targets by GR CTL (HLA B27-restricted NP-specific) but not by JM CTL (HLA A2-restricted Matrix-specific). The most likely explanation for this result is that CPET and CDTa are superantigens with specificity for the TCR Vßs expressed by GR CTL (Vß7, 1, 5, 6, 13, 15) but not JM CTL (Vß17, 13, 6, 14). This was supported by further experiments using several NP-specific HLA B27-restricted clones (described in chapter 5), derived from a single HLA B27-restricted NP-specific SD CTL line. One clone, SD13, using Vß21, reacted with CPET, whereas others derived from the same line, such as SD16 (Vß7.1), did not (data not shown). Fig. 3.6b shows that CPET-induced target cytolysis by the SD CTL line was not HLA restricted. Whilst SD CTL killed only HLA B27-positive targets (T2 transfected with HLA B27) in the presence of cognate peptide, HLA-mismatched allogeneic B cell lines were killed in the presence of CPET and SEB. Both CPET and CDTa induced killing at nM concentrations and in a dose dependent manner, shown for CPET with GR CTL in fig 3.6c. Notably CPET was consistently less potent than SEB (fig. 3.6b and data not shown). This might reflect the purity of the preparation used, or alternatively reactivity with fewer of the Vß elements used by these CTL (see below).

Glutaraldehyde-fixed cells can present CPET
Figure 3.7 shows that PBL from donor NW proliferated in a dose-dependent manner when incubated with CPET at nM concentrations. Thymidine uptake was much greater than with purified protein derivative or tetanus toxid, and, like SEB, was maximal at three days, when proliferative responses to tetanus toxoid and PPD were almost undetectable (data not shown). Proliferation induced by CPET was consistently less than that observed with SEB. Figure 3.7 also shows that neither SEB- nor CPET-induced proliferation of PBLs was inhibited by glutaraldehyde fixation of APCs, unlike that induced by PPD or tetanus toxoid.

HLA B27-restricted influenza A-specific GR CTL, but not HLA A2-restricted JM CTL, recognize CPET and CDTa

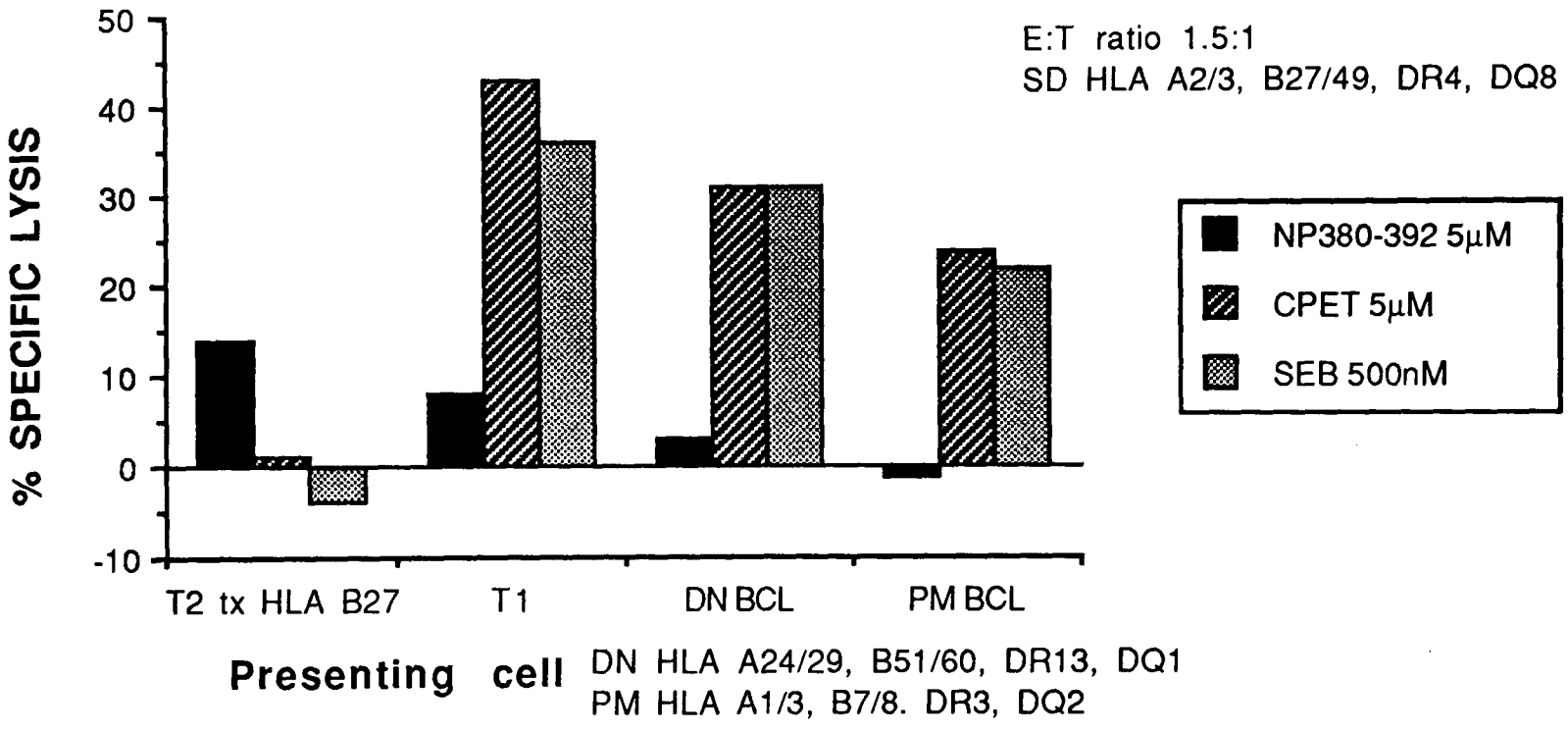
Figure 3.6a



Peptides M57-68 (JM CTL), NP380-391 (GR CTL) at 1μM. SEA, TSST, CDTa at 10nM. CPET at 25nM.
Autologous BCL targets

Lysis by SD CTL of HLA mismatched targets but not HLA class 2 negative targets with CPET and SEB

Figure 3.6b



Titration of cytolysis of autologous targets by GR CTL with CPET and NP380-391

Figure 3.6c

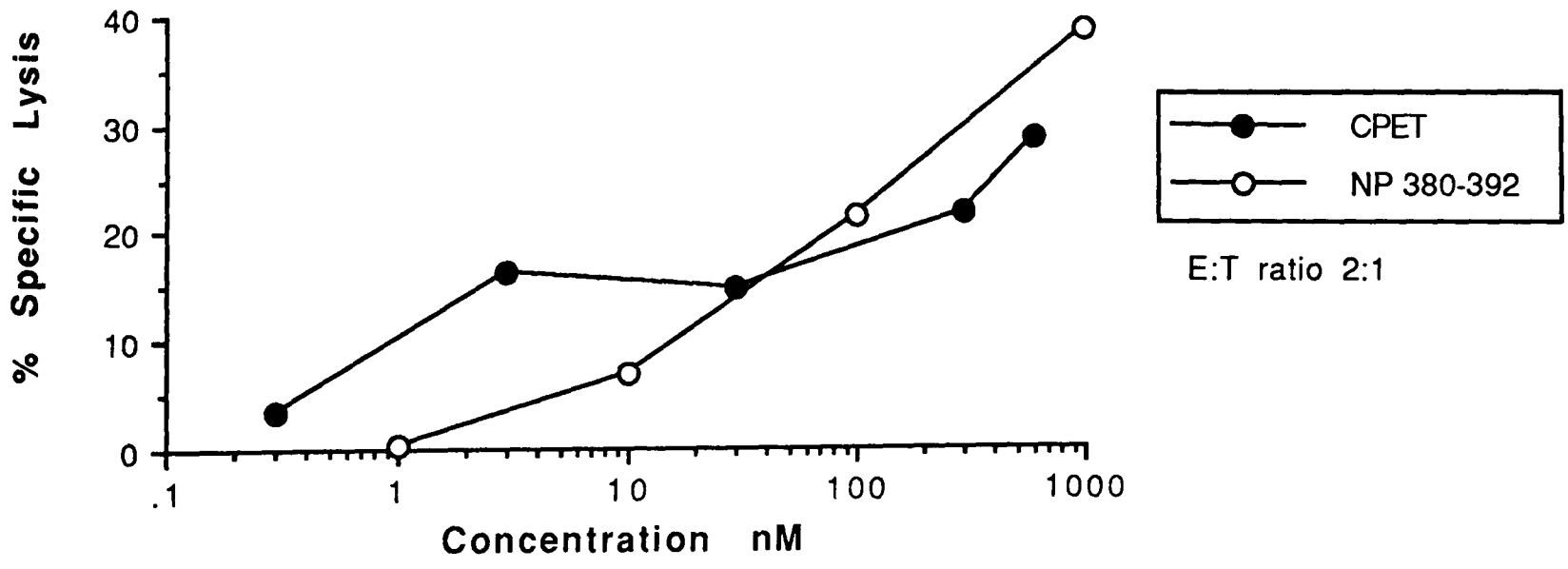


Figure 3.6. C. perfringens enterotoxin induces CTL-mediated cytolysis

Figure 3.7

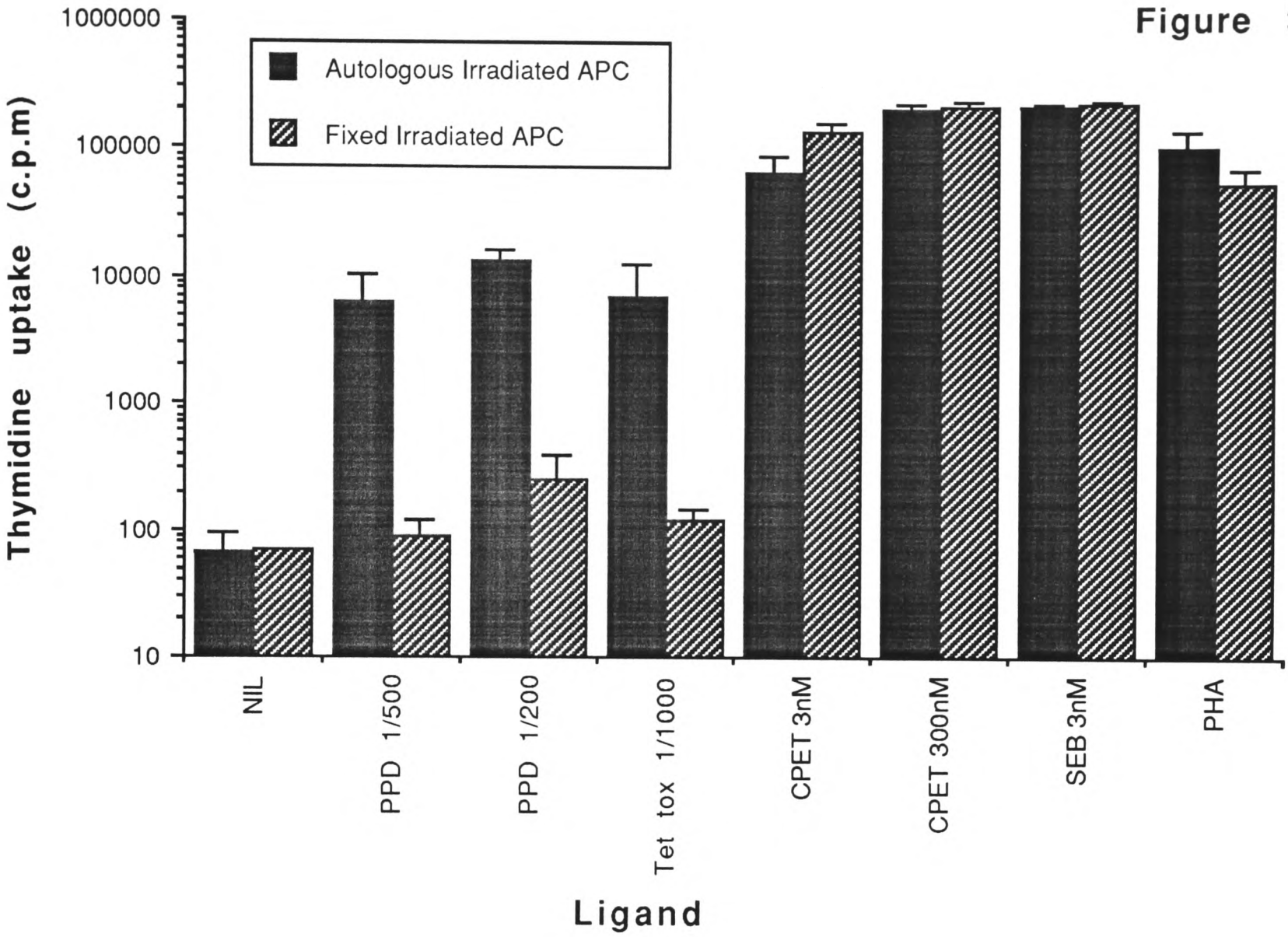


Figure 3.7. Proliferation of NW PBL after 5 day culture with CPET, SEB, PPD, tetanus toxoid and PHA.

Mean and standard deviation of 3H thymidine uptake of quadruplicate cultures are shown. 105 PBL were cultured with 6.6x104 irradiated or glutaraldehyde fixed autologous PBL.

AnPCR analysis of PBL stimulated by CPET and CDT. CPET and CDTa strongly stimulate TCRs Vß6.9 and Vß22, and weakly stimulate Vß18, Vß21 and Vß24

In order to confirm that CPET and CDTa stimulated T cells in a TCR Vß-specific manner, PBL were cultured for 72 hours with these toxins, and activated lymphocytes were then selected for IL2-receptor expression using dynabeads (Dynal, UK). TCR Vß usage of activated T cells was estimated by sequencing cloned PCR products following anPCR amplification of TCR sequences from lymphocyte cDNA. The Vß distribution of both CPET- and CDTa-stimulated lymphocytes from donor WR, shown in table 3.1 and figure 3.8, was very similar, although markedly different to that of unstimulated PBL. Two Vß elements, Vß6.9 and Vß22, were used by almost half of the stimulated lymphocytes. Although members of the Vß6 family (the largest family with at least 11 subfamily members) were common in both toxin-stimulated lymphocytes and unstimulated controls, CPET and CDTa appeared to stimulate Vß6.9-bearing T cells selectively. Vß6.9 made up 61% of Vß6 sequences following toxin stimulation compared to only 39% of PBL Vß6. Whilst no Vß6.6/7 sequences were found in the stimulated group, Vß1, Vß6.2/3, Vß6.4/11 and Vß6.5 were all detected**. The most striking difference between toxin-stimulated and control PBL was in the frequency of Vß22 usage, which made up 18% of sequences from both CPET and CDTa stimulated cells, as compared to less than 2% of unstimulated PBL. The Vß22 amino acid sequence, described by Ferradini and colleagues (1991), is shown in appendix 4. Three other rare Vß families, Vß18, 21, and 24 (Ferradini *et al.* 1991), also appeared to be selectively stimulated by CPET/CDTa. Thus although the numbers following toxin stimulation were small, these families were not found in the 51 PBL sequences obtained (approximately 6 months earlier) by Rosenberg *et al.* (1991).

** The Vß6 subfamily classification of Kimura *et al* (1987) is used. Some subfamily members differ only in N terminal sequence and were not distinguished by the limited sequence obtained. A new W.H.O. TCR nomenclature will shortly be available.

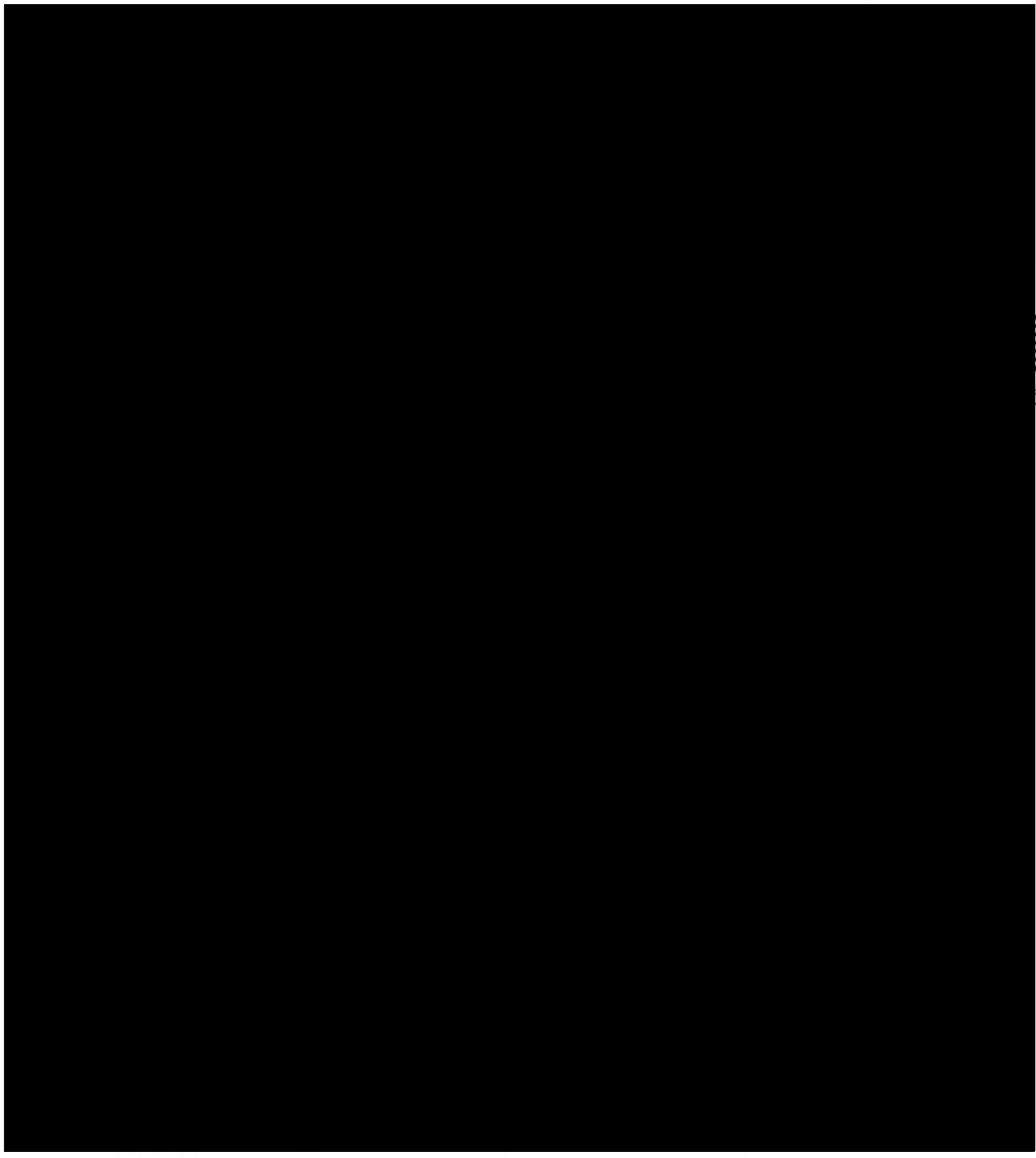


Table 3.1 and Figure 3.8. TCR Vß usage of CPET- and CDTa-stimulated PBL from WR. Activated lymphocytes were selected for IL2-receptor expresion after 72 hour incubation with CPET 30nM or CDTa 10nM. Numbers and percentages of sequenced clones of anPCR products are shown, using the nomenclature of Ferradini *et al (1991)*. The Vß6 family is divided into Vß6.9 and others. TCR Vß usage of WR PBL from Rosenberg *et al* (1991), Vß6.9 usage of WR PBL from W. Rosenberg (unpublished data).

In order to confirm these findings, PBL from WR and 4 other unrelated donors were stimulated with CPET or PHA (and in one case also SEB and TSST), and cDNA made from activated lymphocytes. Recombinant M13 plaques, containing TCRß chains amplified by AnPCR, were screened with oligonucleotide probes specific for Cß and for the three TCR Vß which appeared most strongly stimulated — Vß6.9, Vß22 and Vß24. The results are shown in Table 3.2. Vß6.9 and Vß22 were significantly stimulated by CPET in all 5 individuals tested ($p<0.05$ to $p<0.0005$ as compared to PHA-stimulated controls). Vß24 numbers were significantly increased following CPET stimulation in one of two donors tested. In the one individual tested, TSST stimulated neither Vß6.9 nor Vß22. For this donor SEB was found to stimulate Vß22 but not Vß6.9. The statistical significance of this last finding is uncertain since no allowance was made for the multiple comparisons made.

3.4. Discussion

Data presented in this chapter show that bacterial toxin superantigens are recognized by a variety of influenza-specific CTL lines and clones in a manner that is independent of their (classical) antigen specificity, resulting in target cell lysis. HLA-mismatched human BCL were killed in the presence of SA, but not the T2 cell line (or T2 cells transfected with MHC class 1 molecules), which express little or no HLA class 2. These results strongly suggest that, like CD4+ cells, CD8+ MHC class 1-restricted CTL recognize SA in the context of MHC class 2 molecules. Although Zehavi-Wilner and Berke (1986) concluded that SEB was mitogenic for CTL but could not mediate cytolysis, MHC class 2-negative EL4 cells were used as targets. Three groups have subsequently reported the SA-mediated lysis of MHC class 2 positive targets (Fleischer and Schrezenmeier 1988, Hedlund *et al.* 1990, Herrmann *et al.* 1990). It should be noted that MHC class 2 molecules are not essential for the interaction of SA with the TCR (Hewitt *et al.* 1992). Antigen processing was formally shown not to be required for the stimulation of PBL by SEB and CPET, although could have occurred during the course of the CTL assays.

Table 3.2

[redacted]

Table 3.2. TCR Vß usage of CPET-activated lymphocytes from 5 unrelated donors. PBL were cultured for 72 hours with 100nM CPET, SEB or TSST, or 5ug/ml PHA, and activated lymphocytes selected for IL2-r expression. TCR Vß usage was assigned by oligonucleotide probing of M13-cloned AnPCR products. Toxin culture and anPCR amplification of donor 5 PBL were performed by P. Moss and P. Catlin in J. Bell's laboratory. The donor HLA types were: donor 1 (WR) -A11,33 B35,65 DR1,4; donor 2 (GR) -A3,31 B27 DR4; donor 3 (PM) -A1,3 B7,8 DR3; donor 4 (PB) -A2,29 B44 DR7; donor 5 (HR)-A1,2 B7,8 Cw7 DR1,17 DQ2,5. $^{\#}P>0.05$ compared to PHA, $*p<0.05$ (Fisher's exact probability); $**p<0.005$, $***p<0.0005$ (Chi squared with Yates' correction). From Bowness *et al.* (1992).

The finding that CD8 positive cells recognize SA in the context of MHC class 2 does not exclude a role for accessory molecule interaction. Indeed mAb inhibition experiments suggested an important role for the LFA3/ICAM1 interaction, which has been shown recently using CHO transfectants (Gjorloff *et al.* 1992).

This chapter describes the use of two techniques not previously applied to identify or characterize superantigens. Firstly potential superantigens were screened for their ability to induce lysis of HLA class II bearing targets by Influenza A specific CD8-positive oligoclonal CTL lines bearing different Vß elements. The CTL assay used, which identified CPET and CDTa as potential SAs, was rapid and, when CTL lines with different TCR Vßs were used, could detect Vß specificity. The disadvantages of this technique are that CTL must be maintained in culture, and that the CTL lines used do not encompass all possible Vß. These disadvantages could to some extent be overcome by using a large panel of T cell clones (or TCR transfectants as described in chapter 7), or by use in combination with standard proliferation assays. It should be noted that the failure to identify superantigenic activity from culture supernatants does not exclude the possibilities either that the culture conditions were unsatisfactory for SA production, or that a cell-associated SA might be produced.

A second novel technique, developed by P. Moss in J. Bell's laboratory, was used to characterize the TCR Vß specificity of two candidate SA, CPET and CDTa. cDNA was made from toxin-activated lymphocytes, which had been cultured with SA and selected for IL-2R expression. TCR Vß sequences were then amplified by anPCR. Using this method, P. Moss had previously found that, following culture of PBL with TSST-1, over 40% of TCR ß sequences derived from activated lymphocytes were Vß2 (unpublished data). Unlike the "V region family specific" PCR techniques used previously (Choi *et al.* 1989), anPCR gives quantitative information about all Vß families and subfamilies. However the technique is very time-consuming and will be largely superce ded as more TCR Vß-specific mAbs become available.

Superantigens are currently poor probes for TCR Vß usage.
SA including TSST, SEB, and the candidate SA CPET and CDTa, were shown to induce cytolysis by some CTL lines and clones but not others. Thus the finding that TSST was recognized by GR CTL but not JM CTL was interpreted as suggesting that these CTL lines used different TCR Vß elements, although many other explanations are possible. Since the TCR usage of CTL lines and clones was not known at the start of the study, it was hoped that it might prove possible to analyse their TCR Vß usage using a panel of SA. However it became clear not only that SA TCR Vß specificity was in fact poorly characterized, but also that reactivity was not an "all or nothing" phenomenon, as indicated by variable enrichment of different Vß families with CPET. The failure of JM CTL, which used mostly Vß17, to recognize TSST, was consistent with the reported specificity of TSST for Vß2 (Choi *et al.* 1990). However the GR CTL line, which recognized TSST, did not use Vß2. Interestingly, comprehensive analysis, using anPCR, of TSST-stimulated PBL indicated that Vß5 family members, as well as Vß2, were stimulated (P. Moss, unpublished data). The TSST-induced cytolysis obtained with GR CTL is thus consistent with a weak affinity of this SA for Vß5. In addition, if only a percentage of GR CTL TCRs were TSST reactive this would also explain why higher E:T ratios were required under optimal conditions than for cognate peptide (figure 3.1 and data not shown). Thus SA were found to interact with a wider range of Vß elements than previously thought, and hence were relatively poor probes for the TCR Vß usage of CTL.

Superantigenicity and Vß specificity of CPET and CDTa
This study provides strong evidence that the enterotoxins produced by *Clostridium perfringens* and *C. difficile,* CPET and CDTa respectively, are superantigens. Both toxins were shown to induce cytolysis by some but not all CTL lines, and to cause Vß-specific activation of PBL from one individual. Furthermore presentation by glutaraldehyde-fixed APC implies that CPET, like SEB, does not require antigen processing in order to stimulate T cells. Proliferation with CPET was consistently less than that observed for SEB, as were the numbers of activated lymphocytes obtained after 5 days culture (data not shown). The data suggest either

that CPET is a less potent mitogen or, more probably, that it reacts with fewer or rarer TCR Vß elements. These data are consistent with those shown for TCR Vß specificity (and PBL Vß frequencies) in table 3.1, and taken together suggest that approximately 15% of peripheral blood lymphocytes are CPET/CDTa reactive.

In this study, Vß6.9 and Vß22 were found to be strongly stimulated by CPET. Statistically significant enrichment of these Vßs by CPET, compared to PHA controls, was shown in five individuals. Vß6.9 and Vß22 and also appeared to be stimulated by CDTa in the one individual studied. Different Vß elements appeared to be stimulated to different extents, with Vß22-bearing TCR sequences most dramatically enriched, by 3 to 11 times compared to PHA-stimulated PBL in five individuals. Lymphocytes bearing TCRs Vß24, Vß21 and Vß18 also appeared to be weakly stimulated by both CPET and CDTa. However for these rarer families the numbers of sequences obtained were small and control data insufficient. It was also noticeable that Vß6.6/7 TCRß chains were not found following toxin stimulation, whereas Vß6.2-5 were, suggesting that they are also weakly stimulated. Since the numbers of activated lymphocytes obtained without toxin incubation were insufficient for RNA preparation (data not shown), it is probable that virtually all the TCRß sequences obtained were from cells that had been activated by toxins. These findings support the proposition that one SA may have different affinities for different Vß elements (Fleischer *et al.* 1991).

Since a relatively small number of sequences was analysed, it was not clear if the frequencies of these TCR Vß elements were different following CPET and CDTa stimulation. Nevertheless it is important to point out that, despite possible inaccuracies (for example, biased PCR amplification of different Vß families), this study represents the most comprehensive published analysis of Vß-specificity of any SA (Bowness *et al.* 1992). AnPCR has not previously been used to study the TCR repertoire of SA-stimulated PBL. Indeed the two Vß elements most strongly stimulated by CPET and CDTa (Vß6.9 and Vß22) would not have been detected by any of the monoclonal antibodies currently available, nor specifically by the

oligonucleotides used in previous studies (Choi *et al.* 1989, 1990). For this reason CPET and CDTa are the only SAs for which a complete profile of Vß stimulation has been obtained.

There are two explanations for the finding that CPET and CDTa demonstrate an almost identical TCR Vß specificity. Firstly, the observed effects may have been due to the presence of a single novel SA, made by both *C. difficile* and *C. perfringens*, contaminating one or both preparations. However such contamination is unlikely because the toxins were active at nanomolar concentrations in CTL assays, and both preparations gave only a single visible band after SDS gel electrophoresis under non-denaturing conditions ((Reynolds *et al.* 1986) and Roberts A. unpublished data). Nevertheless it is planned to repeat these experiments using recombinant CPET, which should shortly be available. It is important to note that the observed results could not have been due to contamination with any known SA, as the Vß specificity found was entirely novel. Alternatively both toxins may be SA that interact with the TCR in a similar manner. Although CDTa and CPET differ markedly in size and show little overall similarity, there is a 28 amino acid stretch of 25% amino acid homology between CPET 319-345 and CPET 249-276, which may represent a TCR-binding domain (H. Tranter, personal communication).

Implications for mapping TCR superantigen interactions

Whilst the structural basis for the superantigen/TCR interaction is not known, site-directed mutagenesis suggests that solvent exposed residues around residues 17-22 and 69-74 of the Vß chain are critical for interaction with some superantigens (Choi *et al.* 1990, Pullen *et al.* 1991). Although Vß6.9, Vß22 and Vß24 show marked homology in this region, shown in table 3.3, there are also quite marked differences, for example Vß6.9 and Vß22 have K and D respectively at position 71. It is of interest that Vß6.6, which is CPET and CDTa unreactive, shares five residues with Vß6.9, but lacks a proline at position 70, suggesting a possible role for this residue in the interaction with CPET and CDTa.

	TCR Vß RESIDUE		RESONSE TO	
	16 to 22	70 to 74	CPET	CDTa
Vß Vß6.9	GQNVTFR	RPKGSF	+++	+++
Vß22.1	GQEVILR	RPDGSN	+++	+++
Vß24	GKPVTLS	RPNTSF	++	(++)
Vß21	KQPVAFW	RLKGVD	(++)	(++)
Vß18.1	GQEARLR	RPKEGF	(++)	(++)
Vß6.5	GQNVTFR	RPKGSL	(+)	(+)
Vß6.4/11	GQDVALR	RPEGSI	(+)	(+)
Vß6.2/3	GQDVALR	RPEGSV	(+)	(+)
Vß6.6/7	GKDVELR	RTGGSV	-	-
Vß5.1	GQQVTLR	ESNS	-	+/-
Vß8	GQEVTLR	PNAS	-	-

Table 3.3. Differences in amino acid sequence of CPET/CDTa responsive and non-responsive TCR Vß elements. Only regions thought to be involved in superantigen binding, residues 16-22 and 69-74 ("CDR4"), are shown. Indications of responsiveness, approximate only: +++ >4-fold enrichment. ++ 1.5-3 fold enrichment. + enriched. +/- detected. () indicate small numbers of sequences obtained.

Implications for human disease

CPET and CDTa are both important pathogenic toxins in humans. *Clostridium perfringens* is the second or third most frequent cause of food poisoning in the USA (Horowitz 1977), and the characteristic clinical features of abdominal pain and diarrhoea are thought to be caused by CPET. CPET has also been implicated in some cases of antibiotic-induced diarrhoea, infantile diarrhoea and sudden infant death syndrome, reviewed by McClane *et al.* (1988). It is also possible that the superantigenic properties of CPET may contribute to the shock that may accompany clostridial sepsis, although it should be noted that many other toxins are also produced. CDTa causes pseudomembranous colitis (Bartlett *et al.*

1978). Although the mechanism of action is unknown, both a peripheral leucocytosis and leucocyte infiltration into the colonic mucosa are recognized features (Sumner and Tedesco 1975). It is not known if the superantigenic properties of CPET and CDTa are responsible for their diarrhoeal action. However the fact that the staphylococcal enterotoxins, which have an emetic action on the gut, are also superantigens, suggests that the two properties are closely linked. Whilst SA are known to bind HLA class II, other ligands may also be important *in vivo.* HLA class II-negative colon carcinoma cells have recently been shown to present SAs to T cells (Dohlsten *et al.* 1991). The carbohydrate antigens I, X, and Y, to which CDTa binds (Tucker and Wilkins 1991), might fulfill a similar function.

It has previously been noted that the ability of SA to stimulate T cells in a manner independent of their antigenic specificity, constitutes a potential mechanism for breaking immunological tolerance and inducing autoimmune disease (Tumang *et al.* 1990). CPET and CDTa are the first SA described that are produced by gastrointestinal tract commensals. They thus could act as chronic immune stimulants. The association of enteric bacteria with the seronegative spondylarthropathies is well documented. It is of interest that *C. perfringens* has been associated with rheumatoid arthritis (Olhagen and Mansson 1968), a disease in which perturbation of TCR Vß repertoire consistent with SA effect has been reported (Paliard *et al.* 1991).

CHAPTER 4

T CELL RECEPTOR USAGE OF HLA B27-RESTRICTED CTL LINES

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4.1 Introduction

The specificity of T lymphocytes for antigen is mediated by the T cell receptor (TCR), which is an alpha/beta heterodimer in over 95% of T cells. In order to further understand the molecular basis of recognition of both antigen and superantigen it is thus necessary to characterize lymphocyte TCR usage. In this chapter the TCR usage of HLA B27-restricted CTL lines specific for Influenza A virus and HIV will be described. Although information about cell surface TCR expression can best be obtained using monoclonal antibodies directed towards defined elements of the TCR, the limited availability of reagents specific for human TCRs and the requirement for primary sequence information from individual cells dictated the use of PCR of lymphocyte cDNA. Although a variety of techniques, discussed in chapter 2.4, were considered, "anchored" PCR (AnPCR) was chosen as potentially the most informative. AnPCR was preferred to "inverse" PCR only because the technique, originally described by Loh and colleagues (1989), had successfully been developed for the study of human TCR sequences by W. Rosenberg and P. Moss in J. Bell's laboratory (Rosenberg *et al.* 1991).

Using AnPCR to study antigen-specific HLA B27-restricted CTL lines, it was hoped to obtain information about elements of the TCR involved in recognition of peptide and MHC. The only human HLA class I-restricted response previously characterized at the level of TCR usage was the HLA A2-restricted response to Influenza matrix peptide M58-66, where marked conservation of both alpha and beta chains had been found (Moss *et al.* 1991). HLA B27 provided an ideal second class I restriction element to study because resolution of its crystal structure gave a clear indication of the side-chain positions of bound peptides (Madden *et al.* 1991, Madden *et al.* 1992). At the time of carrying out this study, the only HLA B27-restricted immune responses for which peptides had been defined were those directed against Influenza NP383-391 (SRYWAIRTR) and HIV gag 265-274, a decamer KRWIILGLNK, ((Nixon *et al.* 1988, Huet *et al.* 1990) and S. Rowland-Jones, unpublished data). Comparison of TCR usage in these two immune responses might reveal common features of HLA B27-restricted responses. Such findings might have relevance to the role of HLA B27 in HLA B27-associated autoimmune disease, and might enable identification of those lymphocytes involved in disease pathogenesis from amongst, for example, synovial lymphocytes from the joints of affected individuals.

CTL lines were initially studied for three reasons. Firstly human CTL clones had proved extremely difficult to culture *in vitro*. Secondly the CTL lines studied by Moss *et al.* (1991) had proved more informative than the single CTL clone, as several closely related sequences had been obtained from single PCR amplifications of CTL lines. Thirdly knowledge of the TCR usage of CTL lines was felt to be of use in the screening and characterization of superantigens described in chapter 3. In fact, owing to difficulties in interpreting the results obtained with CTL lines, it ultimately proved necessary to generate CTL clones — this will be described in chapter 5.

4.2. TCR usage of an HLA B27-restricted Influenza virus-specific CTL line from donor HF.

The TCR alpha and beta chain sequences, obtained by AnPCR amplification and M13 cloning of cDNA made from $2x10^6$ HF CTL, are shown in table 4.1. Although the TCR usage appeared relatively polyclonal, three alpha and three beta sequences were represented more than twice. One Vα11/Jα15.3 sequence made up 50% of TCR alpha and one Vß2.3/Jß1.2 sequence 27% of TCRß sequences, suggesting that these might be paired. Both these chains used charged residues in their CDR3-equivalent regions, glutamic acid at position 93 of the alpha chain, and two arginines at ß97 and ß99. One TCRß sequence, represented only once in the HF CTL line and marked with an*, was identical at the nucleic acid level to one of two ß chain sequences obtained independently by P. Moss in J.I. Bell's laboratory from an oligoclonal HF CTL line 2B9 (HF1.2 in figure 5.4b). 2B9 had been derived by S. Rowland-Jones from a vial of donor HF PBL, incubated with Influenza virus at a different time to that used to make the HF CTL line studied here (although from a common blood sample). This demonstrates clonal expansion *in vivo* and also suggests that the TCR repertoire for HLA B27+NP383-391 is limited in this individual. Notably neither of the alpha chains obtained from 2B9 by P. Moss were represented in the HF CTL line, nor were the alpha or beta chains of a single HF clone studied (HF2 in figure 5.4).

4.3. TCR usage of HLA B27-restricted HIV-specific CTL lines from donors 007 and 049

Bulk culture HIV gag 265-274-specific CTL lines were generated by S. Rowland-Jones. They had been derived by culture of PBL with autologous PHA-activated PBL from HIV positive haemophiliac donors 007 and 049, following the protocol of Nixon *et al.* (1988). CTL were analyzed after two weeks *in vitro* culture, except for the cDNA used to amplify 049 TCR alpha chains, which was prepared by P Moss after 4 weeks culture. At this time the bulk CTL lines recognized cognate peptide but also exhibited non-specific "background" cytolysis of autologous targets. The TCR alpha and beta chain sequences, obtained by AnPCR amplification and M13

HF CTL TCR ALPHA — INFLUENZA A NP 383-391

no. sequences	Vα		N		Jα
8	11	DAVYYCA	VEAP	SGGSYIPTFGR	15.3
1	11	DAVYYCA	VGRGSN	DYKLSFGA	1.3
2	31	DSATYLCA	V	GGNNRLAFGK	9.9
2	18	DSATYLCA	RSN	DYKLSFGA	1.3
1	23	DSATYLCA	VYE	GTALIFGK	9.5
1	10.2	DTGHYLCA	GD	SGGSNYKLTFGK	9.3
1	34	DSAVYY		GGSQGNLIFGK	9.11
total 16					

HF CTL TCR ß CHAINS

no.sequences	Vß		ND		Jß
6	2.3	CSA	VNRRGG	YGYTFGS	1.2
1	2.3	CSA	RGAGGP	TDTQYFGP	2.3
4	5.4	CASS	F	TDTQYFGP	2.3
3	6.6	CAS	LLLGTGS	SYEQYFGP	2.7
1 *	6.9	CASS	LEVGD	EAFFGT	1.1
1	6.7	CASS	VDRGGEV	QETYQFGP	2.5
1	6.9	CASS	STWRLH	FGS	1.2
1	6.9	CASS	SDAGGY	FGS	1.2
1	12.3	CASS	GVTRVG	QPQHFGD	1.5
1	12.3	CASS	YLWQLAGG	NTGELFFGE	2.2
1	12.3	CASS	EPSHGLAD	EQFFGP	2.1
1	7.2	CASS	QAFQG	EQTQYFFGP	2.5
total 22					

Table 4.1 TCR usage of an HLA B27-restricted influenza A-specific CTL line from donor HF.
V and J region usage is shown together with predicted amino acid sequences of C terminal V, N (DN), and N terminal J regions. Vα and Jα nomenclature after Moss *et al* (1993). * indicates TCRß sequence identical to that found by P. Moss in oligoclonal line 2B9 from donor HF.

007 CTL TCR Alpha HIV GAG 265-274

no.sequences	Vα	D	Jα	
1	11-- YYCA	AT	SGNTPLVFGK	9.2
1	11-- YYCA	SWN	NNARLMFGD	3.2
1	11-- YYCA	VAD	IIGFGN VLHFGS	16.4
1	11-- YYCA	VDS	WG KLQFGA	1.2
1	11-- YYCA	VEAL	LDTGRRALTFGS	16.6
1	11-- YYCA	VEENT	GFQ LKVFGT	17.6
1	11-- YYCA	VEDR	GNN RLAFGK	9.9
1	11-- YYCA	VGDN	FN KFYFGS	16.7
1	11-- YYCA	V	RDDKIIFGK	9.4
1	11-- YYCA	VEDQ	GGATN KLIFGT	17.1
1	11-- YYCA	VEDL	SNDYKLSFGA	1.3
1	11-- YYCA	VAN	DYKLSFGA	1.3
1	4.5- YLCA	VGAD	SNDYKLSFGA	1.3
1	7.1- YFCA	VKQ	SGGYN KLIFGA	1.5
1	2.8- YLCV	VNH	SGGYQ KVTFGT	17.2
1	2- YLCA	MSA	GNAG KSTFGD	3.1
1	8.1- YFCA	ASGG	DDKIIFGK	9.4

total 17 (also 5 out of frame)

Table 4.2a

007 CTL TCR BETA CHAINS

no.sequences	Vß		ND		Jß
3	12.3	CAS	KTGTEV	SYEQYFGP	2.7
1	12.3	CASS	KTVGRDAY	EQYFGP	2.7
1	12.3	CASS	GDLG	YEQYFGP	2.7
1	12.3	CASS	PRDS	STDTQYFGP	2.3
2	12.3	CASS	FQLNT	EAFFGO	1.1
2	6	CASS	LDPL	YEQYFGP	2.7
1	6.9	CASS	FKTGGD	YEQYFGP	2.7
1	6.9	CASS	VR	YEQYFGP	2.7
1	6.9	CASS	LGGAG	SPLHFGN	1.6
1	6.1	CASS	LYREPSV	AFFGQ	1.1
1	6.1	CASS	PSGVY	TGELFFGE	2.2
2	2.3	CSA	EEGG	NQPQHFGD	1.5
1	2.3	"	REGGDGL	YGYTFGS	1.2
1	2.3	"	RERVKH	EQYFGP	2.7
1	2.3	"	RDFSLRDK	SYNEQFFGP	2.1
1	5.4	CASS	FSPRDRE	SPLHFGN	1.6
1	5.4	CASS	LLE	NTEAFFGQ	1.1
1	1.1	CASS	VTGGG	NEQFFGP	2.1
1	1.1	CASS	SGD	TEAFFGQ	1.1
1	8.1	CASS	FGTT	SPLHFGN	1.6
1	3.1	CASS	FPDGY	QPQHFGD	1.5
1	16.1	CASS	GRDTGD	IQYFGA	2.4
total 27					

Table 4.2b

Table 4.2a and b. TCR usage of an HLA B27-restricted HIV gag specific CTL line from donor 007.

049 CTL TCR ALPHA **HIV GAG 265-274**

no. sequences	Vα		N	Jα	
9**	15	TGDSAICA	EIS	SSGGSYIPTFGR	15.3
5	1.2	DAAEYFCA	VR	SSYKLIFGS	16.5
2	12	DSAVYFCA	LSEA	YNFNKFYFGS	16.7
2	31	DSATYLCA	VQAV	SRDKIIFGK	9.4
1	17	DSATYLCA	LNYG	GSQGNLIFGK	9.11
1	2.6	DSATYLCV	VNIR	GNKLTFGT	17.9
1	6	DSAMYFCA	MRVP	SRDNFKFYFGS	16.7

total 21

049 CTL TCR BETA

no.sequences	Vß		NDN	Jß	
5**	6.6/7	CASS	FGTGG	EQYFGPG	2.7
1	6.2/3	CASS	LKGGQDV	YFGPG	2.5
1	6.2/3	CASS	FPGRQ	TEAFFGQG	1.1
1	6.4	CASS	YRE	SYEQYFGPG	2.7
1	6.9	CASS	TGQPSA	GELFFGEG	2.2
1	6.9	CASS	LSSP	EAFFGQG	1.1
3	5.1	CASS	WSGRGS	NTEAFFGQG	1.1
1	5.1	CASS	LDGTPIL	NEQFFGPG	2.1
1	5.1	CASS	CRQGS	NTEAFFGQG	1.1
1	5.3	CASS	LDPV	TEAFFGQG	1.1
1	1.1	CASS	VRAGED	NEQFFGPG	2.1
1	2.1	CSAS	FGFGV	TDTQYFGPG	2.3
1	3.1/2	CASS	NR	QETQYFGPG	2.5
1	3.1/2	CASS	PAIPPRQGLP	NYGYTFGSG	1.2
1	4.3	CSVE	SVGA	QETQYFGPG	2.5
1	8.1/2	CASS	TYLATT	NTGELFFGFG	2.2
1	8.1/2	CASS	YGQENW	ETQYFGPG	2.5
1	12.3	CASS	DRG	NTEAFFGQG	1.1
1	12.3	CASS	YSMDSM	NTEAFFGQG	1.1
1	18	CASS	PPTDRA	TEAFFGQG	1.1

Total 26

Table 4.3. TCR usage of HLA B27-restricted HIV gag-specific CTL line 049.

Figure 4.1a. TCR Vß usage of NP-specific HLA B27-restricted HF CTL, compared to the PBL of an HLA B27-positive individual GR.

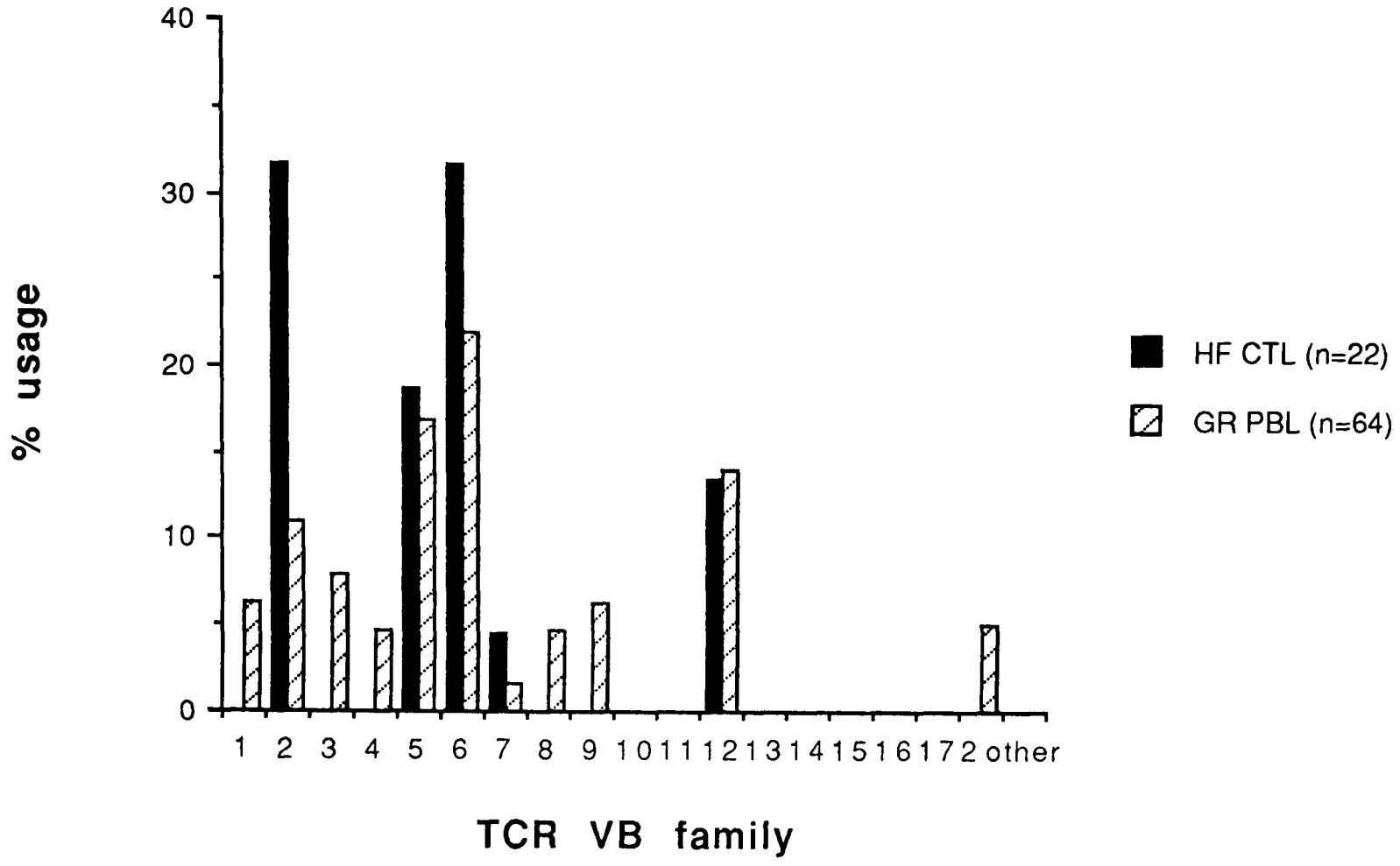


Figure 4.1b. TCR Vß usage of bulk 007 and 049 HIV GAG-specific CTL resemble that of PBL from an HLA B27 donor.

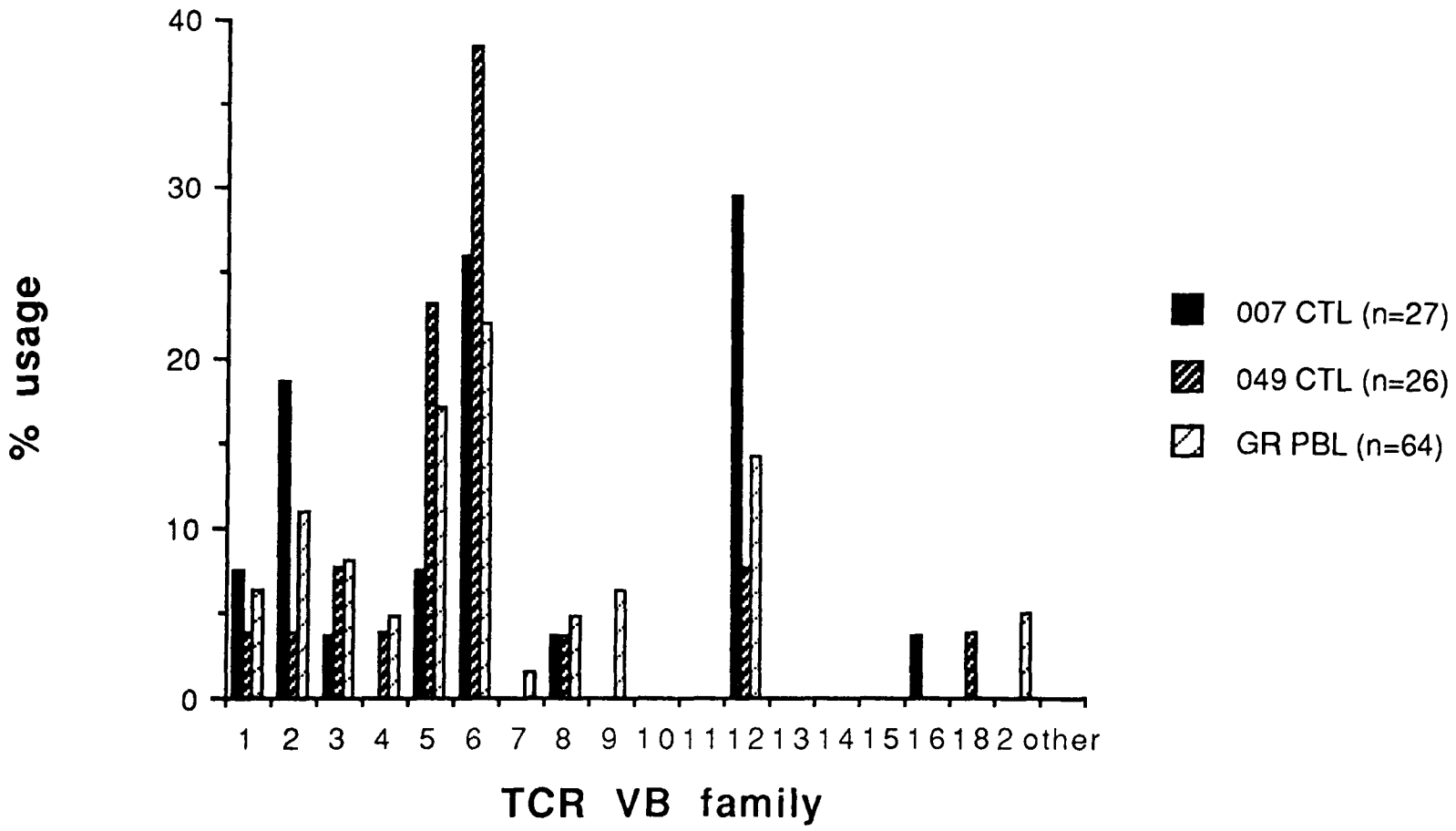


Figure 4.1. TCR usage of early bulk CTL lines resembles that of PBL. HLA B27-restricted CTL lines are compared to PBL TCR usage from donor GR (from figure 5.2).

cloning of cDNA from 10^6 CTL from donors 007 and 049, are shown in tables 4.2 and 4.3 respectively. TCR usage of both lines appeared relatively polyclonal, and whilst certain alpha and beta sequences predominate, they are quite different in the two donors. The 049 CTL line was subsequently sampled by P. Moss and, after a number of months *in vitro* culture, the sequences marked ** made up approximately 90% of both alpha and beta chain sequences obtained (P. Moss manuscript in preparation). In the case of 007 CTL line there appeared to be polyclonal expansion of Vα11 sequences, the majority of which had E at position 93, rather than a single dominant alpha chain. These sequences were not found by P. Moss after a further month of culture *in vitro*, when a single dominant TCR alpha chain was found (P. Moss manuscript in preparation).

4.4. Discussion

The TCR usage of early bulk HLA B27-restricted CTL lines specific for influenza and HIV is polyclonal

TCR usage of one influenza NP383-391-specific CTL and two HIV gag 265-274-specific CTL lines, as estimated by anPCR, appeared to be polyclonal. This could have been due to genuine TCR diversity of epitope-specific HLA B27-restricted CTL, or to amplification of TCR sequences from lymphocytes growing with other specificities in the bulk cultures. Three subsequent lines of evidence strongly suggest, although do not conclusively prove, that the latter explanation is correct. The approach used here, and described in chapter 5, was to generate influenza-specific CTL clones, confirm their antigenic specificity, and then analyse their TCR usage. HLA B27-restricted NP-specific CTL clones, cloned from early (2-4 week) CTL lines, were found to use remarkably similar TCRs. A second strategy was adopted by P. Moss and S. Rowland-Jones. They sampled the TCR usage of the same gag-specific HLA B27-restricted 049 CTL line after further *in vitro* culture. One alpha/beta pair made up approximately 90% of sequences obtained at 2 months, when the CTL line was killing specifically at a very low effector to target ratio, strongly suggesting that these chains pair and confer specificity for HLA B27+gag, and that the *in vivo* repertoire is limited (P. Moss manuscript in preparation). M. Callan

has similarly shown by FACS analysis of an influenza-specific CTL line, that Vα12 and Vß7 specific antibodies stain less than 10% of cells for the first 10 days, but greater than 30% after 4 weeks. These results are consistent with the dominance of specific oligoclonal CTL as T cells with irrelevant specificity die, although the possibility of selection by culture conditions *in vitro* cannot be excluded. This question has been partly addressed with the finding by P. Moss of high frequencies of the dominant TCRß CDR3 sequences of gag-specific CTL lines in donor PBL, (P. Moss manuscript in preparation). Figures 4.1a and b show that the Vß TCR family usage of both the influenza- and HIV-specific CTL lines, studied at an early stage, shows considerable similarity with the TCR Vß usage of the PBL obtained from an HLA B27 donor (detailed in chapter 5). It thus seems reasonable to conclude that at 2-3 weeks the CTL lines still contain viable PBL. This also suggests an explanation for the widespread observation that CTL lines exhibit "non-specific" killing at this stage.

The study of CTL lines also revealed a number of potential problems of AnPCR. Firstly, it is possible that the predominant finding of Vα11 sequences from HF and 007 CTL might be due to preferential amplification of this V segment, rather than its preferential use in the responses studied. Notably subsequent analysis of the 007 CTL line (and of HLA B27-restricted CTL clones) did not confirm the Vα11 data, raising the possibility of a technical artifact. Whilst Vα11 dominance was not found in any other anPCR amplifications, and study of PBL TCR α sequences by Moss *et al* (1993) suggests that such bias is not a major problem, it is not possible to be certain in the absence of suitable monoclonal antibody reagents. Secondly, when studying mixed populations it is particularly hard to exclude the presence of TCR sequences that are in fact PCR contaminants. Indeed this possibility must always be considered whenever identical nucleic acid sequences are obtained from different samples (and cannot be excluded for the HF ß sequence *). The study of CTL clones, where usually only one sequence is expected, reduces the risk of failing to identify PCR contamination. Finally, during the course of sequencing cloned PCR products from an second influenza-specific CTL line GR, a number of short Vα14 sequences

were obtained. It was then noted that Vα14 contains an internal Bgl 2 site, one of the enzymes used to digest the PCR products before cloning. All subsequent amplifications were carried out using 5' primers containing a Sal 1 restriction site, and products were cloned into an appropriately cut vector. However Vα14 products may not have been detected in the HF 007 and 049 CTL lines, since digested PCR products were size-selected.

Dominant TCR sequences are present in HLA B27-restricted CTL lines, but show little similarity between influenza NP- and HIV gag-specific lines

Although TCR usage of the lines was relatively heterogeneous, for reasons already discussed this apparent heterogeneity probably reflects the very early stage at which the CTL lines were sampled. Nevertheless certain dominant TCR sequences were observed, suggesting that the HLA B27 restricted influenza- and HIV gag-specific repertoires may be limited. Thus one alpha and one beta chain predominated in the 049 HIV gag CTL line, even after a very short period of culture. Although this might reflect a rapidly growing clone *in vitro*, it is important to stress that the CTL line became more specific and efficient at cytolysis with time as this sequence pair became predominant (S. Rowland-Jones and P. Moss, unpublished data).

There did appear to be certain features in common between the different HLA B27-restricted CTL lines studied, in both alpha and beta chain TCR usage. Thus one NP- and one HIV gag-specific line appeared to preferentially use Vα11, and a glutamic acid residue, coded for at least in part by N region addition, was found in TCR alpha sequences obtained from all three lines. One attractive explanation for the presence of this negative charge at position 92 or 93 of the alpha chain CDR3, is that this residue may be interacting with one of the positively charged residues of HLA B27 or peptide. It may also be significant that Jα15.3 was used by the dominant alpha chains of both the HF influenza-specific CTL line and the 049 gag-specific line.

Although no beta chain CDR3 region conservation was apparent, Vß6 family members were used in a significant proportion of beta chain sequences obtained from all three lines studied. This may not be significant as the Vß6 family is the most frequently used by PBL (Rosenberg *et al.* 1991), including those from HLA B27-positive individuals (fig.s 4.1, 5.2, and data not shown). Thus Vß6 might be favoured by HLA class-1 restricted responses in general, rather than by HLA B27 in particular, although two recent studies have not found evidence of Vß6 skewing towards the CD8+ T cell population (Akolkar *et al.* 1993, Hawes *et al.* 1993). Furthermore the majority of the NP-specific CTL clones described in chapter 5 used Vß7. Nevertheless HLA B27-restricted TCR undoubtedly can use Vß6 family members, as suggested by the independent isolation of the HF Vß6.9 sequence marked * in the biclonal line 2B9, and subsequently shown for the NP-specific CTL clone SD17 (figure 5.4).

Thus a wide variety of TCR sequences were obtained from early CTL bulk cultures, suggesting that at this stage the lines are largely made up of PBL. However even at an early stage certain sequences were over-represented, and subsequent evidence has shown that the TCR repertoires of HLA B27-restricted CTL lines and clones specific for both influenza A NP383-391 and HIV gag 265-274 are limited. Further study of the TCR usage of HLA B27-restricted CTL lines and clones, specific for a variety of different epitopes, is required to determine if there are structural features common to all HLA B27-restricted TCRs. Since certain problems of reliability and interpretation of AnPCR-derived TCR sequences obtained from CTL lines became apparent, subsequently only CTL clones were studied, and are described in chapter 5.

CHAPTER 5

T CELL RECEPTOR USAGE OF HLA B27-RESTRICTED INFLUENZA-SPECIFIC CYTOTOXIC T CELL CLONES

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5.1. Introduction

This chapter describes the TCR usage of seven HLA B27-restricted Influenza A nucleoprotein-specific CTL clones, studied using anchor PCR amplification of cDNA, followed by M13 cloning and sequencing cloned products. Whilst the generation of the CTL clones will be described briefly here, analysis of their fine specificity for peptide and HLA, and correlation with TCR usage, will be detailed in chapter 6.

Although the initial study of TCR usage in the HLA A2-restricted influenza A-specific immune response used primarily oligoclonal CTL lines (Moss *et al.* 1991), study of T cell clones gives more precise information. Thus the unequivocal association of particular TCR primary sequences with antigen recognition (and fine specificity) can be made. The study of clones also permits pairing of α and ß chains, of relevance both to the interpretation of functional data and also for TCR expression. Concommitant with this, T cell clones can only be defined for functional studies by TCR analysis and demonstration of a single coding TCRß transcript.

5.2. Generation of CTL clones

Five Influenza A nucleoprotein-specific CTL clones were derived and studied from a healthy HLA B27 positive laboratory donor SD, and two clones from an individual with Ankylosing Spondylitis GR. At the same time a CTL clone and a biclonal line were obtained in the same manner by S. Rowland-Jones, from a third donor (HF) with Reactive Arthritis following enteropathic *Salmonella enteritidis* infection. TCR usage of the HF clone and line, studied by P. Moss in J. Bell's laboratory, have been included in figure 5.4 for comparison.

The cloning method used, described in chapter 2.1, was based on a technique developed by H. Spitz *et al.* (1982), and used mixed irradiated allogeneic PBMC as "feeder cells", together with peptide-pulsed irradiated autologous BCLs. Table 5.1 shows the numbers of clones derived and tested in the two successful cloning attempts using this method.

Donor	seeding cells/well	no. wells	no. positive	cloning efficiency	no. HLA B27+NP-specific clones*
GR	1	170	5	2.9%	2
	0.3	170	2	3.9%	0
SD	0.6	250	14	9.3%	4**
	0.3	250	3	4%	1

Table 5.1. Cloning of GR and SD CTL. * Clonality confirmed by demonstration of a single TCRß chain. **One HLA-B27-restricted NP-specific positive well SD11 was excluded after showing reactivity in preliminary studies with all superantigens tested (SEA, SEB, SEE, TSST and CPET), suggesting that it was almost certainly not a true clone. SD CTL were cloned at a slightly earlier stage than GR, after two rather than four weeks culture *in vitro*, possibly explaining the slightly higher cloning efficiency.

Approximately one third of positive wells yielded CTL clones showing HLA B27-restricted and NP383-391 specific cytolysis (data not shown). These CTL were shown to be CD8 positive by FACS staining with the mAb MF8 (fig. 5.5 and data not shown), which also inhibited cytolysis in a dose dependent manner (figure 7.1). The phenotype of clones that were not HLA B27-restricted and NP383-391-specific was not further studied, although subsequent work using this protocol (for different donors) by P. Lehner in L. Boryziewic's laboratory has found that both CD4 clones and Influenza-specific CTL restricted by other donor HLA types are generated (P. Lehner, unpublished data). Notably it proved impossible to culture clones for more than approximately six weeks or obtain more than $2x10^8$ cells, even when clones were stimulated weekly with the addition of fresh mixed irradiated allogeneic PBMC.

5.3. TCR rearrangement in human CTL clones

The TCR transcripts identified and sequenced from the seven HLA B27-restricted NP383-391-specific CTL clones are detailed in table 5.2.

Clone	Alpha transcripts			Beta chain transcripts			
	no. sequences	Vα	Jα	no. sequences	Vß	Dß	Jß
GRb	11	14	9.3	6	7.1**	2	2.3
	1	-	6.1	3	-	1	1.1
GRc	7	12	9.3	4	7.2***	2	2.5
	1	-	17.1				
SD2	5	22	16.1	4	8	?2	2.3
	1	7	9.11	1	-	-	2.3
SD10	4	22	16.1	4	7.1	1	1.4
SD13	4	12	16.2	4	21	1	1.3
	1	-	17.6				
SD16	4	12	16.2	5	7.1	1	1.3
	1	-	- +	1	12*		
SD17	5	14	9.3	11	6.6/7	1	2.5
	1	25*	9.11				

Table 5.2. TCR transcripts amplified from seven CTL clones. Vα and Jα segments are classified according to (Moss *et al.* 1993). *out of frame. **IGRb19 (Ferradini *et al.* 1991). ***IGRb17 (Ferradini *et al.* 1991). + unidentified transcript without recognizable J segment.

A number of features of TCR rearrangement are apparent. The finding of T cell clones with a single ß chain but two α chains is now well documented (Malissen *et al.* 1988, Casanova *et al.* 1991), indicating that allelic exclusion does not occur for alpha chains, at least at the level of transcription. However the CTL clones studied here also appear frequently to produce transcripts comprising Jα/Cα alone or (Dß)/Jß/ Cß. This phenomenon has previously been described for DJμ products of Ig genes, where the translated product has a role in maturation arrest (Gu *et al.* 1991), and for DJCß TCR transcripts of unknown function that are transcribed from a promoter upstream of the D region. Current models of TCR rearrangement assume allelic exclusion of ß but not α chains and one rearrangement attempt for each chromosome (in fact more

than one may be possible for ß). Predicted frequencies of productive alpha transcripts (α^+) in individual cells are thus 4 $\alpha^+\alpha^-$: 1 $\alpha^+\alpha^+$, where α^- indicates failed rearrangement at one chromosome (and $\alpha^-\alpha^-$ always results in cell death). In contrast, ß transcripts should be found at a ratio of 3 $ß^+ß^0$: 2 $ß^+ß^-$, where $ß^0$ indicates no attempted rearrangement. Hence failed alpha transcripts should be present in 80% of T cells and failed ß transcripts in 20%. If the short (D)JC transcripts do indeed represent a failed rearrangement attempt, the findings of 4 of 7 (57%) of clones containing α^- transcripts and 2 of 7 (29%) containing $ß^-$ is broadly consistent with this model. However this is by no means certainly the case, as similar transcripts may be produced both prior to gene rearrangement, and downstream of VJ rearrangements. Given that non-productive transcripts were invariably less abundant than productive, it is possible that the relatively small number of sequences obtained underestimated the frequency of these species. The finding of two productively rearranged alpha chains in 1 of 7 clones, or 2 of 8 if clone HF1 is included, is however in agreement with current models.

These findings suggest that alpha chain allelic exclusion is leaky or absent in human CTL clones, confirming previously reported studies in the mouse (Malissen *et al.* 1988, Casanova *et al.* 1991). Interestingly it has recently been reported that thymocytes frequently undergo multiple alpha chain rearrangements, even once an expressed transcript has been produced, which may continue until a positive selection event occurs (Petrie *et al.* 1993). It also appears that short transcripts of unknown function and comprising only JCα (in addition to (D)JCß transcripts) are commonly produced.

5.4. TCRα chain usage of HLA B27-restricted NP383-391-specific CTL clones

Limited heterogeneity of TCR Vα and Jα usage; Predominant usage of Vα12, 14, and 22, and Jα9.3 and 16.5

The TCR usage of the five clones derived from donor SD and two from GR is summarized in Table 5.3.

		Vα	Jα	Vß	Jß
Clones with unique TCR alpha chains					
Donor GR					
clone	**GRb**	14	9.3	7.1	2.3
	GRc	12	9.3	7.2	2.5
Donor SD					
clone	**SD 10**	22	6.1	7.1	1.4
	SD 16	12	16.5	7.1	1.3
	SD 13	12	16.5	21	1.3
	SD 17	14	9.3	6.6/7	2.5
Clones with two alpha chains					
Donor SD					
clone	**SD 2**	22 7	16.1 9.11	8	2.5

Table 5.3. Summary of TCR usage of seven HLA B27-restricted NP383-391-specific CTL clones.

The NP 383-391-specific HLA B27-restricted CTL clones showed limited TCR alpha chain heterogeneity, with conservation of both Vα and Jα segments. Of eight full length in frame alpha chain sequences obtained, three were Vα12, two Vα14 and two Vα22. A CTL clone derived from a third donor HF by S. Rowland-Jones and sequenced independently by P. Moss in J.I. Bell's laboratory (HF1.2 in figure 5.1b) also used Vα12. Vα12 and Vα14 are closely related, showing approximately 40% homology at the amino acid level. Vα12

is found on 2-4% of peripheral blood lymphocytes (DerSimonian *et al.* 1991), and the Vα14 and 22 segments each comprise about 6% of alpha chain sequences obtained from PBL (Moss *et al.* 1993). As discussed in section 5.2, clone SD2 (and also the clone HF1) was found to have two TCR alpha chains. Since only one of the transcripts appears to be involved in antigen recognition (Malissen *et al.* 1988), the most interesting interpretation of these data would be that, for clones with two alpha chains, the one that was neither Vα12, 14, nor 22 was redundant. Such sequences have been speculatively separated in figure 5.2. However, in the absence of appropriate monoclonal antibody reagents, it would only be possible to prove which alpha chain is involved in HLA B27+NP recognition by expressing the beta chains with both alpha chains separately in an expression system that permitted functional assays.

Jα usage by the CTL clones was also restricted, with three clones using Jα9.3 (also known as JαK), and three using Jα16.5, segments used by only 1.3% and 0.7% of PBL respectively (Moss *et al.* 1993). Jα16.5 was always paired with Vα12. Two clones using Jα9.3 also used Vα14, but one used Vα12, and Jα9.3 was also found to pair with Vα10.2 in one of the TCR sequences obtained from the HF CTL line (table 4.1).

The region of greatest variability in TCR sequences, the third hypervariable region (CDR3), is generated by the combinatorial association of V, D, (for ß chains) and J segments together with N-region nucleotide addition. Chothia and colleagues have predicted that the CDR3 equivalent loops will be formed by residues 93-104 in the alpha chain and 95-106 in the beta, based upon homology of framework residues with the F and G loops of immunoglobulin domains (Chothia *et al.* 1988). The predicted CDR3 region alpha chain amino acid sequences of the clones are shown in Figure 5.1a and b.

Fig 5.1 a

CLONE	Vα					Jα			
			93	CDR3	104				
SD13	Vα12	DSAVYFC	A	**L**	**Q**	-	-	DSS YKL IFG	Jα 16.5
			gct	ct T	CAG	-	-	gatagc	
SD16	Vα12	DSAVYFC	A	**L**	**V**	-	-	DSS YKL IFG	Jα 16.5
			gct	ct G	GTG	-	-	gatagc	
GR c	Vα12	DSAVYFC	A	**C**	**G**	-	-	GSNYKL TFG	Jα 9.3
			gcG	TGC	gga	-	-	ggtagc	
SD17	Vα14	DAAMYFCA		**W**	**I**	S	G	GSNYKL TFG	Jα 9.3
				gct	TGG	ATT	agt	gga	ggt
GR b	Vα14	DAAMYFCA		**Y**	**F**	S	G	GSNYKL TFG	Jα 9.3
				gct	TAT	TTT	agt	gga	ggt
SD10	Vα22	DSAVYFC	A	**L**	**T**	**S**	N	NAGNML TFG	Jα 6.1
			gct	CTG	ACC	TCT	aat	aat	
SD2.1	Vα2	DSAVYFC	A	**L**	-	-	-	-SAS KI IFG	Jα 16.1
			gct	CTG	-	-	-	-agtgct	
SD2.2	Vα7	DSASYLC	A	**V**	**R**	**R**	-	-NQGNL IFG	Jα 9.11
			gct	GTG	AGG	AGG	-	-aaccaa	

Figure 5.1a and b. TCR alpha chain usage and amino acid homology of HLA B27-restricted Influenza A NP383-391 specific CTL clones.

Nucleic acid residues that may be derived by N-region addition are shown in bold. Vα families and CDR3 nucleic acid (fig 5.1a) and predicted amino acid sequences (single letter code) are shown together with the Jα families. Jα families after Moss *et al* (1993) are also known as follows: Jα16.5: HAVP42, Jα9.3: HAVP44, Jα1.8: AD17, Jα6.1: AC17, Jα16.1: HJGA, Jα16.6: wRa53, Jα9.11: IGRJa06 Kimura *et al* (1986), and Yoshikai *et al* (1986) Residues with a probable contribution from N-region addition are shown in bold, although the germline nucleic acid sequences of the Vα segments are not known. Amino acids are numbered according to Chothia *et al* (1988). Where two in frame alpha transcripts were obtained from an individual clone they are numbered .1 and .2. In Fig 5.1b the clone HF1 studied by P. Moss and S. Rowland-Jones is included for comparison, and sequences SD2.2 and HF2.1 have been separated as they may be redundant (see text).

CLONE	-----Vα-----9 1	Junction				------Jα----------	
SD13	Vα12	DSAVYFCA	L	Q		DSSYKLIFGS	Jα16.5
SD16	Vα12	DSAVYFCA	L	V		DSSYKLIFGS	Jα16.5
HF1.2	Vα12	DSAVYFCA	R	M		DSSYKLIFGS	Jα16.5
GR c	Vα12	DSAVYFCA	C		G	GSNYKLTFGK	Jα 9.3
SD17	Vα14	DAAMYFCA	W	I	S G	GSNYKLTFGK	Jα 9.3
GR b	Vα14	DAAMYFCA	Y	F	S G	GSNYKLTFGK	Jα 9.3
SD10	Vα22	DSAVYFCA	L	T	S N	NAGNMLTFGG	Jα 6.1
SD2.1	Vα22	DSAVYFCA	L			SASKIIFGS	Jα16.1
SD2.2	Vα 7	DSASYLCA	V	R	R	NQGNLIFGK	Jα 9.1
HF1.1	Vα32	DSAVYYCL	L	G		DPPMNYGGSQGNLIF	Jα9.11

Figure 5.1b

Several features are apparent from figures 5.1a and b:

1) A number of distinct clones use almost identical TCR alpha chains. Two clones, SD13 and SD16, used Vα12 together with Jα16.5, their alpha chains differing at only a single amino acid residue from each other. Furthermore these clones differed at only two residues from clone HF1. The finding of identical Vα and Jα usage from clones from unrelated donors is remarkable and implies very strong selection. Clones SD17 and GRb both used Vα14 and Jα9.3, and also differed at only two residues. Thus there appears to be selection for either of two possible Vα/Jα combinations.

2) Jα segments contribute the majority of residues to the alpha chain third hypervariable region (CDR3). According to the model of Chothia *et al.* (1988), the TCRα CDR3 equivalent region spans residues 93-104, with framework residues at positions 92 and 105, and a highly conserved cysteine at 90 and glycine at 107. Notably, since TCR CDR3 differ in length, numbering is not necessarily consecutive. In this thesis Vα.and N residues are numbered forwards from 90, and Jα residues numbered backwards from 107. As indicated in figure 5.1, the Jα segments contribute between 5 and 8 residues to CDR3 regions of 5-9 amino acids lenght. In particular four residues contributed by the Jα segments, serine at position 100, tyrosine at 102, lysine at 103 and leucine at 104, were found in all of the clones using Vα12 or 14. Residues S100 and Y102, present in both Jα9.3 and Jα16.5, are rarely found in other Jα segments.

3) Amino acids resulting from N region addition show little homology. Charged, hydrophobic and aromatic residues all found, in contrast to NDN region residues of the TCRß chain CDR3 (see below).

4) The length of the third hypervariable region also shows relative conservation. All chains using Vα12 having an identical CDR3 length of seven amino acids, and all Vα14 alpha chains a length of nine. In the case of clone GRc, in which Vα12 is paired with Jα9.3 instead of

Jα16.5, CDR3 length appears to segregate with Vα and not Jα usage.

These findings can readily be compared to those for the HLA A2-restricted Influenza matrix protein-specific CTL response. Here CTL lines and clones similarly show conserved TCR alpha chain usage, with one predominant Vα (Vα10.2), and a dominant Jα segment (Jα9.11) contributing a conserved portion of the CDR3 ((Moss *et al.* 1991) and P. Moss unpublished data). Furthermore no conservation of residues derived from N region addition was found. These data suggest that in both these CTL responses the Jα segments, which are both more numerous and diverse than the Jß segments, may themselves have a greater role in determining TCR specificity than amino acids contributed by N region addition (Moss *et al.* 1991, Bowness *et al.* 1993).

5.5. TCRß chain usage of HLA B27-restricted NP383-391-specific CTL clones

Vß7 is used by HLA B27 restricted CTL clones from three unrelated donors

The TCR ß chain usage of the seven NP383-391-specific clones are shown in table 5.3 (and in figures 5.3 and 4). Four clones used Vß7 family members, three IGRb19 (Ferradini *et al.* 1991), hereafter known as Vß7.1, and one IGRb17 (Vß7.2). The Vß7.1 and Vß7.2 amino acid sequences, given in appendix 3, are approximately 85% homologous. Vß7.1 was also used by a clone from a third individual studied by S. Rowland-Jones and P. Moss (HF1, see fig 5.4). Vß7 was also detected by "Vß family-specific" PCR amplification of cDNA from an HLA B27-restricted NP 383-391 specific CTL line that had been obtained from donor GR one year previously (data not shown), suggesting that Vß7 usage was consistent over time in this individual. Vß7 family members usually make up only 5-8% of TCR Vß sequences obtained from PBL (Rosenberg *et al.* 1991). However high levels of Vß7 have been reported in some individuals (Malhotra *et al.* 1992), and hence the TCR Vß repertoire of resting PBL from one of the individuals in this study (GR) was ascertained using AnPCR, The GRPBL sequences, sequenced together with P. Moss, are shown in table 5.4, and the Vß usage in figure 5.2.

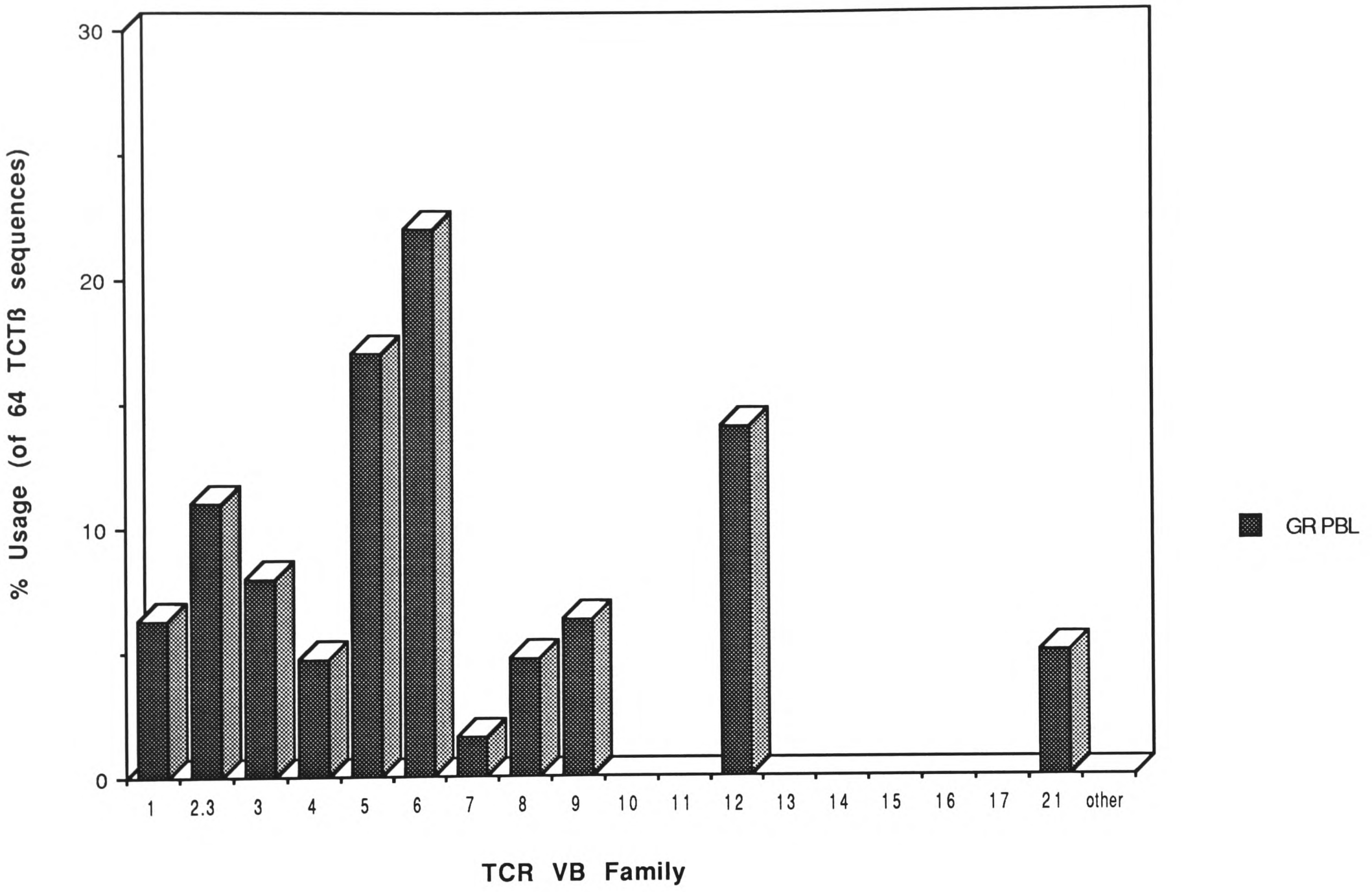


Figure 5.2. TCR Vß usage of unstimulated PBL from donor GR, estimated by AnPCR.

sequence	Vß	Vß C term	NDN	Jß N terminus	Jß	CDR3 length amino acids
1	4.1	CSVV	VGLAA	SYNEQ	2.1	10
2	8	CASS	HGQPIY	PQH	1.5	9
3	3	CASK	EGGIA	EQY	2.7	8
4	6.9	CASS	YGTSG	NIQY	2.4	9
5	5.1	CASS	QGQT	YEQY	2.7	8
6	12	CASN	GPGVD	EQY	2.7	8
7	21	CASS	GGQLP	YNEQF	2.1	10
8	12	CATS	DWGN	NQPQH	1.5	9
9	6.2/3	CASS	LVTGRG	NEQF	2.1	10
10	12.2	CAIS	AKQGSG	TDTQY	2.3	11
11	2.1	CSAS	ILAGAL	SYEQY	2.7	11
12	12.2	CAIT	SGQR	QEQY	2.5	9
13	5	CASS	QYAGLS	SYNSPLH	1.6	13
14	2	CSAN	RLAG	EYNEQF	2.1	10
15	6.6	CASS	PTGRS	SYEQY	2.7	10
16	4	CSVE	DPWGAK	ETQY	2.5	10
17	9.1	CASS	RPGPGAFG	NIQY	2.4	12
18	6.1	CASS	FGRGLE	NTIY	1.3	10
19	5.4	CASS	FPGAG	EQY	2.7	8
20	4	CSVE	EGQGAG	ELF	22	9
21	1	CASS	VDGRT	QETQY	2.5	10
22	12	CASS	YQRENN	EQY	2.7	9
23	6	CASS	LGGR	DTQY	2.3	8
24	21	CASS	LVVGGD	TGELF	2.2	11
25	5	CASS	LQGN	YNSPLH	1.6	10
26	6	CASS	FRAGFN	NEQF	2.1	10
27	2	CSAR	DPTGST	YEQY	2.7	10
28	6	CASS	SRLRG	SNSPLH	1.6	12
29	5.4	CASS	LGRGESN	NEQF	2.1	11
30	8	CASS	STDSY	QPH	1.5	8
31	9	CASS	QVRQGALG	ETQY	2.5	12
32	2	CSAS	LPRGD	YEQY	2.7	9
33	3	CASS	LGGVPS	PQH	1.5	9
34	9	CASS	PRNVVGGGPG	ETQY	2.5	14
35	3	CASS	PPGEV	YEQY	2.7	9
36	6	CASS	LDYN	GELF	2.2	8
37	5	CASS	FWSPFAAGLKD	EQF	2.1	14
38	12	CASS	YSIFVTRRG	SPLH	1.6	13
39	3	CASS	SLAGGSAG	DTQY	2.3	12
40	1	CASS	REPGA	TDTQY	2.3	10
41	9	CASS	PEFRRG	NEQF	2.1	10

sequence	Vß	Vß C term	NDN	Jß N terminus	Jß	CDR3 length amino acids
42	6	CASS	HIRDLR	YNEQF	2.1	11
43	12	CASS	EAKGP	ETQY	2.5	9
44	6	CASS	FQGG	TDTQY	2.3	9
45	1	CASS	AGTSGRR	ETQY	2.5	11
46	2	CSAR	TSGT	YEQY	2.7	8
47	7	CASS	PIGTGAY	EQF	2.1	10
48	5	CAIS	SGDL	YNEQF	2.1	9
49	12	CASS	YSSSGSFLYK	QF	2.1	12
50	5	CASS	LNLRTLAGSS	GELF	2.2	14
51	2	CSAR	AGSF	TDTQY	2.3	9
52	3	CASN	LAGGARA	KNIQY	2.4	12
53	6.4	CASS	RGT	TDTQY	2.3	8
54	5	CASS	LDRG	EQY	2.7	7
55	5.1	CASS	LEGREH	NEQF	2.1	10
56	1.1	CASS	AQGAE	TEAF	1.1	9
57	21	CASS	LTMTGGR	SPLH	1.6	11
58	6.9	CASS	STREA	T	1.2	6
59	6.6	CASS	HRATAG	DTQY	2.3	10
60	6.9	CASS	VPGQGE	DTQY	2.3	10
61	8	CASS	TGTSGG	YNEQ	2.1	11
62	12.3	CASS	YGMVR	EKLF	1.4	9
63	5.2	CASS	IG	TGELF	2.2	7
64	2	CSAS	SIPRFPT	NEQF	2.1	11

Table 5.4 Vß, Jß, predicted CDR3 amino acid usage and CDR3 length of 64 TCRß sequences, obtained from unstimulated PBL from donor GR (HLA B27 positive).

Fig 5.3

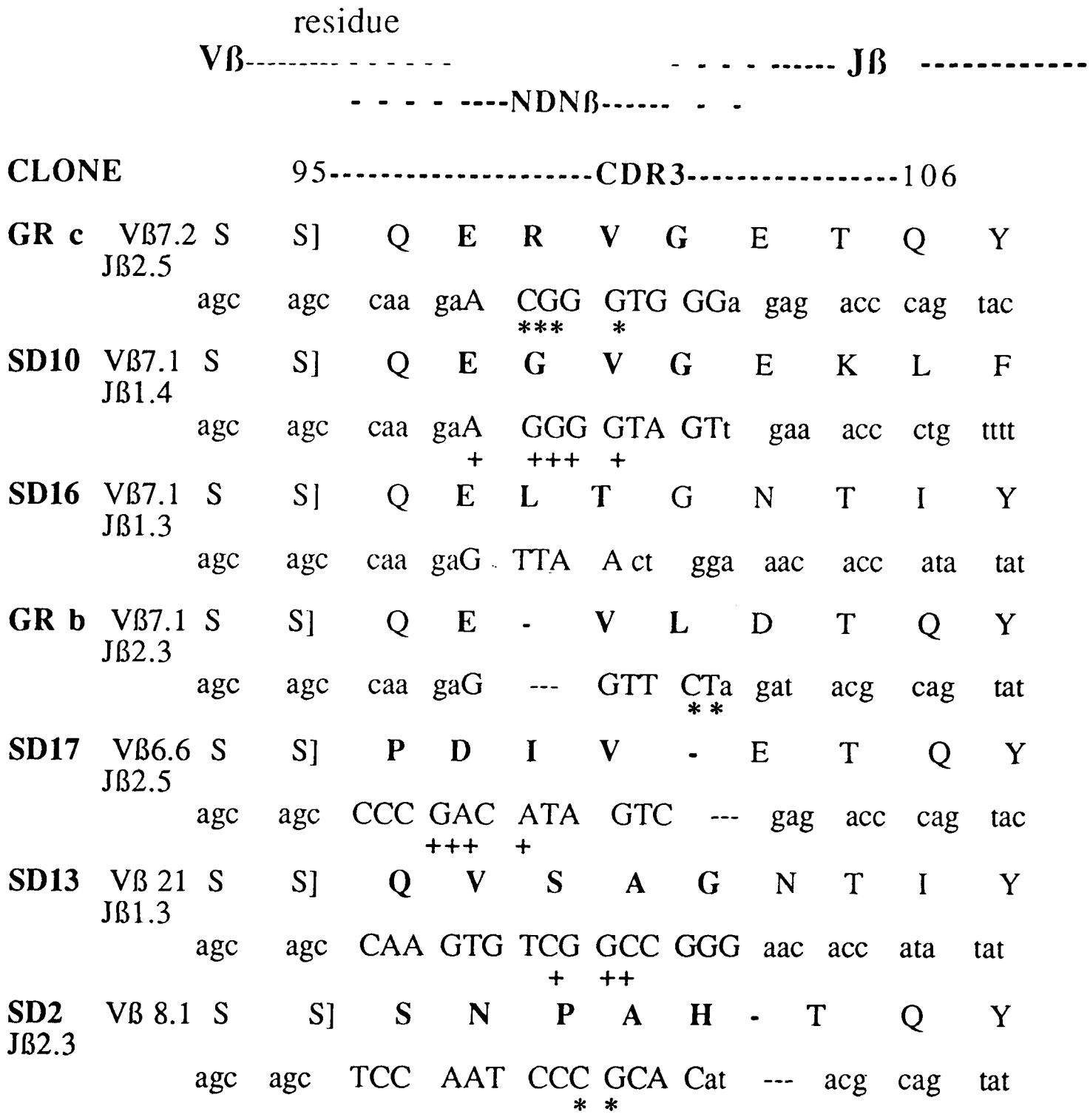


Figure 5.3. Nucleotide and predicted amino acid sequence of TCR beta chain CDR3 regions of HLA B27-restricted influenza-specific CTL clones.

The CDR3 loop is predicted to span residues 95-106 according to Chothia *et al* (1988). Since TCRs vary in length CDR3, Vß and NDN residues are numbered 95 to 99 with reference to a framework cysteine at position 92, and Jß residues with reference to a framework glycine residue at 109. Consequently for GRb, L99 is followed by D104. Probable NDN-derived nucleotide residues are shown in capitals and amino acids in bold. + + indicates possible Dß1 derived nucleotides and ** possible Dß2.] indicates the usual 3' serine derived from the Vß element, however nucleic acid conservation suggests that up to five additional nucleic acids may be contributed by the Vß7 segments. The full sequences of the TCR alpha and beta chains of clone GRb are shown in appendices 5 and 6.

CLONE	---Vß----	95	Junction					101 ------	Jß-------	
GRc	Vß7.2	CASS	Q	E	R	V	G	ETQYFG	Jß2.5	
SD10	Vß7.1	CASS	Q	E	G	V	G	EKLFFG	Jß1.4	
HF2	Vß7.1	CASS	Q	E	T	V	G	EKLFFG	Jß1.4	
SD16	Vß7.1	CASS	Q	E	L	T	G	NTIYFG	Jß1.3	
GRb	Vß7.1	CASS	Q	E	-	V	L	DTQYFG	Jß2.3	
SD17	Vß6.6	CASS	P	D	I	V	-	ETQYFG	Jß2.5	
HF1.2	Vß6.9	CASS	L	E	-	V	G	DEAFFG	Jß1.1	
HF1.1	Vß1	CASS	V	S	V	G	G	GGEQFFG	Jß2.1	
SD13	Vß21	CASS	Q	V	S	A	G	NTIYFG	Jß1.3	
SD2	Vß8	CASS	S	N	P	A	H	TQYFG	Jß2.5	
Consensus			Q	E	x	V	G			

Figure 5.4. TCRß chain CDR3 amino acid homology of HLA B27-restricted NP383-391-specific CTL clones. The HF sequences were obtained by P. Moss from a clone and biclonal line made by S. Rowland-Jones, and included for comparison.

Only 1 of 64 TCRß sequences obtained from GR PBL was Vß7 (IGRb19), showing that the Vß7 usage of the HLA B27-restricted Influenza NP-specific clones was not due to a bias in PBL TCR usage. M. Callan recently confirmed the Vß7 dominance of HLA B27-restricted NP-specific CTL, using a Vß7-specific monoclonal antibody, which stained approximately 40% of cells from an NP-specific CTL line but less than 2% of PBL from laboratory volunteers (M. Callan, unpublished data).

The TCR beta chain third hypervariable regions (CDR3) show marked conservation of amino acid composition

The beta chain third hypervariable regions of the seven CTL clones, made up by the combinatorial association of V, D, and J segments together with N region addition, showed remarkable similarities in amino acid sequence. The nucleotide and predicted amino acid sequences of the TCR ß chains CDR3 regions are shown in figure 5.3 and the CDR3 homology in figure 5.4. Jß usage was not significantly different from that of GR PBL, with five of the eleven Jß segments being used (table 5.5).

	CTL clones (n=8)	PBL (n=64)
CDR3		
Length (amino acids)	8.8 +/- 0.75	10.0 +/- 1.7
Conserved Residues		
96Q	6** (75%)	3 (4.5%)
97E	5** (63%)	4 (6%)
99V	5** (63%)	4 (6%)
99G	0+ (0%)	19 (30%)
100G	5* (63%)	15 (23%)
Jß usage		
Jß1	4+ (50%)	12 (20%)
Jß2	4+ (50%)	51 (80%)

TABLE 5.5. COMPARISON OF TCR BETA CHAIN CDR3 AMINO ACID AND Jß FAMILY USAGE OF INFLUENZA NP-SPECIFIC HLA B27-RESTRICTED CTL CLONES AND GR PBL.
Amino acid utilization at residues 96-100 is shown (single letter code).
** $p< 0.005$, * $p<0.05$, + $p>0.05$ (Fisher exact).

In contrast the CTL clones show at least 60% amino acid homology for four ß CDR3 residues contributed to by N region addition, ß96, 97, 99, and 100, giving a consensus sequence of QExVG. When compared to the CDR3 regions of ß chains amplified from the peripheral blood of GR and illustrated in table 5.4, table 5.5 shows that these consensus amino acids were significantly more frequent in the CTL clones. This analysis also shows that glycine was commonly found at positions 99 and 100 in GR PBL, probably due to the contribution of D segments and the preference of terminal deoxynucleotidyl transferase for deoxyguanidine triphosphate. The most strikingly conserved residue in the ß chain CDR3 regions is the glutamic acid residue at position 97. Because the genomic sequence of Vß7 is not yet known, and the Vß7 clones have identical nucleic acid sequences encoding 96Q and the first two nucleotides of 97E, it is possible that germline nucleotides 3' of the normal splice site (after the CASS) encoded these residues (see figure 5.3). By comparison the Vß7 sequence derived from the HFCTL line also used 96Q (Table 4.1), but the single GR PBL Vß7 sequence used neither 96Q nor 97E (see table 5.4). However of ten Vß7 sequences of T cells stimulated to grow by an anti Vß7.1 mAb, recently analyzed by M. Callan, all had 96Q and one had 97E (Callan *et al.* 1993 submitted). Notwithstanding this possibility, both 96Q and 97E were also found in clones using different Vß chains, implying active selection of these residues. Indeed it is possible that the ability of the Vß7 germline to encode these residues has contributed to its selection in this immune response.

Notably SD2, the only clone with a ß chain lacking any of the conserved junctional residues, also used a distinct Vß element (Vß8). Staining of this clone with the Vß8-specific monoclonal antibody MX6 (Boylston *et al.* 1986), shown in figure 5.5, excluded the possibility of a contaminating cell population, although experiments to demonstrate inhibition of cytolysis by MX6 were not performed.

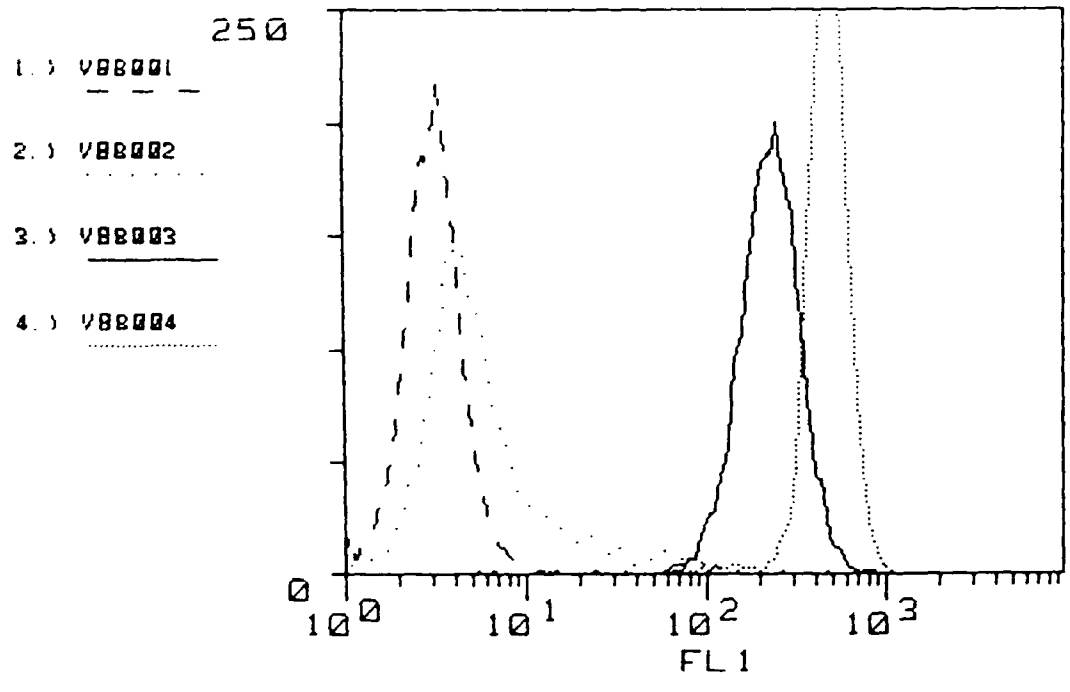


Fig. 5.5a. CTL clone SD2 stains with the mAb MX6 (anti-Vß8).
1): second layer antibody only. 2) 421C1. 3) MX6 4) MF8 (anti-CD8).

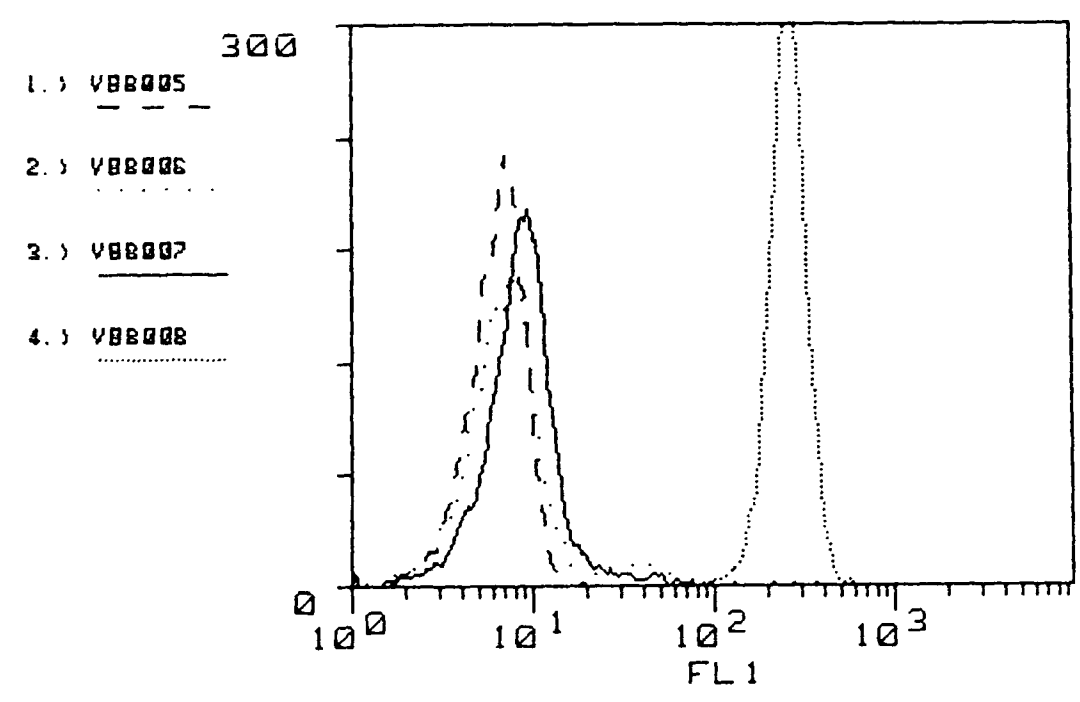


Fig. 5.5b. CTL clone SD16 does not stain with the mAb MX6 (anti-Vß8) or 42 1C1 (anti Vß5.2/3).
5): second layer antibody only. 6) 421C1. 7) MX6 8) MF8 (anti-CD8).

Figure 5.5. FACS staining of the CTL clones SD2 and SD16 with the monoclonal antibodies MX6 and MF8.

5.7. Discussion

HLA B27-restricted NP383-391-specific CTL clones use a restricted TCR repertoire

HLA B27-restricted NP383-391-restricted CTL clones from two unrelated individuals showed conservation of both TCR alpha and beta chain usage, with a majority of clones using Vß7 and part of an NDNß amino acid motif QExVG, together with Vα12 or Vα14 and Jα9.3 or Jα16.5. This implies that only a limited repertoire of TCR are used to recognize the HLA B27+NP383-391 combination *in vivo.* Indeed for the groups of clones using Vα12 and Vα14, Vα usage (and CDR3 length) correlated with two distinct groups of fine specificity, suggesting two alternative recognition patterns (see chapter 6). Whilst it appears that almost any given Vα or Vß segment can be employed by both MHC class 1 or class 2-restricted T cells, recent reports have found skewing of Vß7 and Vα12 to the CD8 as opposed to CD4 lymphocyte population, as estimated by "family-specific PCR" (Alkolar *et al.* 1993, Hawes *et al.* 1993). Since the individuals in these studies were not necessarily HLA B27 positive, this suggests that Vß7 and Vα12 may also be positively selected on other HLA class I molecules.

Interestingly clone SD2 showed no homology with any other clones in either TCR alpha or beta chain usage. This raises the possibility that this clone recognizes the HLA B27+NP complex in a different manner to the other clones (see chapter 6).

Conservation of glutamic acid at position 97 of the TCRß chain

The conserved negative charge found at position 97 of the TCR ß chain in almost all clones suggests that it plays an important role in recognition by these CTL. Although the three dimensional structure of the TCR is not known, the ß chain CDR3 is predicted to contact MHC-bound peptide (Chothia *et al.* 1988). The importance of the equivalent position of the murine TCR ß chain has been demonstrated. A conserved aspartic or glutamic acid was found in I-E $^{k/s}$-restricted clones recognizing pigeon (and moth) cytochrome c (Hedrick *et al.* 1988). Engel and Hedrick (1988) introduced site-directed mutations into this residue of the I-E^{s}-restricted PCC-specific clone D6, showing that a D to N change altered specificity

for peptide (and also alloreactivity), whereas a D to L substitution abolished recognition entirely. This experiment suggested that this CDR3ß residue has a critical role in peptide recognition. Recently amino acid usage at this position in T cell hybridomas from mice with transgenic TCRa chains has been shown to vary following immunization with cytochrome c peptide analogues (Jorgensen *et al.* 1992). Thus whilst there is no evidence that this position contacts MHC-bound peptide in MHC class1-restricted TCRs, and no data on human TCRs, this residue may well be involved in peptide contact. Notably a conserved arginine residue was found at this position in the beta chains of HLA A2-restricted M58-66-specific CTL TCRs (Moss *et al.* 1991).

Conservation of Vα, Jα, Vß and NDNß may reflect a general pattern for MHC class I-restricted virus-specific responses

The conservation of both TCR α and ß chains, exhibited by the Influenza NP-specific HLA B27-restricted CTL clones, although similar to that found by Moss *et al.* (1991), has not been found in all previous studies. For example, several MHC class 2-restricted immune responses have exhibited either diverse TCR usage (De Magistris *et al.* 1992, Giegerich *et al.* 1992, Martin *et al.* 1992), or Vß conservation alone (Urban *et al.* 1988, Danska *et al.* 1990, Boitel *et al.* 1992). The pattern for class 1-restricted responses is, in general, no clearer. For example murine H-2K^d restricted CTL show striking TCRα and ß chain homogeneity in response to human HLA-Cw3 (Casanova *et al.* 1992), but only Vß conservation in response to Plasmodium berghei (Casanova *et al.* 1991), although the results of the latter study are difficult to interpret as six different immunization protocols were used. There is however evidence of restriction of both TCR α and ß chain usage in MHC class-1 restricted responses following infection with natural viral pathogens, such as LCMV in the mouse (Aebischer *et al.* 1990, Brandle *et al.* 1991) and in the human HLA A2-restricted Influenza response (Moss *et al.* 1991). Data from previous studies of TCR usage in MHC class-restricted responses is summarized in Table 5.6.

			TCR alpha			TCR beta			
Infection	**Epitope**	**Restriction**	**Vα** (frequency)	**Nα**	**Jα**	**Vß**	**NDNß**	**Jß**	**Ref**
HUMAN 'FLU A	NP383-91	B27	12 (4/10) 14 (2/10)	N.C. N.C.	16.5(3/10) 9.3 (3/10)	7 (5/8)	QExVG	N.C.	Bowness 1993
'FLU A	M58-66	A2	10.2*	N.C.	9.11	17*	IRSS	N.C.	Moss 1991
RHESUS SIVmac	gag	mamu-A*01	N.C.	N.C.	N.C.	23§ (5/6)	96/7D (4/6)	new (4/6)	Chen 1992
MURINE LCMV	GP275-89 (278-286)	D^b	4 (5/5) 4 (6/6)	n.t. 93D(3/4)	n.t. HL228 (2/4)	10(5/5) 10(2/4)	n.t. N.C.	n.t. 2.1(3/4)	Aebischer 1990
	GP 32-42	D^b	2*	L(3/5)*	TA31(5/5)*	8.1/3*	N.C*	N.C.	Brandel 1991
artificial	HLA-Cw3 170-179	K^d	8 (11/30) 4 (10/30) 3 (6/30)	94 D/E 95G/R/ Q/H	pHDS58 (30/30)	10 (30/30)	97G (30/30)	1.2 (18/30)	Casanova 1992 1993
	HLA-A24 170-179	K^d	8 (9/24) 3 (6/24)	95G/R	pHDS58 (22/24)	10 (23/26)	97G (24/26)	(N.C)	Casanova 1993
	P.berghei CS252-60	K^d	N.C	N.C.	N.C.	13 (15/23)	N.C.	N.C.	Casanova 1992
	tum^-	L^d	42 (3/7) 8 (3/7)	N.C.	31 (3/7) 34 (2/7)	8 (12/17)	N.C.	N.C.	Solheim 1993

Table 5.6. TCR usage in published MHC Class-1 restricted responses. N.C.: Not conserved. n.t.: not tested. * includes data from CTL lines. # shares 94% nucleotie homology with Vß22/23

Two studies of hapten-specific CTL have not been included. Hochgeschwender *et al.* (1986) studied the TCR beta chains of H-2K^b-restricted TNP-specific clones, and found conservation of Vß, Dß, and Jß. Iwamoto *et al.* (1987) studied four AED-specific clones restricted by H-2K^b or H-2D^b and found that two used identical Jα (and Jß) gene segments. It is striking that the pattern of conservation found in the HLA A2-restricted influenza matrix-specific response, of Vα10.2 and Jα9.11 but not Nα conservation, and of Vß17 conservation with an NDN junctional consensus amino acid sequence (IRSS), but not Jß conservation, is the same as that found in the current study. Similarly conservation of Vα and Jα, but not the junctional residue, together with Vß, is seen in the H-2D^b restricted response to LCMV GP275-289 (Brandle *et al.* 1991). Yanagi *et al.* (1990) obtained similar results although conservation of Vα and Vß were less marked. The murine H-2K^d restricted response to HLA-Cw3 similarly shows absolute conservation of Jα and position 97 of the ß chain, but more variation of Nα and Jß. Thus for MHC class I restricted responses to viruses at least there is evidence for a pattern of conservation of Vα, Jα, Vß and usually NDNß, suggesting a common topology for the ternary complex. This may differ from that involved in class 2-restricted recognition. Based on previous models (Chothia *et al.* 1988, Davis and Bjorkman 1988) these data would suggest that critical TCR residues involved in peptide contact may be located in the Jα and NDNß contributions to the α and ß chain CDR3 regions respectively. Since there are only approximately 60 Jα segments, this would imply that the TCRα CDR3 repertoire may be more limited than previously estimated, at least for these immune responses.

The homogeneity and pattern of TCR usage found in the HLA B27-restricted response to Influenza A may be determined by several factors. The number and nature of exposed side chains of MHC-bound peptide, available for TCR interaction, may be the critical determinant of TCR repertoire. Crystallographic data of peptides

bound to HLA B27 suggests that the side chains of residues 1, 4 and 8 of bound peptide are available for TCR interaction (Madden *et al.* 1991, 1992). These are, respectively, serine, tryptophan, and threonine for NP383-391. Peptides bound to MHC in a tightly constrained orientation may limit the range of possible TCR interactions. The salt bridge overlying residues two and three in peptides bound HLA B27 may be important in this respect, as may the water molecules which pack the MHC groove (Madden *et al.* 1992). Other determinants of TCR repertoire probably include the nature of immunization and the similarity of the immunizing epitope to self peptides (Casanova and Maryanski 1993).

Comparison with allospecific HLA B27-directed responses suggests that there may be no characteristic features of HLA B27-restricted TCRs

Comparison with the preliminary data detailed in chapter 4, suggests that there is surprisingly little similarity in TCR usage between the NP-specific and HIV gag 265-274-specific responses. Whilst further studies of TCR repertoire in both the gag-specific and other HLA B27-restricted responses are required, these findings are in agreement with the different TCRs used by murine CTL recognizing different LCMV epitopes presented by H-2D^b, summarized in table 5.6 (Pircher *et al.* 1987, Brandle *et al.* 1991, Aebischer *et al.* 1990, Yanagi *et al.* 1990, Yanagi *et al.* 1992). Whilst MHC is clearly critical for peptide selection and positioning (and thymic selection), peptides themselves may be the critical determinants in TCR repertoire selection, with MHC merely permissive. In support of this proposition it is notable that TCR conservation has been reported for T cells recognizing the same peptide presented by different HLA class 2 molecules (Boitel *et al.* 1992), and it is striking that murine T cells, such as those recognizing pigeon cytochrome c, use very limited TCR repertoire, yet can see peptide in the context of a variety of class 2 molecules (Hedrick *et al.* 1988).

TCR usage of influenza NP-specific CTL was also more highly conserved, and used different elements, to that found previously

for HLA B27 allospecific CTL clones. These used predominantly Vß4, 13 and 14, and showed apparently random Vα usage (Bragado *et al.* 1990, Lauzurica *et al.* 1992). This is probably due to the number of different peptides involved in allorecognition, and is again consistent with the idea that bound peptide, rather than presenting MHC molecule, is the critical determinant of T cell TCR repertoire in a given immune response. An alternative explanation is that TCR with high affinity for HLA B27 are eliminated in HLA B27 positive individuals. Vß4, but not Vß13 or 14, was identified in TCR Vß sequences from PBL of one of our subjects.

Implications for HLA B27-associated autoimmune disease

The CTL clones from the healthy donor (SD) and from individuals with ankylosing spondylitis (GR) or reactive arthritis (HF) showed no obvious difference in TCR usage, suggesting that, for this immune response at least, antigen presentation by HLA B27 and the TCR repertoire are normal in the these conditions. This will be discussed further in chapter 8. Furthermore the finding of a limited TCR repertoire for the Influenza-specific HLA B27-restricted response suggests that, if HLA B27 acts as a restriction element for a specific peptide or peptides in autoimmune HLA B27-associated diseases, a restricted TCR repertoire may also be found and may provide a target for immunotherapy. However available data does not allow prediction of any elements of this repertoire, and indeed suggests that the TCR usage of different HLA-B27-restricted responses will be quite distinct.

CHAPTER 6

FINE SPECIFICITY OF HLA B27-RESTRICTED NP383-391-SPECIFIC CTL CLONES FOR PEPTIDE AND MHC

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6.1. Introduction

This chapter describes the fine specificity of the HLA B27-restricted NP383-391-specific CTL clones for variants of the native optimal nonamer peptide SRYWAIRTR. Some differences in specificty for HLA B27 subtypes were also observed and are described in section 6.5. Synthetic peptides containing amino acid substitutions were made and tested in cytolysis assays in order to identify residues important for TCR recognition. It was expected that the CTL clones tested would exhibit differences in fine

specificity, which might correlate with variation in their TCR usage. Furthermore an indication might be given as to the extent of contact and orientation of the TCR with respect to peptide and MHC. Lastly some idea of the microvariation of the human immune response to a given peptide/MHC combination in naturally immune donors might also be obtained, with the proviso that the cloning protocol used might introduce bias.

In order for a given peptide to be recognized by the CTL TCR, firstly the pepide must bind to HLA B27 (and remain bound), and secondly the CTL TCR must contact the HLA B27/peptide combination, forming a ternary complex. Specific recognition usually leads to target cytolysis, but can result in T cell anergy (Jameson *et al.* 1993). Whilst antigen processing by presenting cells is important in determining which epitopes are presented to T cells, such effects were specifically excluded in this study by sensitizing targets for cytolysis with nonamer peptides added exogenously at the time of assay. Peptides added in this way are able to bypass the normal class 1 antigen processing pathway, probably either by displacing peptides bound in loosely or improperly folded complexes with MHC on the cell surface (Townsend *et al.* 1989), or by binding to "empty" cell surface HLA B27 molecules (Benjamin and Parham 1991). This protocol also enabled accurate quantitation of pepide concentrations available to bind to HLA B27 (albeit in a somewhat artificial manner), permitting comparison between large numbers of peptides.

Using recombinant Vaccinia viruses and pools of synthetic peptides, (Huet *et al.* 1990) had previously shown that all influenza immune HLA B27 positive individuals responded to a single peptide epitope derived from the influenza nucleoprotein (NP). The minimal peptide epitope SRYWAIRTR corresponding to NP residues 383-391, was subsequently defined (Sarah Rowland-Jones, unpublished data). Whilst it has not been formally shown that this nonamer is the exact epitope presented *in vivo*, as has been demonstrated for H-2K^b and VSV infection by peptide elution from the MHC molecules of infected cells (Van-Bleek and Nathenson 1990), not only is this nonamer active at lower concentrations than other overlapping

peptides, but it also conforms exactly both in length and at anchor positions to peptides eluted from HLA B27, shown in table 1.1 (Jardetzky *et al.* 1991).

S. Rowland-Jones had also confirmed the importance of the arginine residue at position two of the peptide, the predicted major anchor for HLA B27 binding, showing that the conservative substitution of this residue to lysine (i.e. 2K), completely abrogated recognition by a bulk CTL line (Colbert *et al.* 1993). It was subsequently shown that this residue was important for HLA B27 binding. The peptide 2L did not bind HLA B*2705 but bound an HLA B27 molecule into which six mutations had been introduced to reproduce the HLA A2 B pocket (H9F, T24A, E45M, I66K, C67V, K70H). Furthermore this mutant peptide/mutant MHC combination was recognized by a bulk HLA B27-restricted NP383-391-specific CTL line, and by one of the clones described here, showing that R at position two is not important in T cell recognition (Colbert *et al.* 1993).

6.2. Choice of substituted NP383-391 peptides

Nonamer synthetic peptides were synthesized with amino acid substitutions at positions 1, 4 , 7 and 8 (shown in table 6.1). The crystal structure of HLA B27 with pooled peptides (Madden *et al.* 1991, Madden *et al.* 1992) gave excellent resolution of side chains of peptide positions 1-4, and 8-9, enabling selection of substitutions at positions likely to affect T cell recognition. The observed and modelled peptide electron densities seen in the HLA B27 crystal structure are shown in figure 6.1a (Madden *et al.* 1992), and a cartoon of the working model for the orientation of NP383-391 in figure 6.1b. Figure 6.1a shows that the side chains of residues 1, 4, and 8, S, W, and T respectively in the NP peptide, "point upwards" towards the TCR. In contrast residues 2, 3, and 9 were seen to be almost entirely buried within the B, D, and C/F pockets respectively, and so were not studied. The position and side chain orientation of residues 5-7 was less clear from the crystal structure, although the side chains of positions 5 and 6

										Assembly of HLA B27
NP383-391	**S**	**R**	**Y**	**W**	**A**	**I**	**R**	**T**	**R**	++
SUBSTITUTIONS										
Position 1										
NAME										
1A*	**A**	-	-	-	-	-	-	-	-	n.t.
1F*	**F**	-	-	-	-	-	-	-	-	++
1K+	**K**	-	-	-	-	-	-	-	-	n.t.
1L	**L**	-	-	-	-	-	-	-	-	n.t.
Position 2										
2L -	**L**	-	-	-	-	-	-	-		–
Position 4										
4A**	-	-	-	**A**	-	-	-	-	-	++
4E*	-	-	-	**E**	-	-	-	-	-	++
4F*	-	-	-	**F**	-	-	-	-	-	++
4H*	-	-	-	**H**	-	-	-	-	-	+
4K*	-	-	-	**K**	-	-	-	-	-	++
4L*	-	-	-	**L**	-	-	-	-	-	++
4Y*	-	-	-	**Y**	-	-	-	-	-	++
Position 7										
7A*	-	-	-	-	-	-	**A**	-	-	++
7D*	-	-	-	-	-	-	**D**	-	-	++
7E**	-	-	-	-	-	-	**E**	-	-	n.t.
7I+	-	-	-	-	-	-	**I**	-	-	n.t.
7K	-	-	-	-	-	-	**K**	-	-	n.t.
Position 8										
8A**	-	-	-	-	-	-	-	**A**	-	n.t.
8E**	-	-	-	-	-	-	-	**E**	-	n.t.
8F*	-	-	-	-	-	-	-	**F**	-	++
8K*	-	-	-	-	-	-	-	**K**	-	++

Table 6.1. Substituted influenza A NP 383-391 peptides made by fmoc chemistry. Peptides marked * were synthesized on 14/4/1993. Peptides marked ** synthesized Aug1992. Peptides marked + were purchased from CRB (Cambridge) Jan 1992, peptide 2L was a gift from B. Colbert, the remainder were obtained from other laboratory members (previously purchased from CRB).



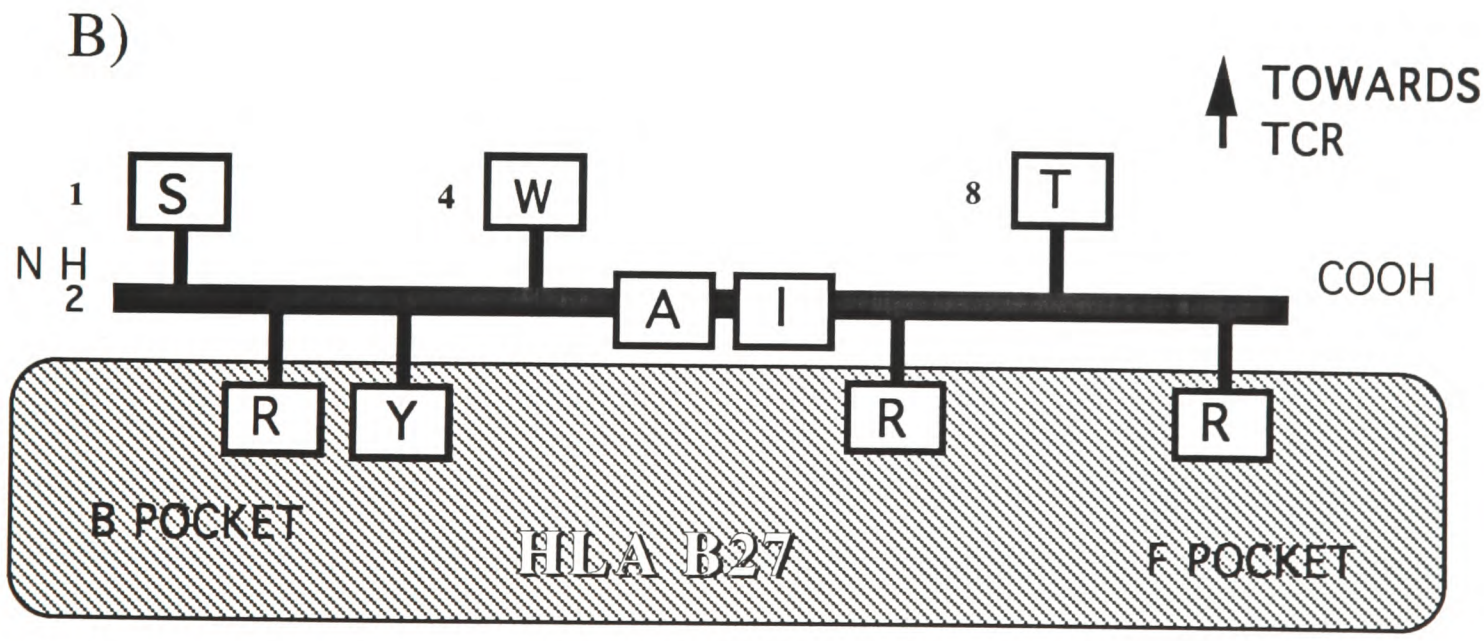


Figure 6.1. A) Observed peptide electron density for peptides bound to HLA B27, and B) Possible orientation of NP383-391 peptide bound to HLA B27. Fig. 6.1a from madden *et al.* 1992. Observed electron density shown in blue, and peptide model RRIKAITLK, built into this density and refined, in yellow.

were predicted to lie horizontally, contacting both MHC and potentially also TCR, with the side chain of residue 7 buried in the E pocket. Substitutions of residues 5 (alanine) and 6 (isoleucine) were not studied because of anticipated difficulties in quantitatively distinguishing differences in MHC and TCR binding, and because substitution of hydrophobic residues (particularly alanine) can be relatively uninformative. Substitutions at position 7, arginine in the NP peptide, were studied because of the unique length and flexibility of the side chain of this amino acid. In addition, although in the HLA B27 crystal (with diverse peptides) the visible electron density for this side chain was orientated downwards into the E pocket (near residue 152), for the Influenza matrix M58-66 peptide bound to HLA A2, both functional data (H. Zwerink, personal communication) and more recent crystallographic data (D. Madden, manuscript in preparation) have shown that this residue is TCR accessible.

6.3. Binding of NP variant peptides to HLA B*2705

Peptide substitutions were made at positions in which a variety of amino acids were found in the peptides eluted from HLA B27 (Jardetzky *et al.* 1991), and which, for positions 1,4, and 8 at least, were not predicted to be important for HLA B27 binding (Madden *et al.* 1991, Madden *et al.* 1992). Nevertheless failure of recognition might still have been due to failure of binding to HLA B27, for example through steric hindrance or effects on adjacent amino acids. Binding to HLA B27 was therefore confirmed by promotion of HLA B27 folding in cell lysates (Townsend *et al.* 1990). All substituted peptides tested, with the exception of 2L, assembled HLA B27 (figure 6.2 and table 6.1). Figure 6.2 also shows that peptides 7A and 7D induced HLA B27 assembly at comparable concentrations to NP383-391. HLA B27 assembly was quantified by densitometry (data not shown), and by counting 35 S methionine incorporation of excised bands, shown in figure 6.3. These results therefore imply that any reduction or loss of recognition with these peptides is due to failure of recognition by the TCR.

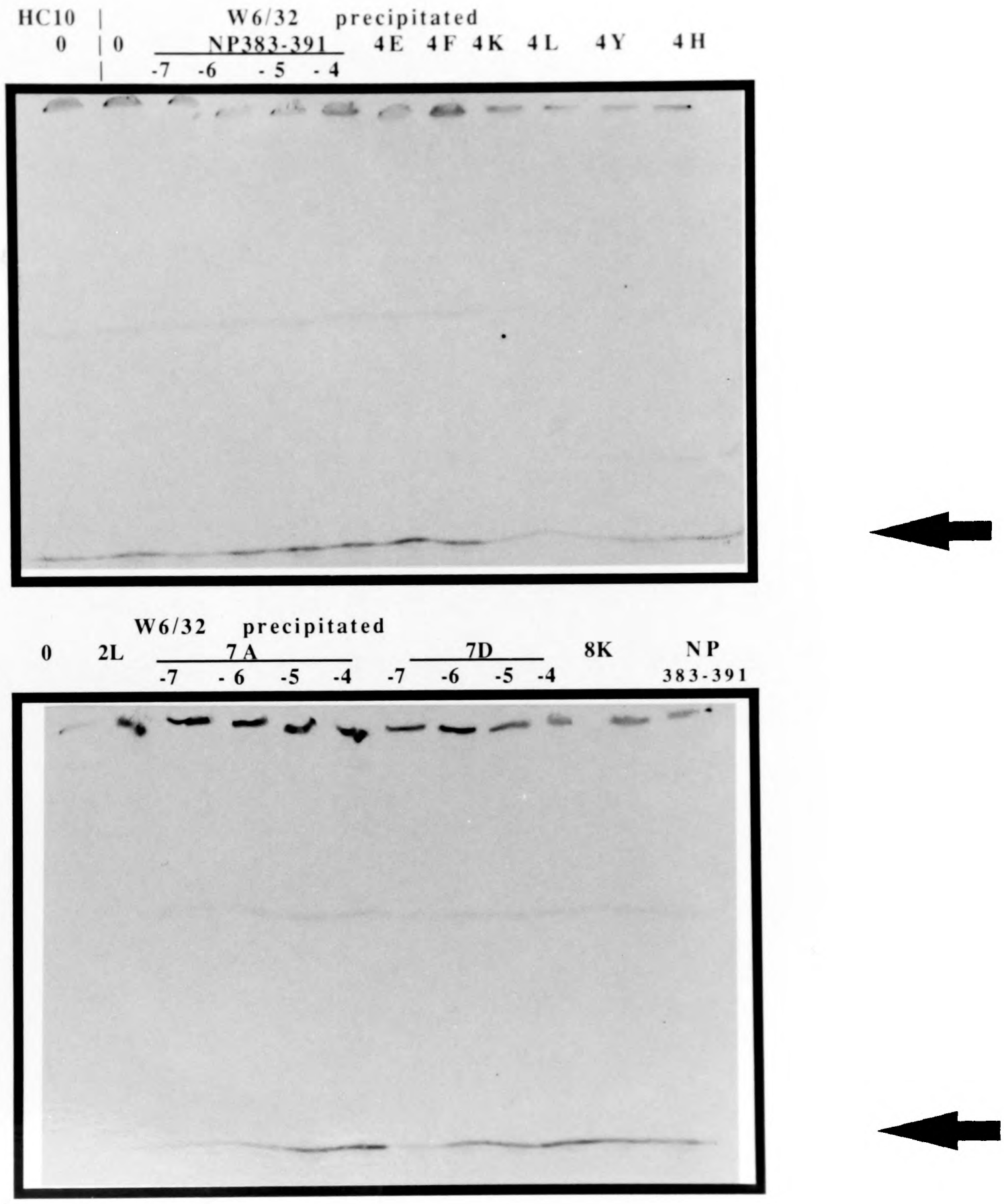


Figure 6.2. Assembly of HLA B27 promoted by NP383-391 and NP analogue peptides. Autoradiographs are shown of isoelectric focussing gels from one experiment. Arrows indicate the position of the HLA B27 band (ascertained from previous gels). 35S methionine-labelled T2 cells transfected with HLA B27 were lysed and peptides added at the concentrations indicated. The lysates were then heated and precleared overnight. Finally W6/32 was used to immunoprecipitate assembled HLA class 1 molecules.

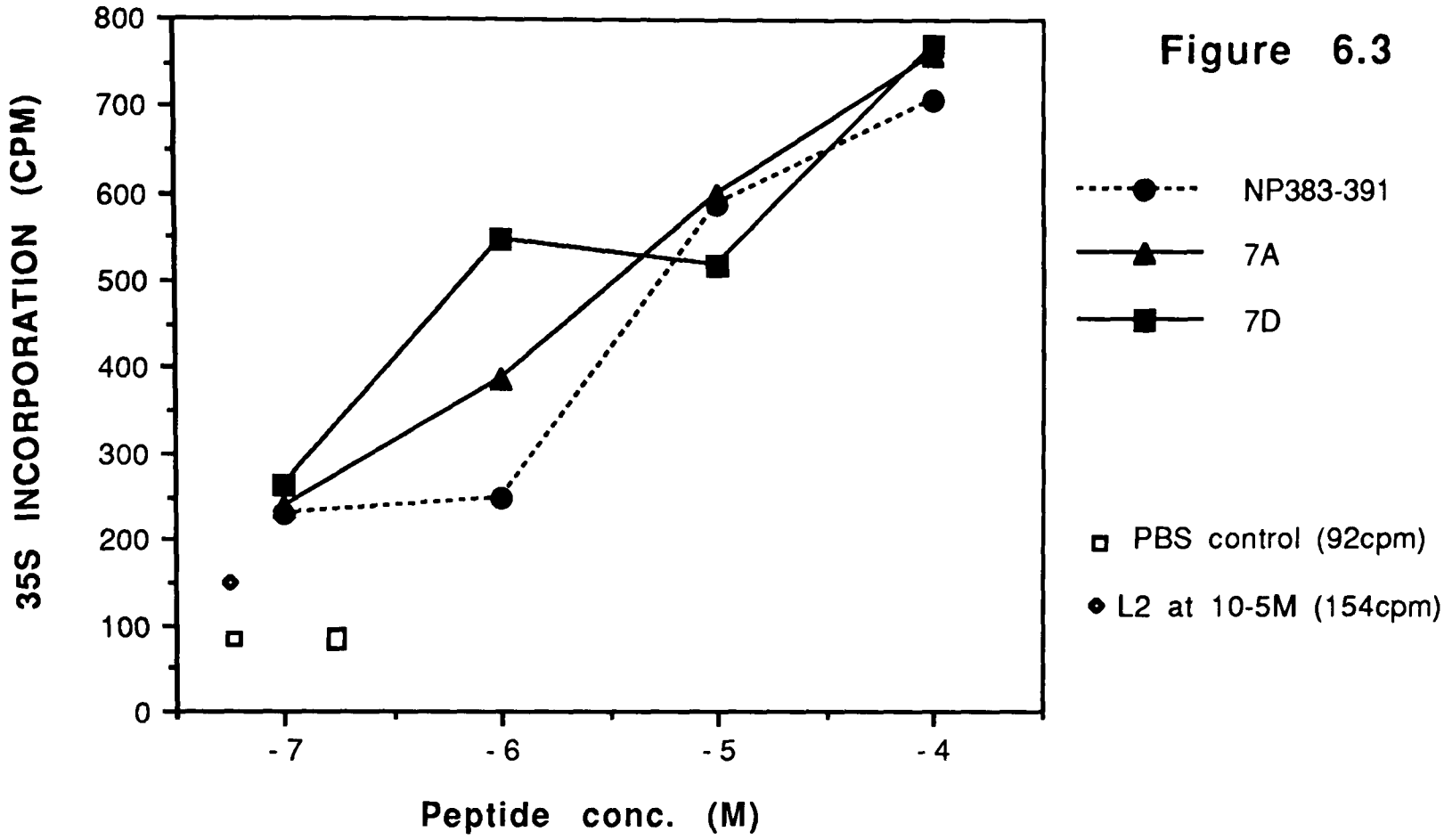


Figure 6.3

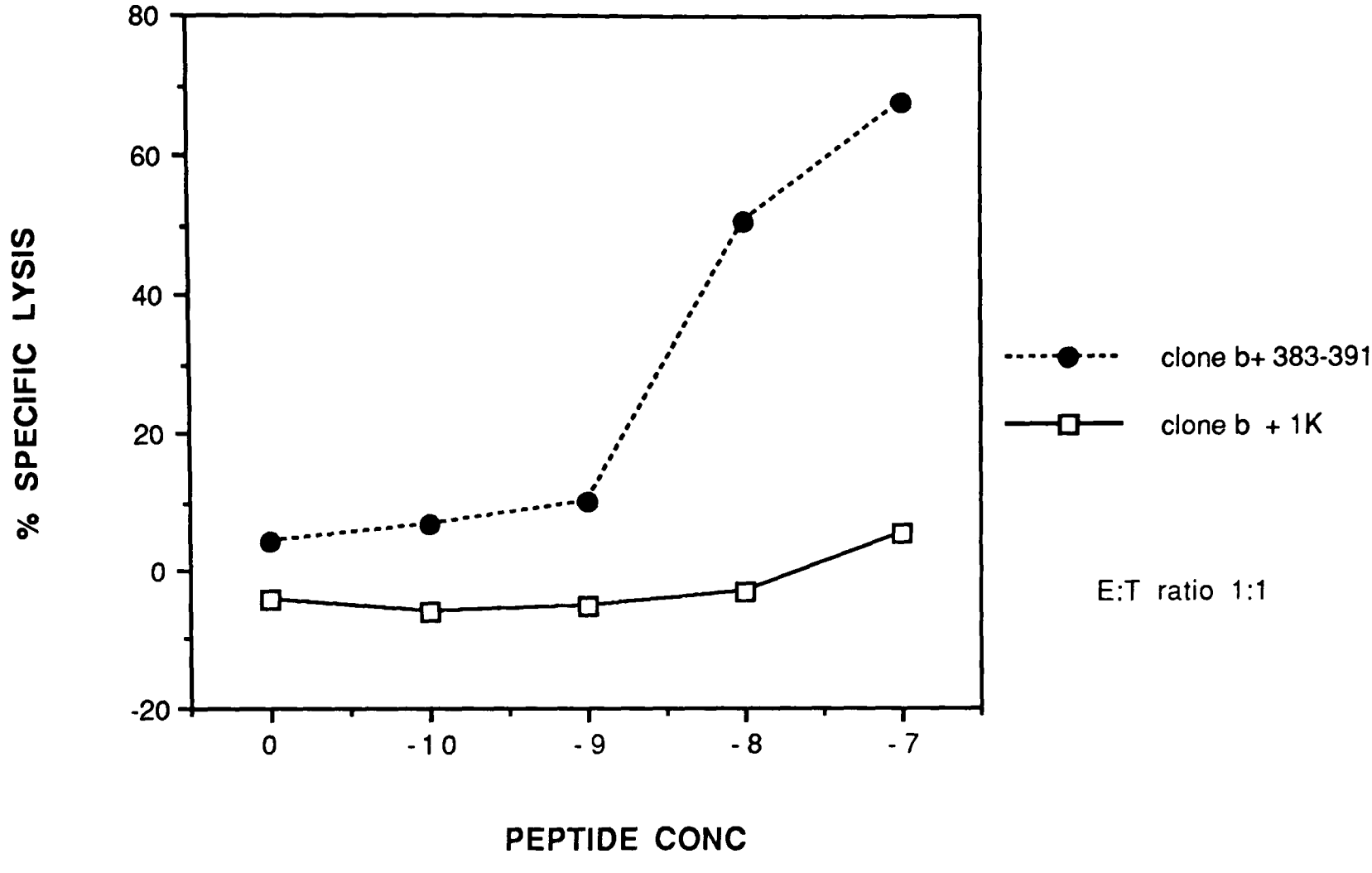
Figure 6.3. Quantitation of assembly of HLA B27 with NP383-391 and analogues7A and 7D. Gel bands, corresponding to HLA B27 on autoradiography of isoeletric focussing gels (fig. 6.2) were cut out and counted on a ß-plate counter.

6.4. Recognition of analogue NP383-391 peptides by CTL clones

Recognition of position 1 variants

Although the N terminus and main chain carbon atom of the first residue of peptides bound to HLA B27 are completely inaccessible to solvent (Madden *et al.* 1992), peptide modelling suggests that an arginine (Madden *et al.* 1991) or lysine (M. Smith and P. Driscoll, personal communication) at this position could reach solvent and hence interact with TCR. Whilst position 1 is serine (S) in the NP peptide, R and K were commonly found here in the eluted peptides shown in table 1.1 (Jardetzky *et al.* 1991), and indeed the HIV gag 265-276 epitope has a lysine at 265. For this reason the first substitution made was for a lysine at position 1, i.e. the peptide 1K. In initial experiments the 1K analogue sensitized HLA B27 positive targets for cytolysis by the CTL clones GRc, SD2, SD13, and SD16 but not GRb or SD17, as shown in figures 6.4 and 6.5.

CTL clone GRb fails to recognize peptide 1K **Figure 6.4a**



Clone GRc recognizes peptide substitution 1K **Figure 6.4b**

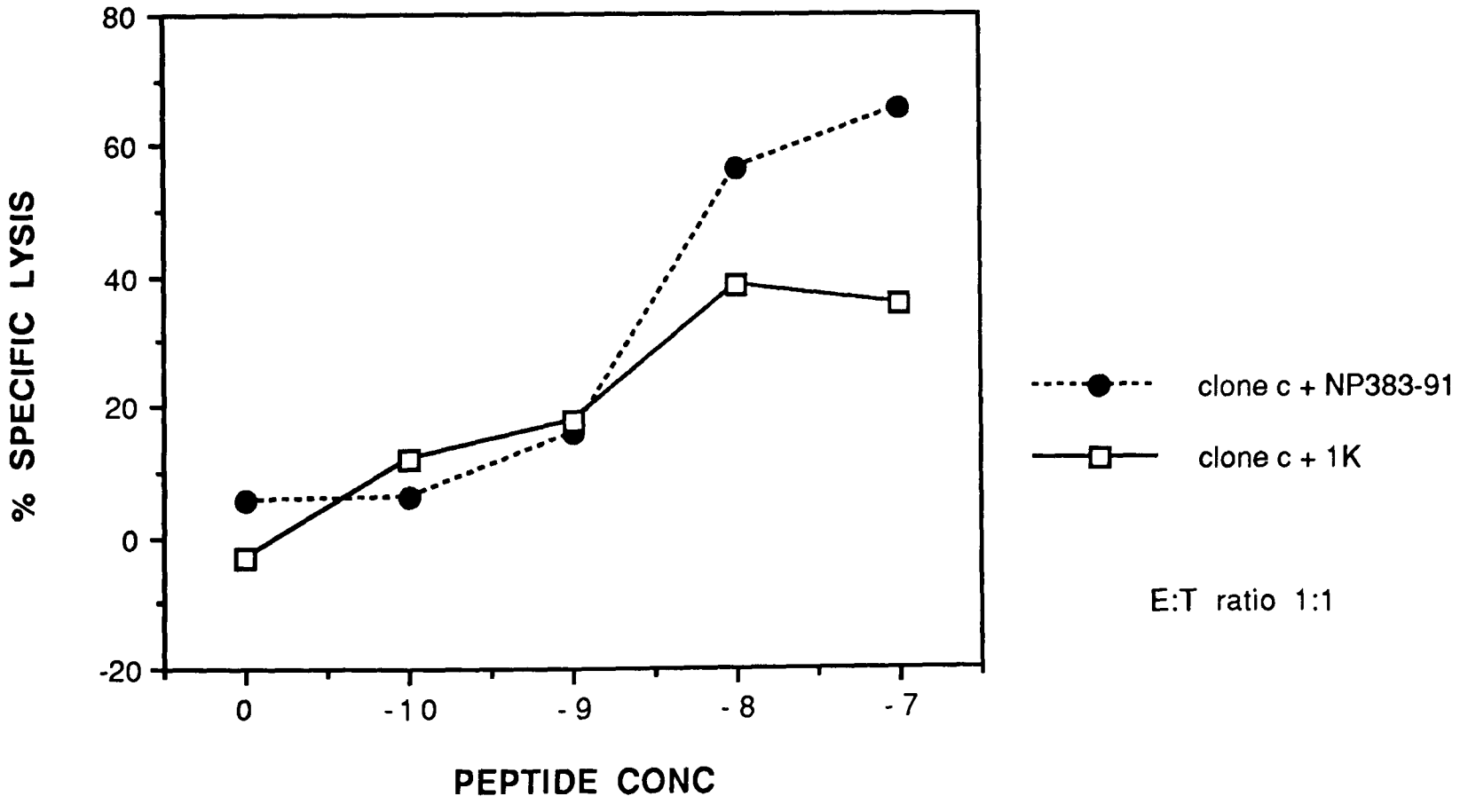


Figure 6.4. Recognition of NP 383-391 postition 1 substitution 1K by CTL clone GRc (6.4b) but not GRb (6.4a) Data from expt. "a". C1R B2705 targets

CTL clone SD2 sees peptides 1F and 1K poorly

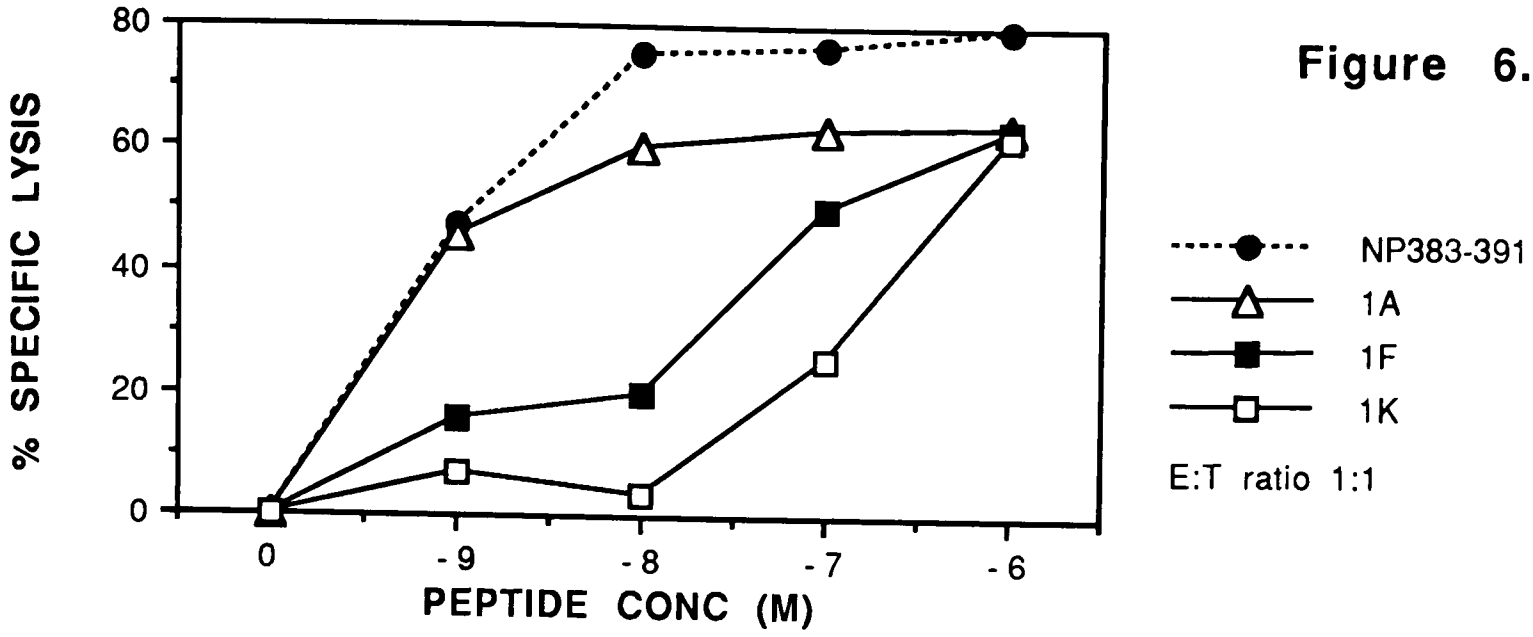
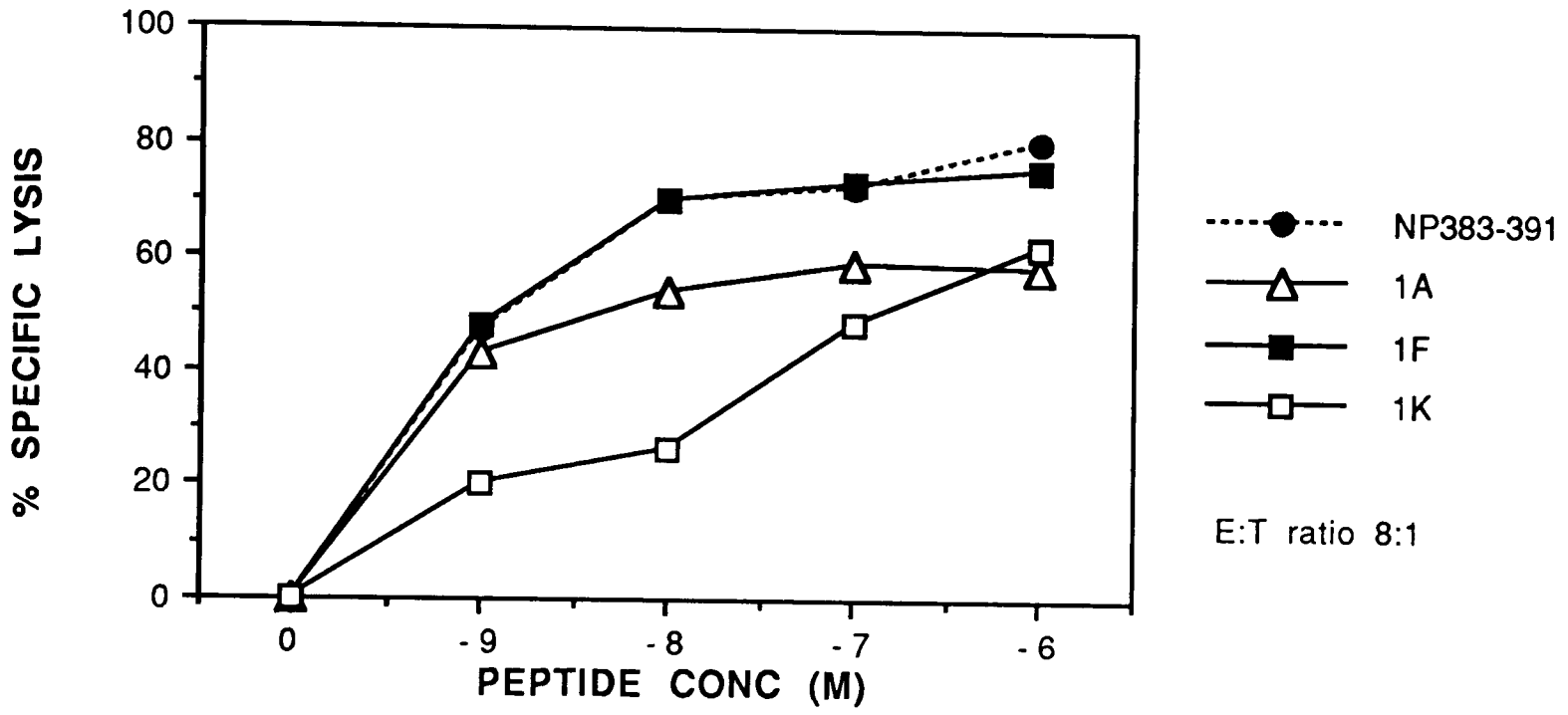


Figure 6.5a

CTL clone SD16 sees NP383-391 position 1 variants

Figure 6.5b



CTL clone SD17 does not recognize peptide 1K

Figure 6.5c

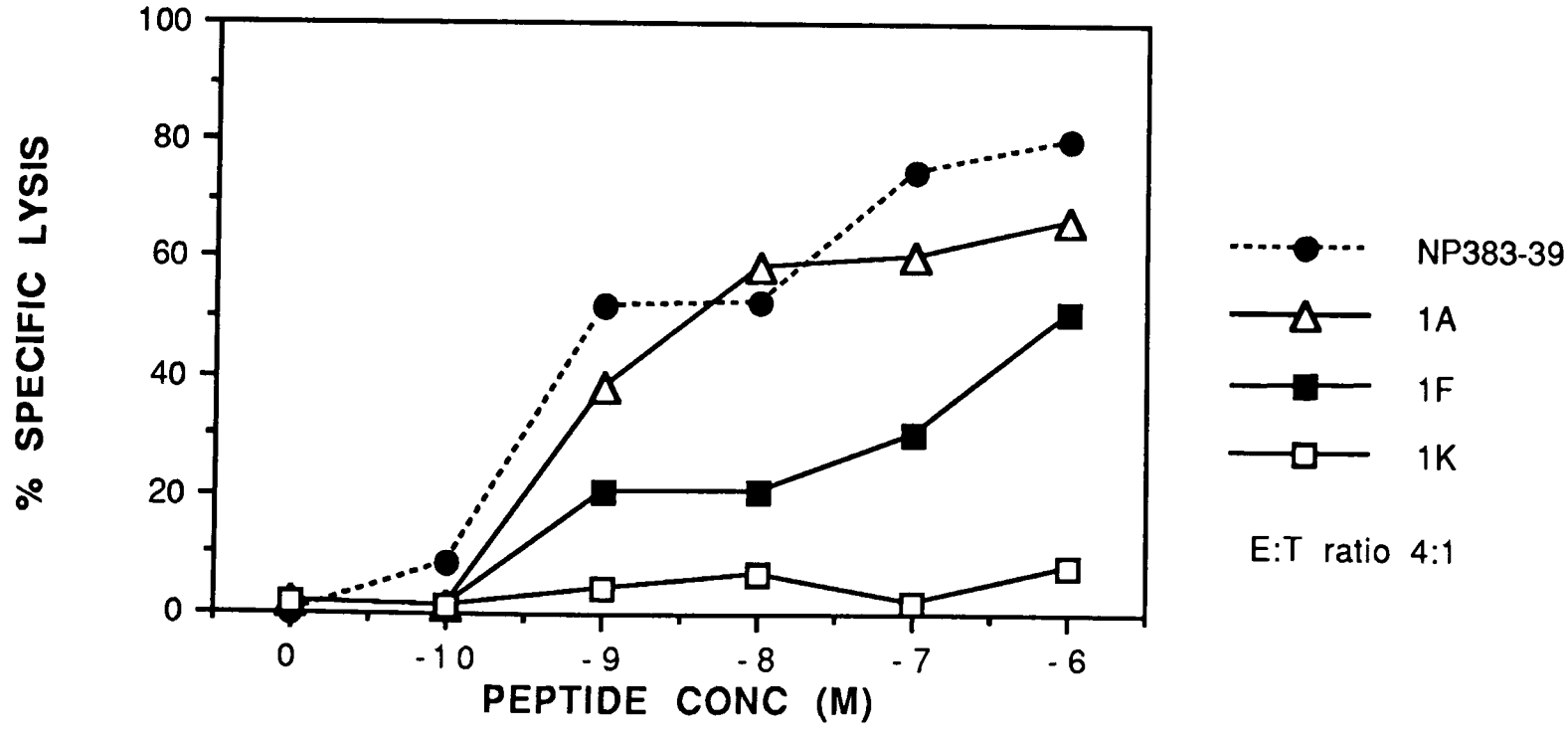


Figure 6.5. Recognition of postition 1 substitutions by CTL clones SD2 (fig 6.4a), SD16 (fig 6.4b), and SD17 (fig 6.4c). Data from expt "n".

These results show not only that 1K is able to bind to HLA B27, but also that a change at this position can dramatically reduce recognition by one CTL clone but not another. Three further substitutions, 1A, 1L, and 1F, were made and the results of a number of cytolysis assays titrating these peptides are shown in table 6.2 and summarized in table 6.3. Cytolysis was expressed as the concentration of peptide required to give half the maximal killing observed with native peptide (usually found at 10^{-6} M) in a given experiment, as detailed in the table legends. In most experiments the NP peptide gave half maximal lysis at 10^{-8} M, and so a score of +++ shows recognition equivalent to native peptide. As illustrated for the 1A substitution in figure 6.5, peptides 1A and 1L were seen by all CTL clones as well as native peptide. The results obtained with peptide 1F were less straightforward, but paralleled those obtained for 1K. Thus clones GRb and SD17 (fig. 6.5c) recognized 1F very poorly, whereas clone SD16 did not distinguish 1F from wild type peptide (fig. 6.5b). Clone SD2 exhibited an intermediate phenotype with definite but reduced responsiveness to both 1F and 1K (fig. 6.5a).

Recognition of position 4 variants

As shown in figure 6.6 and tables 6.2 and 3, most substitutions at this position completely abrogated recognition by all clones tested, even when peptides were used at 10^{-6}M in CTL assays. The only exceptions were peptide 4Y, seen by all clones tested although requiring approximately 100-fold greater concentrations than the NP peptide, and peptide 4F, which was seen only by clone SD2, which also recognized 4Y at a lower concentration than the other clones tested. Notably all the position 4 variants, except for 4H, induced HLA B27 assembly comparable to native peptide at 10^{-5}M, as shown in figure 6.2 and table 6.1.

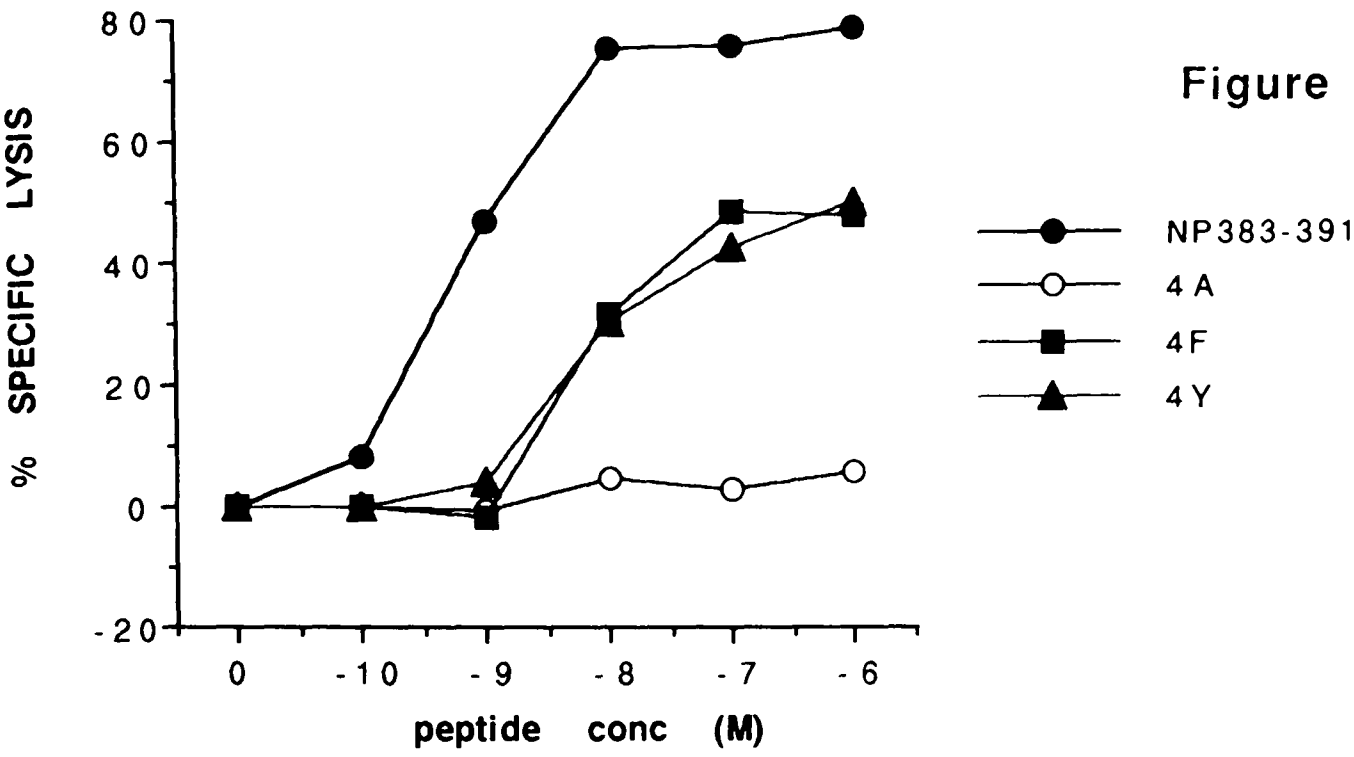


Figure 6.6a

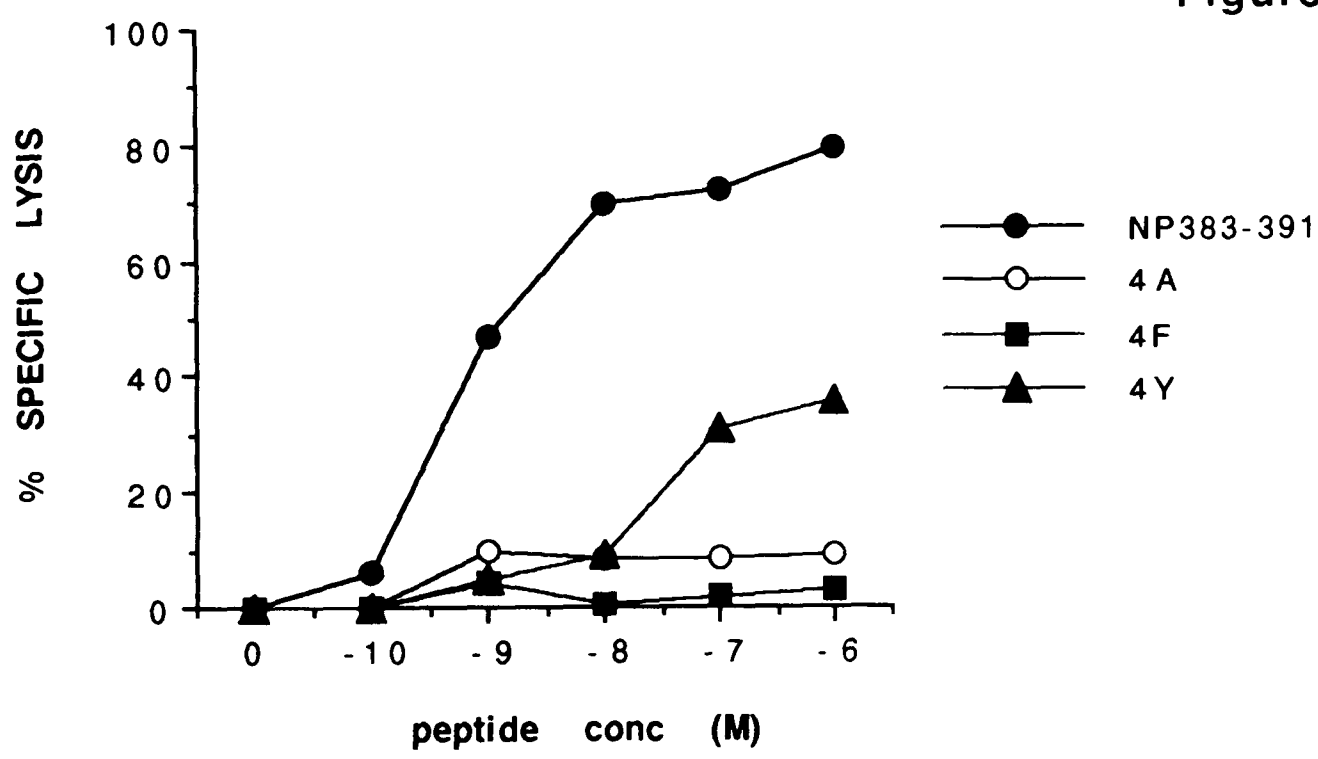


Figure 6.6b

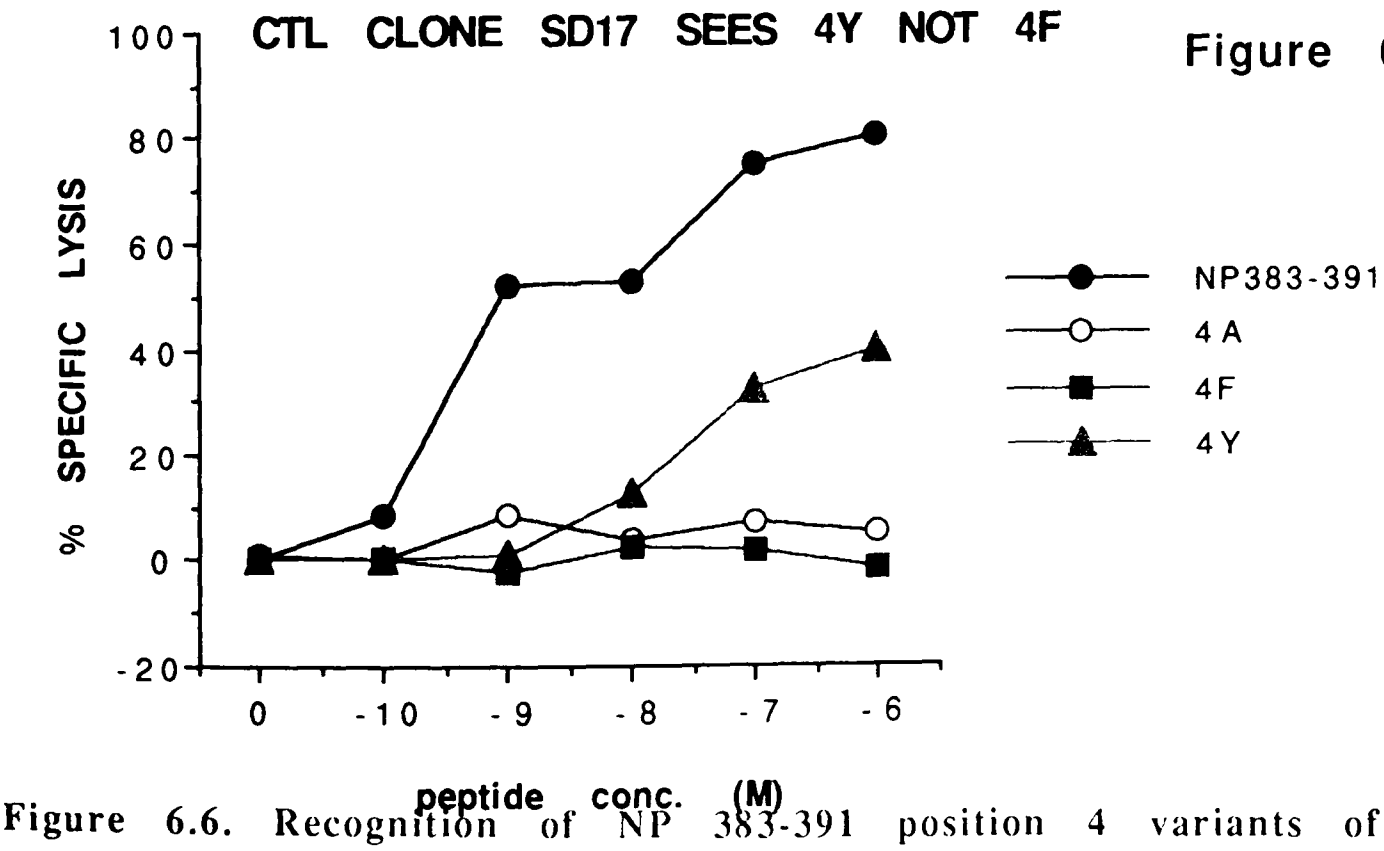


Figure 6.6c

Figure 6.6. Recognition of NP 383-391 position 4 variants of by SD CTL clones.

Recognition of position 7 variants

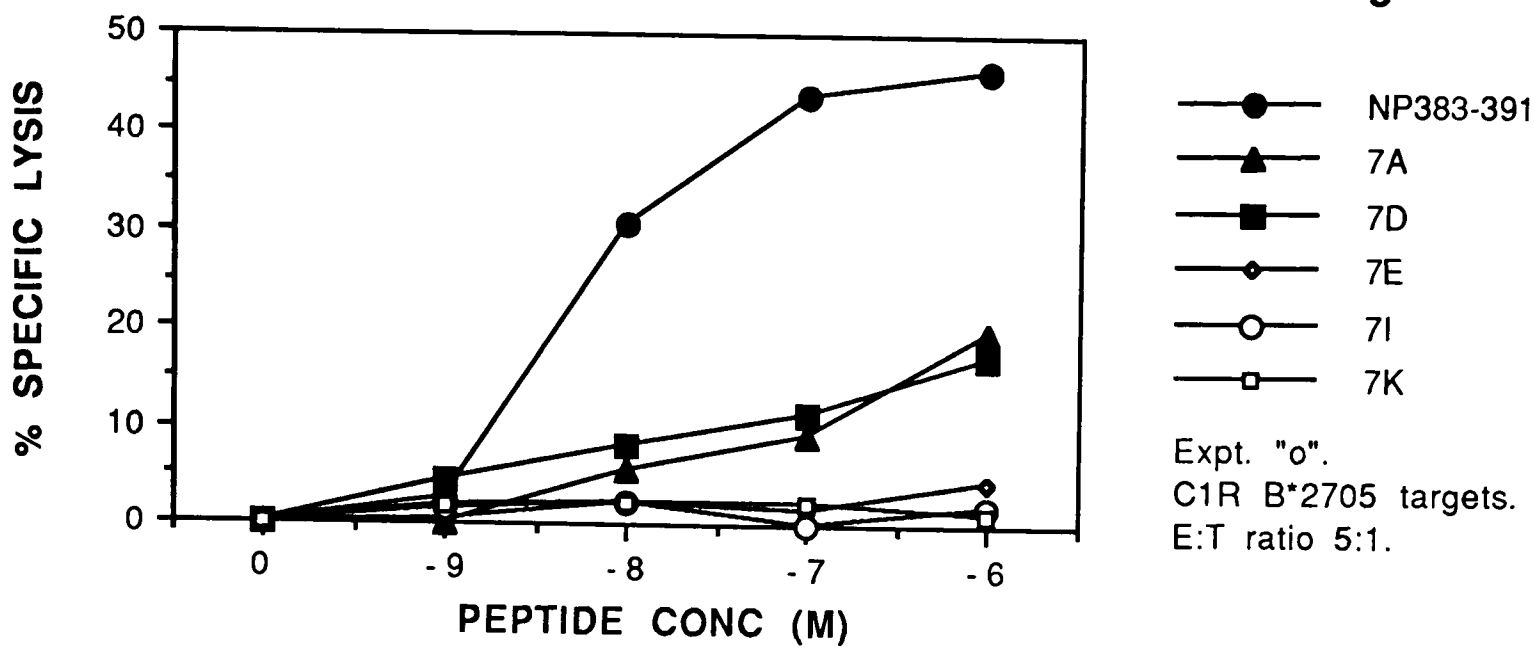
Recognition of position 7 variants is shown in tables 6.2/3 and examples of individual experiments in fig. 6.7a-c. All substitutions dramatically reduced recognition; 7E and 7K were not seen by any clones, although S. Rowland-Jones had previously demonstrated recognition of 7K at a higher concentration (10^{-5}M) by a bulk CTL line from donor HF (Colbert *et al.* 1993). Clones GRb, SD16 and SD17 did however see peptide 7D and, to a lesser extent, 7A at high concentrations (10^{-6}M or greater), and clone SD2 saw both 7A and 7D equally well. Peptides 7A and 7D were tested for their ability to assemble HLA B27 *in vitro*, and were virtually indistinguishable from the wild type peptide (figures 6.2 and 6.3).

Recognition of position 8 variants

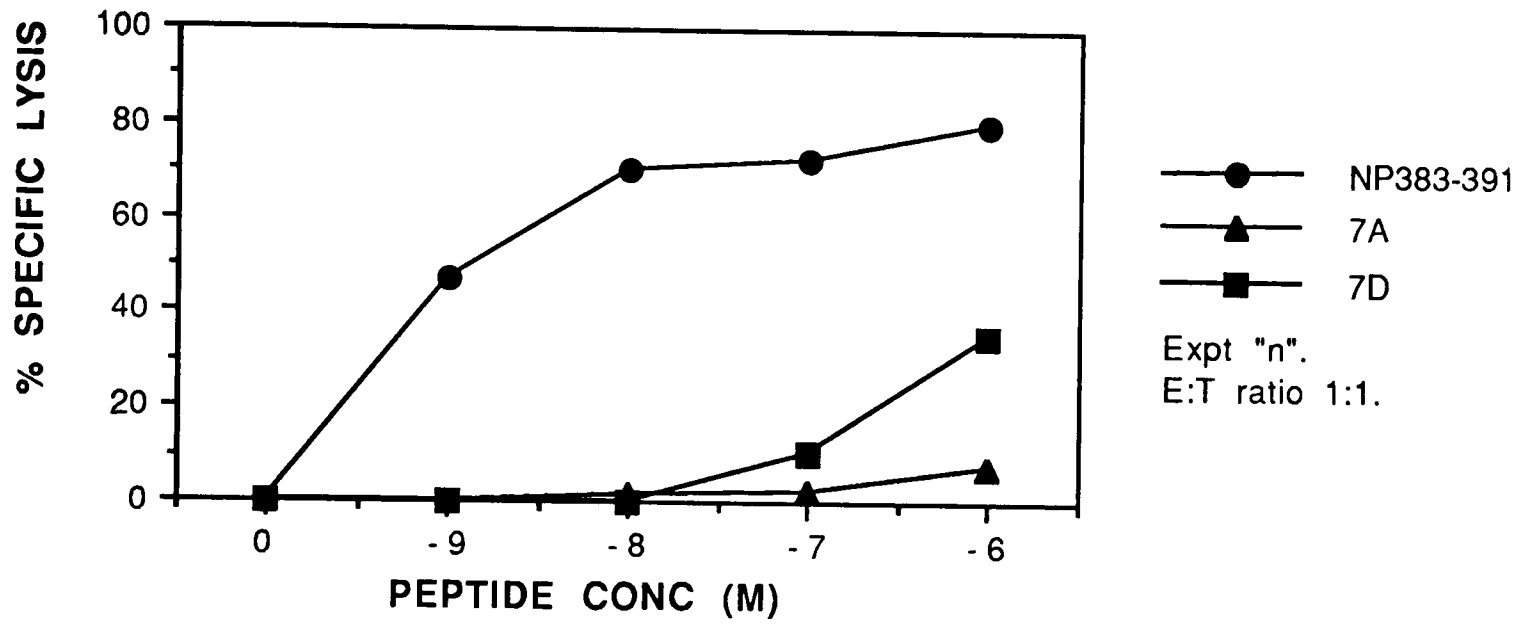
Substitution of threonine at position 8 for A, E, K or F produced profound effects on recognition by the CTL clones, as summarized in table 6.3. These substitutions had varying effects on the different clones, although cytolysis by every clone was adversely affected by at least one substitution. This is confirmed by the finding that 8F and 8K bind HLA B27 well (table 6.1). Substitution 8E reduced recognition by all clones tested, whereas 8K had less profound effects. Indeed recognition of 8K by clone SD17, was comparable to that for native peptide, as shown in fig.s 6.8a and b.

Unlike the other clones, recognition by clone SD2 was profoundly reduced by every position 8 substitution, as illustrated for peptides 8F and 8K in figure 6.8c.

Clone SD2 sees 7A and 7D but not 7E, 7I, or 7K. **Figure 6.7a**



Clone SD16 sees peptides 7D and 7A at high concentration. Figure 6.7b



Clone SD17 sees peptides 7A and 7D at high concentration. Figure 6.7c

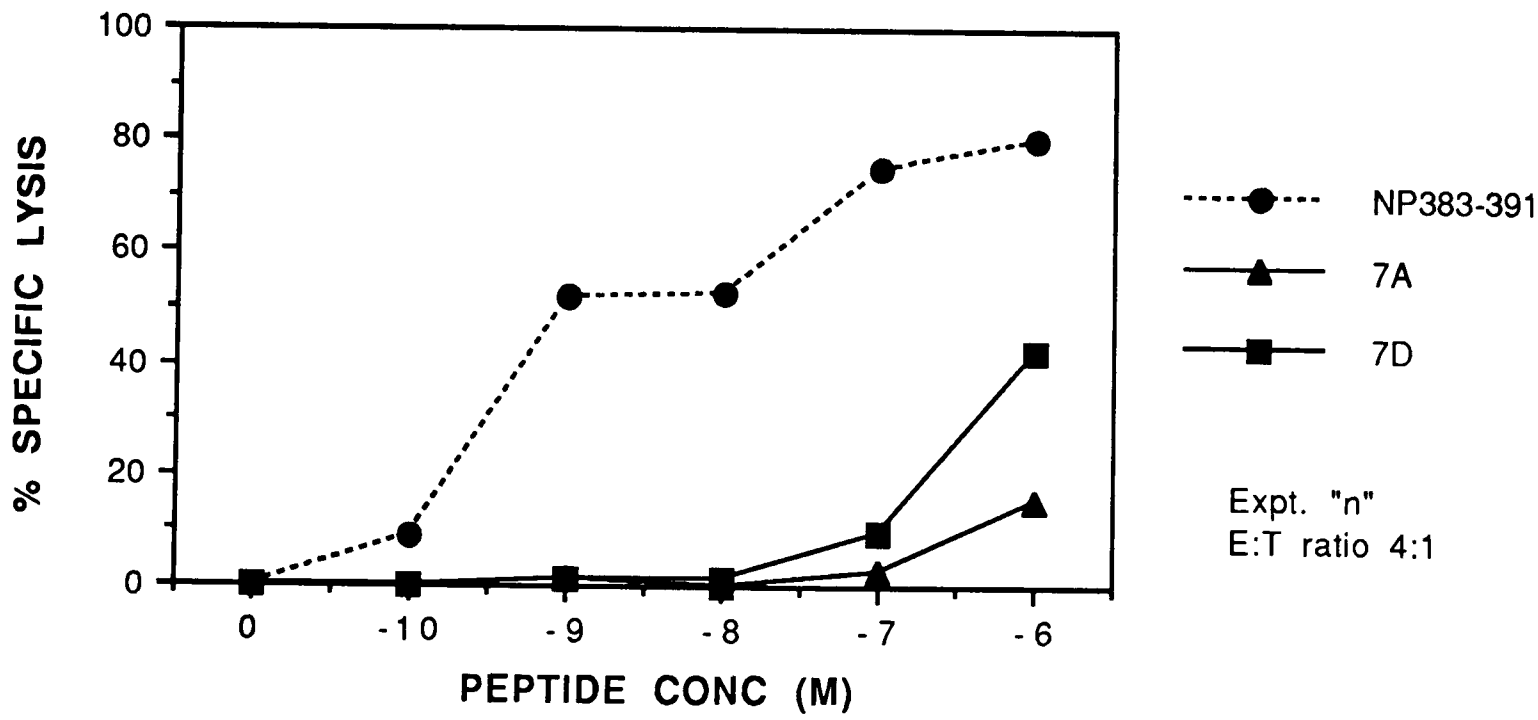
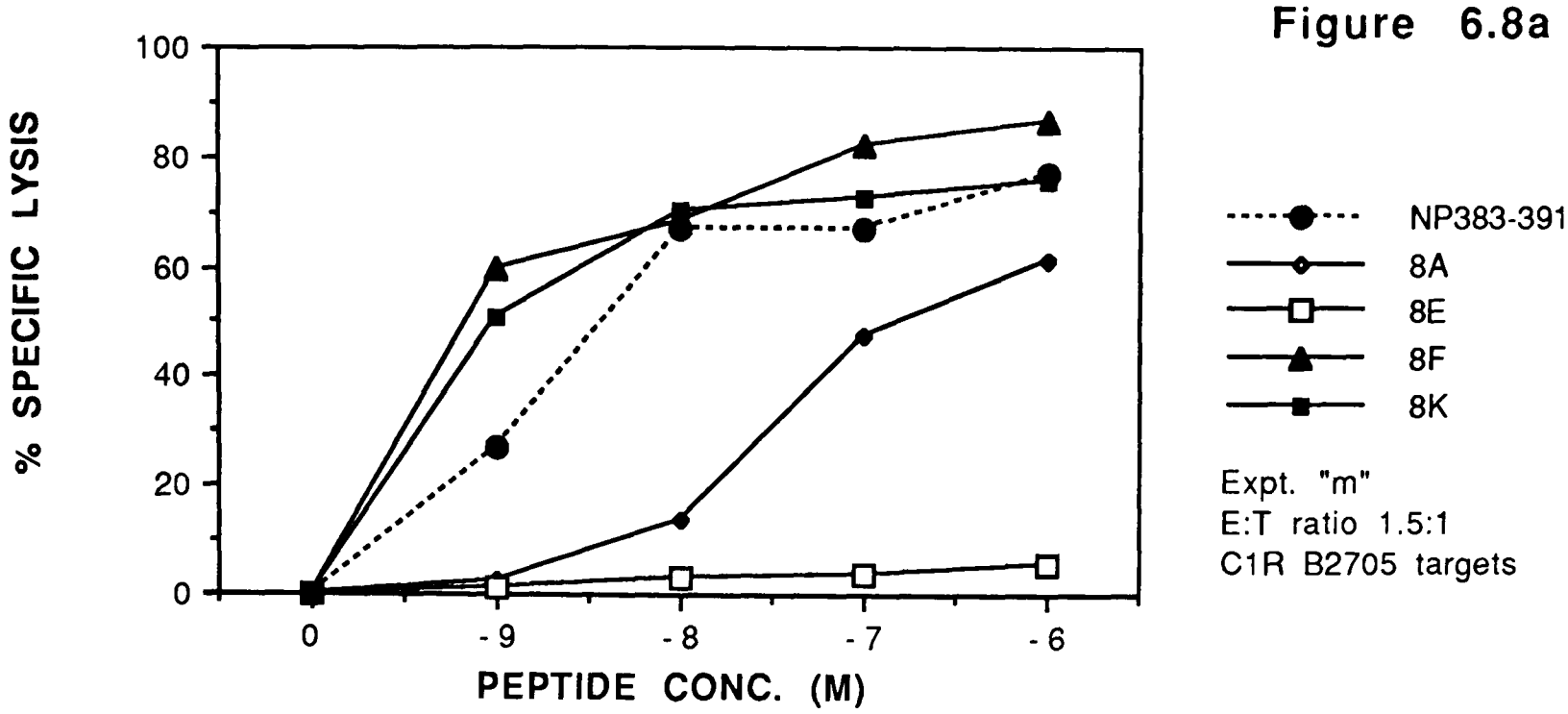


Figure 6.7a-c, Recognition of position 7 substitutions of NP383-391 by SD CTL clones.

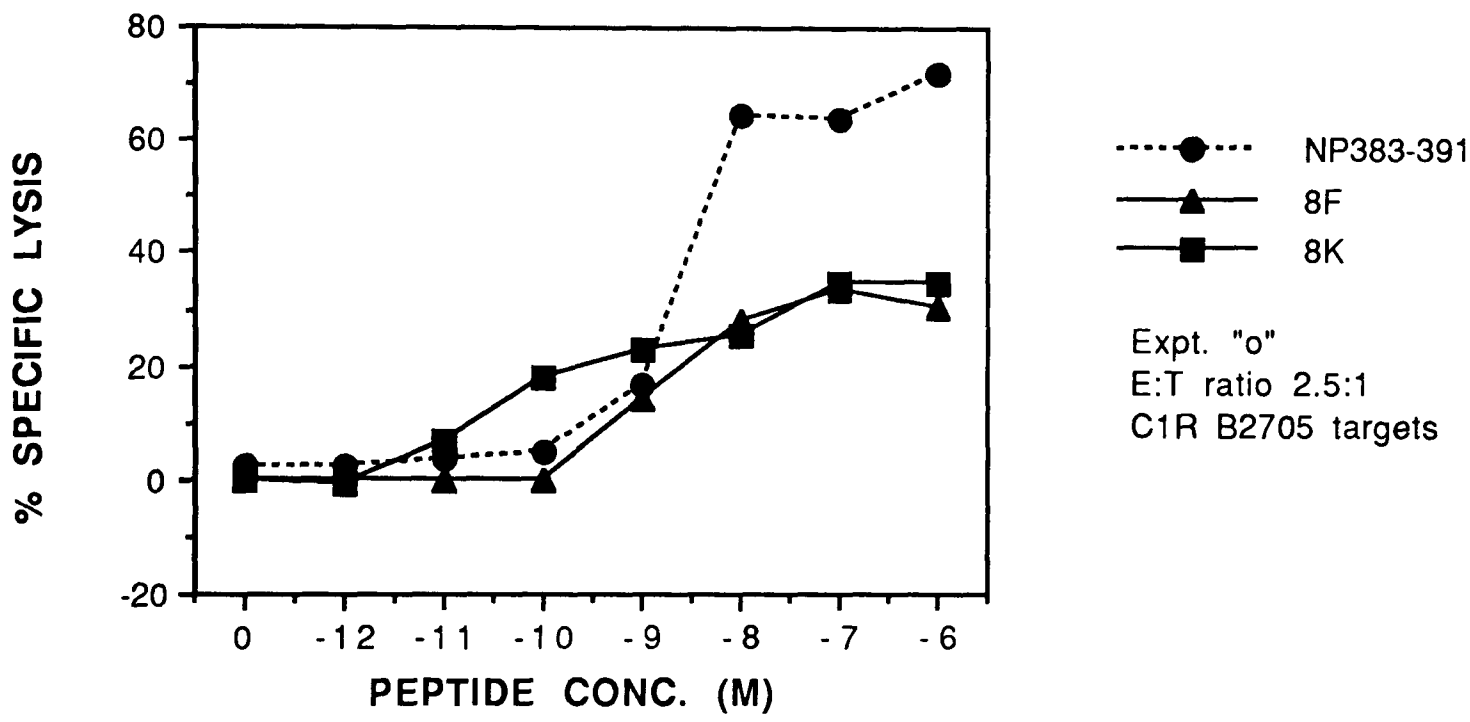
Clone SD17 sees peptides 8F and 8K

Figure 6.8a



Reduced recognition of 8F and 8K by clone SD17

Figure 6.8b



Poor recognition of 8K and 8F peptides by clone SD2

Figure 6.8c

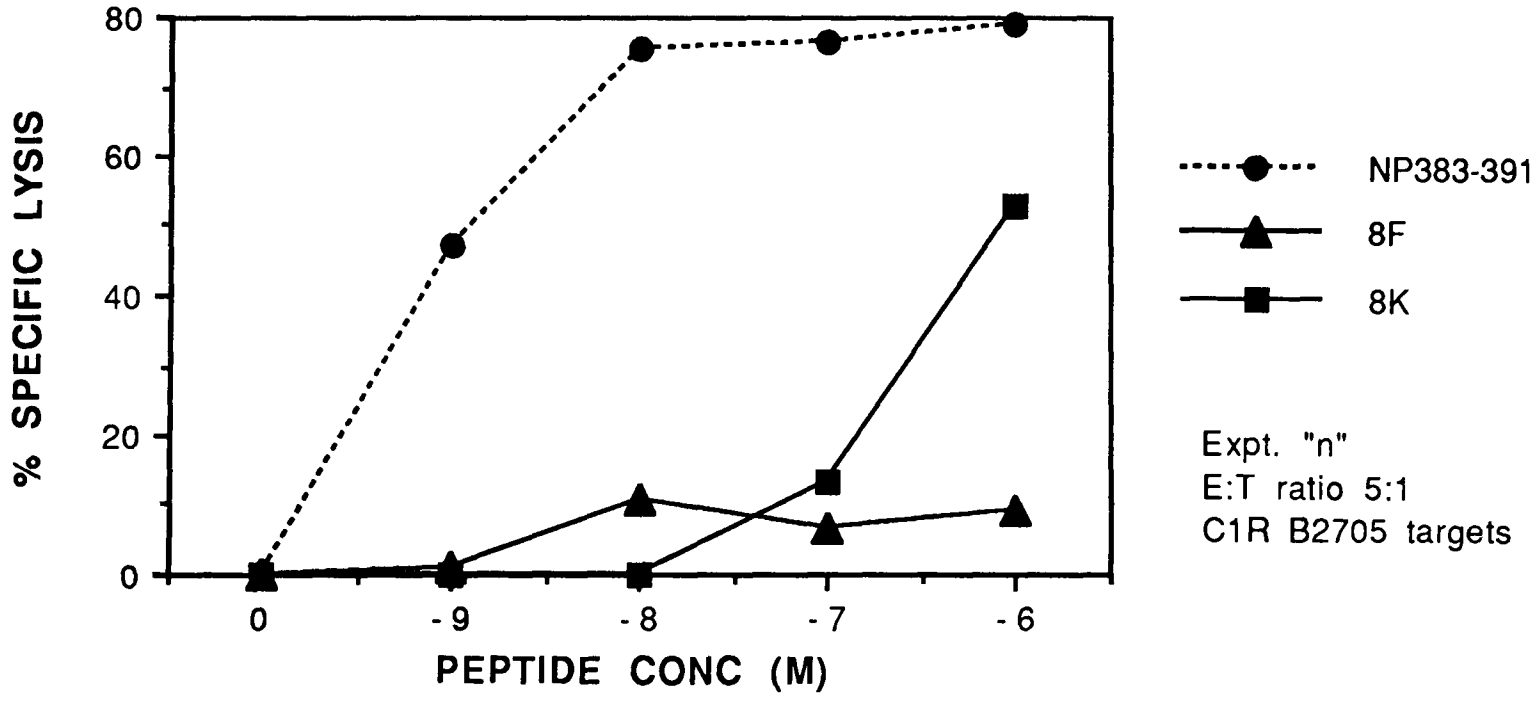


Figure 6.8a-c. Recognition of position 8 substitutions of NP383-391 by SD CTL clones SD17 and SD2.

Clone		GRb	SD17	SD2	GRc	SD16	SD13
PEPTIDE Position1	1 A	n t	++++o ++n	++++n,o	n t	++++n	n t
	1 L	++e	++i,j	++e ++i	++e	++e,j	++++j
	1 K	- a,e	-i,j,n	+b,e,i,n ++c	++a,b	++i,n +d,e	+++c,d
	1 F	trace o	+n,o ++m	++n +o	n t	++++n +++o	n t
Position4	4 A	- u	- n	- f,n	n.t.	- f, n	n.t.
	4 L	n.t.	- m,o	- o	n.t.	- o	n.t.
	4 E	n.t.	- m,n	- n	n.t.	- n	n.t.
	4 K	n.t.	- n	- n	n.t.	- n	n.t.
	4 F	- o	- m,n	++ n,o	n.t.	- n,o	n.t.
	4 Y	+ o	+ m,n	+++ o ++ n	n.t.	trace n + o	n.t.
	4 H	n.t.	- p,q	n.t.	n.t.	n.t.	n.t.
Position7	7 A	n.t.	- n,o	trace o - n	n.t.	- n,o	n.t.
	7 D	trace o	+ n trace o	trace n,o	n.t.	+ o trace n	n.t.
	7 E	- u	- o	- o	n.t.	- o	n.t.
	7 I *	- a	- o	- g,o trace c	trace a	- c,g,o	+ k trace d
	7K**	- o	- o	- o	n.t.	- o	n.t.
Position	8 A	++ s,t +u	- j,r(h) + m	- g(h,j)	++j	trace g ++ (h)	n.t.
	8 E	- t,u trace s	- j,m,r(h)	- g(h,j)	trace j	++g +(h)	n.t.
	8 K	++o	++++m,n ++ o,r	+ n,o	n.t.	++ n + 0	n.t.
	8 F	trace o	trace n,o,r +++ m	- n,o	n.t.	trace n,o	trace d,k

Table 6.2. RECOGNITION OF VARIANTS OF NP 383-391 BY CTL CLONES. Lower case letters a-u after killing indicate the experiment. Recognition scored as concentration required for half of maximal killing with native peptide: ++++ 10^{-9}M, +++ 10^{-8}M, ++ 10^{-7}M, + 10^{-6}M, trace 10^{-5}M (or 25% maximal lysis at 10^{-6}M), n.t. not tested * seen at "trace" by bulk CTL lines GR and HF in expt. g. ** seen weakly by bulk HF CTL, Colbert *et al* (1993).

Clone		GRb	SD17	SD2	GRc	SD16	SD13
TCRα	Vα	1 4	1 4	2 2 / 7	1 2	1 2	1 2
	Jα	9.3	9.3	6.1 / 9.1	9.3	16.5	16.5
	CDR3 /length	FSGGSNYKL/9	ISGGSNYKL/9	SASKI/5 RRNQGNL	GGSNYKL / 7	VDSSYKL / 7	QDSSYKL / 7
TCRß	Vß	7	6	8	7	7	2 1
	Jß	2.3	2.5	2.5	2.5	1.3	1.3
	CDR3 /length	SQEVLDTQ/8	SPDIVETQ/8	SSNPAHTQ/9	SQERVGETQ/9	SQELTNTI/9	SQVSAGNTI/9
PEPTIDE	1 A	N.T.	■	■	N.T.	■	N.T.
	1 L	▒	■	■	▒	■	■
	1 K	□	□	▒	▒	▒	■
	1 F	▒	▒	▒	N.T.	■	N.T.
	4 A	□	□	□	N.T.	□	N.T.
	4 L	N.T.	□	□	N.T.	□	N.T.
	4 E	N.T.	□	□	N.T.	□	N.T.
	4 K	N.T.	□	□	N.T.	□	N.T.
	4 F	□	□	▒	N.T.	□	N.T.
	4 Y	▒	▒	■	N.T.	▒	N.T.
	4 H	N.T.	N.T.	□	N.T.	N.T.	N.T.
	7 A	N.T.	□	▒	N.T.	□	N.T.
	7 D	▒	▒	▒	N.T.	▒	N.T.
	7 E	□	□	□	N.T.	□	N.T.
	7 I	□	□	□	▒	N.T.	▒
	7 K	□	□	□	N.T.	□	N.T.
	8 A	▒	□	□	▒	▒	N.T.
	8 E	▒	□	□	▒	■	N.T.
	8 K	▒	■	▒	N.T.	■	N.T.
	8 F	▒	▒	□	N.T.	▒	▒

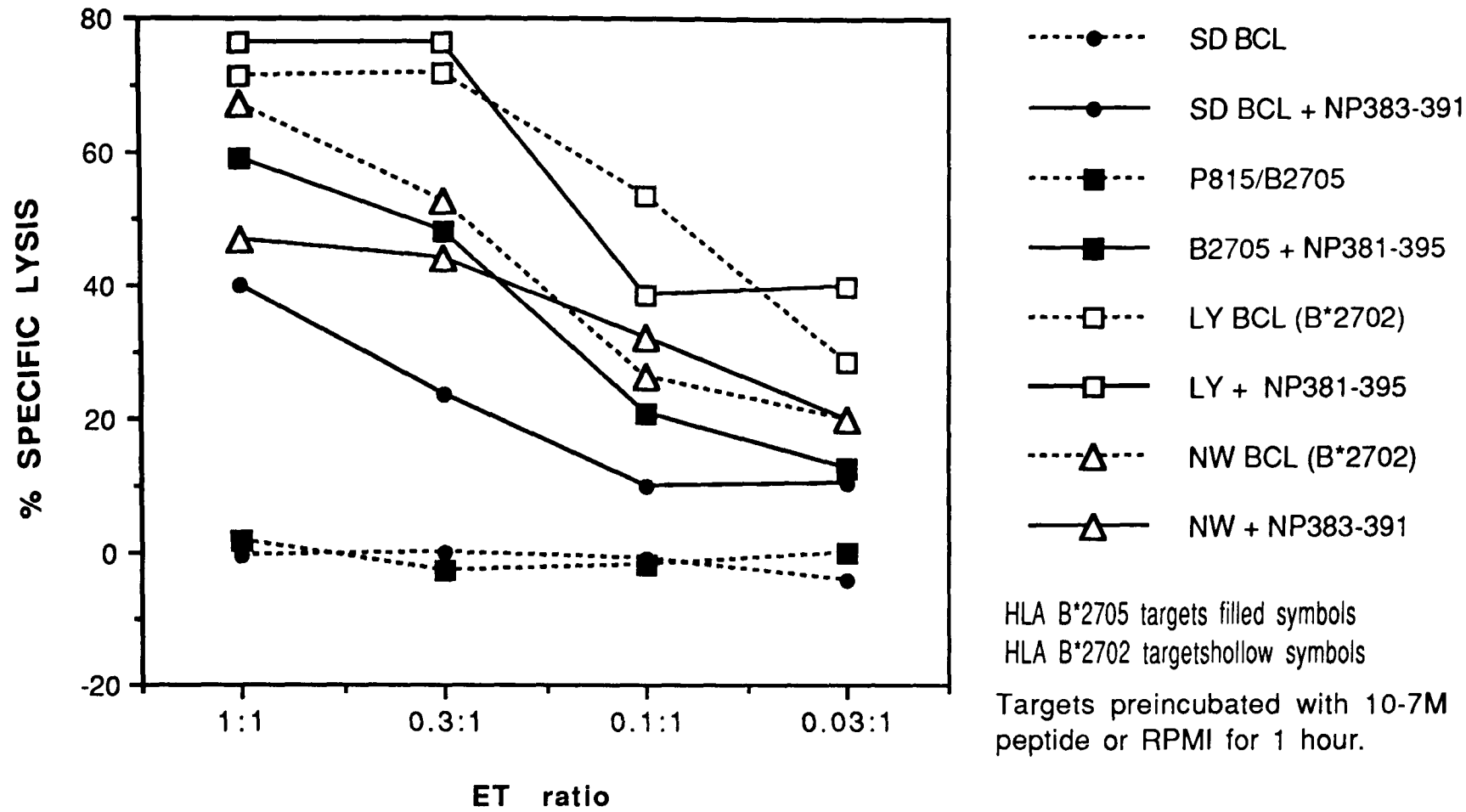
Table 6.3. SUMMARY OF RECOGNITION OF NP VARIANTS OF NP 383-391 BY CTL CLONES. Recognition scored as most frequent concentration (or lower of two equally frequent results) giving half of maximal killing found with NP383-391 ■ 10^{-8}M or 10^{-9}M, ▒ 10^{-7}M, ▒ 10^{-6}M, ▒ 10^{-5}M (or 25% maximal lysis at 10^{-6} M), □ no cytolysis at 10^{-6} M.

6.5. Recognition of HLA B27 subtypes by CTL clones

All CTL clones were tested at least once for cytolysis of HLA B*2702-bearing targets, with and without NP383-391. HLA B*2702 is the second most frequent Caucasian B27 subtype and is associated with autoimmune disease. No cytolysis was observed with or without peptide for clones GRc, SD2, SD10, SD13 or SD16 (data not shown), but clones GRb and SD17 killed HLA B*2702-positive cells, with or without peptide, in 5 out of 5 and 2 of 3 experiments respectively (e.g. fig. 6.9a). Clone GRb killed without distinction the human HLA B*2702 positive BCLs LY, NW, R34, and R39, as well as two cell lines transfected with HLA B*2702: HMy2.C1R cells (two experiments) and the murine mastocytoma line P815 (one experiment, fig. 6.9b). In some but not all experiments (e.g. that shown in fig. 6.9b), alloreactive killing of HLA B*2702 targets was comparable to that observed for HLA B*2705 targets incubated with NP383-391. Notably no other alloreactive specificities were observed for any clones, in experiments using BCLs from at least four unrelated donors (data not shown).

Clones GRb (fig. 6.9c), SD16 and SD17 were also tested and found to recognize NP383-391 presented by the Gambian HLA B*2703 B cell line UYTIU.

Alloreactive cytolysis of HLA B*2702 targets by clone GRb **Figure 6.9a**



The HLA B*2703 positive BCL B809 presents NP383-391 to clone GRb **Figure 6.9b**

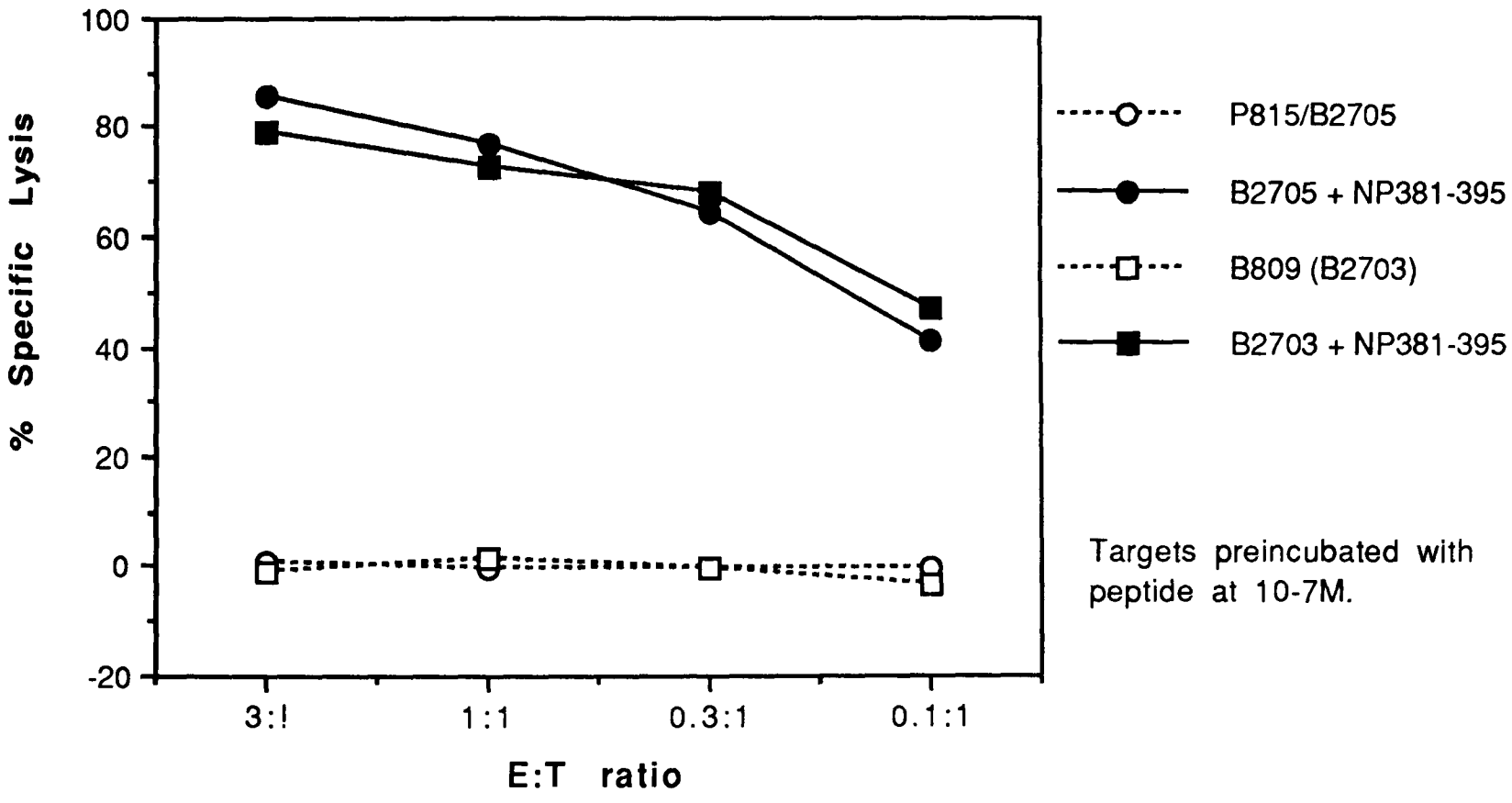


Figure 6.9. Allo killing of HLA B*2702, and peptide presentation by HLA B*2703, for CTL clone GRb.

6.6. Discussion

Peptide binding to HLA B27

Peptides that did not induce target cytolysis by any CTL lines or clones, or were seen poorly by all clones, were tested and shown to promote *in vitro* assembly of HLA B27 at dilutions comparable to the native peptide NP383-391 (fig.s 6.2 and 6.3, table 6.1). This result was expected as substitutions of positions thought to affect binding (such as L2, fig. 6.2) were not studied. The assembly assay employed was felt to be more physiological than other methods used to measure peptide binding to HLA class 1 molecules. Alternative methods include indirectly demonstrating binding by inhibition of cognate peptide-induced cytolysis with excess competitor peptide e.g.(Huet *et al.* 1990), however vast molar excesses of competitor were required. Direct binding of radiolabelled peptides to HLA B27 molecules can be measured e.g. Benjamin and Parham (1991), Buxton *et al.* (1992), however it was felt that radio-iodination of Y391 could in itself significantly alter binding. Consequently it was decided to measure binding by quantifying the ability of peptides to assemble and stabilize correctly folded HLA B27 molecules in solution. This method was developed using the RMAS and T2 cell lines in which almost all HLA molecules are thought to be in an unstable conformation (Townsend *et al.* 1989). Surprisingly, when this protocol was followed for T2 cells transfected with HLA B27, most HLA B27 molecules appeared to be in a W6/32 precipitable form even in the absence of peptide (data not shown), although they could be dissociated by heating to 60°C for 15 minutes (see chapter 2.3). Recently alternative methods of obtaining unfolded HLA B27 heavy chains have been described using either CHO transfectants, in which poor association of hamster ß2 microglobulin with human HLA is accentuated by intracellular excess of the transfected molecule (Gao *et al.* 1993), or alkali denaturation of HLA B27 from BCL (Fruci *et al.* 1993, Tanigaki *et al.* 1993).

Although the behaviour of HLA B27 in assembly assays may not reflect that found *in vivo*, some features do appear different to those of other HLA molecules studied previously. Firstly in the assembly mutant cell line T2, a relatively high proportion of the molecules appear to be in a W6/32 precipitable form (data not

shown). One possible explanation for this is that HLA B27 might be stable in a folded but "empty" configuration, as suggested by the report of peptide binding to "empty" cell surface molecules (Benjamin and Parham 1991). It is not clear how widespread this phenomenon might be. Secondly the concentrations of NP and analogue peptides required to assemble HLA B27, approximately 10^{-6} to 10^{-5} M for half maximal assembly, are higher than those used for HLA A2, B8, B35 or B53 ((Elvin *et al.* 1991) A. Hill, S. MacAdam, unpublished results). Similar results have recently been observed with a variety of peptides using both the assembly deficient cell line LCL 5.2.4 (A. Daser, personal communication), and CHO cells transfected with HLA B27 (X-M Gao, pers. comm.). It is notable that the concentration of exogenously added NP peptide required for half maximal lysis in CTL assays with both clones and bulk lines, usually about 10^{-8}M, was similarly one to two logs higher than that required for HLA A2-restricted CTL with the Influenza matrix peptide M58-66 (Morrison *et al.* 1992). There is as yet insufficient data available to determine if this property is unique to HLA B27, or of any significance *in vivo*, where peptide binding to transporters or chaparones may be rate-limiting.

Recognition of position 1 variants

Substitution of the first residue of the NP383-391 peptide to alanine (or leucine) had no effect on CTL recognition, suggesting that that the hydroxyl group of 1S is probably not important for T cell recognition. The introduction of lysine at position 1 into the NP383-391 peptide abrogated recognition by two clones, GRb and SD17 (fig.s 6.4a and 6.5c). Lysine has a long positively charged side chain that is predicted to reach solvent (Madden *et al.* 1991)(M. Smith and P. Driscoll, personal communication), and so might directly interfere with the interaction of TCR with MHC in this region, through either steric or charge effects. In contrast this substitution had no effect on recognition by clone SD13, and resulted in only mild to moderate reduction by clones GRc and SD16, as shown in fig.s 6.4b and 6.5b respectively. Since there is no naturally occuring analogue of lysine lacking an NH3+ group, the substitutions 1L and 1F were made. Recognition of 1L equivalent to that of wild type NP peptide was seen for all clones, and of 1F for

clone SD16 (fig. 6.5b). In contrast the 1F substitution dramatically reduced, although did not abolish, recognition by clones GRb and SD17 (fig. 6.5c). The possibility that peptide 1F binds poorly to HLA B27 but leads to improved recognition by some clones is ruled out by demonstration of an ability to assemble HLA B27 comparable to that of the native peptide (data not shown). It is however possible that these substitutions have effects on the local conformation of the HLA B27 molecule or indirectly on the other main chain atoms — with large side chains reducing the freedom of rotation about the psi angle (Madden *et al.* 1991).

The CTL clones thus fall into two distinct groups according to their recognition of position 1 peptide variants 1K and 1F. Clones GRb and SD17 cannot tolerate these substitutions, whereas clones GRc, SD13, and SD16 are little affected. Clone SD2 has a distinct phenotype at positions 1, 4 and 8 and will be considered separately. The fine specificity of the clones for position 1 variants does not correlate with TCR Vß, NDNß or Jß usage, but does correlate with Vα usage and α chain CDR3 length (and inversely with ßCDR3 loop length). Thus the two clones sensitive to substitution with a bulky side chain at position 1, GRb and SD17, use Vα14 and have a long CDR3 loop of nine residues (see figure 5.1). In addition, and unlike the other clones tested, they both have bulky aromatic residues at position α92, and, for GRb, at 93 as well. In contrast clones GRc, SD13 and SD16 all use Vα12 and have a shorter CDR3 region of 7 amino acids lacking aromatic residues. It seems reasonable to propose that the TCR alpha chain contacts the HLA B27/NP383-391 in this region, with clones using Vα12 and a short CDR3 interacting less closely or more flexibly in this region. Indeed the TCR of clone SD13, unaffected by the position 1 mutations tested, might simply not contact the peptide/HLA B27 complex in this region. The finding that cytolysis by clones GRb, SD17, and particularly SD16 was reduced more by the substitution 1K than 1F suggests that for all these clones interaction with MHC in this region may be of a hydrophobic nature, with, as already suggested, the polar side chain of residue 1, serine in NP, solvent inaccessible.

The fact that clone GRc shares an identical Jα with clones GRb and SD17, yet behaves like the the other Vα12 clones, suggests that it is the Vα or the first two residues of the CDR3 that determine this phenotype, and may overlie this region (see section 6.7). If this model is correct, the fact that the side chain of the first residue of the NP peptide is almost certainly buried might thus explain the lack of amino acid conservation observed at the start of the TCRα CDR3 in the HLA B27-restricted NP383-391-specific clones. In particular this might explain the failure of conservation at residue 93, which has been identified as conserved peptide contact residue in the $IE^{k/s}$-restricted response to moth cytochrome c (Jorgensen *et al.* 1992).

Notably clone SD2, which had an intermediate reactivity for position 1 substitutions, used a very different ß chain (Vß8) and two distinct alpha chains (Vα 22 and Vα7), compared with the other clones (see table 5.3).

Recognition of position 4 variants.

The crystal structure of pooled peptides bound to HLA B27 shows the side chain of the amino acid at position four to be orientated directly towards solvent, indeed, because of the "kinked" nature of bound peptide, this side chain appears the most TCR accessible (Madden *et al.* 1991). The results summarized in table 6.3, together with the binding data of figure 6.2 (table 6.1), clearly show that the tryptophan at position 4 is critical for recognition of the NP peptide by all CTL clones studied. With the exception of SD2, substitution for tyrosine resulted in a >100 fold reduction in recognition, and no other amino acid tested was seen at all. This suggests a specific requirement for T cell recognition of a benzene ring containing a hydrogen bond donor. Furthermore their exact conformation is probably also important, because the substitution 4H was not seen by the one clone tested, SD17. However the possibility cannot be excluded that the loss of the polar OH group between Y and F abrogates recognition indirectly by forcing a conformational change in the HLA B27 molecule.

One possible candidate residue of the TCR that might interact with 4W is Y102 of the TCR alpha chain, conserved in GRb (and GRc), SD 16, and SD17. The equivalent residue has been shown to contact MHC-bound peptide in the H-2K^d-restricted response to a photoreactive variant of the *Plasmodium berghei* circumsporoite peptide 253-260 (Romero *et al.* 1993, Romero *et al.* 1993). It is quite possible that the TCR beta chain CDR3 loop simultaneously contacts 4W, and in this case the protruding tryptophan might be seen in a "lock and key" configuration as with certain antibody/hapten interactions (Amit *et al.* 1986). Indeed such a phenomenon might explain the findings of (Casanova *et al.* 1993), who observed differences in both α and ß chains between H-2K^d-restricted CTL clones raised against HLA-derived peptides differing only at this position.

Recognition of position 7 variants.

All position 7 substitutions dramatically reduced CTL killing (table 6.3), and peptides 7E and 7K were not seen by any clone tested at 10^{-6}M, although the latter has been shown to induce cytolysis by an HF CTL line at 10^{-5}M by S. Rowland-Jones (Colbert *et al.* 1993). Peptides 7A and 7I induced low levels of killing by some but not all CTL at 10^{-6}M, and peptide 7D was seen weakly by all clones tested, although at least 100 fold less well than native peptide. The consistent reduction of killing with a variety of different amino acid substitutions implies that R7 is critical for either binding or CTL recognition. The former proposition seems unlikely for a number of reasons. Firstly the crystal structure of HLA B27 shows weak electron density for the side chain(s) of the P7 residues, accommodated in a relatively large E pocket, suggesting a lack of physical constraint (Madden *et al.* 1991). Secondly peptides eluted from HLA B27 show considerable variation at this position, with the following residues identified -T, I, V, R, G, D, and E. Finally, as illustrated in fig.s 6.2 and 6.3, peptides 7A and 7D assembled HLA B27 at comparable concentrations to wild type NP383-391. Thus this data shows that the arginine side chain of position 7 of NP383-391 is involved in TCR contact and, although it does not exclude an indirect effect on HLA B27 conformation, suggests that part of the arginine side chain is solvent exposed. This finding is in apparent

contradiction to the downward orientation of the P7 side chain in the HLA B27 crystal structure (Madden *et al.* 1992), although the position 7 side chain is solvent accessible in a variety of peptides bound to HLA A2 (D. Madden, manuscript in preparation). The likely explanation resides in the uniquely long and flexible side chain of arginine, which might either "dip" into the E pocket, or might rotate to point out of the site, as originally suggested by Madden and colleagues (Madden *et al.* 1991). Since the HLA B27 crystal contained a variety of peptide species, weak electron density corresponding to up-pointing side arginine chains, which would in any case be highly mobile, might well not have been observed. Preliminary modelling of the NP peptide in the HLA B27 groove by M. Smith and P. Driscoll (Biochemistry dept. Oxford University) indicates that both orientations may be energetically similar. It is conceivable that the same peptide might stably bind to the same HLA molecule in two conformations, and likely that some flexibility is possible once bound, with considerable implications for the responding TCR repertoire. These possibilities will be clarified as more crystal structures of individual peptide/MHC complexes are solved.

Thus these data suggest that R7 makes an important contact with the TCR of the CTL clones. The most attractive possibility is that this residue might interact with the conserved glutamic acid residue at position 97 in the TCRß CDR3. Since the affinity of TCR for peptide+MHC is probably only of the order of 10^{-5}M (Weber *et al.* 1992), the energy loss associated with the disruption of one such salt bridge (approximately 4Kcal) could account for the dramatic loss of recognition observed when R7 is substituted. Although those clones lacking E97, with the possible exception of SD13, did not appear to tolerate position 7 substitutions better, it is possible that D97, N97 and Q96 fulfill similar roles in clones SD17, SD2 and SD13 respectively.

Recognition of position 8 variants.

Recognition by all clones was reduced by one or more of the four substitutions made for threonine at position 8, implying that this is probably a TCR contact residue, and one that is used by all CTL

clones. Although all clones exhibited different fine specificities, it is notable that clone SD16 was relatively tolerant of the 8E substitution, whereas clones GRb and SD17 were not. It is possible that reactivity correlates with the different amino acid usage in the Jß contribution to the CDR3ß, for example residue 104, which is D in clone GRb and E in SD17, but N in SD16. All these clones are relatively tolerant of the 8K substitution, unlike clone SD2, which has H at 104. It is difficult to compare recognition by SD2 to that of other clones, because it uses very different TCR alpha and beta chains, and shows a distinct fine specificity at positions 1, 4 and 8. It is interesting that clone SD2, which was least affected by changes at position 4, is most sensitive to changes at position 8. One possibility is that the orientation of the TCR of clone SD2 with respect to peptide and MHC is different to that of all the other clones.

*Clones vary in fine specificity for HLA B*2702.*

Clones GRb and SD17 were found to kill HLA B*2702 targets even without added NP383-391. It is well established that a single TCR heterodimer can confer both antigen/MHC specificity and also one or more alloreactive specificities (Kaye and Hedrick 1988, Malissen *et al.* 1988). Alloreactivity is frequently but not invariably peptide dependant {e.g.(Clayberger *et al.* 1987, Marrack and Kappler 1988, Grandea and Bevan 1992)}, and in this case would necessitate the same peptide(s) being presented by all cell lines tested (including the murine cell line P815). In one experiment allocytolysis was not inhibited by 10^{-5}M of HIV gag 265-274 (data not shown), suggesting that either peptide is not important, or that it is tightly bound.

These experiments were performed in the light of two observations made by by S. Huet and S. Rowland-Jones, firstly that HLA B*2702 could present the NP peptide to some HLA B*2705-restricted NP-specific CTL lines, and secondly that BCL from one HLA B*2702-positive individual NW failed to present Influenza virus or peptide (Pazmany *et al.* 1992). Whilst the first finding suggests that cross-reactive clones exist, none were found in this study, implying that the NP/HLA B*2702 complex must appear quite different to the CTL TCR. HLA B*2703 was found to present the NP peptide to

all clones tested. This implies that the single amino acid difference (Y59H in B*2703) does not significantly affect peptide binding or the conformation of the peptide/MHC complex for this peptide. Position 59 lies close to the position 1 side chain of bound peptide and might have a greater effect on presentation of peptides with bulky first residues such as HIV gag 265-274.

6.7. Conclusions

Exquisite specificity of the TCR for key peptide residues

The data presented here show that positions 4, 7 and 8 of the Influenza NP383-391 peptide are critical for recognition by all NP383-391-specific HLA B27-restricted CTL clones tested, with even conservative amino acid substitutions profoundly reducing cytolysis. In addition, substitution of serine with bulky residues at position 1 inhibited recognition by some clones. These findings confirm the prediction of Madden and colleagues based on the HLA B27 crystal structure (Madden *et al.* 1991, Madden *et al.* 1992), that positions 4 and 8 would be involved in TCR contact. Indeed the nature of the side chains of positions 4 and 8, W and T respectively in NP383-391, was critical for recognition by all CTL clones tested. Whilst other HLA B27 restricted peptide-specific immune responses must be studied, the TCR specificity shown here for these dominant "flag" residues is comparable to that of MHC for the "anchor" residues involved in peptide binding.

Whilst it is likely that, for all peptides binding to a given MHC molecule, the side chains of "flag" residues at certain positions are always available for TCR contact, the side chains of other residues may be important for specific peptides, either through direct contact with TCR or indirectly by altering the peptide or MHC conformation. Thus in the HLA B27-restricted response to NP383-391 studied here arginine at position 7 is crucial for T cell recognition whereas serine at position 1 can be substituted with other small residues (i.e. a permissive site). The importance of R7 would not have been predicted from existing data, emphasizing that every peptide/MHC combination may have unique features.

It is remarkable that even relatively conservative changes at any one of three positions abrogated recognition. Recent studies have suggested that such changes might have an antagonistic effect on TCR recognition through an unknown mechanism (De Magistris *et al.* 1992, Jameson *et al.* 1993, Sloan Lancaster *et al.* 1993). It is planned to test the peptides used in the current study for antagonistic properties.

It is possible that bulky "flag" residues, such as W at position 4 of NP383-391, may be seen by TCR in a manner akin to that of haptens by antibody. This proposition is supported by the finding that certain TCRs appear to be able to recognize antigen even in the absence of MHC (Ganju *et al.* 1992). On the other hand a peptide in which the side chains of positions 4 and 8 were very small, would, bound to HLA B27, present a relatively flat surface for TCR interaction and might elicit a very different TCR response. Clarification of these possibilities must await the solution of crystal structures of TCR/peptide/MHC ternary complexes.

The TCR can span all accessible side chains of MHC class 1-bound peptide.

Recognition of the NP383-391 peptide by clones GRb and SD17 is abrogated by changes at either position 1 (S to K) or 8 (T to E). Indeed all clones were sensitive to changes at both these positions, with the possible exception of clone SD13 at position 8. Whilst these changes could have distant effects on the conformation of the peptide, this result suggests strongly that the TCRs of these clones engage both ends of the peptide (assuming the 9th amino acid is buried). Since both these clones are also sensitive to changes at positions 4 and 7 it is likely that their TCRs span the entire length of MHC-bound peptide. This has not been demonstrated previously using human CTL clones with defined TCRs, although modelling based on immunoglobulin loops suggests that the alpha and beta CDR3 loops could span the 2.5nm groove length (Chothia *et al.* 1988). It is also possible that the greater length of peptide bound to MHC class 2 might exceed the possible TCR contact area. This is suggested by the mapping of both TCR alpha and beta chain contact residues to the C-terminal half of the MCC

bound to I-E^k (Jorgensen *et al.* 1992), and would have implications for the heterogeneity of TCR repertoire specific for a given peptide/MHC combination.

CTL clones exhibit at least three fine specificities which correlate with TCR usage

The HLA B27-restricted NP383-391-specific CTL clones described here show at least three different fine recognition patterns for variant peptides. Differing fine specificities for HLA B27 subtypes were also identified. The most marked difference in phenotype was of recognition of position 1 substitutions, with three clones, GRc, SD13 and SD16, relatively unaffected by radical substitutions. These three clones used Vα12, had a short CDR3α loop and a long CDR3 ß loop, in contrast to the two clones sensitive to position 1 changes, GRb and SD17, which both use Vα14, have a long CDR3α loop (containing aromatic residues) and a short CDR3ß loop. This suggest that the TCR alpha chain interacts with MHC in this region, and that the TCRs of the Vα12 clones interact less strongly or more flexibly. Furthermore clones GRb and SD17 exhibited different fine specificities for the position 8 variants 8A and 8E (table 6.3), with GRb, and also the other Vß7-bearing clones, relatively tolerant of these substitutions.

Clone SD2 exhibited a unique fine specificity and used very different alpha and beta chain elements to the other clones. It is possible that this clone sees peptide and MHC in an entirely different orientation (e.g. at 180%, see below) to the other clones.

A possible orientation of the TCR is suggested for HLA B27-restricted NP383-391-specific CTL

These data provide the most detailed information about the nature of the ternary complex of a human HLA class 1-restricted immune responses, and permit preliminary modelling of this interaction. A number of models of TCR structure have been proposed based on that of immunoglobulin (Chothia *et al.* 1988, Davis and Bjorkman 1988, Claverie *et al.* 1989). All predict that the CDR3 equivalent regions of both alpha and beta chains contact MHC-bound peptide, however Chothia goes further to suggest which CDR3 residues may

be involved, although he stresses that the relative positions of the alpha and beta chains are not defined. These results are thus entirely consistent with the proposition that the TCR alpha chains of all clones, with the possible exception of SD2 (see below), interact with HLA B27 in the region of the peptide N terminus, and that this interaction is more tightly constrained for the Vα14 bearing clones GRb and SD17. A possible orientation of the TCR for clones GRb and c, and SD 13, 16, and 17 is outlined in figures 6.10. Figures 6.11 and 6.12 show the predicted amino acid usage of the CDRs of the TCRs of clones GRb and SD16 respectively, using the model of Chothia *et al.* (1988). The results of recognition of substitutions at other positions of the NP peptide are consistent with this model. This data cannot however be conclusive, because no two clones have identical beta chains. Furthermore little is understood of the exact conformation or interaction of the alpha and beta chains, particularly in the CDR3 equivalent regions. Although speculative such models allow future experiments to be designed. In this case the proposed orientation could be tested by TCR expression and either mutagenesis (Engel and Hedrick 1988) or alpha chain substitution, or better still by demonstrating peptide or MHC interaction with TCR directly using chemical cross-linking reagents (Romero *et al.* 1993). This and other orientations, their implications and future experimental approaches will be discussed further in chapter 8.

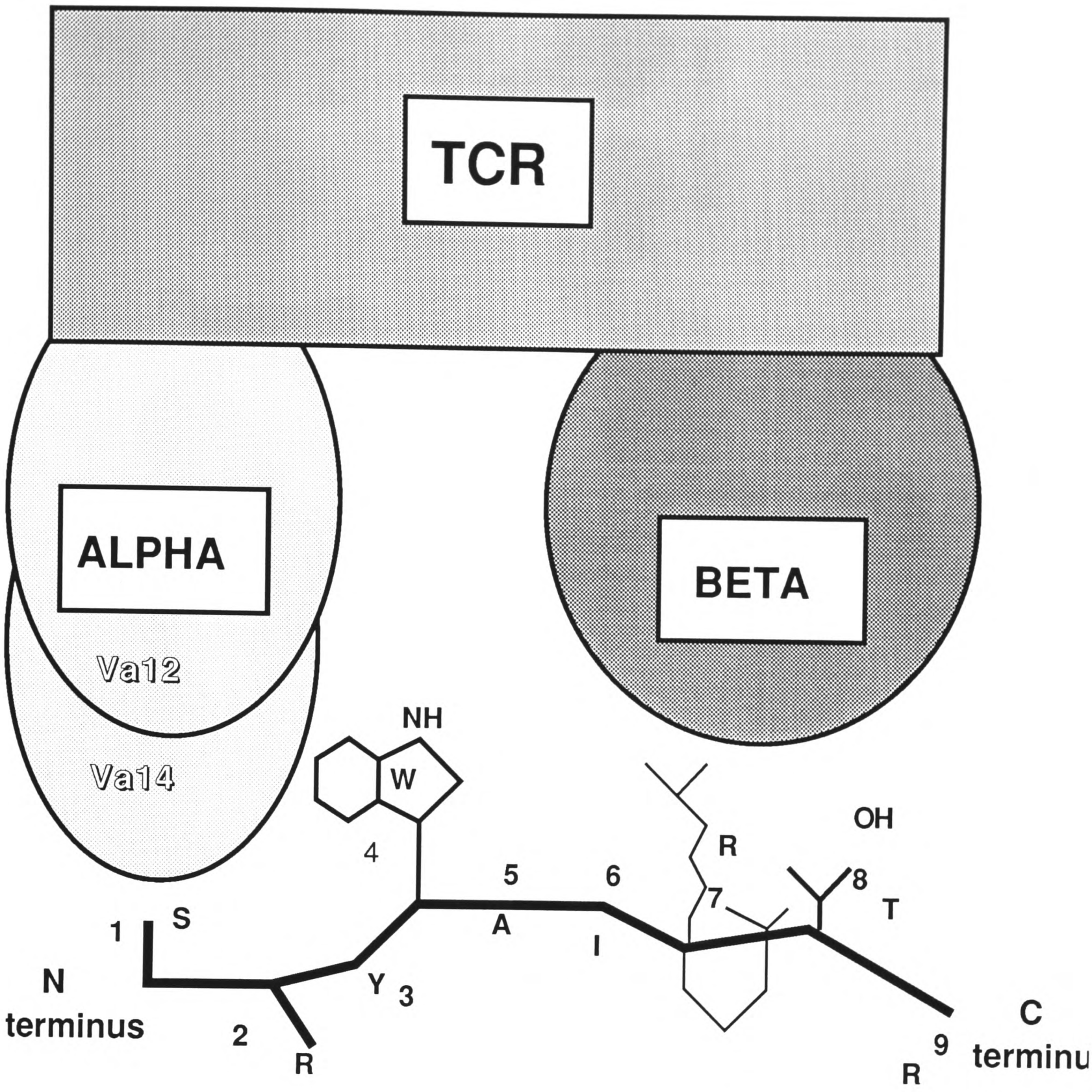


Fig. 6.10. Possible orientation of CTL TCRs recognizing NP383-391 + HLA B27. Side view, not to scale.



Fig. 6.11. Predicted amino acid usage of GRb TCR CDRs. After Chothia *et al* (1988).

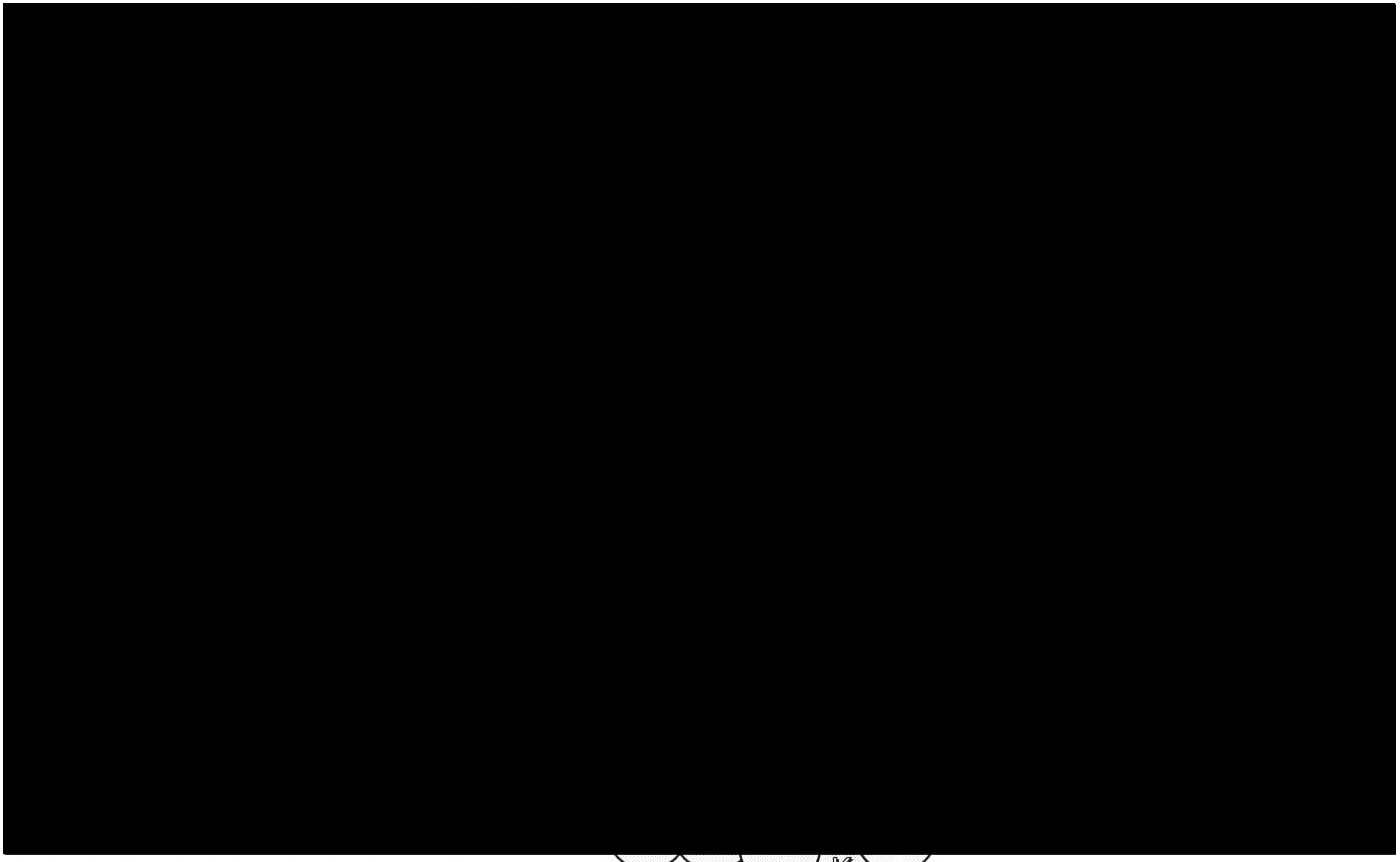


Fig. 6.12. Predicted amino acid usage of SD16 TCR CDRs. After Chothia*et al* (1988).

CHAPTER 7

EXPRESSION OF THE TCR OF CTL CLONE GRb IN FUNCTIONAL FORM IN RBL-2H3 CELLS

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7.1. Introduction

The TCR of one of the HLA B27-restricted influenza NP383-391-specific clones was expressed primarily in order to obtain material for crystallization and structural study, and as a means of generating monoclonal anti-TCR antibodies. It was also hoped to carry out functional studies, since the murine 2B4 TCR had retained specificity for I-E^k and cognate peptide when expressed in this form, as demonstrated by degranulation mediated by ζ signalling (Engel *et al.* 1992). Hence it was hoped that the GRb TCR would also retain antigenic specificity when transfected in this form, confirming that the correct α and ß chains had been cloned and were correctly folded. Not only would this be important for the interpretation of structural studies, but it would permit the introduction and analysis of site directed mutations into the constructs.

The expression vector and expression system has been described in chapter 1.5 and detailed in chapter 2.6, and reported by (Engel *et al.* 1992).

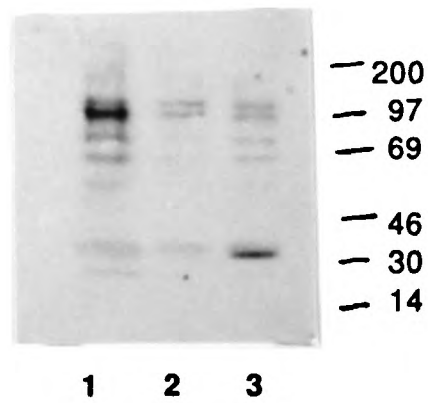
Full length sequences of GRb TCR α and ß chains.
The GRb TCR α and ß chain nucleic acid and predicted amino acid sequences are shown in appendices 5 and 6. The 5' untranslated, V, N(DN) and J regions were sequenced from M13 clone(s). The V, N(DN), J and C regions were sequenced after independent cDNA PCR and cloning into the SRαζ plasmid. The V, J and C sequences obtained concur with published sequences, except for a non-coding change at position 90 of the Cß2 of the SRαζ GRß6.3 plasmid used for expression, of TGT-TAT (coding for valine). This presumably represents a PCR-related error. The Vα14 5' untranslated sequence was obtained from a single full-length M13 clone and so could not be independently confirmed.

7.2. Confirmation of expression of the GRb TCR αß heterodimer by Western blot and FACS analysis.

At the time of transfection no mAb reagents were available that stained the intact TCR of CTL clone GRb, which used Vα14 and Vß7. Therefore Western blotting of transfected cells was carried out, using the anti-ζ mAb TIA-2 (Coulter UK), in order to demonstrate expression of a heterodimer of appropriate size, as described in chapter 2.6. The Western blot of a 10% SDS gel run under non-denaturing conditions is shown in figure 7.1. In the jurkat positive control lane (3) a band of approximately 35 kD can be seen, corresponding to native CD3ζ in T lymphocytes. A band of approximately 100kD is present in the RBL cells transfected with GRb αζ and ßζ constructs (lane 1), but not in the control lane 2 (untransfected RBL). Whilst confirming expression of the fusion protein, this band is compatible with the presence of αζ/ßζ hetero- and/or homodimers in the transfected cells. Immunoprecipitation performed subsequently by M. Callan and H. Reyburn with ßF1 gave the same result (Callan *et al.* 1993).

Confirmation of cell surface TCR ß chain expression was obtained by FACS staining with the antibodies JOVI 1 and ßF1. Figure 7.2 shows the staining of unsorted and FACS sorted RBL transfected with the

TCR/ZETA construct is expressed as a 95 Kd dimer



Western Blot of transfected rat basophil leukemia cells (RBL) using the anti-zeta monoclonal antibody TIA-2 (non-denaturing SDS-PAGE stained with peroxidase-conjugated anti-mouse antibody).

1. RBL transfected with GRb alpha/zeta and beta/zeta constructs.
2. untransfected RBL.
3. Jurkat

Figure 7.1. Western blot of transfected TCR heterodimer from GR clone b.

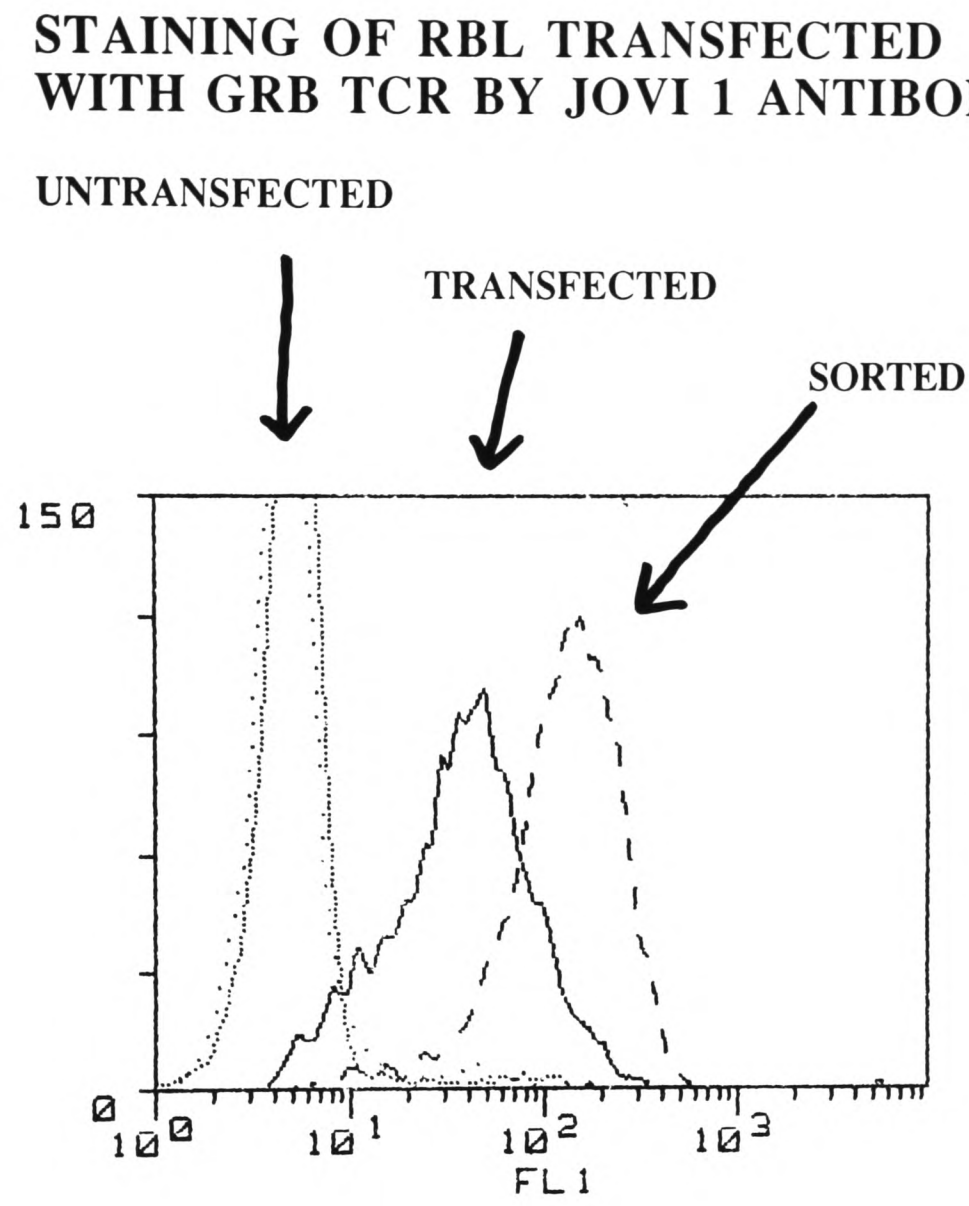


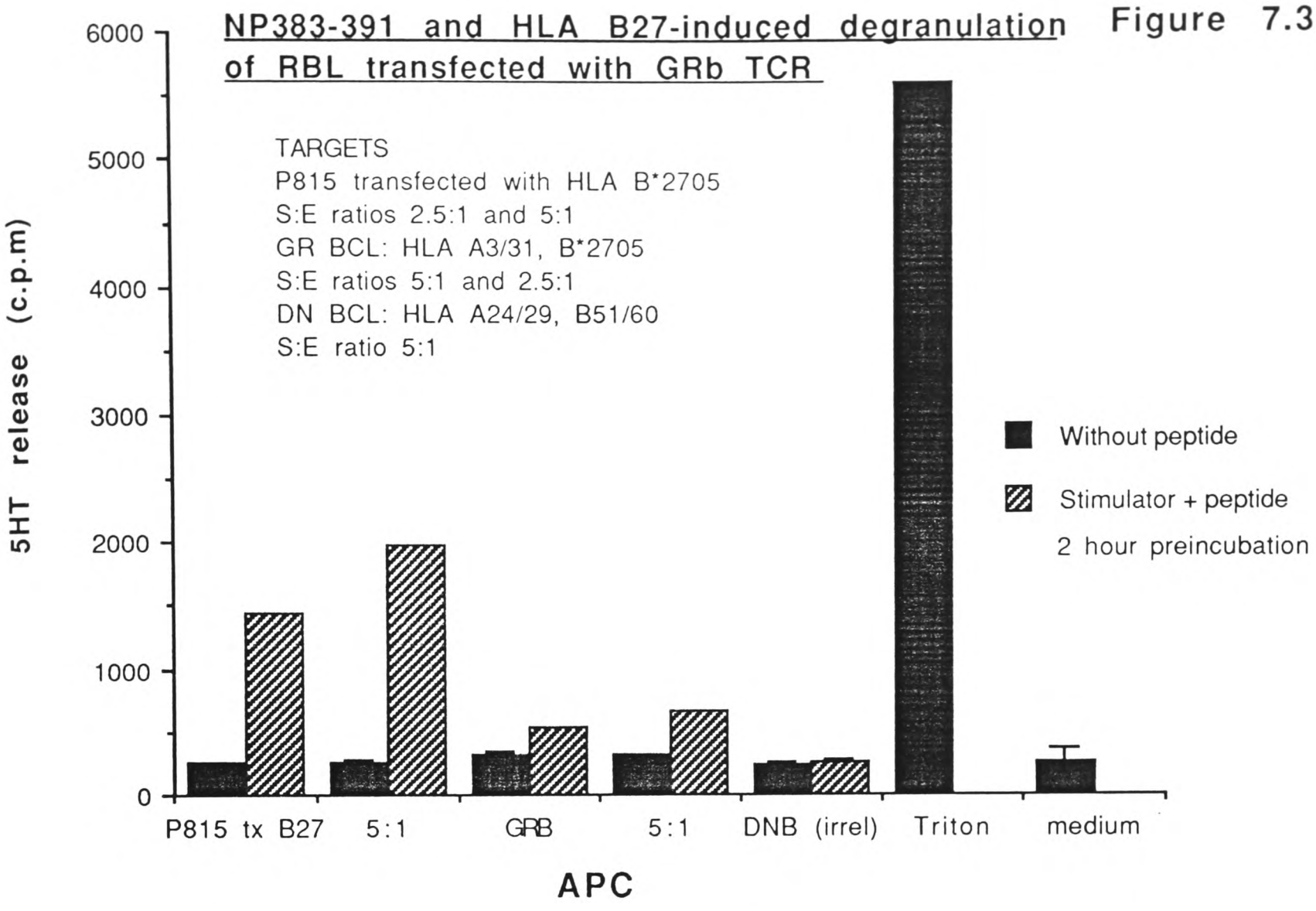
Figure 7.2. FACS analysis of RBL-2H3 cells transfected with GR$\alpha\beta$ TCR. $2x10^5$ cells were stained with Jovi 1 or control antibody. Dotted line: untransfectd RBL, solid line: transfected RBL, dashed line: facs sorted RBL, fine dotted line: isotype matched control antibody staining of sorted RBL.

GRαβ TCR. JOVI 1, an IgG2a mAb that stains 60% of CD3 positive PBL, was a kind gift from J Viney and M. Owen (ICRF, London). βF1 stains the Cβ segment of fixed but not viable lymphocytes. The staining of RBL transfectants with βF1 was also observed with other expressed human TCRs (T. Ottenhof, H. Reyburn personal communications), and might reflect exposure of Cβ determinants unmasked by the lack of associated CD3 elements.

7.3. Antigenic specificity of GRb TCR transfectants.

*Expressed GRb TCR αβ retains specificity for HLA B*2705 and NP383-391, but not allospecificity for HLA B*2702.*

Degranulation assays were performed as described by (Engel *et al.* 1992), with the modifications detailed in chapter 2.7. The result of a degranulation assay using unsorted uncloned GRαβ RBL transfectants is shown in figure 7.3a. In this experiment the maximum 3H 5-HT release for untransfected controls was 279 c.p.m. (data not shown, see also controls in fig. 7.4b). Thus specific 5-HT release occurred only when GRαβ RBL transfectants were incubated with HLA B27-positive APC that had been pulsed with NP383-391. Peptide pulsing with irrelevant peptides such as the HIV gag 265-274 epitope, known to bind HLA B27, did not result in degranulation (figure 7.5). Notably BCL were poorer APC than either C1R cells or murine P815 transfected with HLA B27, and in some experiments did not induce significant specific release after peptide pulsing (e.g. figure 7.4a). Cell surface HLA B27 expression, assessed by ME1 fluorescent staining, was no greater for P815 transfectants than for GR BCL (data not shown). It is possible that P815 cells carry additional costimulatory or adhesion molecules that enhance the interaction with RBL cells. FACS staining with the antibody OX 45 showed that RBL cells bear CD48, an LFA3 ligand (data not shown), and also rat ICAM1 (IA29 mAb, L. Wedderburn, ICRF, London, personal communication). RBL cells did not stain with antibodies to rat LFA1α (WT1), CD5 (OX19) or CD2 (OX34) (data not shown), or to MAC1 or LFA1β (OX42 and WT3, L Wedderburn unpublished data). Stimulator:effector (S:E) titration of P815 B27 transfectants showed optimal degranulation at an S:E ratio of 5:1 (data not shown). C1R transfectants were slightly poorer APC at



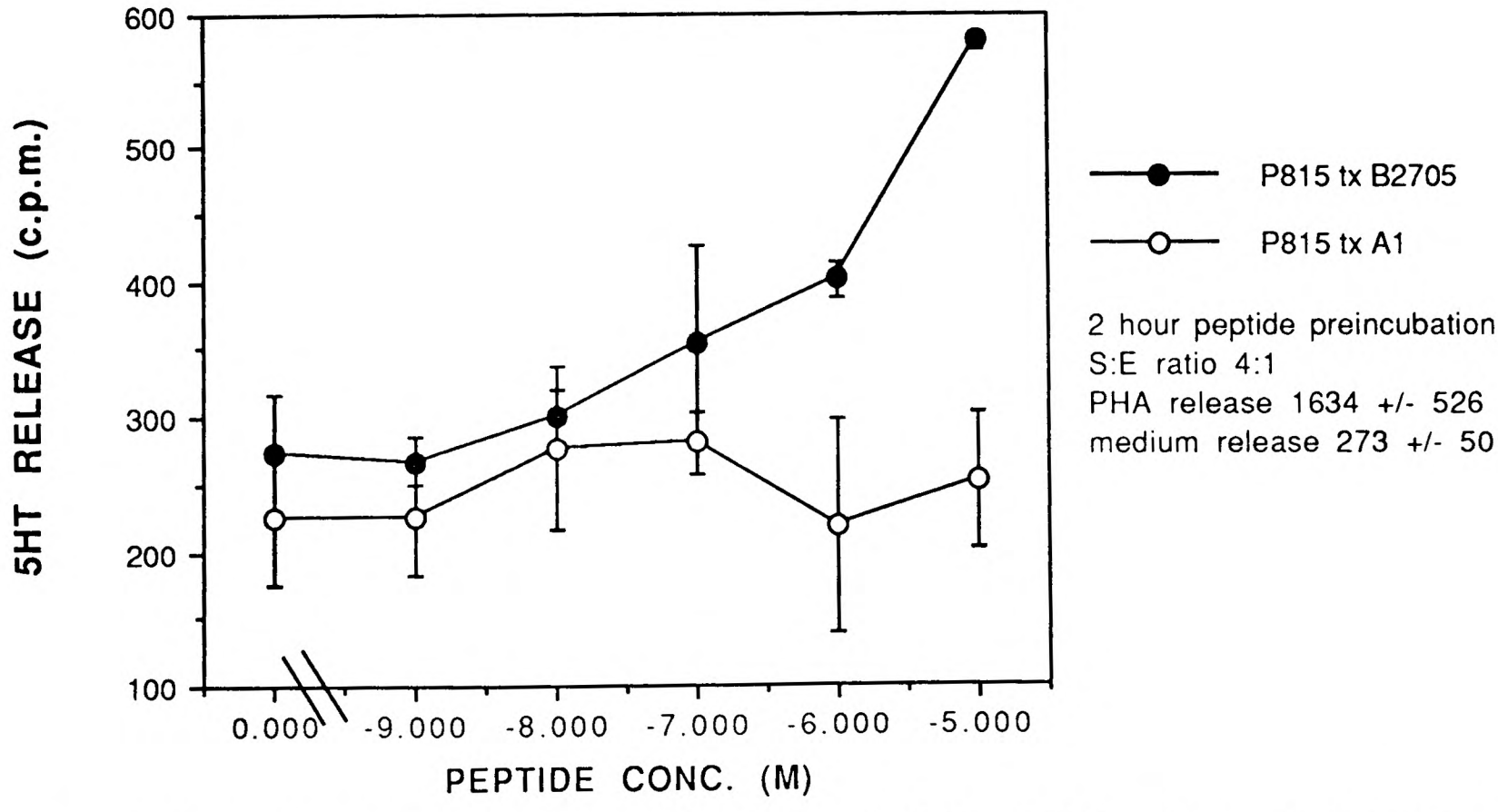


Figure 7.2. Degranulation of GRb TCR RBL transfectants wi HLA B27 and NP383-391.

HLA B*2705 specificity of GRb TCR transfectants

Figure 7.4a

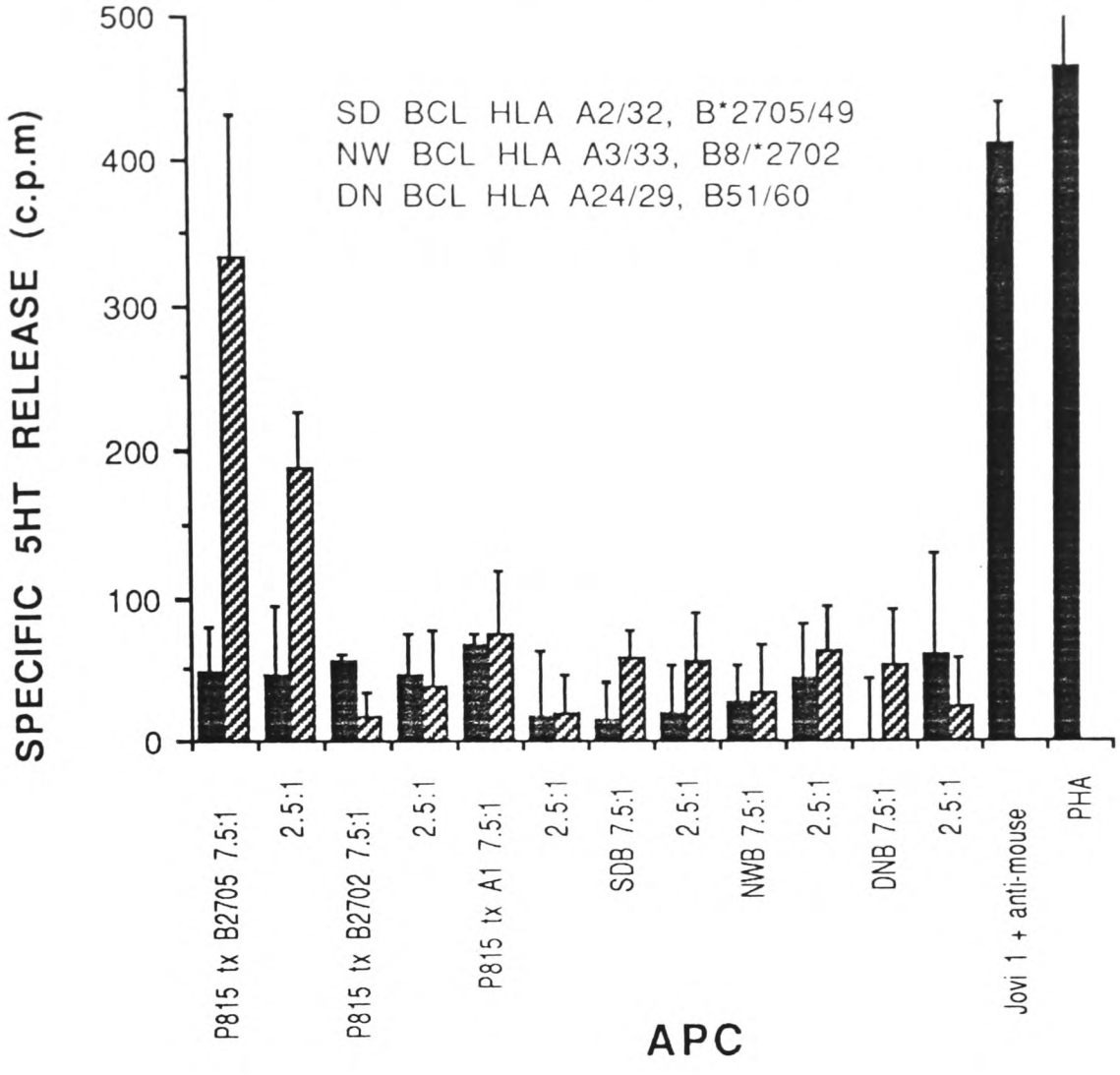
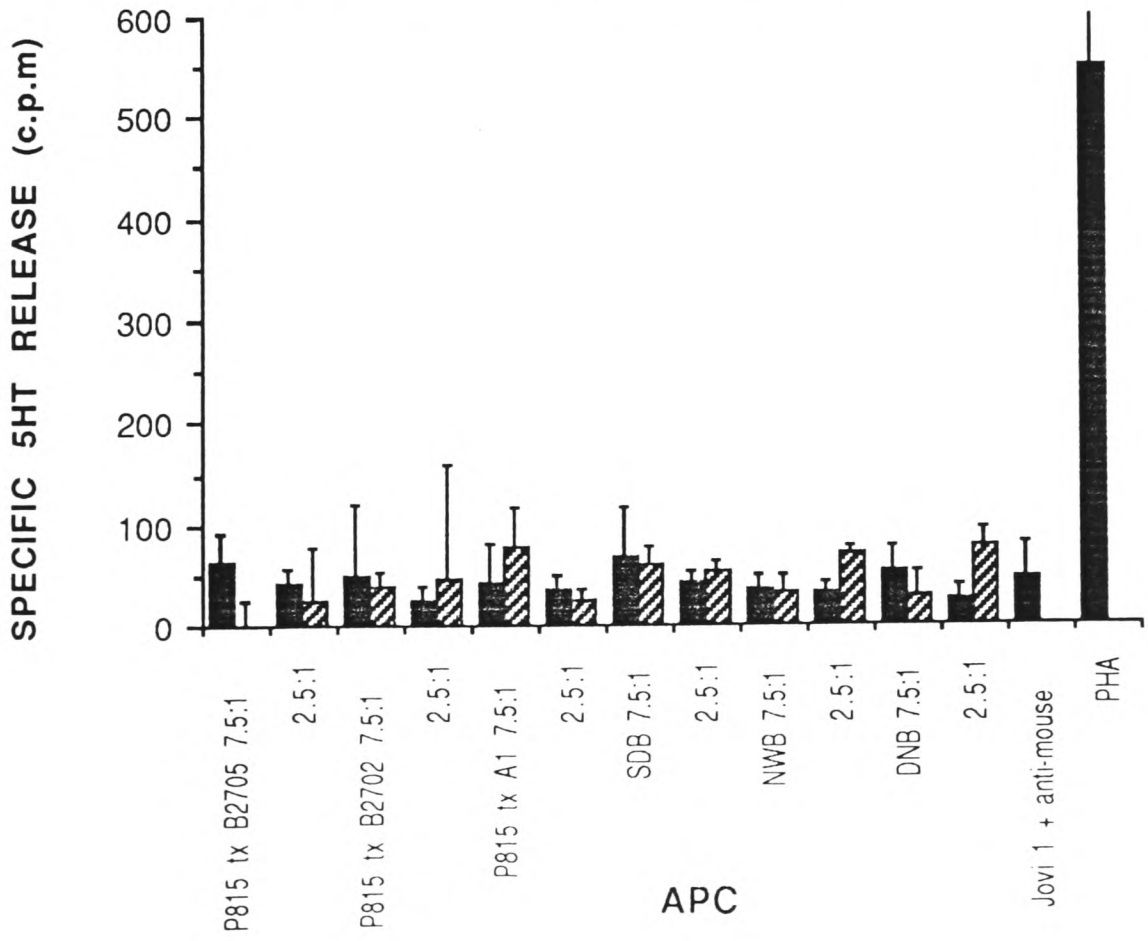


Figure 7.4b

Untransfected RBL do not degranulate with HLA B27 + NP383-391



this ratio, but were comparable APC at a higher ratio of 7.5:1. This might simply reflect the smaller size of C1R cells.

As shown in figure 7.3b, recognition of NP383-391 by the GRαß RBL transfectants, at optimal S:E ratios, could be titrated down to 10^{-7}M, and was not saturated at the highest concentration of peptide used, 10^{-4}M (data not shown). Thus, although the readout was entirely different, the titration curve for peptide recognition appeared to be shifted 2-3 logs to the right, as compared to cytolysis of the parental clone GRb (shown in fig.6.4).

HLA B*2702-expressing transfectants, either P815 (figure 7.4) or C1R (data not shown), and the HLA B*2702-positive BCL NW (figure 7.3), did not induce degranulation of GRαβ. These experiments were performed using uncloned FACS-sorted GRαß RBL transfectants (which gave the greatest degranulation), and unsorted and cloned unsorted transfectants.

Fine specificity of expressed GRb TCR αß for NP383-391 peptide variants

An example of a degranulation assay using peptide variants of NP383-391 is shown in figure 7.5. The position 1 substitutions 1S and 1L reproducibly caused significant degranulation for the GRb aß transfectants, but not untransfected controls (data not shown), although 5-HT release was markedly less than for NP383-391. The 4Y and 7D peptides gave degranulation marginally above background in some experiments. The other peptides tested, 1F, 4E, 4F, 4K, 4L, 7A, 7E, 8A, 8E or 8F, were not recognized (figure 7.5 and data not shown). These findings are broadly similar to those obtained for the GRb CTL clone in cytolysis assays, and shown in tables 6.2 and 6.3.

7.4. Production of a Vß7.1 mAb using the GRαβ RBL transfectant as an immunogen

A number of mAbs were generated by M. Callan and H. Reyburn (J. Bell's laboratory), from an SJL mouse immunized with the GRαβ TCR RBL transfectant (Callan *et al.* 1993). Two antibodies, 3G5 and 3C5, both of the IgG2b isotype, were shown to stain 100% of

Figure 7.5.

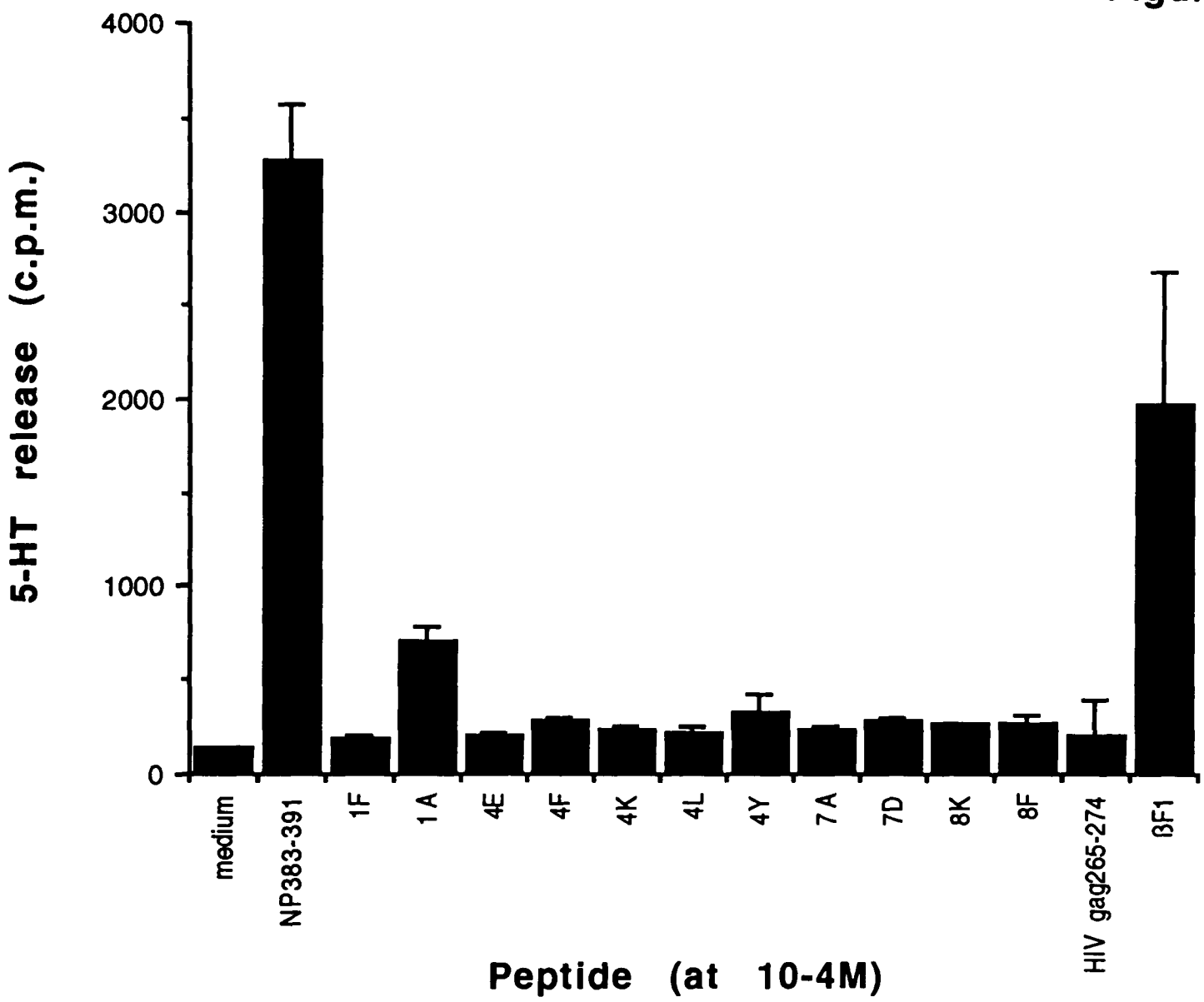


Figure 7.5. RBL GRαβ TCR transfectant recognize the 1A variant of NP383-391. HIV gag 265-274, included as a negative control, is known to bind to HLA B27. A bulk FACS sorted transfected line was used. Mean and S.D. of triplicate 5-HT release (raw counts) are shown for a 45 minute degranulation. Peptide 1A release with untransfected RBL controls was 371 +/- 65 c.p.m. Triton X release was 8000 c.p.m.

CTL clone GRb (Vα14, Vß7.1, Jß2.3), and also SD16 (Vα12, Vß7.1, Jß1.3), but not SD17 (Vα14, Vß6.6). Specificity of the 3C5 mAb for the Vß7.1 subfamily was confirmed by M. Callan by sequencing anPCR amplified TCR sequences from PBL cultured for 4 weeks with 3C5 (Callan *et al.* 1993).

7.5. Discussion

The TCR α and ß chains of the CTL clone GRb, expressed as a fusion heterodimer with the CD3 ζ protein and transfected into RBL cells, conferred specificity for HLA B27 (B*2705) together with cognate peptide NP383-391. However approximately 100-1,000 fold greater peptide concentrations were required to induce degranulation by GRαβ transfectants than cytolysis by CTL clone GRb. The fine specificity of the transfectant cell line for variants of NP383-391 was comparable to that observed for the CTL clone, although some peptides recognized by clone GRb (albeit less well than NP383-391) were not seen by the GRα β transfectant. In addition the allospecificity, shown by CTL clone GRb for HLA B*2702, was lost by the transfectant cell line. Notably no new specificity was observed. It is possible that these differences in specificity reflect a qualitative difference in antigen recognition between the CTL clone and the GRα β transfectant. However it is perhaps more likely that they reflect a quantitative difference in signalling, or merely a lack of sensitivity of the RBL degranulation readout. This is supported by the failure of recognition by the GRαβ transfectants of NP peptide-pulsed HLA B27-positive BCL in some experiments — when cell lines transfected with HLA B27 were seen. Thus it may be that if the GR αζ/βζ heterodimer was transfected into a different cell line, specificity for peptide variants and HLA B*2702 would exactly parallel that of the parental CTL clone GRb. It is planned to investigate this possibility by transfection of the GR αζ/βζ construct into a murine TCR negative cell line. Alternatively it is possible that other molecules are required for recognition. It appears that the TCR peptide/MHC interaction is of low affinity (Weber *et al.* 1992), and it has been suggested that coreceptors such as CD8, and adhesion molecules such as LFA 1 and 3, may be important in increasing the "avidity" of the

interaction *in vivo* (Davis and Chien 1993). It is perhaps surprising that recognition could be transferred at all in the absence of CD8, given that cytolysis by clone GRb could be completely inhibited by the anti CD8 mAb MF8 (figure 7.6), although it should be noted that this experiment was performed with suboptimal peptide concentrations, and that a very high anti-CD8 concentration was required for complete inhibition. Notably the TCR studied by (Engel *et al.* 1992) was from the CD4-independent murine clone 2B4, although other human TCRs have subsequently been functionally expressed (T. Ottenhof, unpublished data). This phenomenon will be further investigated by super- or co-transfection of GRαβ–expressing cells with CD8. It is also planned to attempt to inhibit degranulation using antibodies to human and mouse LFA3 and 1.

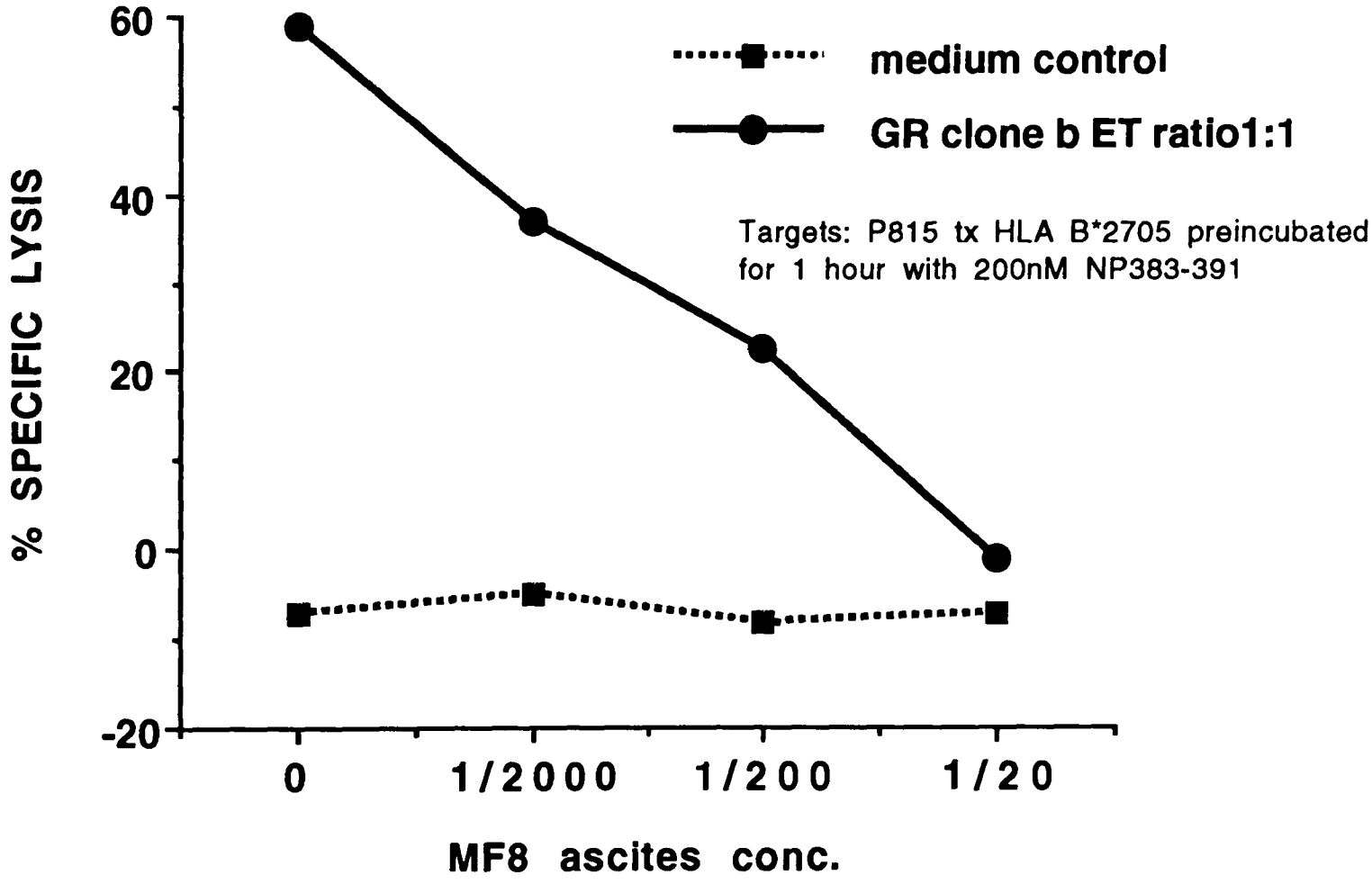


Fig. 7.6. Inhibition of cytolysis by CTL clone GRb with MF8 ascites (anti-CD8).

The expression in functional form of the TCR alpha and beta chains, identified from the CTL clone GR, formally demonstrates that recognition of HLA B27 and the NP383-391 peptide can be

conferred by this heterodimer alone. The ζ fusion protein/RBL transfection system used provides a useful approach to the generation of mAbs, and a tool for studying TCR recognition. This may be particularly important as the human CTL clones described in chapter 5 appear to have only a limited lifespan. Finally, expression of the αζ/βζ construct in different cell types may prove to be useful for studying TCR affinity and signalling requirements, both in the presence and absence of accessory molecules.

CHAPTER 8

CONCLUSIONS

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Work presented in this thesis details the molecular characterisation of cytotoxic T lymphocyte (CTL) recognition of antigen and superantigen, concentrating on the immune response to two viral peptides presented by HLA B27, and on the identification and characterisation of a novel superantigen. This work was carried out in order to address two broad immunological questions. Firstly: How does the TCR recognize antigen? or, more precisely: What determines the fine specificity of the T cell receptor for a specific peptide/MHC combination? And secondly: Why does possession of HLA B27 predispose to autoimmune disease? In particular, does the recognition of HLA B27 by the TCR in defined anti-viral immune responses give any indication of its autoimmune function?

8.1. Summary of results

HLA B27-restricted influenza A-specific CTL lines and clones were generated from the peripheral blood of three unrelated individuals. The rearranged T cell receptor (TCR) alpha and beta chain genes of the CTL clones showed remarkable conservation of Vα and Jα, and Vß and NDNß usage, implying strong immune selection. Most TCR alpha chains used either Vα12 together with Jα16.5, or Vα14 with Jα9.3. Four of the seven beta chains

sequenced used Vß7 together with an NDN junctional amino acid motif QExVG. This pattern of conservation, found between clones from unrelated individuals, exactly parallels that previously demonstrated for the HLA A2-restricted CTL response to the Influenza A matrix peptide M58-66 (Moss *et al.* 1991).

Two HLA B27-restricted CTL lines specific for HIV gag, and one specific for influenza NP, were found to express polyclonal TCRs, probably derived from non-specific T cells remaining in the cultures. Dominant TCR sequences were however observed, although no striking common features were evident..

The CTL clones showed remarkable fine specificity for cognate peptide NP383-391. In cytolytic assays clones failed to recognize synthetic variants of NP383-391 containing single conservative amino acid substitutions at positions 4, 7 or 8, even though these variants were shown to bind to HLA B27. The two CTL clones using Vα14, but not the three that used Vα12, also failed to recognize synthetic peptides with bulky residues (K or F) at position 1. This shows, for the first time, that a single TCR can span all accessible side chains of MHC class 1-bound peptide. Correlation of fine specificity for position 1 substitutions of NP383-391 with TCR alpha chain usage further suggests a possible orientation for the TCR/HLA/peptide ternary complex in this immune response.

The TCR heterodimer from the CTL clone GRb was expressed in a rat basophil cell line and shown to confer specificity for HLA B*2705 + NP383-391. Recognition of HLA B*2702, shown by the parental CTL clone in the absence of peptide, could not be demonstrated for the transfected TCR.

Influenza A-specific CTL were also shown to recognize bacterial superantigens in an MHC-unrestricted but class II-dependent manner. A rapid screening assay for candidate superantigens was developed and used to identify a novel superantigen made by *Clostridium perfringens*. This superantigen was found to have a unique specificity for human TCRß chains. Preliminary data

suggested that *Clostridium difficile* also produced a superantigen with a related or identical TCR specificity.

8.2. TCR recognition of peptide and MHC

The human HLA B27-restricted CTL response to influenza A uses a very limited TCR repertoire and shows remarkable fine specificity for antigen

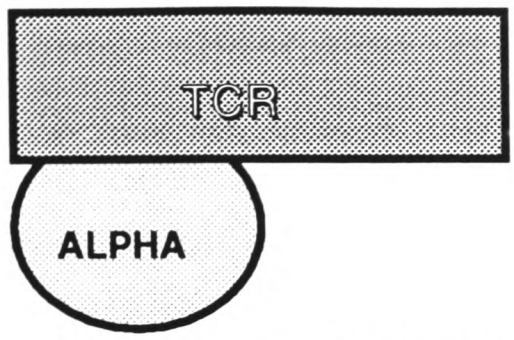
The molecular anatomy of the ternary complex of TCR, MHC and peptide is the key determinant of specificity in the cell-mediated immune response. The exact nature of the TCR ligand has become clear during the course of this work, and it is now known that T cells see short peptide fragments, tightly bound in extended conformation in the grooves of HLA molecules (Rammensee *et al.* 1993, Madden *et al.* 1991, Brown *et al.* 1993). The polymorphism of MHC molecules can now be seen to have a physical correlate in the amino acid variation within key pockets lining the peptide-binding groove, which in turn leads to selection of peptide "anchor" residues. In contrast, relatively little is known about the exact conformation of the TCR and, without structural data, much of the evidence remains indirect. The approach adopted in this thesis entailed an analysis of TCR usage of a number of HLA B27-restricted NP383-391-specific T cell clones (and lines). Clones from unrelated individuals used TCR α and ß chains of striking similarity, often differing at only a single amino acid residue. Conservation of Vα, Jα, Vß and NDNß regions suggested that these regions might be important for peptide/MHC contact. The Nα and Jß regions were not conserved. The same pattern of conservation, but using different TCR elements, had been found previously for the HLA A2-restricted response to influenza matrix M58-66 by Moss *et al.* (1991). This suggests that similar parts of the TCR may make important peptide and MHC contacts in these (and other) MHC class 1-restricted responses.

Comparison with other immune responses suggests that a number of different TCR/MHC orientations may be possible

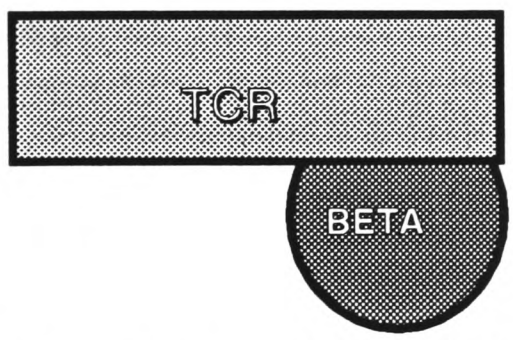
Unlike the HLA A2-restricted M58-66-specific response, in which a single dominant Vß element pairs with a single Vα/Jα combination, the NP-specific HLA B27-restricted CTL clones were found to use two principle Vα and two Jα segments, usually

but not invariably in the same combination. The fine specificity of the NP383-391-specific clones for the N terminal residue of bound peptide appeared to correlate with TCR alpha chain usage, enabling preliminary orientation of the ternary complex. However since no two clones shared either identical alpha or beta chains, this model, outlined in figures 6.10-12, must be provisional. Nevertheless clear predictions are made which will be tested by introducing selected mutations into the alpha and beta CDR3 regions and looking for complementation with substituted peptides.

As previously described, TCR usage has now been characterized for a number of specific immune responses, some of which are summarized in table 5.6. Some of these studies have suggested an orientation for the TCR, relative to peptide and MHC, by correlation of TCR usage with fine specificity. Thus Taylor *et al.* (1990), studying T hybridomas specific for influenza A HA 110-120 presented by I-E^k, identified three clones with identical beta chains but different fine specificities for N terminal substitutions of the peptide. This finding implies that the (different) alpha chains interact with the peptide N terminus, although strictly does not rule out an indirect effect on the beta chain. Furthermore, if the TCR CDR loops adopt the same configuration as V_H and V_L, as proposed by Chothia and colleagues (1988) and illustrated in figures 6.11 and 6.12, the TCR beta chain CDR1 and CDR2 can be roughly orientated to interact with the Eß alpha helix. In contrast, Danska and colleagues (1990) found differences in fine specificity of T cell clones, with identical TCRß chains, for the C terminal residue of the sperm whale myoglobin 110-118, presented by I-E^d. Similarly Yanagi and co-workers (1990), studying the D^b-restricted response to LCMV GP 272-293, found two clones with identical alpha chains that differed in fine specificity for the N terminal position of the peptide. A similar orientation was proposed by Casanova *et al.* (1993) for the K^d-restricted response to two HLA-derived peptides differing only at their fourth position. Their data could, however, be interpreted to suggest that both chains of the TCR

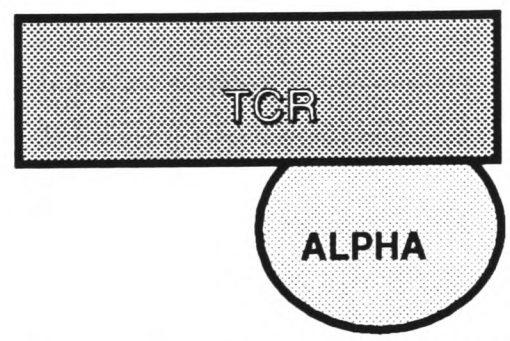


N **383-------391** NP383-391
HLA B27 Influenza NP383-391
E^k 'flu HA 110-120 Taylor (1990)

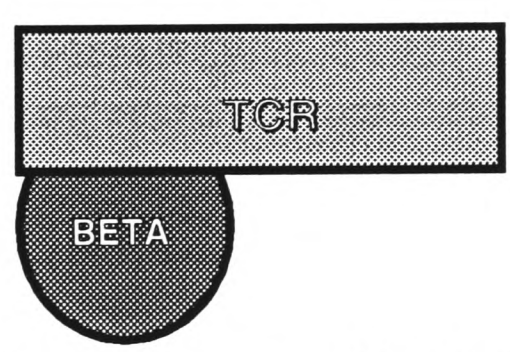


N **-------259- PbCS**

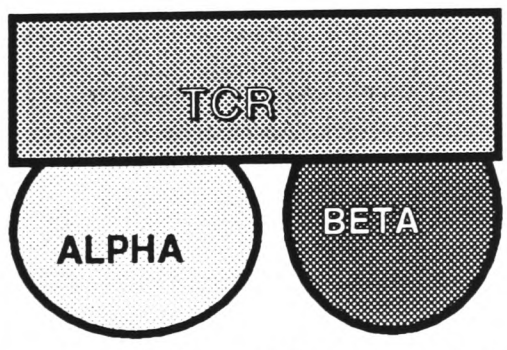
K^d PbCS 259 IASA derivative 253-260. Romero (1993)



N **-------259- PbCS**
K^b ova 262 of 257-264 Carbone (1992)
E^d SPWM 110-118 Danska (1990)
K^d PbCS 259 IASA derivative 253-260. Romero (1993)



N **---173----** Cw3/A24
D^b LCMV GP278-86 Yanagi (1990)
K^d HLA Cw3/A24 170-9 Casanova (1993)



N **----------99--102** MCC
E^k MCC88-103 Jorgenson (1992)

Figure 8.1. Orientations for TCR recognition of peptide and MHC suggested by different studies. Peptide is assumed to be orientated with the N terminus on the right in all cases (See text).

can interaction with this peptide residue. Since constraints at the N and C termini appear to force all peptides to bind MHC in the same orientation (Latron *et al.* 1991, Madden *et al.* 1992), it would thus appear that the TCR must be able to take up at least two orientations. These results are summarized in figure 8.1. A variety of other approaches, used to study TCR specificity, have provided support for this proposal. These include TCR transfection and mutagenesis, analysis of immune responses in TCR "hemitransgenic" mice, and, most recently, direct cross-linking experiments. Saito and Germain (1987) transferred E^b restriction from one PCC-specific clone to another by transfection of its Vß3 TCRß chain. This unusual result is consistent with the Vß CDR1 and 2 interacting with the Eß chain. More frequently heterodimers have been transfected into TCR-negative cell lines. This permits one chain of the TCR to be paired with a variety of partners from other clones, or with its natural partner into which point or segmental mutations have been introduced. Mutagenesis of residues 93α and 97ß, using the numbering of Chothia *et al.* (1988), of another PCC-specific clone restricted by I-E^k showed that both these positions together determined specificity for a substituted peptide T102S (Kaye *et al.* 1991, 1992). This important result suggests that both TCR α and ß CDR3 regions can interact with the side chain of a single peptide residue, although could be explained by an indirect effect of one CDR3 on the other. This result also emphasizes the difficulties of interpreting mutagenic studies — where single amino acid changes may have effects reaching beyond their nearest neighbours.

Recently an elegant approach has been adopted by Jorgensen and colleagues (1992). Mice, made "hemitransgenic" for one or other chain of the TCR from a MCC-specific I-E^k-restricted T cell clone 5C.C7, were immunized with variants of MCC 88-103. Alpha but not beta chain transgenic mice responded poorly to the peptides K99E or K99Q, whereas for peptides substituted at 102, the reverse was found. These data indicate that the 5C.C7 beta chain normally interacts with a C-terminal residue side chain, with the alpha chain more N-terminal, as shown in figure 8.1. These

contact points were precisely mapped to the TCRß CDR3 residue 97, and CDR3α residue 93, by demonstrating complementary amino acid changes in responding T hybridomas. In contrast Carbone *et al.* (1992), studying the K^b-restricted response to ovalbumin 257-264 in TCRß hemitransgenic mice, have suggested that alpha chain pairing determines specificity for residue 262. Notably the reciprocal hemitransgenic mouse was not studied, nor was TCR sequence data given for either of the two responding T cell hybridomas studied.

Recently direct crosslinking of the TCRs of CTL clones using photoaffinity labelling has proved possible (Romero *et al.* 1993a and b). CTL clones were raised against an derivative of the PbCS peptide 253-260, synthesized with a photoreactive iodo, 4-azido salicylic acid moiety at the 7th position. Two clones were labelled on the alpha chain, one on the beta, and one on both. Alpha chain reactivity could be correlated with usage of a particular Jα segment containing a tryptophan at position 102 (the same position as the conserved tyrosine found in the HLA B27-restricted NP response). Finally the photoreactive group was shown preferentially to label tryptophan.

Thus the data presented in this thesis, taken together with the studies summarized in figure 8.1, suggest that the TCR may interact with peptide bound to MHC in at least two conformations. The CDR3 regions might both lie along the groove in two possible orientations, with the α chain CDR1–2 overlying the MHC α1 (or α) helix, and the ß chain CDR1-2 the α2 or ß helix of MHC, or *vice versa*. Furthermore, since the TCR-exposed surface of MHC is relatively flat, additional alignments at right angles to this and even in intermediate positions might also be possible. This model has certain implications. It predicts that TCR are likely to exist which recognize a given peptide/MHC combination with great precision (as demonstrated in this thesis), but which can also recognize a very different peptide/MHC combination — in a different orientation. This would help explain the very high frequency of alloreactive specificities shown by T cells (as would cross-reactive low affinity TCRs). Furthermore this model

would permit the crossreactive TCR of an autoreactive T cell to recognize a foreign "triggering" peptide in one orientation, and a self peptide, perhaps in combination with a different MHC molecule, in another. Of course most such specificities for self peptide/MHC would usually be eliminated during thymic negative selection. This model would thus predict not only that self and autoimmune-provoking peptides need not be highly homologous, but also that certain autoimmune diseases might be associated with combinations of two MHC molecules.

8.3. Does the immune function of HLA B27 explain its association with autoimmunity?

The findings presented in this thesis suggest that CTL responses to viral peptides presented by HLA B27 are not substantially different to responses mediated by other HLA or H-2 class 1 molecules, at least in terms of fine specificity and TCR conservation. Thus the pattern of TCR conservation observed for HLA B27-restricted influenza NP-specific CTL clones was similar to that described by Moss *et al.* (1991) for influenza matrix-specific HLA A2-restricted CTL, although different gene segments were used. Furthermore no characteristic "B27-specific" TCR motifs could be identified by comparison with two HLA B27-restricted HIV gag CTL lines. Consequently it is suggested that bound peptide is the principle determinant of the TCR repertoire for a given peptide/MHC complex, although it should be stressed that further analysis of TCR usage in HLA B27-restricted antigen-specific responses is required to test this proposal. It remains possible that one TCR element, for example Vß7, is preferentially used in HLA B27-restricted responses, and somehow in itself predisposes to disease. Such a theory, the somewhat controversial "V-region" hypothesis of (Heber-Katz and Acha-Orbea 1989), has been put forward to explain the frequent usage of Vß8.2 in both murine and rat autoimmune diseases, including experimental allergic encephalomyelitis (Acha-Orbea *et al.* 1988), experimental allergic neuritis, and experimental allergic uveitis. However no indication is given as to how Vß8 usage might predispose to disease. Overall the findings presented in this thesis would suggest that there is nothing strikingly

abnormal in the TCR repertoire selected in anti-viral HLA B27-restricted responses. This does not exclude the possibilities that thymic selection on HLA B27 produces an "arthritogenic TCR repertoire", or that the particular TCR usage of lymphocytes, recognizing an arthritogenic self peptide in the context of HLA B27, results in susceptibility to SA stimulation. It is planned to study the TCR usage (and antigenic specificity) of both CD4+ and CD8+ synovial lymphocytes and PBL from patients with Reactive Arthritis, in order to address the latter possibility.

It remains probable that HLA B27 is directly involved in disease pathogenesis, as shown when similar symptoms develop when rats are made HLA B27-transgenic, albeit with multiple gene copies (Hamer *et al.* 1990). Given recent support for the "arthritogenic peptide" model of disease pathogenesis — with the demonstration of *Salmonella*- and *Yersinia*-specific HLA B27-restricted CTL in the joints of individuals with ReA (Hermann *et al.* 1993), two possibilities, compatible with the data presented in this thesis, suggest themselves. Firstly, the most striking structural feature of HLA B27, its unique B pocket amino acid composition, could alone account for disease, if no other HLA molecule was able to present a specific pathogenic peptide epitope or epitopes, presumably containing an arginine at the second position. Basic proteins such as histones would be expected to be good providers of HLA B27-restricted epitopes.

A second property of HLA B27, which may or may not be related, is its apparent stability within peptide transport deficient cells. This may explain the observation made by (Benjamin and Parham 1991), that empty HLA B27 molecules can bind peptides at the cell surface. Those authors suggested that this might allow HLA B27 to bind extracellular peptides, for example derived from collagens, not normally accessible to the MHC class 1 antigen-processing pathway.

More is now known about the structure and immune function of HLA B27 than about almost any other MHC molecule, giving clues as to its possible role in autoimmunity. However until we obtain

a greater understanding of the behaviour of other MHC molecules, and of the structure and function of T cell receptors that recognize them, it remains unclear whether HLA B27 is a paradigm or a maverick in the field of antigen presentation to T cells.

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