A Molecular Analysis of the T-Cell Receptor

D.Phil. Thesis

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Contents

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Thesis Abstract

The recognition of MHC-peptide ligands by the T cell receptor (TCR) is central to the induction of the adaptive immune response. This thesis describes the development of a bioassay for TCR recognition which was then used to undertake a molecular analysis of the TCR/MHC-peptide interaction.

1. A TCR-CD3ζ chimeric receptor was stably expressed in the cell line RBL-2H3 to give the transfectant RBL-008. RBL-008 was shown to exhibit MHC-restricted peptide-specific responses to both cellular and multimerised recombinant HLA-A2-pol peptide targets (Chapter 3).

2. By competitively inhibiting the response of RBL-008 to HLA-A2 pol complexes with monovalent soluble recombinant MHC-peptide complexes it was confirmed that the TCR makes significant contact with both the MHC and peptide parts of its ligand. Furthermore it was found that only a few peptides in a random mixture can prevent contact between the TCR and HLA-A2. This has implications for positive selection since it supports evidence suggesting that some TCRs can be selected on a wide range of unrelated peptides (Chapter 4).

2. The bioassay was used to examine the flexibility of TCR-peptide interactions using a panel of variant peptides designed on the basis of the previously published HLA-A2-pol peptide structure (Chapter 5). Several variant peptides were recognised by the TCR and interestingly one of these altered peptide ligands was actually recognised better than the index peptide, raising the prospect of designing 'improved epitopes'.

3. By mutating the β chain of TCR-CD3ζ chimeric receptor it was shown that allelic variation in the TCR genes can have a significant effect on antigen recognition and may therefore be disease susceptibility candidates genes (Chapter 6).

4. The structural relationship between the V and C domains of the TCR was examined and found to be of considerable functional significance since disruption of this relationship resulted in loss of expression of the TCR-CD3ζ receptor.

Chapter 1
Introduction

1.1 Adaptive Immune Responses

The immune system is the means by which the body defends itself against infection by the multitude of pathogenetic organisms to which it is exposed. Broadly speaking the components of the immune system can be subivided into those providing innate and adaptive immunity. Innate immunity employs the primarily non-specific activities of complement, natural killer cells and the different classes of phagocyte. In contrast adaptive immunity is distinguished by the induction of a response which is **specifically directed** towards the infecting pathogen. Early demonstrations of adaptive immunity were provided by Von Behring, Kitasato and Ehrlich who showed that animals previously unexposed to diphtheria could be protected by the serum of those who had recovered from infection. This form of adaptive response, now known to be due to antibodies, is termed humoral immunity. A second form of adaptive immune response was later demonstrated by Mackaness who identified cell mediated immunity by showing that resistance to an intracellular bacterium could be adoptively transferred by cells but not serum. Humoral and cell mediated adaptive immune responses are now recognised to be primarily effected by B and T cells respectively although the initiation of both arms of the adaptive immune response relies heavily on the phenomenon of T cell help.

An underlying mechanism for adaptive immune responses was provided by the theory of clonal selection as proposed by

F. Macfarlane Burnett in the 1950s. This theory predicted the pre-existence of a vast array of lymphocytic cells each having the ability to respond to a distinct antigen. Exposure of a lymphocyte to its particular antigen was thought to provoke cellular proliferation resulting in multiple identical clones of the original cell line. Once expanded the selected clone was then able to mount a specific and effective response against the invading pathogen. An inevitable implication of clonal selection is that there must be a mechanism for eliminating those cells which recognise self antigens from the wide panoply of lymphocytes. This phenomenon, known as immunological tolerance, was first demonstrated by Medawar and together with clonal selection it underpins the adaptive immune responses initiated by both B and T lymphocytes.

The ability of lymphocytes to respond to foreign antigens requires a means of specific antigen recognition. In B cells this is achieved through cell surface immunoglobulins which have the same specificity as the soluble antibody molecules produced by the cell. Recognition of pathogenic material by the cell surface molecule triggers expansion and maturation of the B cell clone. T cells also recognise antigen through an immunoglobulin like cell surface molecule, the T cell receptor (TCR). However the nature of the antigen recognised by T cells is quite distinct from that recognised by B cells being in the form of small peptide fragments which are presented in the context of major histocompatability complex (MHC) molecules. This thesis represents an attempt to examine the molecular details of the interaction between one particular TCR and its cognate MHC-peptide ligand.

1.2 The Major Histocompatibility Complex

The MHC was first discovered as a consequence of its influence on foreign tissue graft rejection in mice (Snell 1958) and it was not until later that it became apparent that it also has a vital part in physiological immune responses to invading pathogens. Initial indications came from studies in which B cell responses were shown to be controlled by immune response (Ir) genes that mapped to the MHC (Benacerraf et al 1967, McDevitt & Tyan 1968). Subsequently it was shown that this was an indirect effect and that the MHC-linked Ir genes were infact influencing activation of T helper (Th) cells which then stimulated B cells to produce antibody. The fundamental inter-relationship between the MHC and T cell activation was finally elucidated by the demonstration that these lymphocytes can only recognise foreign antigens in conjunction with self-MHC, a phenomenon known as MHC restriction (Zinkernagel & Doherty 1974a&b). A structural basis for MHC restriction was then provided by a variety of studies showing that T cells recognise peptides and not whole proteins (see next section) and that the self MHC acts a vehicle for the presentation of these peptide antigens to the T cell receptor (discussed below).

The human MHC, also known as the Human Leukocyte Antigen (HLA) system is located on chromosome 6 and consists of hundreds of genes and pseudogenes (Klein 1986, Campbell & Trowsdale 1993) which are divided into three different classes (Fig 1.1). The main class I MHC genes encoded at the HLA-A,-B and -C loci and the class II MHC genes encoded at the HLA-D loci are now known to be those which perform the function of

peptide presentation to T cells. However there are important differences between the class I and II proteins. Class I proteins are expressed on the surface of virtually all cells and present short (8-10 amino acid) peptides derived from cytosolic proteins to CD8 positive cytotoxic T cells (CTL) (reviewed in Townsend & Bodmer 1989, York & Rock 1996). In contrast class II MHC molecules are expressed on 'professional' antigen presenting cells and present longer (12-24 amino acids) peptides derived from extracellular proteins to CD4 positive Th cells, (reviewed in Germain & Margulies 1993, Germain 1994). The different sources of the peptides presented by MHC class I and II molecules represent an important divergence of function for these molecules, allowing both intracellular and extracellular pathogens to be processed and presented to T cells which then initiate an appropriate immune response.

Both MHC class I and II molecules are members of the immunogobulin superfamily. MHC class I consists of a 44kDa membrane bound heavy chain non-covalently associated with a single domain 12kDa β_2-microglobulin (Bjorkman & Parham 1990). The heavy chain of class I comprises three extracellular domains (α1,2 and 3) and a cytoplasmic domain. By contrast the class II molecule consists of a pair of membrane spanning α and β subunits each of which has two extracellular domains. The relationship between MHC class I and II structure and their function as peptide receptors was elucidated with the publication of high resolution crystal structures of representative molecules (MHC class I Bjorkman et al 1987a, Saper et al 1991, and MHC class II Brown et al 1993, reviewed in Madden 1995, shown schematically Fig 1.2a&b). In both

cases the two most membrane distal domains were shown to form a cleft or groove into which the antigenic peptide is bound ready for presentation to T cells. In the case of the class I molecules crystallised to date the peptide binding groove has been shown to be closed at each end commensurate with the role of these molecules in presenting short peptides of relatively finite length. In addition it has been shown that the groove contains a series of six pockets (A to F) which act as binding sites where specific interactions occur between the MHC and peptide side chains (Garret et al 1989 [HLA-A68], Madden et al 1992 [HLA-B27], Smith et al 1996a&b [HLA-B35 and B53], Reid et al 1996 [HLA-B8]). The A and F pockets, located at the ends of the peptide binding groove, are conserved among class I molecules and bind the amino and carboxyl terminals of the peptide respectively (Madden et al 1991). The remaining pockets are located in the central part of the groove and are highly polymorphic (Parham et al 1989). This polymorphism, by modulating the size, shape and polarity of the pockets, determines allele specific differences in the fine structure of the groove and affects the peptides which can be presented by each class I allele. The constraints on peptide binding provided by the presence of the pockets in the groove are reflected in allele specific motifs of peptides eluted from purified class I molecules (Falk et al 1991, Hunt et al 1992a, Henderson et al 1992).

The peptide binding groove in class II MHC molecules is distinct from that of class I in that it is open at both ends reflecting the longer peptides which are bound by these molecules. Although there are also pockets in the groove of

these molecules peptide binding motifs have been harder to define because peptide length is not restricted making alignment difficult (Rudensky et al 1991a, Rudensky et al 1992, Hunt et al 1992b, Rammensee et al 1995, Chicz et al 1992).

Structures of distinct peptides coupled to the same MHC molecule such as those reported for five peptides bound to HLA-A2 (Madden et al 1993) have shown that peptides may adopt widely differing conformations within the peptide binding groove. These variations in structure are predicted to account for the antigenic identity of MHC-peptide complexes by forming part of the potential TCR binding site. The remainder of the TCR binding site is provided by the upward pointing residues on the MHC α-helices which border the peptide binding groove. This supposition is supported by multiple mutational analyses of the MHC which show that antigen recognition by T cells is highly sensitive to changes in the proposed T cell contact residues (Allen et al 1984, Nathenson et al 1986, Jaulin et al 1992, Sun et al 1995, see also Chapter 5). Confirmation of the molecular details of the TCR binding site has recently come with the resolution of the crystal structures of both human and murine TCR/MHC-peptide co-complexes (Garbozci et al 1996, Garcia et al 1996; see in the section on TCR below for further discussion).

1.3 The Generation of T Cell Antigens

Initial studies using the influenza A virus as a model system demonstrated that although CTL were able to lyse cells expressing influenza nucleoprotein the intact protein could not be detected on the surface of these target cells using antibodies

(Townsend & Skehel 1984). The resulting proposition that CTL might recognise protein fragments was confirmed in a series of elegant experiments showing that target cells could be sensitised for CTL lysis either by expressing minigenes encoding protein fragments (Townsend et al 1985) or by being pulsed with peptides (Townsend et al 1986, Bastin et al 1987). These findings, coupled to the structural confirmation that MHC class I bound short peptide fragments (see preceding section for details), predicted an intracellular pathway for the degradation of cytosolic proteins and their subsequent transport into the ER lumen for association with MHC class I molecules. Studies with the murine cell line RMA-S (Townsend et al 1989), which has a defect in antigen presentation, provided the starting point for the identification of the molecular components of the antigen processing pathway. RMA-S cells demonstrated normal synthesis of MHC class I in the ER but a dramatically reduced level of expression on the cell surface. Importantly cell surface expression could be increased by the addition of appropriate peptide epitopes or by culturing the cells at 25°C. These data suggested that MHC class I is unstable in the absence of peptide and that the antigen presenting defect in these cells was due to a failure in either the production of peptides or their transport into the ER. That the defect lay in peptide transport was confirmed by experiments in which class I expression could be restored by infecting cells with a peptide fused to an ER transport signal sequence (Anderson et al 1991). A human cell line 721.174 with a similar phenotype to RMA-S was then found to carry a large deletion in the class II MHC region (Cerundolo et al 1990)

thereby indicating where the missing processing genes were likely to be encoded. Two potential candidate genes, now termed TAP-1 and TAP-2 (transporter associated with antigen presentation) were identified in this region (Spies et al 1990, Trowsdale et al 1990, Deverson et al 1990, Monaco et al 1990) and their role in antigen processing was confirmed when it was shown that transfection of these genes could restore antigen presentation by mutant cell lines (Spies et al 1991, Spies et al 1992). The sequences of these genes showed them to be members of a family of ATP-dependent transporter proteins supporting their predicted role in antigen transport. This prediction was further supported by demonstrations of TAP 1 and 2 heterodimers in the ER (Kleijmeer et al 1992) and confirmed by experiments using streptolysin-O permeabilised cell membranes (Neefjes et al 1993, Androlewicz et al 1993) and microsome fractions. Analysis of antigens transported by the TAPs indicates that these take the form of peptides of fairly restricted length (Schumacher et al 1994, Momburg et al 1994a&b, Heemels & Ploegh 1994) which bind to TAP in an ATP independent fashion and are then released when ATP is bound. Subsequent studies have further elucidated the process of antigen presentation by showing that partially folded MHC class I in the ER is associated with several chaperone proteins such as BiP and calnexin (Degen & Williams 1991) and that the presence of peptide allows complete folding of MHC class I followed by transfer to the cell surface (Degen et al 1992).

Accepting that the TAPs are responsible for transferring peptides into the ER for assembly with MHC class I still leaves open the question of how peptides are generated in the cytosol.

The major route for degrading proteins in the cytosol is ATP-dependent and catalysed by proteasomes and this seems a good candidate for the pathway generating antigenic peptides, particularly since two proteasome subunits (LMP-2 and LMP-7) are encoded in the MHC (York & Rock 1996). The basic active unit of the proteasome is a 20S cylindrical particle composed of four rings (Rechsteiner et al 1993). In mammalian cells, the outer rings are composed of seven distinct α subunits, likely to be primarily structural and regulatory, while the inner rings are composed of seven distinct β subunits that form the catalytic sites (Lowe et al 1995). Strings of unfolded proteins are thought to be inserted into the cylinder and cut by active centres, the resulting peptides are then released into the cytosol. Experiments using purifed proteasomes in vitro have shown that they can generate class-I presented peptides from intact antigens (Dick et al 1994) although this does not prove a role in antigen processing in vivo. Stronger evidence for an in vivo processing role for the proteasome has been obtained by blocking its function with tripeptide and dipeptide aldehydes. These agents inhibit class I presentation of peptides derived from intact proteins without affecting presentation of peptides injected into the cytoplasm (Rock et al 1994).

The MHC encoded proteasome components LMP2 and LMP7 are members of the β subunit family. Although most cells constitutively express low levels of these components, γIFN upregulates their expression implying a function in the immune response. The role of these subunits in antigen processing has been investigated in mutant cells (Arnold et al 1992, Momburg et al 1992, Cerundolo et al 1995) and mice (Fehling et al 1994,

Van Kaer et al 1994) lacking LMP2 or LMP7. The results of these studies have been mixed with initial reports on mutant cells suggesting that neither LMP2 or LMP7 affected antigen presentation (Arnold et al 1992, Momburg et al 1992). More recently transfection of LMP7 into T2 cells has suggested that some defects in the ability of this cell line to present antigen can be corrected by this subunit (Cerundolo et al 1995). Studies with transgenic mice have been more convincing with the LMP7 knockout showing a moderate reduction in class I expression (Van Kaer et al 1994) and the LMP2 deficient mouse having a reduction in CD8 T cells possibly reflecting a defect in thymic selection due to an alteration in the array of thymic peptides (Fehling et al 1994). Analysis of the effect of LMP2 and LMP7 on the cleavage pattern of the proteasome shows that there is an increase in the number of peptides with a basic or hydrophobic N-terminus which should favour the production of peptides likely to be bound by class I molecules. More recently another γIFN inducible proteasome subunit has been identified, PA28 (Dubiel et al 1992, Ma et al 1992). A role for this subunit in antigen processing has been supported by experiments in which it was found that the production of dominant MHC ligands by purifed 20S proteasome was enhanced in the presence of PA28 (Dick et al 1996, see Fig 1.3a for schematic representation of antigen processing and presentation by MHC class I).

While viruses and some bacteria replicate in the cytosol and are processed and presented through the class I pathway other pathogens replicate in the intracellular vesicles of macrophages and are inaccesible to the proteasome. In addition intracellular

vesicles contain proteins internalised from extracellular pathogens. Proteins in these sites are are processed by a second pathway and peptides are presented at the cell surface by MHC class II molecules (reviewed in Germain & Margulies 1993, Germain 1994, see Fig 1.3b for schematic representation of antigen processing and presentation by MHC class II).

Newly synthesised class II α and β chain molecules associate with the invariant chain, li, forming a nonameric $(\alpha\beta li)_3$ complex in the ER (Roche et al 1991). li binds to the class II peptide-binding groove through the CLIP (class II associated li peptide) segment (Frieswinkel et al 1993, Romagnoli et al 1994, Riberdy et al 1992, Vogt et al 1995) thereby protecting the peptide binding groove of class II molecules from premature occupancy by endogeous peptides or by unfolded polypeptides in the ER (Roche & Cresswell 1990, Roche & Cresswell 1991). In addition a targeting motif in the cytosolic domain of li is responsible for delivery of class II αβli complexes from the trans-Golgi network to acidic endosomal-lysosomal organelles (Bakke & Doberstein 1990, Lotteau et al 1990). In these compartments class II molecules encounter peptides generated by enzymatic degradation of self proteins and internalised exogenous antigens (Steinman et al 1983, Chain et al 1988, Takahashi et al 1989, Van Noort et al 1991, Diment 1990, Chicz et al 1993). However loading of class II molecules can not proceed until li has been proteolytically degraded (Roche and Cresswell 1991) and the intermediary associated fragment CLIP removed (Avva et al 1994), an event facilitated by the non-classical class II molecule HLA-DM (Fling et al 1994, Morris et al 1994). Unlike classical class II molecules HLA-DM does not

seem to function as a peptide receptor and it carries a cytoplasmic internalisation signal preventing it from being expressed on the cell surface (Marks et al 1995). Instead, HLA-DM is localised to MHC class II-containing endocytic compartments, termed MIIC, where it transiently binds to HLA-DR (Sanderson et al 1996) and removes CLIP (Denzin & Creswell 1995, Sherman et al 1995) allowing association with antigenic peptides and transport of loaded class II to the cell surface.

1.4 The T Cell Receptor

The demonstration that T cell responses are MHC-restricted (see section on MHC) resulted in considerable speculation about the nature of the TCR. One early model proposed that T cell antigen recognition required two independent receptors for MHC and antigen while a second hypothesis held that only a single receptor existed which was capable of interacting with MHC and antigen simultaneously. The first steps in solving this dilemma and characterising the TCR relied on the production of monoclonal antibodies (mAbs) directed against cloned T cell lines (Staerz et al 1984). Since some of these mAbs reacted only with the immunizing clone it suggested that they were binding a unique molecule on the T cell surface, the function of which was indicated by showing that the clonotypic antibodies could specifically inhibit antigen recognition. The antibodies were then used to immunopreciptate the antigen receptor molecule and show that it was a heterodimer consisting of polypeptide chains termed α and β. The genes encoding these chains were then cloned by a subtractive hybridisation method which

highlighted cDNAs that were present in T cells but not B cells (Hedrick at al 1984a&b Chien et al 1984). Finally, transfection of T cells with the TCR genes was shown to transfer specificity for both MHC and antigen (Dembic et al 1986, Saito et al 1987). This confirmed that a single αβ receptor molecule was sufficient for the recognition of both MHC and foreign antigen and complemented the growing body of evidence indicating that MHC molecules presented small peptide antigens thereby forming a composite T cell ligand.

One important but surprising result of the cloning of the TCR genes was the discovery of a second type of heterodimer, γδ, which is expressed on a small minority of peripheral T cells and the majority of dendritic T cells (Saito et al 1984). The function of the γδ TCR remains unknown while the αβ TCR appears to be responsible for the specificity of the great majority of cytotoxic and helper T cells.

Once the TCR genes were cloned it became possible to make predictions about both the structure of the TCR and the mechanisms for generating sufficient diversity in the TCR repertoire to cover the potentially enormous range of antigens. Perhaps unsurprisingly, given their roles as antigen receptors, the TCR and the Immunoglobulin (Ig) were found to be very closely related (reveiwed in Jorgensen et al 1992a, Davies & Bjorkman 1988). Initial structural impressions of the TCR, based on sequence comparisons, implied that it would resemble an Ig Fab fragment with each chain having a variable and constant domain (Chothia et al 1988, Davis and Bjorkman 1988, Claverie et al 1989). As with Igs the antigen combining site of the TCR was thought to correspond to the area of principal

sequence diversity which is concentrated into three complementarity determining regions (CDRs) within the variable domain (the TCR also has two further hypervariable regions αHV-4 and βHV-4). The mechanism for generating TCR sequence diversity also shows a strong relationship to that employed by Igs. Thus, the variable domain of the TCR is encoded in an array of variable (V), joining (J) and diversity (D) gene segments which, like their Ig counterparts, rearrange to produce complete variable domain exons. The diversity achieved by combining multiple gene segments is further extended by the phenomenon of N-region addition whereby nucleotides are added at the point where V and J segments join in α chains and where V,D and J segments come together in β chains. Since the sites of N-region addition correspond to the CDR3 of the α and β chains of the TCR this means that the principal diversity of the TCR is concentrated here rather than in αβCDRs1 or 2 (Davis and Bjorkman 1988, Jorgensen 1992, Elliot et al 1988, see Chapter 6 for a fuller discussion of TCR genes and generation of diversity). An important difference from Igs is the lack of evidence for somatic hypermutation in the TCR genes.

The observation that the αβCDRs3 of the TCR are more diverse than αβCDRs1 or αβCDRs2 suggested that the αβCDRs3 are likely to be the point of contact between the TCR and the antigenic peptide, since this is the most variable part of the MHC-peptide complex. This model received strong support from a number of sources (Engel & Hedrick 1988, Nalefski et al 1992, White et al 1993, Katayama et al 1995) including elegant studies in single TCR chain transgenic mice which showed that

altering the sequence of an immunising peptide caused compensatory changes in the CDR3 of the non-transgenic TCR chain (Jorgensen et al 1992b). Although the consensus is that the αβCDRs3 of the TCR contact the antigenic peptide the precise topological relationship between the TCR and its ligand has remained a point of debate (Chien & Davis 1993). Early models proposed that the αβCDRs 1 and 2 ran parallel to the MHC α-helices (Chothia et al 1988, Davis and Bjorkman 1988, Claverie et al 1989, Hong et al 1992) while more recent data suggest that the TCR lies diagonally across the MHC α-helices (Sun et al 1995, Sant'Angelo et al 1996). Confirmation of the nature of the TCR antigen combining site and the topology of the TCR/MHC-peptide interaction has recently been provided with the long-awaited solution to the TCR crystal structure (Garcia et al 1996a, Garboczi et al 1996, see Fig 1.4) Two TCR/MHC-peptide co-complexes have been solved to date, the first of these shows a murine TCR complexed with an H-2kb-dEV8 peptide ligand (Garcia et al 1996a) and the second a human TCR complexed with an HLA-A2-tax peptide ligand. Both structures confirm that the αβCDRs make up the antigen combining site. As predicted the main TCR-peptide contact involves the αβCDRs3 however there is significant contact between the termini of the peptide and the αβCDRs1 and 2 (with the exception of the βCDR2 in the human receptor). Interestingly both TCRs have a similar diagonal orientation with respect to the MHC which is dictated by the N-terminal peaks of the MHC α-helices (see Chapters 4,5 & 6 for more detailed discussion of TCR structure). This fits reasonably with some of the more recent mutational data (Sun et al 1995,

Sant'Angelo et al 1996) and suggests that there might be a common binding mode between the TCRs and MHC-peptide complexes. In terms of the overall structure of the TCR it does indeed have much similarity with an Ig Fab fragment although the Cα domain is quite distinct from the usual Ig type structure (Garcia et al 1996a).

1.5 T Cell Activation

The TCR is associated on the cell membrane with the CD3 protein complex, which is required for signal transduction since the TCR heterodimer does not have the facility for direct signal transmission (Weismann et al 1988, Weiss & Stobo 1984). Five chains have so far been identified in the CD3 complex and they are referred to as γ,δ,ε,ζ and η (Clevers et al 1988). The CD3 γ,δ,ε chains are closely related proteins and consist of a single extracellular Ig domain and a cytoplasmic tail. In contrast the ζ and η subunits, which are products of a single alternatively spliced transcript, form disulphide linked dimers and consist of a tiny extracellular domain and a large cytoplasmic domain. Mutational analysis of the cytoplasmic domains of the CD3 molecules has identified a tyrosine rich motif called the immunoglobulin receptor family tyrosine-based activation motif (ITAM) which is crucial to the transmission of signals through the TCR (Reth 1988). One copy of this motif is present in the γ,δ,ε chains while three copies exist in the ζ chain. The initial signalling event elicited by the TCR is activation of the src family protein tyrosine kinases (PTKs) $p59^{fyn}$ and $p56^{lck}$, (reviewed in Cantrell 1996, Samelson & Klausner 1992) which phosphorylate the tyrosine residues of the ITAMs (Samelson et

al 1986) leading to recruitment, tyrosine phosphorylation and activation of another PTK, the 70-kD zeta-associated protein (ZAP-70) (Iwashima et al 1994, Weil et al 1995). ZAP-70 binds to the phosphorylated ITAMs through its two SH2 domains (src family kinases have only one SH2 domain, an SH3 domain and a myristylation signal). The subsequent intracellular events following activation of protein kinases are complex and lead to the activation of phosphatidylinositol-phopholipase-C, elevation of cytoplasmic free calcium and activation of multiple serine/threonine kinases (reviewed in Cantrell 1996).

Although recognition of MHC-peptide complexes by the TCR is the central and specific event in T cell activation a number of other accessory molecules also play an essential part in this process. Some of these accessory molecules, such as CD2 (CD=cluster of differentiation) and CD11a/CD18 and their respective ligands CD58 and CD54 promote inter-cellular adhesion thereby facilitating initial contact between the TCR and MHC-peptide (Shaw & Luce 1987, Spits et al 1986, Springer et al 1987, Holter et al 1996) although other data suggest that these molecules may also potentiate intracellular signals (Meuer et al 1984, Bierer & Burakoff 1989). Another class of accessory molecules are the CD8 and CD4 co-receptors which identify subsets of T cells through their ability to bind MHC class I and II molecules respectively (Parnes 1989, Janeway 1992). T cells carrying the CD8 co-receptor recognise MHC class I-peptide complexes and are primarily responsible for cytotoxic T cell function, while those expressing CD4 respond to MHC class II-peptide complexes and have T helper cell activity. The contribution of these co-receptors to TCR/MHC-peptide

interactions is reflected in their ability to increase the sensitivity of T cells to antigenic stimulation (Janeway 1988, Parnes 1989), however there is still debate as to exactly how this occurs. The fact that both molecules are linked to $p56^{lck}$ suggests that they enhance signal transduction (Spits et al 1986) although recent binding data generated using a soluble CD8 molecule shows that this co-receptor also has a stabilising effect on the TCR/MHC-peptide complex (Garcia et al 1996b). A contribution to T cell activation is also made by CD45 an accessory molecule with tyrosine phosphatase activity which is thought to activate $p59^{fyn}$ and $p56^{lck}$ (Janeway 1992). While the accessory molecules described above are critical in the stimulation of all T cells it has been shown that *naive* T cells require the delivery of a TCR-independent signal to differentiate into activated Th cells/CTL which have respective roles in initiating the adaptive immune response and destroying virally infected cells (reviewed in Grewal & Flavell 1996, Van Gool et al 1996). The molecules involved in delivering this so called 'second signal' are the T cell antigens CD40L (Grewal & Flavell 1996) and CD28 (June et al 1990, Harding et al 1992) and their respective ligands on APCs CD40 and CD80/CD86 (Linsley et al 1991). The function of these molecules is summarised by the two step model of T cell activation (Fig 1.5ab&c) in which engagement of the TCR by its MHC-peptide ligand leads to upregulation of CD40L which transmits a signal, via CD40, to the APC. APCs then upregulate expression of the costimulatory molecules CD80 and CD86 which provide a vital secondary signal to T cells through their interaction with CD28. The absence or blockade of the 'second

signal' from CD80/86-CD28 pathway has been shown to result in apoptosis (Webb et al 1990) or anergy (Jenkins et al 1988) instead of activation of T cells.

1.6 Altered Peptide Ligands for the TCR

Recently a series of observations have shown firstly that the TCR is not strictly peptide specific (Bhardwaj et al 1993, Nanda et al 1995, Hagerty & Allen 1995, Evavold et al 1995, Wucherpfennig & Strominger 1995) and secondly that peptide variants can lead to altered T cell responses (reviewed in Evavold et al 1993, Jameson & Bevan 1995, Sloan-Lancaster & Allen 1996). Functionally distinct peptide variants or altered peptide ligands (APLs) were first identified by making single amino acid substitutions in peptide antigens and showing that these peptide variants could still elicit a functional response from T cell clones (Evavold & Allen 1991). However the response of the T cell differed from that provoked by the cognate (agonist) peptide in that some but not all T cell functions could be induced. APLs which partially activate T cells are termed partial agonists. Subsequent to the initial demonstration of partial T cell activation, the phenomenon of T cell antagonism was described in which simultaneous presentation of agonist and certain nonstimulatory peptides to T cells resulted in peptide specific down-modulation of T cell responses to agonist peptides (De Magistris et al 1992). The intracellular mechanism by which partial agonism and antagonism arise is still the subject of investigation (reviewed in Sloan-Lancaster & Allen 1996) however, a comparison of early signalling events after TCR ligation by either agonist or

partial agonist peptides showed that partially activating APL are not able to activate phospholipid hydrolysis but are able to stimulate specific intracellular tyrosine phosphorylation events (Sloan-Lancaster et al 1994). Interestingly the pattern of tyrosine phosphorylation was distinct from that induced by either the agonist peptide or a non-stimulatory control. More specifically it was demonstrated that the three classes of ligand gave rise to distinct phosphorylated forms of the TCR CD3ζ chain. These observations were subsequently confirmed for other partial agonists and also for antagonists (Madrenas et al 1995). On the basis of these observations partial activation of T cells by APLs is proposed to result from inefficient activation/recruitment of src kinases leading to incomplete phosphorylation of CD3 ITAMs resulting in a failure to bind ZAP-70. This leads to activation of some downstream pathways but not others (Fig 1.6a&b).

An important unresolved issue relating to the generation of differential T cell responses/signalling by APLs is how the TCR transmits subtle differences in ligand recognition across the cell membrane. Two non-mutually exclusive models have been proposed; qualitative hypotheses suggest that the TCR must undergo a conformational change in order to transmit a signal across the cell membrane while quantitative hypotheses suggest that partial T cell activation might be related to ligand affinity or kinetic factors (Sykulev et al 1995, Rabinowitz et al 1996). Currently there is no direct evidence to support qualitative models although there is some circumstantial data which has been generated using anti-TCR antibodies (Janeway et al 1989, also see chapter 4). In contrast, quantitative models

have been extensively examined with the elucidation of the biophysical characteristics of TCR/MHC-peptide interactions. It is now established that the affinity of the TCR for cognate MHC-peptide complexes is quite low with a Kd ranging between 10^{-4} and 10^{-7} M and a commensurately rapid dissociation rate (Fremont et al 1996). These values have been obtained by a variety of different methods (see Chapter 3 for a more detailed discussion) including the inhibiton of T cell hybridomas with soluble MHC-peptide (Schneck et al 1989) or soluble TCR (Weber et al 1992), competition between soluble MHC-peptide and anti-TCR Fabs (Matsui et al 1991, Sykulev 1994a), direct binding of soluble MHC-peptide to CTL (Sykulev et al 1994b) and, most significantly, the use of surface plasmon resonance technology (reviewed in Margulies et al 1996, Corr et al 1995, Matsui et al 1994, Khilko et al 1995, al-Ramadi et al 1995). Using surface plasmon resonance technology it has recently been demonstrated that APLs have either a lower affinity for the TCR (Alam et al 1996) and/or a more rapid dissociation rate (Lyons et al 1996) than the agonist peptide although it is not clear which is the better correlation. The relationship between the affinity and kinetics of TCR/MHC-peptide interactions, quantitative models of T cell activation and variable responses to APLs has found a potential explanation in two recent observations. Firstly it was shown that a small number of MHC-peptide complexes on an APC are able to serially engage multiple TCRs leading to TCR internalisation (Valitutti et al 1995). Subsequently it was found that T cell activation can be related to the internalisation of a threshold number of TCRs (Viola & Lanzavecchia 1996). Therefore the low affinity/fast

dissociation rate characteristic of TCR/MHC-peptide interactions favours T cell activation by allowing sequential engagement of multiple TCRs. In contrast high affinity ligands such as anti-TCR mAbs have been shown to be less efficient than MHC-peptide complexes in the activation of T cells because they are unable to disengage from the TCR (Viola & Lanzavecchia 1996). This model can also be extended to account for partial T cell activation by MHC-APL complexes by proposing that the even lower affinity of these ligands means that they are unable to reach the threshold affinity for TCR internalisation despite being able to serially engage multiple TCRs. An additional implication of the studies showing serial engagement of TCRs by MHC-peptide complexes is that the very low numbers of MHC-peptide complexes required for stimulation means that it is numerically very unlikely that T cell activation requires TCR oligomerisation. This goes against much of the early data generated by mAbs (Janeway et al 1989) and non-MHC-peptide TCR ligands (Symer et al 1992) which suggested that the TCR had to be multimerised in order to deliver a signal.

A number of studies have shown that APL have direct biological relevance both in selection of the developing thymocyte and in modulating responses of mature T cells in the periphery. Thymic selection results in the maturation of T cells recognising foreign antigens, but not self peptides, bound to self-MHC molecules. Positive selection allows cell survival (reviewed in Jameson et al 1995, see chapter 4 for a fuller discussion) while negative selection leads to cell death (reviewed in Nossal 1994). Both processes require engagement of the TCR by MHC-peptide complexes but it is the peptide that

determines the outcome. The development of in vitro culture models to study thymic selection has identified peptides that can induce either positive or negative selection. (Hogquist et al 1993, Hogquist et al 1994, Jameson et al 1994, Ashton-Rickardt et al 1994, Sebzda et al 1994). Interestingly the peptides which are most effective in positively selecting a given TCR are frequently those APLs with partial agonistic or antagonistic effects (Hogquist et al 1994) on the mature T cell, while agonistic peptides lead to negative selection. This suggests that the outcome of thymic selection is likely to be the result of the affinity/avidity of TCR/MHC-peptide interactions and supports concurrent findings showing that increasing ligand density and thereby increasing the avidity of TCR/MHC-peptide interactions can convert a positively selecting ligand to a negatively selecting one (reviewed in Allen 1994, Ashton-Rickardt et al 1994). The relationship between TCR-ligand affinity and thymic selection has been further reinforced by surface plasmon resonance experiments demonstrating a positive correlation between the outcome of selection and ligand affinity (Alam et al 1996). The mechanism whereby ligand affinity is coupled to outcome in selection is thought to resemble the situation in the periphery such that low affinity APLs activate a distinct pathway from higher affinity ligands which in the the thymocyte lead to positive and negative selection respectively. In the periphery APLs may have a range of effects on T cell behaviour including modulation of immune responses in autoimmune disease (Windhagen et al 1995), provision of viral escape strategies (Klenerman et al 1994, Bertoletti et al 1994) and maintenance of T cell memory.

1.7 Thesis Aims

There are three principle aims of this thesis

1. The development of a reproducible bioassay for the study of TCR/MHC-peptide interactions (Chapter 3).

2. The use of this bioassay to examine the details of the TCR/MHC-peptide interaction with respect to:

a) The relative contributions of peptide and MHC to TCR engagement (Chapter 4).

b) The flexibility of TCR-peptide interactions and the design of altered peptide ligands (Chapter 5).

c) The effect of allelic variation in the TCRV genes on the recognition of MHC-peptide (Chapter 6).

3. The use of this bioassay to examine the functional implications of the close structural association between the V and C domains of the TCR α and β chains (Chapter 7).

Each chapter contains an introduction, a results section and a discussion of the results. Chapter 2 contains all the methodology and Chapter 8 summarises the contents of the thesis and puts the results in the context of the field of TCR biology.

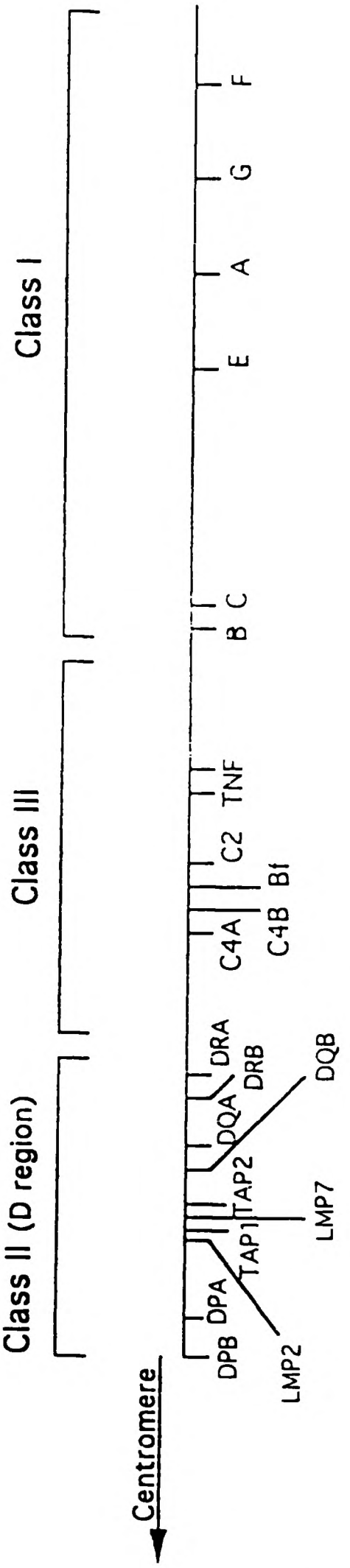


Fig 1.1
The organisation of the human MHC on chromosome 6.
Locations of the the major loci of the class I, II and III regions are shown. (Adapted from Campbell & Trowsdale 1993).

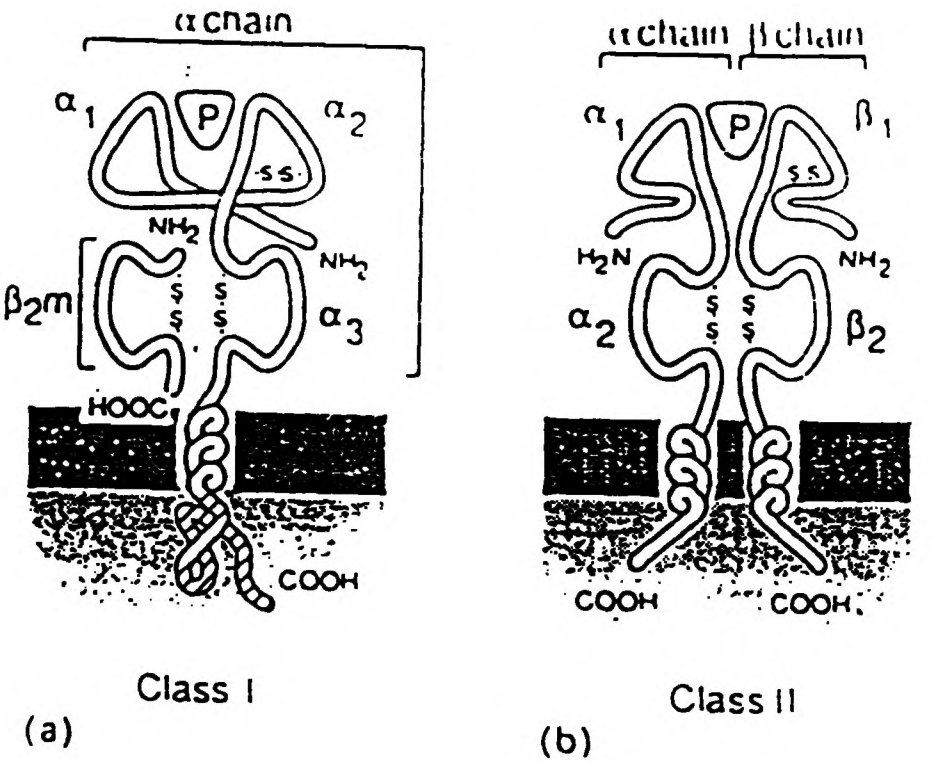


Fig 1.2a&b
Schematic representation of the structures of (a) MHC class I and (b) MHC class II.
Class I molecules are composed of a 45kD heavy chain and the 12kD β2m. The peptide binding site is formed by a groove between the α1 and α2 helices of the heavy chain. Class II molecules are composed of a 33-35kD α chain and a 25kD β chain, each chain contributes an α-helix to the peptide binding groove. (P=peptide).

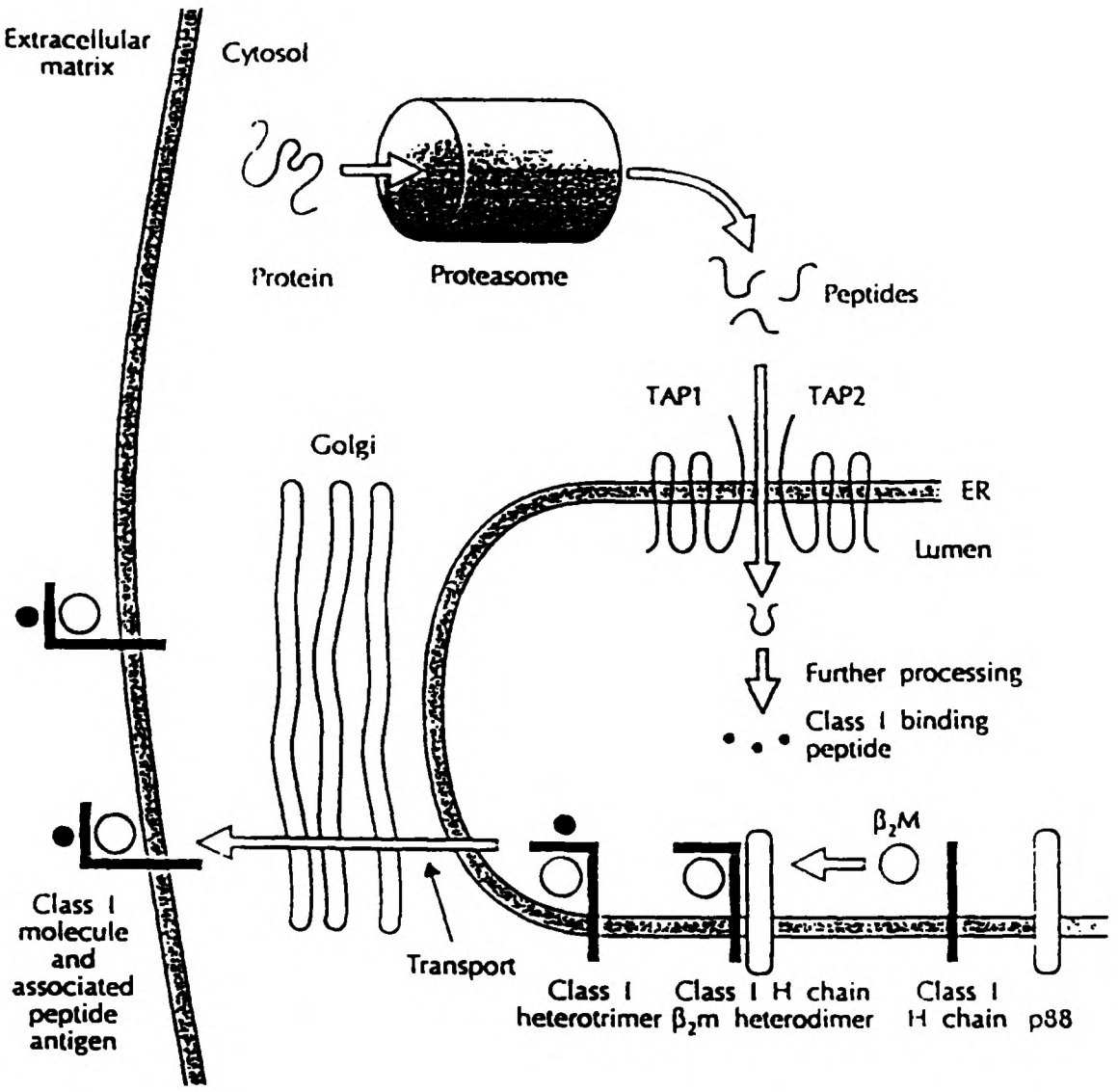


Fig 1.3a
Schematic model of antigen processing and presentation by MHC class I proteins.
Peptides for presentation by class I proteins are generated in the cytosol, most likely through ATP-dependent degradation by the proteasome. The resulting peptides are then transported into the ER by the TAP1/TAP2 heterodimer. The peptides are then loaded onto class I heavy chain/β2m dimers to form a heterotrimer which is transported through the Golgi apparatus to the cell membrane.

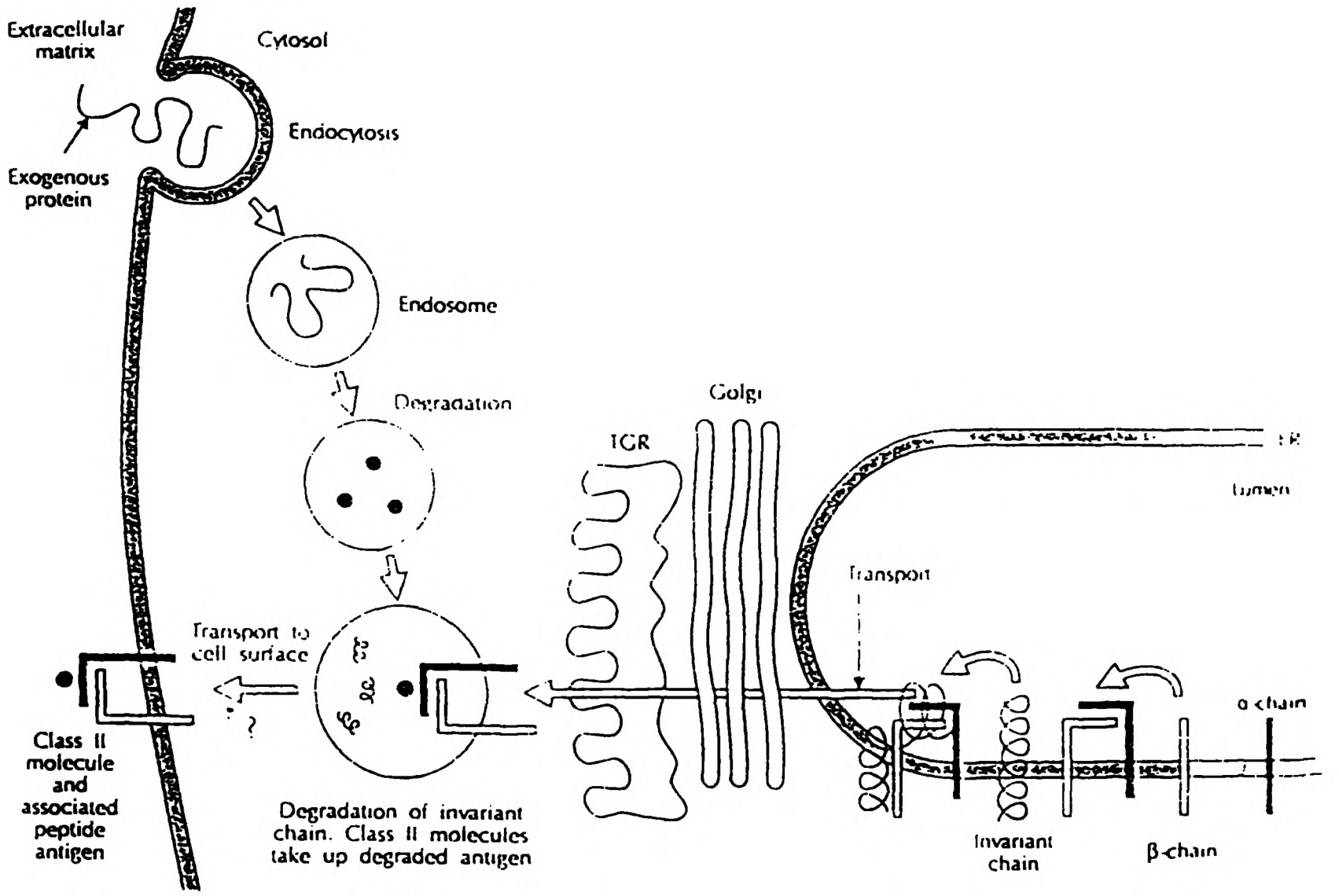


Fig 1.3b
Schematic model of antigen processing and presentation by MHC class II proteins.

In the class II pathway the α and β chains form a heterotrimer in the ER with the invariant chain, which occupies the peptide binding groove. Although it is not shown here three of these heterotrimeric complexes actually associate with one another. Complexes pass through the Golgi apparatus to a compartment in the endocytic pathway where extracellular proteins are degraded to peptides. HLA-DM (not shown) then facilitates removal of the invariant chain derived peptide, CLIP, from the peptide binding groove of the class II molecule which is now free to bind processed peptides and move the the cell surface.

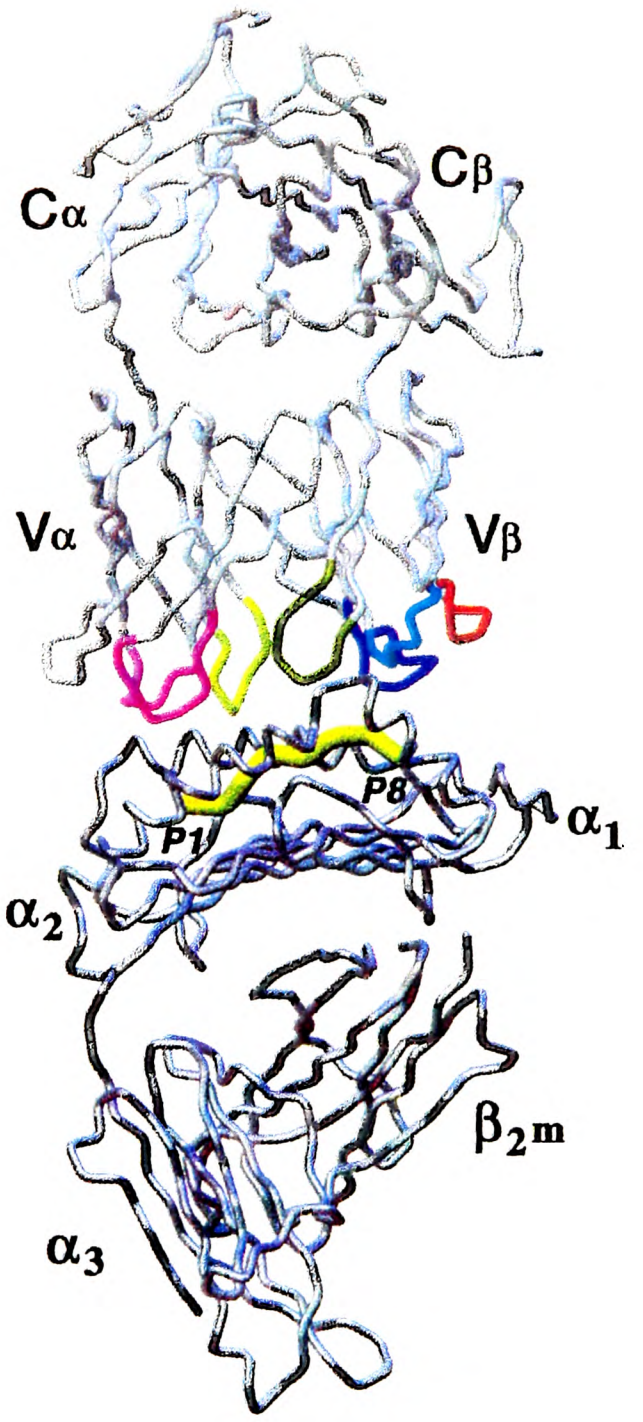


Fig 1.4
Backbone tube representation of the murine 2C-TCR/H-2Kb-dEV8 co-complex.

The peptide is in yellow, the αCDRs 1&2 are in pink, the βCDRs 1&2 in blue, the αβCDRs3 in yellow, the αHV-4 in white and the βHV-4 in orange. (Adapted from Garcia et al 1996a).

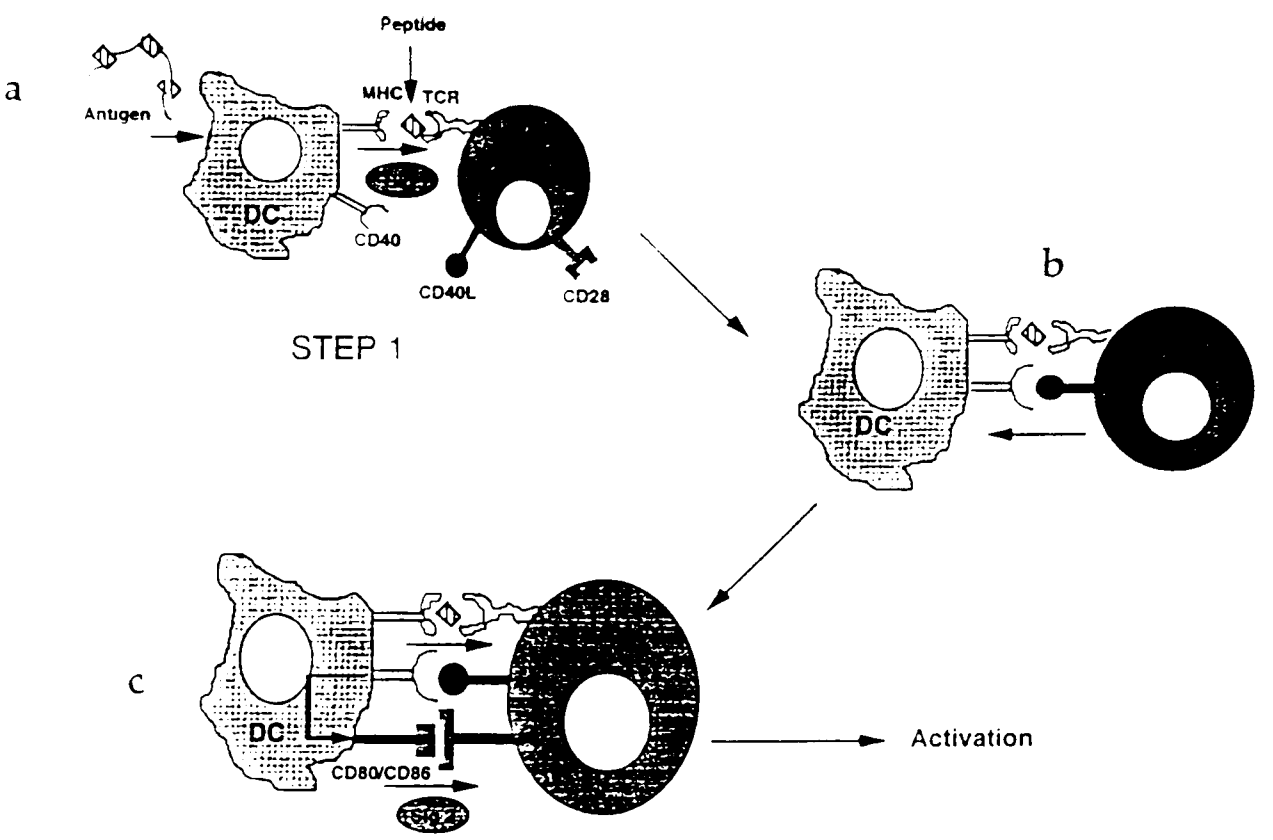


Fig 1.5a,b&c
The two step model of T cell activation.
(a) In the first step exogenous antigens are taken up by professional APCs and presented to naive CD4+ T cells in the context of MHC class II. The interaction between these MHC-peptide complexes and the TCR delivers the first signal to the T cell and leads to upregulation of CD40L. (b) CD40L interacts with CD40 on the APC which induces expression of the costimulatory molecules CD80/86 on the surface of the APC. (c) CD80/86 interact with CD28 on the T cell delivering the second signal and prompting full activation. (Adapted from Grewal & Flavell 1996).

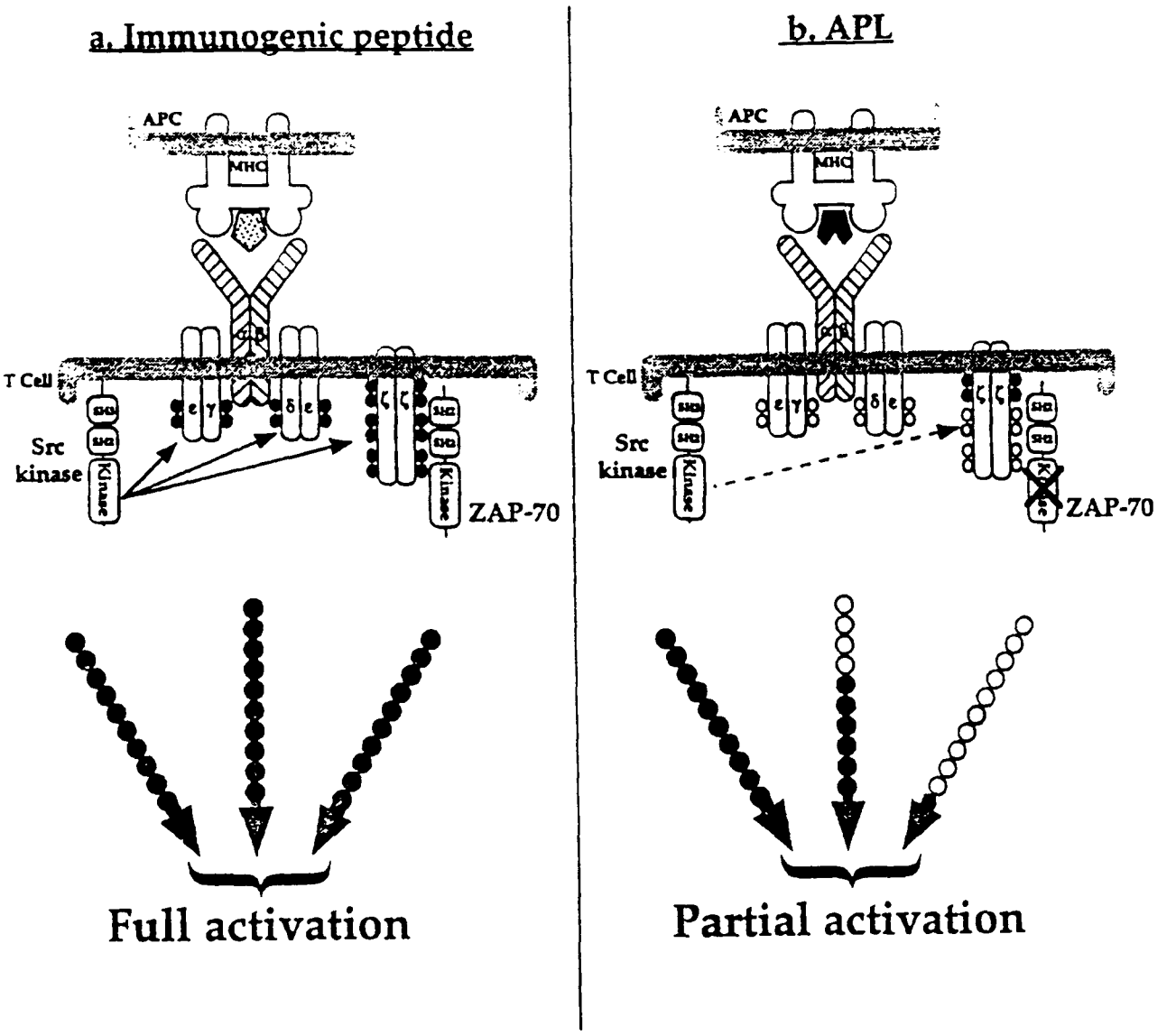


Fig 1.6a&b
Model for partial T cell activation induced by APL.
(a) TCR ligation by the cognate ligand leads to activation of src family kinases, phophorylation of CD3 ITAMs, recruitment of ZAP-70 and full activation. (b) TCR ligation by and APL leads to inefficient activation of src family kinases and incomplete phosporylation of CD3 ITAMs leading to a failure to recruit some SH-2 domain containing proteins such as ZAP-70. (Adapted from Sloan-Lancaster & Allen 1996).

Chapter 2
Materials and Methods

2.1 Bacterial Work

2.11 Media

PBS (phosphate-buffered saline) is 50 mM KH_2PO_4/K_2HPO_4 pH 7.3, 150 mM NaCl. LB (Luria Bertani broth) is 10 g bacto-tryptone, 5 g bacto-yeast extract, and 10 g NaCl per liter. 2xTY broth is 16 g bacto-tryptone, 10 g bacto-yeast extract, and 5 g NaCl per liter. Minimal broth is 2 mls 1 M $MgSO_4$, 20 mls 20% glucose, 0.1 ml 1 M $CaCl_2$, 200 mls M9 salts per liter. M9 salts are 64 g Na_2HPO_4-$7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, and 5 g NH_4Cl per liter. All media was autoclaved before use. LB-agar plates included 15 g/L bacto-agar.

2.12 Bacterial Strains

E. coli strains used were: DH5α for general plasmid preparations and XA90 for protein expression, CJ236 and TG1 for mutagenesis. Frozen stocks of naive, transformed, or infected bacteria were maintained at -70°C with 15% glycerol. Working concentrations of antibiotics were 100 μg/ml ampicillin, and 12.5 μg/ml tetracycline. Unless otherwise specified bacterial cultures were grown at 37°C with rapid shaking (250 rpm). Competent bacteria were prepared by growing bacteria in LB broth until mid-log phase (OD_{600} 0.6). Bacteria were centrifuged and resuspended in 0.2 volumes of ice cold TFB 1 (Transforming buffer 1 30mM KOAc, 50mM $MnCl_2$, 100mM KCl, 10mM $CaCl_2$). Bacteria were centrifuged a second time and

resuspended in 0.2 volumes of ice cold TFB2 (Transforming buffer 2 10mM Na-MOPS pH7.0, 75mM $CaCl_2$, 10mM KCl, 15%v/v glycerol).

2.2 Molecular Biology

2.21 DNA Preparation

Minipreparations of plasmids were performed according to manufacturer's protocols using a kit obtained from Qiagen. Midipreparations of plasmids for transfections were performed by alkaline lysis. A 50ml overnight culture in LB broth was centrifuged for 10mins at 4°C. The pellet was then resuspended in 4ml ice cold GTE (50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA). The bacteria were then lysed in 8ml of 0.2M NaOH, 1% SDS, mixed well and left on ice for 5mins. Next 6ml of neutralising solution was added (29.4g KOAc, 11.5ml glacial acetic acid in 100ml H_20) followed by centrifugation for 15mins at 4°C. The supernatant was collected into a fresh tube and 17ml of propan-2-ol was added. The tube was chilled on dry ice for 15mins. Following further centrifugation for 15mins the pellet was resuspended in TE (10mM Tris-HCl pH7.5, 0.1mM EDTA). Next 2.5ml of 4.4M LiCl was added and this was left on ice for at least 10mins. Further centrifugation at 4000rpm for 10 mins removed precipitated RNA and the supernatant was collected and added to 10ml of absolute ethanol. This was left at room temp for 15mins and then centrifuged as before to collect the DNA. The pellet was resuspended in 400µl of TE and treated with 4units of DNase free RNase (Boehringer) for 15mins at 37°C. The DNA was then extracted once each with phenol, phenol/chloroform/isoamyl alcohol (100:96:4) and

chloroform and then precipitated with 0.1 volumes of 2.5M NaOAc and 3 volumes of absolute ethanol. Plasmid concentrations and purity were determined by their absorbance at 260 nm and 280 nm and were stored in distilled water or TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

2.22 Restriction digests, Gel purification, and Ligations.

Restriction enzymes (New England Biolabs or Promega) were used in 4 hour digestions with the manufacturer's buffers. DNA gels were prepared with 0.8%-2.0% agarose and were run at 80 V in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). 10 µg/ml ethidium bromide was included in the gel so that bands could be visualised with ultraviolet light. Sample buffer was 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol. DNA was gel purified from agarose gels under long wave ultraviolet light by cutting into the gel infront of the DNA and inserting a fragment of moist DEAE-cellulose paper (Whatman) backed with dialysis membrane. The DNA fragment was then electrophoresed onto the paper which was centrifuged to collect the DNA. Finally the DNA was cleaned up in a phenol extraction, excess phenol was removed by passage over a TE equilibrated G25 spin column. Ligations were performed with T4 DNA Ligase (Promega) usually with a 3:1 insert to vector ratio overnight at 16°C. Transformation of plasmids into bacteria was performed by incubating 50 µl competent cells with the DNA for 5 min on ice, heating for 5 min at 37°C and plating on agar plates containing selective antibiotics.

2.23 DNA Sequencing

Both dsDNA and ssDNA sequencing were performed using the dideoxy chain termination method (Sanger et al. 1977) with the Sequenase reagents and protocols (US Biochemicals). dsDNA templates were prepared by minipreparations of plasmids and were then denatured according to the manufacturer's protocols (US Biochemicals). ssDNA templates were prepared from constructs cloned into pBluescript (Stratagene). A single colony was innoculated into 1.6ml of LB containing ampicillin. After 1hr shaking at 37°C as culture became slightly turbid 1µl of helper phage R408 (Stratagene) was added. After shaking for a further 5hrs at 37°C the culture was transferred to an Eppendorf tube and spun in a microfuge for 10mins 1ml of supernatant was then removed to which 250µl of 2.5M Na Cl, 20% PEG was added. After 15mins at room temp the pellet was collected by microcentrifugation. The pellet was then resuspended in 100µl of TE and extracted with 60µl of phenol. The pellet was then precipitated in 6µl of 3M NaOAc and 200µl of absolute ethanol and resuspended in 40µl of TE. DNA sequencing gels (6% acrylamide, 7M urea in TBE buffer [45 mM Tris-borate, 1 mM EDTA]) were prepared with 100 ml SequaGel (Severn Biotech) solution supplemented with 900µl 10% APS (ammonium persulfate) and 30µl TEMED (*N*, *N*, *N'*, *N'*-tetramethylethylenediamine), and were run at 80 W in TBE buffer after a 1 hour pre-run. Gels were fixed in 10% acetic acid, 10% methanol, dried onto filter paper (Whatman 3MM), and exposed to autoradiographic film overnight.

Primer sequences: Oligonucleotides were synthesised by Genosys inc.

ACSEQ1 (5' AAT ATC CAG AAC CCT GAC CC 3')

ACSEQ2 (5' TTC AAC AAC AGC ATT ATT CC 3')

ACSEQ3 (5' GG GTC AGG GTT CTG GAT ATT 3')

ACSEQ4 (5' AGA ATC AAA ATC GGT GAA TA 3')

ACSEQ5 (5' ACA GGA ACT TTC TGG GCT 3')

ACSEQ6 (5' T GTT GCT CTT GAA GTC C 3')

BCSEQ1 (5' GAG GAC CTG AAA AAC GTT TTC 3')

BCSEQ2 (5' GAG AAT GAC GAG TGG ACC CA 3')

BCSEQ3 (5' GAA AAC GTT TTT CAG GTC CTC 3')

BCSEQ4 (5' AG TGT GGC CTT TTG GGT GTG 3')

BCSEQ5 (5' ACA GTC TGC TCT ACC CCA 3')

BCSEQ6 (5' TC ATT GAG GGC GGG CTG 3')

Primers supplied by stratagene with pBluescript

REVERSE (5' GGAAAC AGC TAT GAC CAT G 3')

T3 (5' AAT TAA CCC TCA CTA AAG GG 3')

2.24 DNA Mutagenesis

Mutagenesis was performed according to previously described methods (Kunkel 1985) using the Boehringer mutagenesis kit. Briefly single stranded uracil containing DNA was prepared in bacterial strain CJ236 following the method described above for producing ssDNA (see DNA sequencing). 200pmol of mutagenesis primer were kinased for 30min at 37°C in a volume of 30µl including 3µl of 10X kinase buffer (NEB), 3µl of 10mMATP using 1µl of T4 polynucleotide kinase. 1µl of the kinase reaction was then annealed to 300-400ng of ssDNA by heating in a hot block to 70°C for 5min and cooling to room temperature. The total volume for the annealing step was 20µl including 2µl of 10X annealing buffer (200mmol/l Tris-HCl,

20mmol/l $MgCl_2$, 500mmol/l NaCl, pH7.75). Second strand synthesis was initiated by adding 2µl of synthesis buffer (100mmol/l Tris-HCl, 50mmol/l MgCl2, 20mmol/l Dithierythriol, 5mmol/l dATP, 5mmol/l dCTP, 5mmol/l dTTP, 10mmol/l dATP, pH7.75), 1µl of T4 DNA polymerase and 1µl of T4 DNA ligase to the annealing reaction for 90mins at 37°C. The synthesis reaction was stopped by adding 80µl of stop solution (10mmol/l Tris-HCl, 10mmol/l EDTA pH8.0). 10µl of the final reaction was used to transform E.coli strain TG1. Plasmid DNA was isolated from 10 colonies by minipreparation. Screening for succesful mutagenesis was by restriction digest (where possible) and DNA sequencing.

Primer sequences: Oligonucleotides were synthesised by Genosys inc.

BV1S1A1N1 (5'GGC CTC CAG TTC CTC ATT CAA TAT TAT AAT GGA GAA GAG 3')

AV2S1A2GLY (5'TTG ACT GTC CAT CCA GGA AAT ATC CAG AAG CCT GAC CCT GCC 3')

BV1S1GLY (5' ACC CGG CTC ACA GTT CTC GGA GAG GAC CTG AAA AAC GTC TTC 3')

ACEAG (5' AAT ATC CAG AAC CCT GAC CCG GCC GTG TAC CAG CTG AGA G 3')

BCAFL (5' CTC ACG GTC ACA GAG GAC CTT AAG AAC GTT TTC CCA CCC GAG GT 3')

AV10S2DELC (5' CT AAA CTC TCT GTT AAA CCA GGG CCC GAT CTG GTT CCC CGG GGA TC 3')

BV17S1DELC (5' GC ACC AGG CTC ACG GTC ACA GGG CCC GAT CTG GTT CCC CGG GGA TC 3')

2.25 Polymerase Chain Reaction (PCR)

PCR reactions were performed with *Taq* or *Pfu* thermostable DNA polymerases (Stratagene), synthetic oligonucleotide primers, and templates of cDNA or plasmid DNA. 50 µl reactions included 0.5 µl 1 M Tris-HCl pH 8.3, 0.5 µl dNTP's (20 mM each), 2.5 µl 1 M KCl, 7.5 µl 10 µM $MgSO_4$, 0.5 µl polymerase enzyme, and 2 µl each of the 5' and 3' primers (at 50 ng/µl). A drop of mineral oil was used to overlay the reaction tubes. A Perkin Elmer Cetus temperature cycler was used to heat the samples for 5 min at 94°C and then to cycle 25-30 times at 94°C for 1 min denaturation, 1 min at an annealing temperature 5°C below that of the primer with the lower annealing temperature (according to the formula Tm= 4°C per C/G plus 2°C per A/T), and 72°C for 1 min extension. A final 72°C extension was performed at the end of the cycling steps, and the crude PCR products were gel purified. For PCR using *Pfu* thermostable DNA polymerase the denaturation temperature was 97°C and the extension time was 10min.

Primer sequences. Oligonucleotides were synthesised by Genosys inc. **AV2S1A2F** (5' CAC CGC TCG AGC CGC CAT CAT GAT GAA ATC CTT GAG A 3'),

AV2S1A2B (5' CTG GTA CAC GGC CGG GTC AGG GTT CTG GAT ATT 3'),

BV1S1F (5' ATG CAA GTC AGT CGA CCC GCC ACC ATG GGC TTC AGG CTC CTC T 3')

BV1S1B (5' CAC AGC GAC CTC GGG TGG GAA CAC GTT CTT AAG GTC CTC 3')

AV2S1A2CB (5' AAA CGT TCT TAA GGT CCT CTG GAT GGA CAG TCA AGA TGG 3')

BV1S1CA (5' GTA CAC GGC CGG GTC AGG CTT CTG GAT ATT GAG AAC TGT CAG CCG GGT G 3')

2.3 Protein Chemistry

2.31 Fmoc Peptide Synthesis

Four peptides (pol, polA8, polT8, polE8) were obtained from the Oxford Centre for Molecular Sciences and were purified by reverse-phase HPLC and checked by mass spectrometry. The other peptides were synthesized with the assistance of J.Wyer using standard Fmoc chemistry on a Zinsser Analytic SMPS 350 peptide synthesizer. 0.025 mmol resin carrying the carboxy-terminal amino acid was added to each reaction vessel, and a 7-fold molar excess of the amino acids were used in the reactions. Each cycle of the synthesis included washing the resin 5 times with 750 µl DMF (N, N'-dimethylformamids; Rathburn), deprotecting the Fmoc group on the last amino acid with 500 µl DMF/40% piperidine (Aldrich) for 20 min, washing 10 times with DMF, and coupling 350 µl of the next amino acid (dissolved in DMF/0.5 M HOBT [1-hydroxybenzotriazole; Sigma]) using as a catalyst 200 µl DMF/0.875 M DIC (N, N'-diisopropyl carbodiimide; Sigma). Following the deprotection of the final (amino-terminal) amino acid, the resins were washed 10 times with DMF, 5 times with methanol, 6 times with DCM (dichloromethane), and 4 times with ether. A 10 min vacuum was used to dry the resin after the final wash. Peptides were cleaved from the resin with 3 mls of a solution containing 5% phenol, 5% water, 3% TIPS (triisopropylsilane), and 87% TFA (trifluoroacetic acid) for 3 hours. If the peptide contained arginine, then the cleavage was left overnight. The peptides

were precipitated by the addition of 20 mls ether pre-chilled on a dry ice/methanol bath, vortexed, pelleted by centrifugation for 5 min at 2500 rpm, and washed 4 times in cold ether. At the end of the final wash, the peptide precipitates were air-dried, dissolved in either water or 20% acetic acid, and lyophilized. The final peptides were then analyzed for purity by HPLC reverse-phase chromatography and HPLC purified if necessary. Peptide concentrations were measured by comparing the HPLC trace with that of a standard peptide of known concentration. The random synthetic nonameric peptide library used in Chapter 4 was constructed by M. Davenport by adding equimolar concentrations of all the amino acids (with the exception of a 1.5 molar excess of arginine and no cysteine) to each coupling step of the peptide synthesis (Flynn et al 1991).

Peptide sequences:

HLA-A2 epitopes- pol; HIV pol protein residues 476-484 (ILKEPVHGV) (Tsomides et al 1991). Variants of the pol peptide were produced by substituting the following amino acids for glycine at position 8: alanine (polA8), threonine (polT8), glutamic acid (polE8), phenylalanine (polF8), isoleucine (polI8), leucine (polL8), methionine (polM8), asparagine (polN8), proline (polP8), glutamine (polQ8), arginine (polR8), serine (polS8), valine (polV8), tryptophan (polW8), tyrosine (polY8); substitutions for the isoleucine at position 1: serine (polS1); substitution for the glutamic acid at position 4: asparagine (polN4), glutamine (polQ4); substitution for the valine at position 9; leucine (polL9), alanine (polA9). Influenza A matrix peptide (fmp); matrix protein residues 58-66 (GILGFVFTL)

(Morrison et al 1992, Bednarek et al 1991). gag; HIV gag p17 residues 77-85 (SLYNTVATL) (McMichael and Walker 1991) TLW; a self peptide (TLWVDPYEV) (Hunt et al 1992).
HLA-B8 epitopes- gag; HIV gag p17 residues 24-32 (GGKKKYKLK) (Nixon et al 1988).

2.33 Gel Electrophoresis of Proteins and Western Blotting

Protein gels (Bio-Rad Mini-Protean II System) were run by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) according to standard protocols (Laemmli 1970). Resolving gels were 12-16.5% acrylamide (30:1 acrylamide:methylene-bisacrylamide), 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.05% APS, 0.01% TEMED; stacking gels were 5% acrylamide, 0.125 M Tris-HCl pH 6.8, 0.1% SDS, 0.05% APS, 0.01% TEMED; and running buffer was 25 mM Tris, 250 mM glycine pH 8.3, 0.1% SDS. Samples were boiled for 5 minutes in 62.5 mM Tris-HCl pH 6.8, 10% glycerol, 0.2% SDS, 5% 2-mercaptoethanol, 0.0025% bromophenol blue prior to electrophoresis at 160 V. Gels were stained in 0.1% Coomassie blue R-250, 30% methanol, 10% acetic acid and destained in 15% methanol, 5% acetic acid. For Western blotting protein was transferred to a nitrocellulose membrane (Hybond-C) soaked in transfer buffer (1l contains 14.4g glycine, 3g tris base, 25% methanol) in a semi-dry blotter (Biorad) for 40mins at 80mA. The membrane was then blocked in 'Blotto' (3% dried milk powder, 0.05% NaN_3, 0.05% 'Tween') for a minimum of 1hr. Anti-TCR mAb was then added according to manufacturers instructions and the membrane was incubated at room temp

for a further hour. The membrane was then washed 3 times in PBS and a 1:2000 dilution of horse radish peroxidase conjugated goat anti-mouse antibody (Dako) was added in Blotto. After a further hour of incubation at room temperature the membrane was washed again three times in PBS, developed with an electro-chemical luminescence kit (ECL Amersham) and exposed to autoradiographic film.

2.34 Preparation of Soluble MHC-peptide Complexes

A recombinant, soluble form of HLA-A*0201 consisting of the extracellular domains (residues 1-275) of the molecule was prepared essentially as described (Garboczi et al. 1992). The protein complexes used in Chapter 4 were prepared primarily by D. Barouch with my assistance, those used in Chapter 5 were prepared solely by R. Vessey.

The truncated heavy and β_2m chains, each cloned into a pHN1 expression plasmid (MacFerrin et al. 1990), were transformed into separate cultures of XA90 *E. coli* cells. Small scale cultures were grown overnight in LB containing ampicillin. Large cell cultures were seeded by a 1:40 dilution from an overnight culture into fresh LB/ampicillin. When the cultures reached OD_{600} = 0.5 protein expression was induced for 3 hours with the addition of 1 mM IPTG (isopropyl b-D-thiogalactosidase; CalBiochem). Cells were harvested by centrifugation for 15 min at 3000 rpm and washed once in PBS.

The heavy chain and the β_2-microglobulin chain were expressed as insoluble inclusion body aggregates, which were isolated and purified separately. For a 1 L growth of bacteria, the cells were resuspended in 20 mls sucrose solution (25%

sucrose, 50 mM Tris-HCl pH 8, 1 mM EDTA, 1 mM DTT, 1 mM PMSF). Cells were lysed by the addition of 1 mg/ml lysozyme (Sigma) and 1% Triton X-100, and the lysis was incubated on ice for 30 min until the solution was extremely viscous. In order to cleave the DNA, 30 mM $MgCl_2$ and 50 µg/ml DNAse (Sigma) were added, and the mixture was incubated at room temperature for 30 min until the solution lost its viscosity. 25 mM EDTA was added and the solution was freeze/thawed twice using a dry ice/methanol bath and a vessel containing warm water. Another 25 mM (50 mM final) EDTA was added, and the inclusion bodies were pelleted by centrifugation at 10,000 rpm for 20 min at 4° in an SS34 rotor (Beckman). The pellet was resuspended, homogenised in 25 mls wash buffer (0.5% Triton X-100, 50 mM Tris pH 8, 100 mM NaCl) using a dounce homogeniser, centrifuged as before, and washed repeatedly until the pellet was a "chalky white" color and the supernatant was colourless (usually 3-5 times). The washed inclusion bodies were then homogenized in 50 mM Tris pH 8, 100 mM NaCl in order to remove most of the detergent and then solubilized in 2 mls of 8 M urea, 50 mM MES (2-[N-morpholino] ethanesulfonic acid) pH 6.5, 0.1 mM EDTA, 0.1 mM DTT. Insoluble material was pelleted by centrifugation at 15,000 rpm for 1 hour, and the solubilized inclusion bodies in the supernatant were frozen in aliquots at -70°C and tested for purity and concentration by SDS-PAGE.

Refolding of the trimeric HLA-A2 complexes (heavy chain/β_2m/peptide) was initiated by adding 1 µM heavy chain (approx. 6 mg), 2 µM β_2m (approx. 5 mg), and 10 µM synthetic peptide (approx. 2 mg) into 200 mls of an ice-cold folding

solution containing 400 mM L-arginine, 100 mM Tris-HCl pH 8, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 0.5 mM PMSF. The folding solution was stirred gently in the cold room for 24 hours and then concentrated with a stir cell with a 10 kDa cutoff membrane (Millipore) and a Centriprep-10 (Amicon). The buffer was then exchanged into either 20 mM Tris-HCl pH 8, 150 mM NaCl or PBS, the solution was concentrated again to 10 ml, and the folded HLA-A2/single peptide complexes were purified by FPLC. The final protein solution was concentrated with a Centricon-10 to 5 mg/ml and was stored at 4°C. Protein concentration was determined by the Quantify protein assay system (Promega) and integrity could be confirmed by presentation to RBL/TCR-CD3ζ transfectants in multivalent form (see below) or by an ELISA. The ELISA was performed as follows; 96-well Maxisorp plates (Nunc) were coated overnight at 4° C with 5 µg/ml of a rat monoclonal anti-human class I antibody (YTH 862.2, Serotec) in 50 mM potassium carbonate buffer pH 9.6. The rest of the ELISA was performed at room temperature. The wells were blocked with 2% bovine serum albumin in phosphate-buffered saline (PBS/2% BSA) for 2 hours. HLA-A2, diluted to 10 µg/ml in PBS/2% BSA, was then applied to the wells for 1 hour. Following 3 washes with PBS/0.4% BSA, the primary antibody, diluted to various concentrations in PBS/2% BSA, was applied for 1 hour. Following 3 more washes with PBS/0.4% BSA, a 1:1000 dilution of an alkaline phosphatase-conjugated anti-mouse IgG antibody (Sigma), pre-adsorbed with rat serum, was added. The ELISA was developed with a solution of 1 mg/ml

p-nitrophenol phosphate (Sigma) in 0.1 M glycine pH 10, 1 mM $ZnSO_4$, 1 mM $MgCl_2$, and the absorbance was read at 405 nm.

2.35 Preparation of Biotinylated HLA-A2

This method was developed in conjunction with D.Barouch. Biotinylated HLA-A2 single peptide complexes were prepared by surface biotinylation of 5 mg refolded β2m protein for 1 hour at room temperature in a 1 ml volume using a 3-fold molar excess of NHS-SS-biotin (Pierce). This was followed by gel filtration purification to remove free biotin, and seeding a folding reaction essentially as above using folded biotinylated β2m, denatured heavy chain, and a synthetic peptide. The complex was purified by HPLC gel filtration chromatography. A dynabead immunoassay was developed as a very sensitive assay in order to detect biotinylated HLA-A2. It is essentially an ELISA with dynabeads as the solid matrix, but it is faster and more sensitive due to the larger binding capacity of dynabeads compared with plastic wells. 25-50µl streptavidin coated dynabeads were used per reaction. The beads were washed three times in PBS and incubated with biotinylated HLA-A2 for 10 minutes at room temperature. Following three washes in PBS, the beads were blocked in 1ml PBS/2% BSA (or PBS/2% nonfat dry milk powder). Then a specific mAb at 1µg/ml or less in 0.5ml blocking buffer was added, and the beads were incubated for 30 min rotating at room temperature. Following three washes in PBS, a 1:1000 dilution of an alkaline phosphatase-conjugated anti-mouse secondary antibody was added for 30 min. The beads were washed three times and the

immunoassay was developed with *p*-nitrophenol phosphate as described for the ELISA.

2.36 Multimerisation of MHC Molecules

Recombinant MHC molecules, folded as described above, were multimerised on the surface of dynabeads. Sheep anti-mouse dynabeads (Dynal) were washed three times in PBS, incubated overnight with a saturating concentration of various anti-MHC antibodies (2 µg mAb/mg beads), and washed again three times. The beads were then incubated for 2 hours rotating at 4° with an over-saturating concentration of MHC protein (20 µg MHC/mg beads) in 1 ml PBS and then washed three times in R10 tissue culture medium. Alternatively biotinylated MHC molecules, prepared as described above, were multimerised on the surface of streptavidin dynabeads (Dynal). Dynabeads were washed three times in PBS and incubated for 15 minutes rotating at 4°C with various quantities of biotinylated MHC protein in 1 ml PBS. The MHC-coated beads were then washed three times in R10 tissue culture medium.

2.4 General Tissue Culture

2.41 Cell Lines

Influenza and HIV 1 specific CTL lines were available as frozen stocks having been generated from peripheral blood mononuclear cells by published methods (Nixon et al 1988). The generation of the HLA-A$^-$ and HLA-B reduced cell line C1R from the human B-lymphoblastoid line Licr.Lon.Hmy2 (Edwards et al. 1982) and the mutant B cell line LBL 721.174 (0.174) (Cerundolo et al 1990) which has a defect in antigen

processing have been described previously. The adherent rat basophil leukaemia cell line RBL-2H3 (Metzger 1983, Engel et al 1992) was used for TCR-CD3ζ transfections.

2.42 Growth Conditions and Media

Mammalian cells were grown in sterile conditions at 37°C in an atmosphere of 5% CO_2. R10 and D10 media were prepared by supplementing RPMI 1640 (Gibco or Sigma) or Dulbeccos modified Eagles medium with 10% FCS (fetal calf serum; Globepharm), 100µg/ml streptomycin, 100U/ml penicillin, and 2mM L-glutamine. Pelleting of cells was performed by centrifugation at 1500 rpm for 5 min. Frozen aliquots were prepared by resuspending $5x10^6$ cells in 90% FCS, 10% DMSO and freezing slowly to -70°. Cells were thawed by adding 25 mls R10 dropwise, centrifugation, and resuspension in R10. C1R cells, 0.174 cells and EBV-transformed B cells were grown in liquid suspension. RBL-2H3 cells were grown in D10. Cells were split every 2-3 days. CTL lines were grown on irradiated peptide-pulsed autologous B lymphoblastoid cell lines in R10 supplemented with recombinant IL-2.

2.43 Transfection, Cell Sorting, and Cell Cloning

RBL-2H3 cells were transfected with 10µg of TCR-CD3ζ vector (linearised with *Hin*dIII, see Chapter 3 for details of cloning) by electroporation (500µF, 250mV) and grown for 18 hours in D10. Stable transfectants were selected by the addition of 700µg/ml geneticin G-418 sulphate (Gibco), 15µg/ml hypoxanthine, 10µg/ml aminopterin, 2µg/ml thymidine, 250µg/ml xanthine and 5µg/ml mycophenolic acid (All Sigma).

C1R cells were used as the recipient for transfectant class I genes. The production of mutant HLA-A2 genes and the expression and maintenance of both these and the wild type HLA-A2 and HLA-B8 genes in C1R cells has been previously described (Moots et al 1991, Tussey et al 1995). For previously untransfected mutant HLA-A2 genes C1R cells were transfected by lipofection (LIPOFECTIN-OPTI-MEM, GibcoBRL). Cells were washed in serum free RPMI unsupplemented with antibacterial agents. Cells were then resuspended in a 35mm tissue culture plate at a density of $3x10^6$ cells in 0.8ml of serum free RPMI. For each transfection two solutions were prepared and allowed to stand at room temp for 30-45 mins; solution A, 5µg of DNA was added to 100µl of OPTI-MEM I medium and solution B, 10µl of lipofectin reagent was added to 100µl of OPTI-MEM I medium. Solutions A and B were then combined and incubated for a further 10min and then added to the cells which were incubated for at least 5 hrs before the addition of R10. After a further 24-48 hours selection medium was applied consisting of R10 supplemented with 1.8 mg/ml geneticin G418 sulphate (Gibco).

Stably transfected cell lines were sorted with mAb coated dynabeads in order to enrich for high-expressing cells. Sheep anti-mouse dynabeads (Dynal) were washed three times in sterile PBS, incubated for 2 hours with a sterile solution of mAb at a ratio of 5 µg mAb per mg dynabeads, and washed again three times. 10^7 cells were washed in PBS and incubated rotating for 1 hour at room tempterature with 10^7 antibody-coated dynabeads. The beads were washed three times in PBS and then added to growth media. After two weeks of cell

growth, the beads were removed from the culture. In the case of C1R cells expressing HLA-A2 the mAb used was BB7.2 while for RBLs expressing TCR-CD3ζ the cells were subjected to two rounds of selection firstly using mAb βF1 and then mAb αF1.

Transfectant cells were cloned by limiting dilution in 96-well plates (see Chapter 3). Individual clones were selected from the plate containing an initial concentration of 0.1 or 0.3 cells per well and were screened for surface expression of the transfected class I gene by flow cytometry.

2.44 Flow Cytometry

Flow cytometry was used to monitor surface expression of transfected molecules on mammalian cell lines. For each sample, $5x10^5$ cells were washed twice in blocking buffer (PBS containing 1mg/ml BSA [bovine serum albumin; Sigma RIA grade], 0.1% NaN_3 and 10% normal human serum) and resuspended in 100µl PBS containing 5µg/ml of a specific mAb. Following a 30 min incubation on ice, 1 ml blocking buffer was added, and the cells were pelleted by centrifugation. The second layer was 100µl of FITC (fluorescein isothiocynate) or PE (phycoerithrin)-conjugated polyclonal anti-mouse antibody (DAKO) diluted in PBS according to the manfacturer's instructions. Following a 30 min incubation on ice, 1ml blocking buffer was added, and the cells were pelleted by centrifugation. The stained cells were resuspended in 0.5ml PBS and analyzed by FACScan (Becton Dickinson fluorescence activated cell scanner).

2.45 Monoclonal Antibodies and Bacterial Superantigens

Monoclonal antibodies (mAbs) were available in the laboratory having been prepared from stable hybridoma cell lines according to standard protocols (Harlow and Lane 1988) or purchased from commercial suppliers. Briefly large scale growths used R10 tissue culture media in 2 L roller bottles. The cultures were overgrown for 4 days post-saturation until 80% of the cells had died and then were centrifuged for 20 min at 3000 rpm. To the supernatant (containing the secreted antibodies at approximately 10μg/ml) 1/10 volume of 1 M Tris-HCl pH 8 was added. The antibody solution was passed over a sepharose pre-column and an antibody affinity column of protein A sepharose (Sigma; for IgG2a and IgG2b mAbs) or protein G sepharose (Sigma; for IgG1 mAbs). The binding capacity was approximately 10 mg mAb per ml column resin. The column was washed with 10 column volumes of 100 mM Tris-HCl pH 8 and 10 volumes of 10 mM Tris-HCl pH 8. The antibodies were eluted with 100 mM glycine pH 3, the pH was immediately neutralized with 1/10 volume 1 M Tris-HCl pH 8, and the antibody solution was dialysed extensively against PBS. Purity of the antibodies was checked by SDS-PAGE, specificity of the antibodies was tested in immunoprecipitations and flow cytometry, and concentrations were determined by spectrophotometry at 280 nm (1 OD = 0.8mg/ml). The following hybridomas were used:

BB7.2	anti-A2/A69 (Parham and Brodsky 1981)

W6/32	anti-HLA class I monomorphic (Barnstable et al. 1978; Kahn-Perles et al. 1987)

The following antibodies were purchased from Serotec.

αF1	anti-TCR Cα region.
βF1	anti-TCR Cβ region.
YTH 862.2	anti-human class I antibody (rat)

The following antibody was purchased from Immunotec.

antiTCRBV17S1	anti TCR β variable region BV17S1

The streptococcal superantigen, SPE-C was purchased from Toxin Technology (California USA) and reconstituted in purified water according to the manufacturers instructions.

2.5 Functional Assays

2.51 Class I Peptide Binding Assays

Binding of peptides to HLA-A2 molecules in cell lysates was performed essentially as described (Elvin et al 1993). 10 million 0.174 cells (per lane of the analytical gel) were incubated for approximately 45 mins at 37°C in methionine free medium. ^{35}S Trans-label (Amersham) was added and cells incubated for a further hour at 37°C. Cells were then washed twice in PBS and lysed in 1ml of lysis buffer (50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.5% NP-40, 5mM EDTA, 0.5% Mega 9, 2mM PMSF, 5mM iodoacetamide). After 30 mins on ice the lysates were cleared by centrifugation and 90µl of 10% Staph. aureus immunoadsorbent (Sigma) was added to the supernatant. Peptide was added over a range of concentrations

from 0.06-60μM. Following an overnight incubation at 4°C samples were immunoprecipitated by 10μg of HLA-A2 specific mAb BB7.2 (Parham and Brodsky 1981) and 90μl of protein-A sepharose (Sigma). Immunoprecipitates were washed three times in lysis buffer and analysed on a 15% SDS-polyacrylamide gel under reducing conditions. The gels were fixed, dried, and exposed to autoradiographic film, and the intensities of the heavy chain bands were quantitated by scanning densitometry. Binding of peptides to HLA-B8 and mutant HLA-A2 molecules expressed in C1R cell lysates was assayed by a similar method with the following modifications (Tussey et al. 1994). Peptides were iodinated on either histidine or tyrosine by incubating 1μg peptide in 100μl PBS for 60 seconds with 0.5 mCi of I-125 (Amersham) and 10μl of 5mg/ml chloramine T. The reaction was terminated by the addition of 30μl of 5mg/ml sodium metabisulphite and 2.5ml of 10mM Tris-HCl pH 7.5, 0.15M NaCl, 0.1% NP-40, 0.1% BSA, 0.2% sodium azide, and the labelled peptide was purified on a gel filtration Sephadex G-25 column (Pharmacia) and 2μl aliquots were tested by gamma counting. 10^7 cells expressing a class I allele were then labelled with ^{35}S methionine and lysed as described above. After 30 mins on ice the lysates were cleared by centrifugation and 90μl of 10% Staph. aureus immunoadsorbent (Sigma) was added to the supernatant together with $1x10^5$ to $5x10^6$ cpm of labelled peptide. The concentration of peptide in the assay is estimated to be 100pM ($1x10^5$ cpm) to 5nM ($5x10^6$ cpm). Immunoprecipitations were as described above except that W6/32 (Barnstable et al. 1978) was used for HLA-B8 precipitations. Immunoprecipitates were

analysed on a 15% gel which was fixed and dried as before. The quantitation of peptide binding normalized to the amount of heavy chain present was calculated by the formula: (peptide intensity - background) / (heavy chain intensity - background).

2.52 CTL Chromium Release Assays

Standard ^{51}Cr release assays were performed as described (Gotch et al. 1988) in order to test for CTL recognition of peptide-pulsed target cells (which were either autologous B cells or C1R cells expressing wild-type or mutant HLA-A2 molecules). $2x10^6$ target cells were washed with PBS and labelled with 100µCi of ^{51}Cr (Amersham) in 100µl PBS for 1 hour at 37°C. Following two washes in PBS, the target cells were plated at $5x10^3$/well in U-bottom 96-well plates with the agonist peptide as well as CTLs, media alone, or 5% Triton X-100 in a total reaction volume of 200µl/well. The media alone and triton wells represented spontaneous ^{51}Cr release and maximum ^{51}Cr release, respectively. Peptide concentrations ranged generally from 10^{-6} M to 10^{-12} M, and E:T (effector to target) ratios were generally 3:1 to 15:1. Competition assays as used in Chapter 5 were performed as described (Sutton et al 1993). Briefly autologous B cell targets were labelled with ^{51}Cr as above and then pulsed with 60µM pol, polA8 or gag p17 peptide for a further hour. After washing, $5x10^3$ target cells were plated out in 100µl of media containing either 60µM pol, polA8 or gag p17 together with various concentrations of fmp. Finally $2x10^4$ flu matrix specific CTL were added to each well to make a total volume of 150 µl. All assays underwent a 5 hour incubation at 37°C, after which 20µl aliquots were

harvested onto filter mats. which were dried in the microwave on full power for 5 min and placed in sample bags with scintillation fluid. The radioactivity release was determined using a beta-plate counter (Wallac). The percent specific lysis was calculated according to the formula: (experimental release - media release) / (triton release - media release).

2.53 RBL degranulation assays.

Degranulation of RBL/TCR-CD3ζ transfectants was used as an assay for TCR triggering essentially as described (Engel et al 1992). RBLs were cultured in flat-bottomed 96-well plates (Falcon) at a density of $5x10^4$ cells/well for 12 hours at 37°C in D10 with ^{3}H-hydroxytryptamine creatinine sulphate (DuPont) at a final concentration of 0.2μCi/ml. The RBLs were washed 3 times in warm media, incubated for 20 mins, and then washed once more. Targets were either peptide or bacterial superantigen pulsed cells or multivalent recombinant MHC-peptide complexes. Peptide pulsed target cells were prepared by resuspending C1R cells stably expressing HLA-A2 (or HLA-B8 as a negative control) at $1x10^6$ per 100μl and pulsing with various concentrations of peptide for 2 hours at 37°C. After one wash the antigen presenting cells were resuspended at $5x10^5$ per 100μl, added to the RBLs and coincubated for 60 minutes at 37°C. Two types of multivalent recombinant MHC-peptide complexes were prepared (described in detail above) using either streptavidin coated dynabeads which had been preincubated with varying quantities of biotinylated HLA-A2 peptide complex (range 0.09-2.7μg/10^7 beads) or sheep anti-mouse dynabeads coupled to recombinant MHC-peptide with

the mAb w6/32. In either case 10 million beads were added per well of RBLs in a final volume of 100µl, coincubation was for 60 minutes at 37°C.

For degranulation competition assays ascending concentrations of non-biotinylated monovalent recombinant MHC-peptide complexes were used to compete against a fixed stimulus. The stimulus was either 10^7 streptavidin coated dynabeads linked to a fixed amount of biotinylated recombinant HLA-A2 pol (see Chapter 4; 0.9µg/10^7 beads, per well) or $5x10^5$ C1R cells expressing HLA-A2 pulsed with 100µM pol peptide (see Chapter 5). When multivalent complexes were used as a stimulus the beads and the competing complexes were added to labelled RBLs simultaneously folowed by coincubation for one hour at 37°C. When peptide pulsed target cells were used as a stimulus monovalent competing MHC-peptide complexes were added to the RBLs in 50µl of PBS and allowed to reach binding equilibrium with the TCR over a 30 minute incubation at 37°C, before addition of the target cells and a further incubation of one hour at 37°C. Non-specific inhibition of degranulation by monovalent recombinant MHC-peptide complexes was assessed by repeating the competition assays using 5µl of ascites containing the anti TCR β constant domain mAB βF1 as a stimulus to degranulation in place of multivalent recombinant MHC-peptide or peptide pulsed target cells. All assays underwent a 1 hour incubation at 37°C, after which 20µl aliquots were harvested onto filter mats, which were dried in the microwave on full power for 5 min and placed in sample bags with scintillation fluid for counting on a β-plate counter (Wallac).

2.54 Direct Binding Assays

This method was developed in conjunction with D Barouch. MHC-peptide complexes were iodinated using chloramine T by previously described methods (see under peptide binding assays, Tussey et al 1994, 20µg of MHC-peptide replaced 1µg of peptide). Protein integrity was analyzed by reactivity to the anti-MHC mAb W6/32 and gel filtration chromatography. Transfected or untransfected RBL cells were then plated in 96-well plates at a density of 10^5 cells per well, and the wells were blocked for 20 minutes with PBS/1% BSA. Iodinated HLA-A2 pol complexes (0.1µM final concentration) were added to the adherent RBL cells with or without unlabelled HLA-A2 peptide competitor (10µM final concentration) and incubated for 30 min at room temperature in a volume of 100 µl. The unbound protein was removed, and following one rapid wash, the bound protein was eluted over 5 minutes with 100µl PBS/1% BSA containing 10µM unlabelled HLA-A2 pol, in order to prevent the rebinding of the labelled HLA-A2 pol. The bound counts were determined with a gamma counter.

Chapter 3
Development of a Bioassay for Analysis of TCR/MHC-peptide Interactions

3.1 Introduction

3.11 Biological Systems for Studying TCR/MHC-peptide Interactions

The recognition of MHC-peptide complexes by the TCR is the critical molecular recognition event signalling the activation of specific T lymphocytes in the initiation and regulation of immune responses. Understandably, this interaction has been the subject of intensive investigation and a variety of model systems have been generated for analysing both its topology (reviewed in Chien & Davies 1993) and biophysical parameters (reviewed in Fremont et al 1996). The development of techniques for producing T cell clones and examining their responses in reproducible bioassays has been of great significance since this facilitated the discovery of MHC restriction (see Chapter 1, Zinkernagel & Doherty 1974a&b) and allowed the definition of peptide epitopes (Gotch et al 1987, Gotch et al 1988, Nixon et al 1988). Subsequently, T cell assays were used to examine the effect of substitutions in the MHC (Krieger et al 1991, Jaulin et al 1992, Ehrich et al 1992, Tussey et al 1994, Sun et al 1995) and peptide (Evavold & Allen 1991, Nanda et al 1994, Wucherpfennig & Strominger 1994, Kersh & Allen 1996) on T cell recognition, which has increased understanding of the topology of TCR/MHC-peptide interactions (reviewed in Chien & Davies 1993) and resulted in the discovery APLs (reviewed in Jameson & Bevan 1995, Sloan-Lancaster &

Allen 1996). T cell clones have also been used to measure the affinity of TCRs for a variety of MHC-peptide ligands both directly, using iodinated recombinant MHC-peptide complexes (Sykulev et al 1994b), and indirectly using anti-TCR Fabs that compete for binding with soluble MHC-peptide complexes (Sykulev et al 1994a, Matsui et al 1991). However, despite the usefulness of T cell clones and their obvious relevance to *in vivo* responses there are some disadvantages in their use as a bioassay for T cell recognition. Firstly they can be hard to sustain and they do not always maintain stable function in culture. In addition, despite the use of anchor PCR of TCR genes from T cell cDNA (Loh et al 1989, reviewed in Moss et al 1992), it can be difficult to be certain that a T cell population is truly clonal which may result in the inadvertant investigation of more than one TCR at a time. This concern is further compounded by the expression of multiple TCR α-chains or even functional TCR heterodimers by individual T cell clones (Padovan et al 1993, Valitutti et al 1995). Finally although T cell responses can be used to examine the effect of mutations in the peptide or MHC on T cell recognition, the TCR itself can not be mutated unless it can be transfected into the effector cell. An alternative to using T cell clones is to generate either T cell hybridomas (Schneck et al 1989, Weber et al 1992) or to transfect TCR genes into TCR negative cell lines such as Jurkat (Ohashi et al 1985, Wedderburn et al 1995). These provide stable effector cells for bioassays and, in the case of transfectants, allow for the mutagenesis of TCR genes which aids in the fine mapping of TCR/MHC-peptide interactions (Wedderburn et al 1995). One obvious drawback of these approaches is the potential loss of biological relevance when not studying a native T cell.

Furthermore there are specific problems in that there is no fusion partner for creating human T cell hybridomas and the TCR negative cell lines are CD4 positive, making them less useful for analysing MHC class I restricted TCRs.

An important feature of all the T cell based bioassays described above is that the TCR/MHC-peptide interaction is studied in the context of a multitude of other inter-cellular contacts such as those between adhesion molecules or T cell specific coreceptors (Janeway 1992, Van Gool et al 1996). In some instances, such as the characterisation of T cell responses to APLs, this is clearly what is required because the system needs to be as biologically relevant as possible. However in other circumstances, for example in defining the biophysical parameters of TCR/MHC-peptide interactions, it may be preferable to isolate the TCR and its ligand from other potentially significant cell-cell contacts. Modern techniques of genetic engineering have provided means of studying protein-protein interactions in isolation from cellular responses by allowing the production of soluble protein fragments. The detailed binding kinetics of these protein fragments can then be measured by surface plasmon resonance (SPR) in which the small changes in molecular concentration that occur as solution phase ligands interact with a solid phase receptor can be detected continuously in a flow cell (reviewed in Margulies et al 1996). Data obtained by this method supports that from cell based methods (Schneck et al 1989, Matsui et al 1991, Weber et 1992, Sykulev et al 1994a&b) by showing firstly that the TCR tends to have a low affinity for the MHC-peptide complex (reported range for the Kd of between 10^{-4} and 10^{-7}M) and secondly that the MHC-peptide complex undergoes very rapid

dissociation from the TCR (Fremont et al 1996, Corr et al 1994, Matsui et al 1994, al-Ramadi et al 1995). Although the development of SPR clearly represents a significant technical advance problems still remain with this approach. Firstly, when two soluble binding partners are analysed by SPR there is no way of testing the biological integrity of the molecules concerned and, more specifically, the generation of soluble TCR molecules has proved to be difficult (Traunecker et al 1989, Fremont et al 1996).

In view of the problems with both SPR and T cell based assays it was decided to develop an alternative bioassay which, despite being cell based, succeeds in isolating the TCR/MHC-peptide interaction from the other intermolecular events involved in T cell activation (reviewed in Janeway & Bottomley 1994, Flavell & Grewall 1996, Van Gool et al 1996). The original experiments on which this bioassay is based were performed by Klausner and colleagues who showed that a chimeric molecule comprised of the extracellular components of the TCR α and β chains fused to the transmembrane and cytoplasmic domains of the CD3ζ chain could provoke MHC restricted and peptide specific degranulation of tritium labelled 5-HT when expressed in the rat basophil leukaemia cell line, RBL-2H3 (Engel et al 1992). The molecular basis of these responses lies in the ability of CD3ζ to activate the degranulation process due to its homology with the FCεRI receptor for IgE, which is naturally expressed on RBLs.

This chapter describes how the original methods of Klausner and colleagues were modified and extended to produce RBL cells transfected with a TCR-CD3ζ chimeric receptor specific for HLA-A2 HIV pol complexes. The specificity of the transfectants was compared with the T cell clone from which the TCR was derived.

In addition the transfectants were used in the generation of anti-TCR mAbs. Finally the advantages and disadvantages of this bioassay are discussed in detail.

3.2 Results

3.21 Expression Vectors for TCR-CD3ζ Constructs

The original TCR-CD3ζ transfectants described by Klausner were made by cloning the extracellular portion of the TCR α and β chains immediately distal to the interchain disulphide bond into a mammalian expression vector encoding the CD3ζ chain (Fig 3.1). These vectors were then cotransfected into RBL cells together with a third plasmid containing a selectable marker (Engel et al 1992). The TCR-CD3ζ fusions used in the experiments described here were designed so as to be identical to those of Klausner and colleagues (Fig 3.1). However, the system was significantly modified by cloning TCR-CD3ζ fusions into two separate mammalian expression vectors one incorporating the bacterial XGPRT gene which provides resistance to mycophenolic acid (Fig 3.2a&c) and the other encoding the neomycin phosphotransferase gene for aminoglycoside antibiotic resistance (Fig 3.2b). The perceived advantage of dual selection over the use of a single selectable marker is that a higher proportion of transfectants are likely to express both chains of the TCR-CD3ζ receptor and this premise was supported by the results of multiple transfections in which TCR negative transfectants were not identified (data not shown).

3.22 Identification of TCR Genes and Cloning as TCR-CD3ζ Fusions

Two α/β TCRs were studied, in each case the TCR genes were identified from CTL clones which had been produced by standard dilutional methods (Gotch et al 1988). The CTL clones were specific for either an HLA-A2 restricted flu matrix peptide (CTL clone JM22, cloning performed by P Moss) or an HLA-A2 HIV pol peptide (CTL clone 008A3, cloning performed by S Rowland-Jones, see Chapter 2 for peptide sequences). Identification of the receptors expressed by these CTL clones was undertaken by P Moss using anchored PCR (Loh et al 1989, Moss et al 1992). Briefly, mRNA is derived from CTL followed by first strand cDNA synthesis and 3' poly-G tailing by terminal transferase. The TCR genes are amplified using primers directed to the poly-G tail and the TCR constant domain and PCR products subcloned into M13. Ten individual clones are then sequenced to establish that only one TCR-β chain is present (i.e that the T cell population is clonal). Because of the temporal sequence in which the TCR genes are rearranged during thymic development more than one TCRα chain is frequently identified although often there is only one in-frame sequence allowing easy identification of the functional α chain. The presumed TCRV genes utilised by clone JM22 were BV17S1 and AV10S1A1 and by clone 008A3 BV1S1A2 and AV2S1A2. The JM22 a and b chains already existed as CD3ζ chimeras (Callan et al 1993) and were cloned into expression vectors 2B4-GPT and pMC1neo to create the vectors BJ043 and BJ048 (fig 3.2a&c).

In order to facilitate cloning of future TCR genes a cassette cloning strategy was devised. Firstly the JM22 α and β chains were cloned into pBluescript II(KS-) with the C domains truncated

immediately distal to the interchain disulphide bond (fig 3.3). Then the TCR-Cα region and TCR-Cβ region were silently mutagenised using oligonucleotides ACEAG and BCAFL respectively so that they encoded an EagI site at position 15 of Cα (construct BJ008) and an AflII site at position 10 of Cβ (construct BJ011) (Fig 3.3, see Chapter 2 for oligonucleotide sequences). The introduction of these sites allowed for the simple cloning of the other PCR-generated TCRV fragments without the need to amplify and resequence the C domain (Fig 3.3).

The genes encoding the TCR expressed by the CTL clone 008A3 were available as poly-G tailed cDNA and therefore a fragment of the α and β chain encoding the V domain and a small part of the C domain was amplified by PCR using the primer pairs AV2S1A2F/AV2S1A2B and BV1S1F/BV1S1B respectively (see chapter 2 for oligonucleotide sequences). These DNA fragments were then subcloned into vectors BJ008 and BJ011 to give constructs RV002 and RV001 respectively (see fig 3.3). The DNA sequences of the amplified parts of the TCR sequence were verified by standard dideoxy sequencing and then the Vα-Cα or Vβ-Cβ gene fragments were subcloned into the expression vectors BJ048 or BJ043 respectively to generate A3-TCRα or β -CD3ζ chimeras, RV004 and RV003 (Fig 3.3).

3.23 RBL/TCR-CD3ζ Transfectants

Expression vectors encoding the TCRα and β CD3ζ chimeras were transfected into RBL-2H3 cells by electroporation and stable transfectants were selected in media containing the aminoglycoside geneticin G-418 sulfate, hypoxanthine, aminopterin, thymidine, xanthine and mycophenolic acid (see

Chapter 2 for concentrations). Expression of both chains of each receptor was demonstrated by flow cytometry using antibodies to the constant regions of the alpha and beta chains (αF1 and βF1) as well as a mAb to BV17S1 (Fig 3.4ab&c). The transfectant expressing the HLA-A2 restricted flu-matrix specific receptor was termed RBL-38 while that expressing the HLA-A2 restricted HIV pol specific receptor was designated RBL-008. In addition to dual chain transfectants a panel of transfectants expressing only the α or β chain of each receptor was made (Fig 3.4def&g). As reported previously by Klausner single chain transfectants tend to express less effectively then those transfected with both chains (Engel et al 1992).

3.24 Characterisation of RBL/TCR-CD3ζ Transfectants Responses to Peptide Pulsed Target Cells

The transfectants RBL-38 and RBL-008 were labelled with a tritium containing 5-HT precursor molecule and their degranulation responses tested using anti-TCR mAbs and peptide pulsed target cells. Both cell lines could be induced to degranulate using antibodies to the constant regions of the α and β chains of the TCR and RBL-38 also degranulated in response to the anti-BV17S1 mAb (Fig 3.5a&b). However the response to peptide pulsed target cells varied between the two cell lines. Whereas RBL-38 could not be made to respond reliably to peptide pulsed target cells (Fig 3.5a) RBL-008 responded strongly and with great reproducibility (Fig 3.5b). Further characterisation of the response of RBL-008 was carried out using a panel of HIV pol peptide variants (Fig 3.6a) and comparison with the response of the original CTL clone 008A3 (Fig 3.6b, assay performed by S.

McAdam) shows that the TCR-CD3ζ chimeric receptor of RBL-008 closely mimics the specificity of the original TCR.

3.25 Generation and Characterisation of Monoclonal Antibodies to the A3-TCR

Production of anti TCR mAbs using RBL/TCR-CD3ζ transfectants has been previously described (Callan et al 1993) and these cells have proved to be a highly effective immunogen. Furthermore screening of hybridoma supernatants by provocation of degranulation of the transfectants is rapid and efficient. The rat mAbs described in this chapter were generated in conjunction with A. Necker of Immunotech. The production of a high expressing clone of RBL-008 was undertaken by R Vessey and the characterisation of the mAbs against a panel of single chain transfectants was duplicated in Oxford and at Immunotech and is described here. The rat immunisations, fusions and supernatant screening were performed at Immunotech and are not discussed in detail.

First the mixed population of RBL-008 was cloned by plating cells in a 96 well plate at a density of 0.5 cells per well. The highest expressing clone, named RBL-00802 (Fig 3.7), was then supplied to Immunotech. Following immunisation and fusion several hybridomas were identified which produced mAbs that stimulated degranulation of RBL-008. Next, the recognition site of each mAb was assigned to the variable or constant part of the alpha or beta chain (fig 3.8ab&c) by staining a panel of target cells in flow cytometric analyses. The cells used were untransfected RBL cells, RBL single chain transfectants, RBL-38, RBL-008 and whole blood and a total of three anti-BV1S1A2, three anti-

AV2S2A2 and two anti-Cβ mAbs were identified. Further characterisation of the mAbs is currently being performed by J.Wilson by spectratyping lymphocytes selected by anti-BV1S1 or anti-AV2S2A2 mAb coated immunomagnetic beads.

3.3 Discussion

The biggest potential disadvantage of the RBL/TCR-CD3ζ system is the difference in the mode of activation of RBLs, which are normally stimulated by high affinity FcεRIγ receptor-ligand interactions which promote receptor aggregation (reviewed in Metzger 1983, Metzger et al 1986, Beaven & Metzger 1993), and T cells, which appear to be activated by low affinity interactions in which small numbers of ligands serially engage the TCR (Margulies et al 1996, Valittuti et al 1995, Viola & Lanzavecchia 1996). The homology between CD3ζ and FcεRIγ means that activation of RBL/TCR-CD3ζ transfectants by MHC-peptide complexes is likely to resemble activation of untransfected RBLs by the interaction of IgE-Fc with the FcεRI receptor (reviewed in Metzger 1983, Metzger et al 1986, Beaven & Metzger 1993). Biophysical analysis of the engagement of FcεRI by IgE shows that this interaction is high affinity ($Kd>10^{-10}M$) with a slow dissociation rate ($k_{-1}<10^{-5}s^{-1}$) (Metzger et al 1986). These parameters reflect the requirement for FcεRI aggregation in activating RBLs, although the number of FcεRI complexes which must associate remains uncertain with some studies suggesting that dimerisation is sufficient (Siraganian et al 1975, Segal et al 1977, Kagey-Sobotka et al 1981) while others imply the need for higher order oligomerisation (Fewtrell & Metzger 1980). Following FcεRI aggregation the src family tyrosine kinase (PTK) $p56^{lyn}$,

which is associated with FceRIβ (Minoguchi et al 1994), becomes activated (Eiseman & Bolen 1992) and phosphorylates the tyrosine residues in the ITAMs of FcεRIγ. Phosphorylation of the ITAMs prompts recruitment of another PTK, termed Syk, which associates with FcεRIγ (Kihara & Siraganian 1994) and stimulates phospholipase activity and Ca^{2+} influx (Zhang et al 1996). Although the activation of RBL/TCR-CD3ζ transfectants by MHC-peptide complexes has not been fully characterised it is clear that it does share some of the predicted similarity with activation of RBLs through the FcεRI-IgE interaction. Most notably it has been shown that TCR-CD3ζ molecules must aggregate in order to stimulate degranulation since monovalent recombinant MHC-peptide complexes are ineffective in provoking a response (see Chapters 4 and 5, Vessey et al 1997). As with the FcεRI receptor it is unclear how many TCR-CD3ζ molecules must associate since Kourilsky and colleagues report activation by dimerised MHC-peptide (Lone et al 1994) while this could not be repeated with RBL-008 (data not shown).

Initial studies of T cell activation implied that TCR aggregation is necessary for productive signalling. For example neither anti-TCR Fab' fragments (Rojo & Janeway 1988) nor soluble monomeric MHC-peptide complexes can trigger T cells (Schneck et al 1989), presumably because they fail to cross-link the receptor. In addition a hapten specific TCR expressed in Jurkat cells could only trigger IL-2 release when presented with multivalent arrays of antigen (Symer et al 1992). However recent experiments have challenged the requirement for TCR oligomerisation by showing that the numbers of MHC-peptide complexes required for T cell activation are so small that it is statistically very unlikely that two

or more TCRs can be cross-linked (Valitutti et al 1995). Instead it is proposed that the small number of MHC-peptide complexes serially engage a large number of TCRs with a stoichiometry of 1:1 (Valitutti et al 1995). The result of TCR engagement is receptor internalisation and when the number of internalised TCRs reaches a critical number the T cell becomes fully activated (Viola & Lanzavecchia 1996). The concept of serial engagement has been used to explain the inherent low affinity and rapid dissociation rate ((Kd = 10^{-4}-10^{-7}M, k_{-1}=2.1x10^{-1}-5.5x$10^{-3}s^{-1}$, Fremont et al 1996) of TCR/MHC-peptide interactions since higher affinity ligands such as anti-TCR mAbs are less effcient at stimulating T cells because they can not dissociate rapidly enough to engage multiple TCRs (Viola & Lanzavecchia 1996).

The relatively low affinity of TCR/MHC-peptide interactions may mean that RBL/TCR-CD3ζ transfectants can not be activated by some MHC-peptide ligands, since they will not induce receptor aggregation. Indeed, this may explain why RBL-008 is about two logs less sensitive than the CTL clone 008A3 (Figs 3.6a&b) and also why some ligands such as polS1 (Figs 3.6a&b) are stimulatory in CTL lysis assays but not in RBL degranulation assays. The reduction in sensitivity of RBL/TCR-CD3ζ transfectants is of significance when interpreting the results of mutational analyses since loss of response by an RBL/TCR-CD3ζ transfectant may not equate to loss of response by a CTL clone. An alternative explanation for the reduced sensitivity of RBL/TCR-CD3ζ transfectants is that it is due to the absence of coreceptors and could therefore be viewed as a more accurate reflection of subtle changes in the TCR/MHC-peptide interaction. The failure of RBL-38 to respond to peptide pulsed target cells may also be a function

of the low affinity of TCR/MHC-peptide interactions. It is possible that the affinity of the JM22 TCR for HLA-A2 fmp is below the threshold for triggering RBLs via a TCR/CD3ζ chimera. This in turn implies that the 008A3 TCR may have a higher affinity for HLA-A2 pol than the JM22 TCR for its cognate ligand. One piece of indirect evidence supporting such a supposition is the observation that the CTL clone 008A3 appears to be CD8 independent (S McAdam unpublished observations) a characteristic which is thought to reflect a high intrinsic affinity of the TCR for MHC-peptide (Kwan-Lim et al 1993). An alternative explanation for the failure of RBL-38 to respond is that the alpha chain identified from the JM22 clone is not the active partner for BV17S1. However unpublished observations by P. Moss suggest that this is unlikely to be the case since the AV10S1 gene has been found to be consistently expressed together with BV17S1 in a variety of HLA-A2 restricted flu matrix specific CTL clones from different donors.

Differences in the components of the intracellular signalling pathway between T cells and RBL/TCR-CD3ζ transfectants may give rise to problems in the study of TCR recognition of APLs. Thus in T cells there are multiple signalling molecules within the CD3 complex whereas the TCR-CD3ζ chimera has only the CD3ζ chain. Furthermore although signalling in both cell types requires the activation of PTKs there are differences, for example $p56^{lyn}$ (Minoguchi et al 1994) and Syk (Kihara & Siraganian 1994) in RBLs replace $p56^{lck}$ and ZAP70 in T cells (reviewed in Cantrell 1996). These variations in the signalling machinery may mean that RBL/TCR-CD3ζ transfectants are not capable of subtle

variations in response such as the antagonism of T cells observed with some APLs (De Magistris et al 1992).

Despite the potential limitations of RBL/TCR-CD3ζ transfectants the response of RBL-008 to a panel of stimulatory peptides closely mimics that of CTL clone 008A3, albeit with a reduction in sensitivity. This demonstrates that the extracellular portions of the TCR are correctly folded and suggests that it represents a reasonable model for analysing the topology and affinity of the interaction between the 008A3 TCR and HLA-A2 pol. Furthermore the RBL/TCR-CD3ζ system has a number of significant advantages as a bioassay for TCR/MHC-peptide interactions. One advantage, which is common to all transfection based assays, is the ability to mutagenise the extracellular components of the TCR which allows both mapping of antigen recognition and structure/function analysis of the TCR (see Chapters 6 and 7). Transfection of the TCR also guarantees that a single TCR is being studied which is not necessarily the case when using T cells (Padovan et al 1993, Valitutti et al 1995). In addition the isolation of the TCR/MHC-peptide interaction from the setting of the recognition of APCs by T cells excludes the contribution of coreceptors and other intercellular contacts and allows the analysis of a single molecular interaction, especially when recombinant MHC-peptide is used to stimulate RBL cells in place of peptide pulsed target cells (see Chapter 4 and 5). Further benefits of this system are the simplicity of the assay and the ease with which RBL/TCR-CD3ζ transfectants can be grown, which is an important consideration when studying HIV infected T cells because the CTL clones are often difficult to maintain in culture. Indeed, some of the studies

described in this thesis were hampered by the inability to reliably store and regenerate CTL clone 008A3.

Finally as described in Chapters 4 and 5 the strict requirement for aggregation of the TCR-CD3ζ receptor in activation of RBL transfectants becomes a great advantage in competition assays using recombinant monovalent MHC-peptide complexes to inhibit degranulation in response to a multivalent stimulus. Comparison of the inhibition curves obtained with several different MHC-peptide complexes allows a comparison of the relative ability of these complexes to engage the TCR. Although experiments of this kind have been performed with T cell hybridomas (Schneck et al 1989, Weber et al 1992) they may be flawed because of the previously unrecognised ability of soluble MHC-peptide complexes to induce apoptosis of CTL (Zavazava & Kronke 1996).

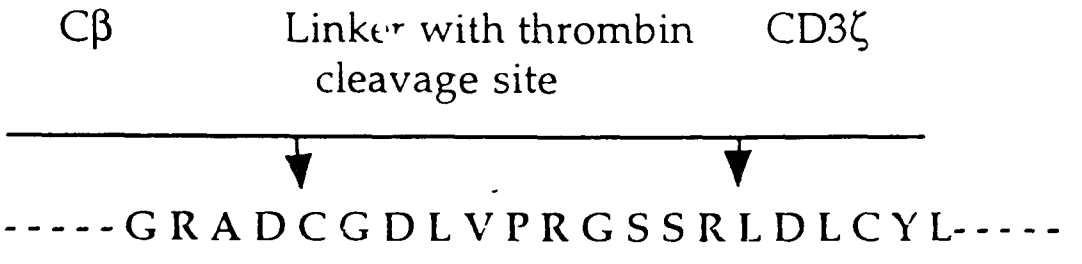
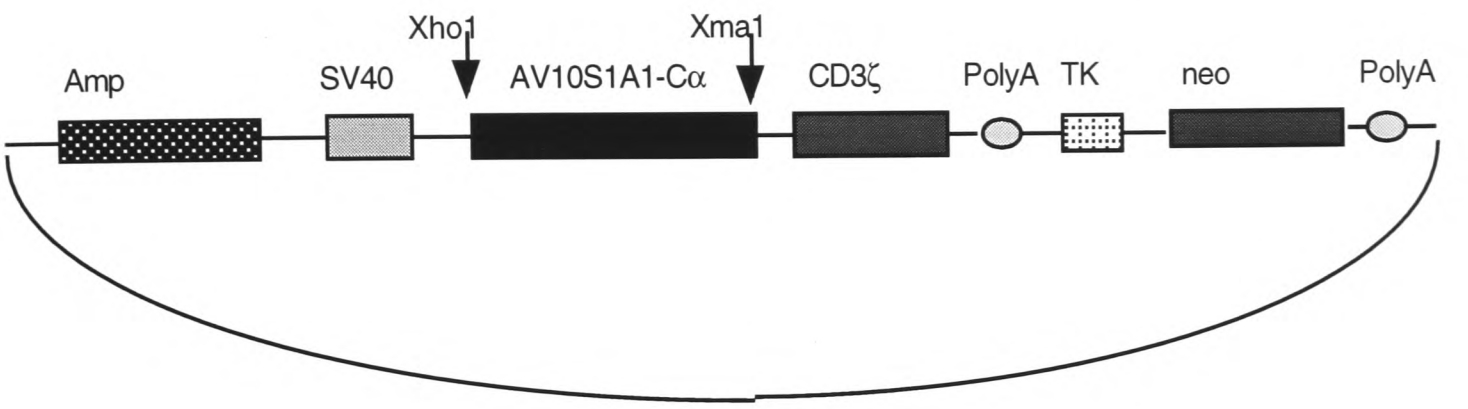


Fig 3.1

The amino acid sequence of the TCR β chain fusion to CD3ζ

As used by Engel et al 1992 and in the experiments described in this thesis. The Cβ domain is truncated at the cysteine which participates in the interchain disulphide bond (the same truncation point was used in the α fusion). The linker contains a thrombin cleavage site since the construct was orignally designed so that the extracellular portion of the TCR could be cleaved form the RBL surface (see Engel et al 1992) (Amino acids are represented in the single letter code).

(a) BJ048



(b) BJ043

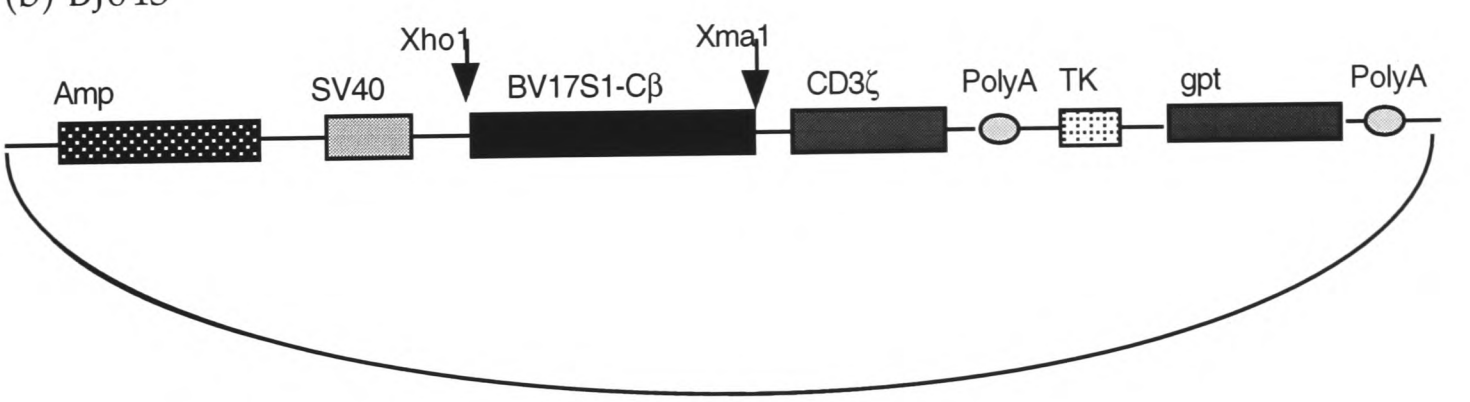


Fig 3.2a&b
Schematic representation of expression plasmids.

(a) pBJ048 was made by cloning an AV10S1A1-Cα-CD3ζ fusion into the expression vector pMC1neo.

(b) pBJ043 was made by cloning a BV17S1-Cβ-CD3ζ fusion into the expression vector 2B4-GPT.

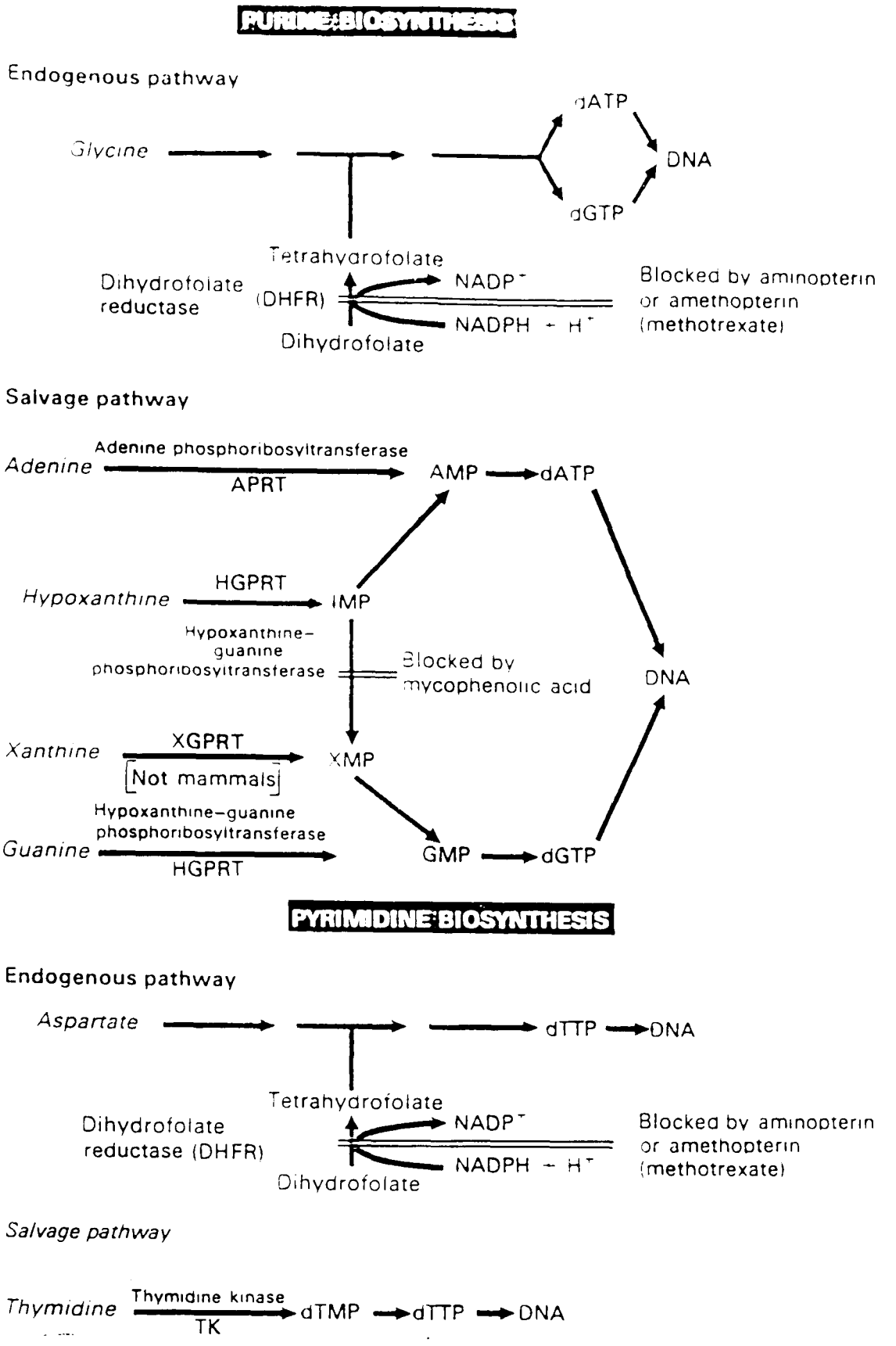


Fig 3.2c.
Selection of stable transfectants expressing the gpt resistance gene.
Mycophenolic acid blocks a step on the pathway that generates dGTP from hypoxanthine. This block can be overcome by expression of the XGPRT gene which allows conversion of xanthine to XMP and ultimately to dGTP. A selection system can then be generated by stably transfecting in the XGPRT gene in the presence of xanthine and hypoxanthine (which allows production of dATP) and mycophenolic acid. The selection can be made more efficient by the addition of aminopterin which prevents the endogenous production of dGTP, dATP and dTTP, although thymidine must then be provided to salvage the production of dTTP. (Adapted from Old & Primrose).

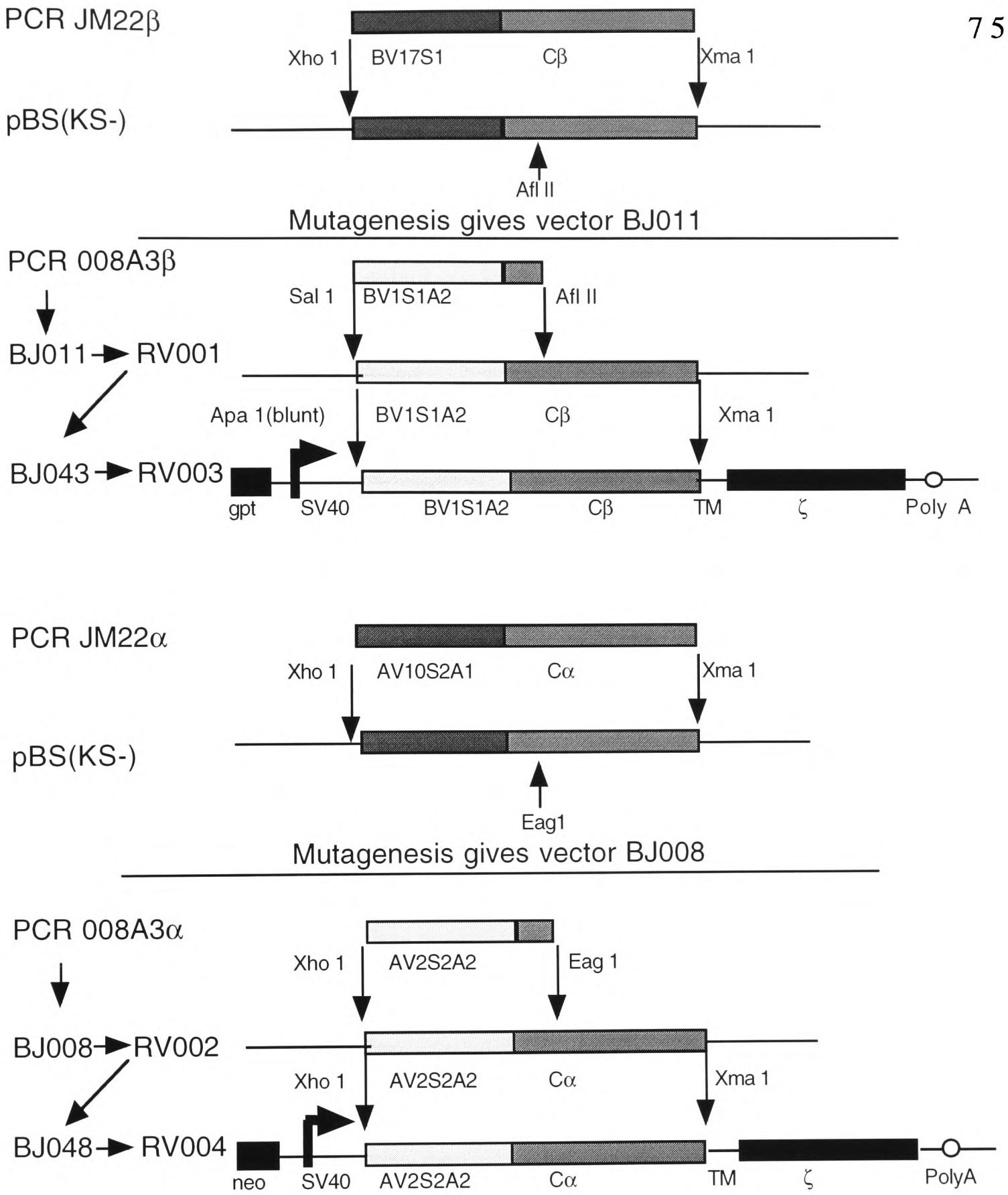


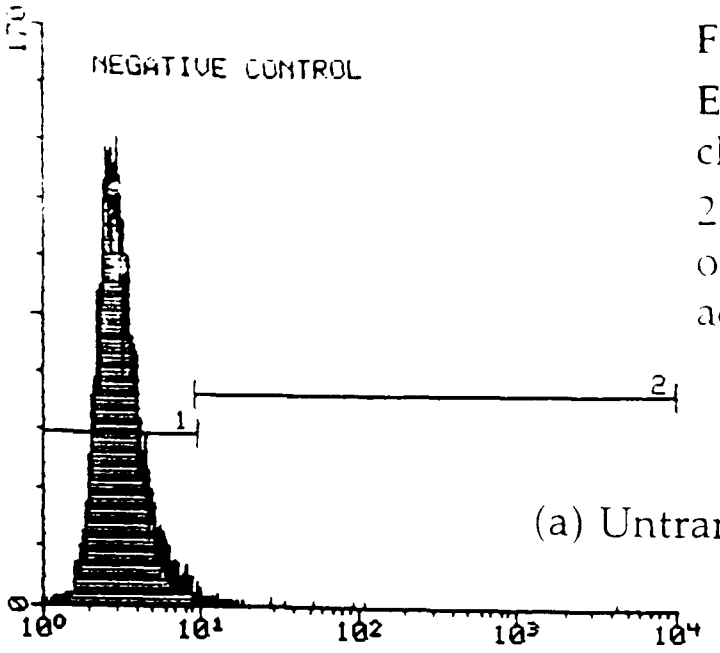
Fig 3.3 Cloning of the A3 TCR-CD3ζ chimeric receptor.

The α and β chains of the JM22 TCR were amplified and cloned into pBluescript (KS-) as fragments truncated immediately 3' to the interchain disulphide bond. These were then silently mutagenised to encode an EagI site at position 15 of Cα and an AflII site at position 10 of Cβ (Constructs BJ008 and BJ011). Portions of the α and β chains of the 008A3 TCR encoding the V domain and a small part of the C domain were then generated by PCR and cloned as SalI-AflII (construct RV001) and XhoI-EagI (construct RV002) fragments respectvely. The V-C portions of both chains were then subcloned into BJ048 and BJ043 as XhoI-XmaI and Blunt-XmaI respectively (Constructs RV003 and RV004).

Fig 3.4abcdef&g

Expression of the A3 TCR-CD3ζ chains by various cell lines.

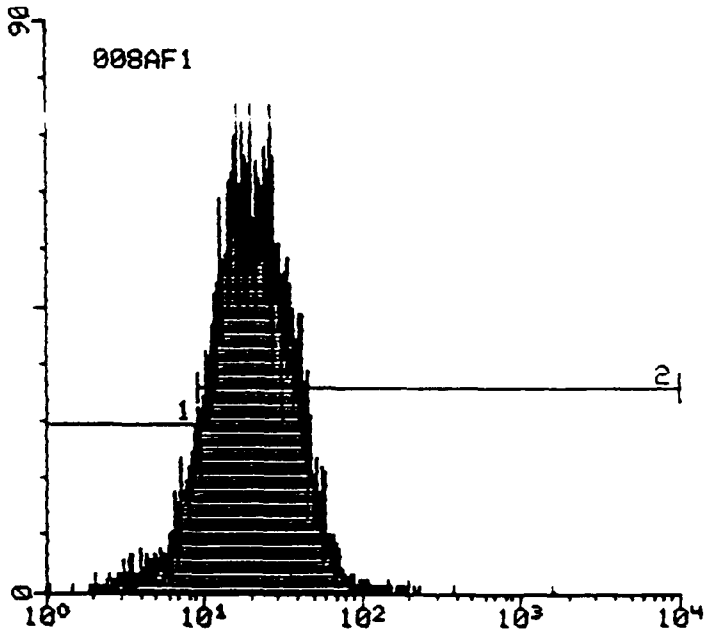
2x10⁵ cells were stained with mAbs βF1, αf1 or (in the case of RBL-38) antiBV17S1 according to methods described in Chapter



(a) Untransfected RBLs stained with βF1 and αf1

--- Arithmetic Histogram Statistics for V3:RV002 ---
Parameter FL1-H FL1-Height Ungated

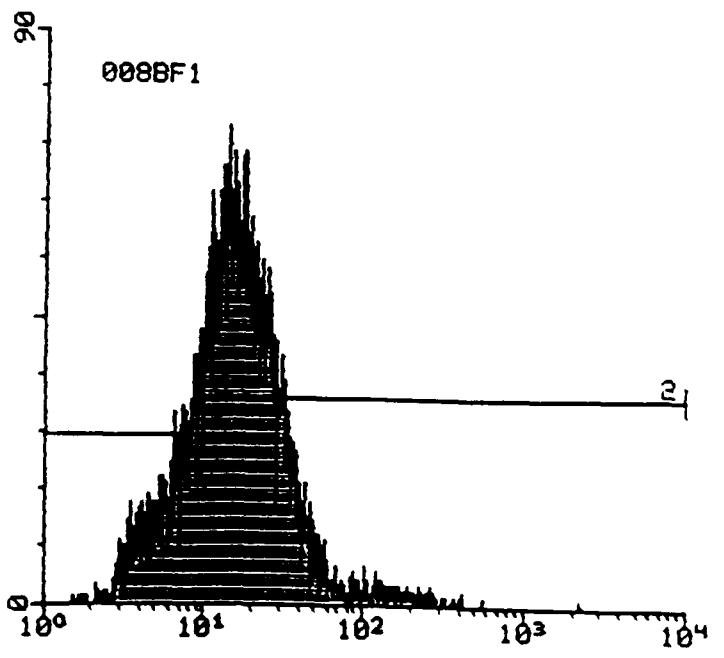
M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV
0	1.00, 9910	9403	100.00	138	3.05	3.78	2.97	43.50	>100.0
1	1.00, 9.73	9352	99.45	138	3.05	3.26	2.97	1.18	36.30
2	9.30, 9910	67	0.71	6	9.73	78.22	10.99	513.55	>100.0



(b i) RBL-008 stained with αF1

--- Arithmetic Histogram Statistics for V3:RV003 ---
Parameter FL1-H FL1-Height Ungated

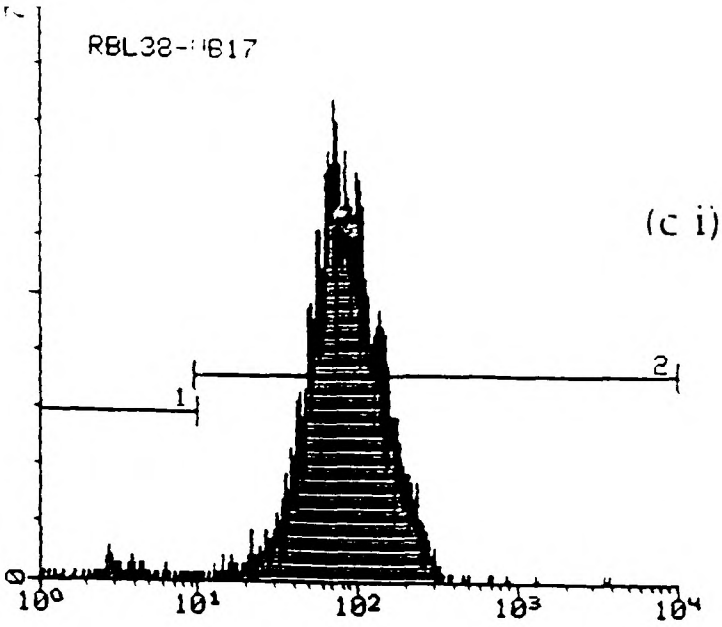
M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV
0	1.00, 9910	10000	100.00	77	16.69	23.70	20.06	22.00	92.84
1	1.00, 9.73	1091	10.91	29	9.30	7.22	7.68	1.93	26.72
2	9.30, 9910	9054	90.53	77	16.69	25.46	21.84	22.40	87.98



(b ii) RBL-008 stained with βF1

--- Arithmetic Histogram Statistics for V3:RV004 ---
Parameter FL1-H FL1-Height Ungated

M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV
0	1.00, 9910	10000	100.00	75	14.07	21.27	15.64	34.19	>100.0
1	1.00, 9.73	2265	22.65	43	9.64	6.64	6.80	2.03	30.56
2	9.30, 9910	7970	79.70	75	14.07	25.08	18.17	37.34	>100.0

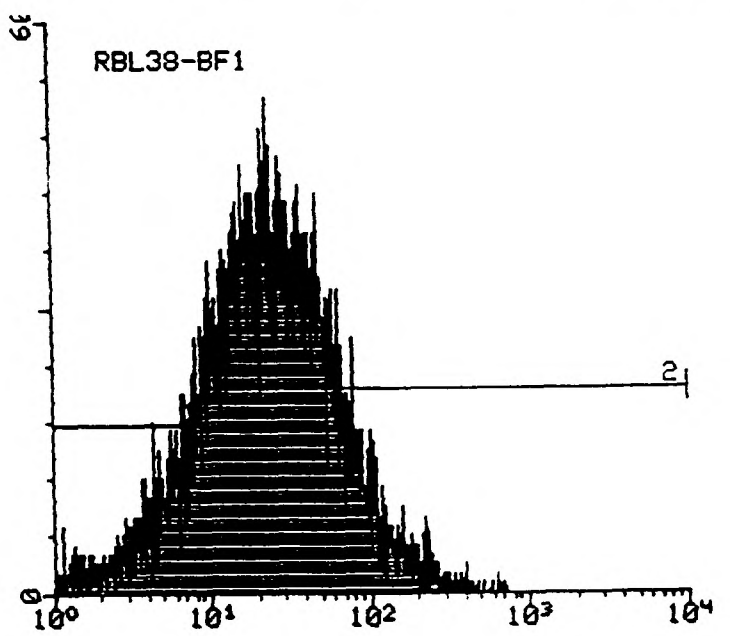


(c i) RBL-38 stained with anti-BV17S1

--- Arithmetic Histogram Statistics for V3:RV007 ---

Parameter FL1-H FL1-Height Ungated

M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV
0	1.00, 9910	6193	100.00	59	72.99	95.28	81.67	85.80	90.05
1	1.00, 9.73	101	1.63	4	2.71	3.99	3.27	2.03	50.99
2	9.30, 9910	6095	98.41	59	72.99	96.75	82.34	85.69	88.57

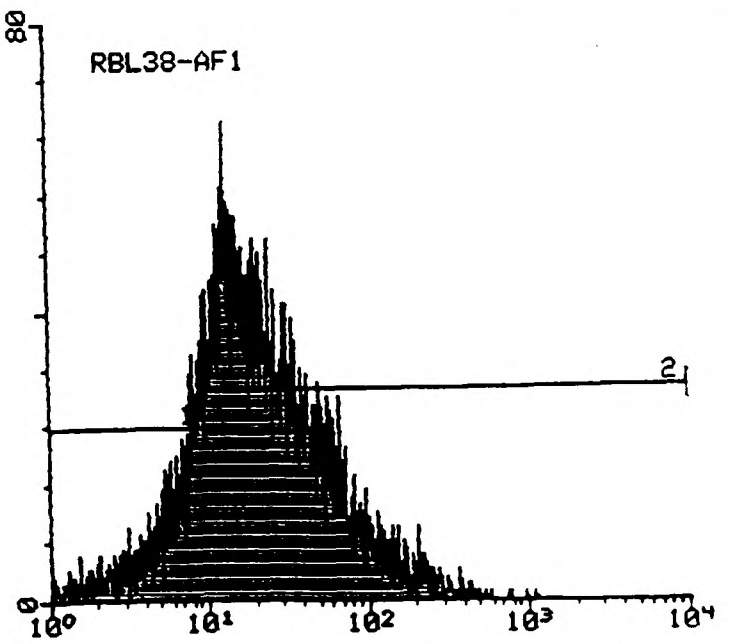


(c ii) RBL-38 stained with βF1

--- Arithmetic Histogram Statistics for V3:/1/RV014 ---

Parameter FL2-H FL2-Height Ungated

M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV
0	1.00, 9910	10000	100.00	52	23.50	35.22	23.17	41.81	>100.0
1	1.00, 9.73	1911	19.10	47	1.00	5.80	5.93	2.56	44.13
2	9.30, 9910	8236	82.35	52	23.50	41.58	28.47	43.49	>100.0

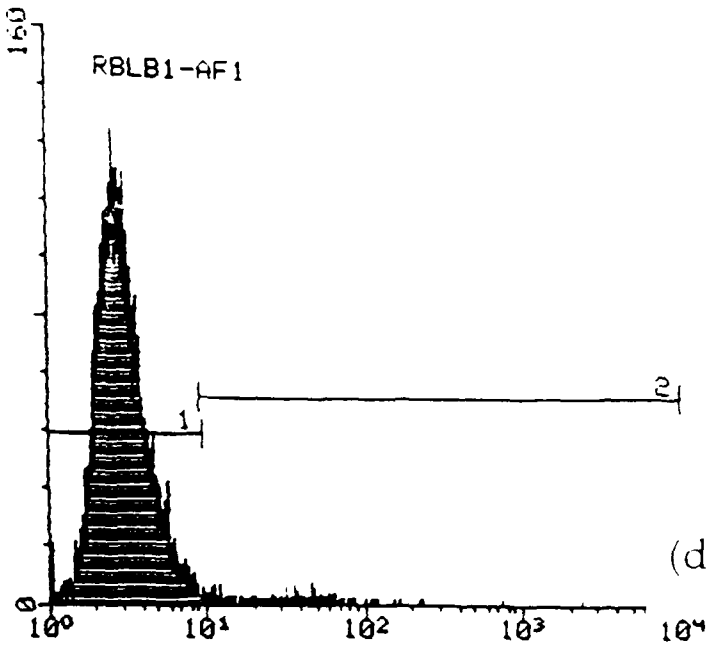


(c iii) RBL-38 stained with αF1

--- Arithmetic Histogram Statistics for V3:/1/RV010 ---

Parameter FL2-H FL2-Height Ungated

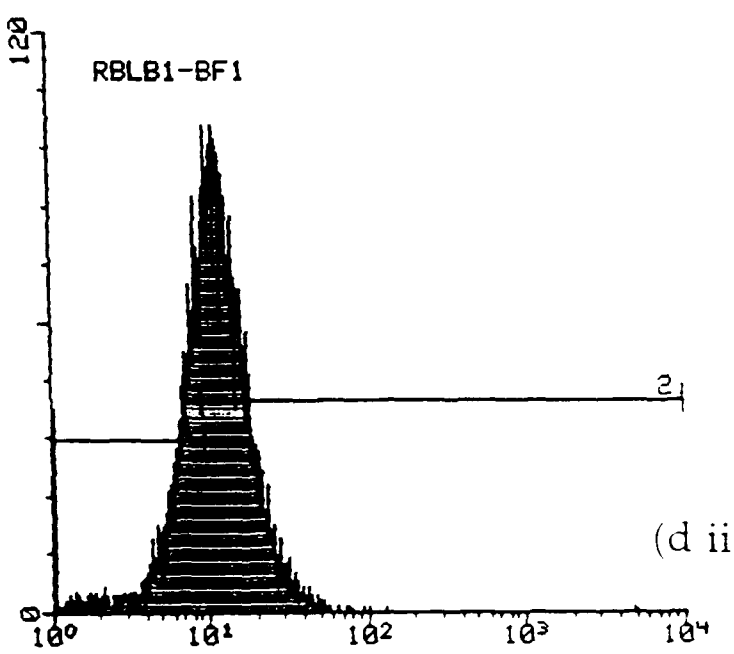
M	Left,Right	Events	%	Peak	PkChl	Mean	Median	SD	CV
0	1.00, 9910	10000	100.00	66	13.45	33.25	18.74	47.18	>100.0
1	1.00, 9.73	2059	20.58	42	9.73	6.19	6.66	2.53	40.95
2	9.30, 9910	8143	81.43	66	13.45	39.50	23.12	50.22	>100.0



Parameter FL1-H	FL1-Height		Ungated
M Left,Right	Events	%	Peak
0 1.00, 9910	10000	100.00	132
1 1.00, 9.73	9759	97.59	132
2 9.30, 9910	255	2.54	6

PkChl	Mean	Median	SD	CV
2.64	4.30	2.94	25.98	>100.0
2.64	3.18	2.86	1.25	39.32
46.97	47.63	29.03	156.79	>100.0

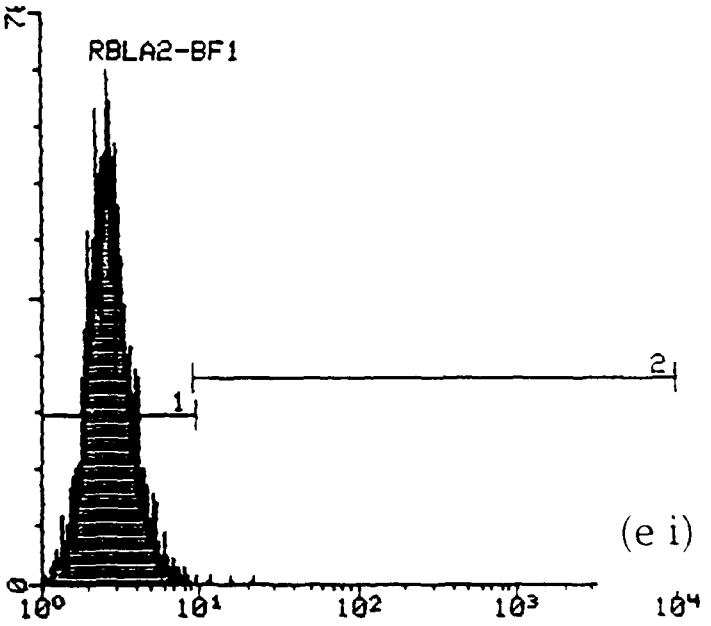
(d i) RBL-β1 (BV1S1A2-CD3ζ only) stained with αF1



Parameter FL1-H	FL1-Height		Ungated
M Left,Right	Events	%	Peak
0 1.00, 9910	10000	100.00	101
1 1.00, 9.73	3718	37.17	88
2 9.30, 9910	6749	67.49	101

PkChl	Mean	Median	SD	CV
10.00	12.83	11.17	49.01	>100.0
9.47	7.06	7.62	2.10	29.83
10.00	15.78	13.43	59.42	>100.0

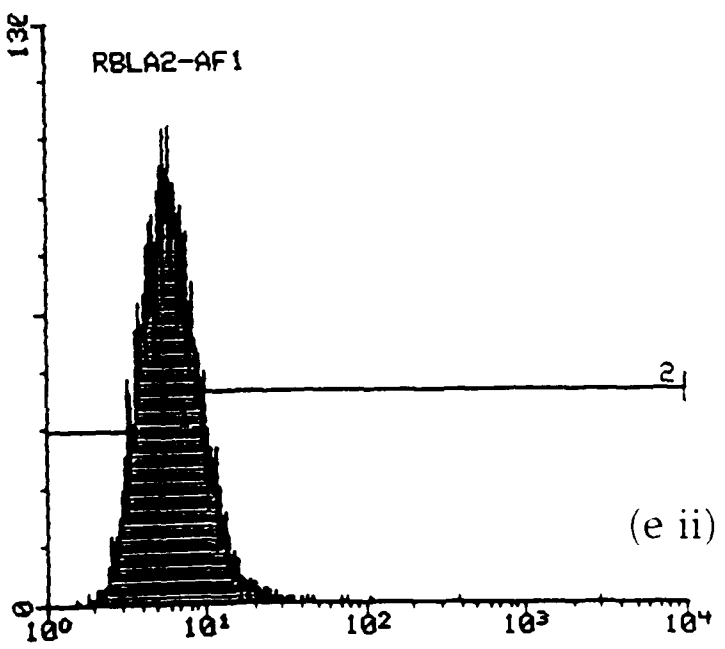
(d ii) RBL-β1 (BV1S1A2-CD3ζ only) stained with βF1



Parameter FL1-H	FL1-Height		Ungated
M Left,Right	Events	%	Peak
0 1.00, 9910	3818	100.00	63
1 1.00, 9.73	3815	99.92	63
2 9.30, 9910	5	0.13	1

PkChl	Mean	Median	SD	CV
2.64	2.86	2.68	1.03	36.10
2.64	2.85	2.68	0.95	33.50
9.56	13.63	9.64	5.09	37.35

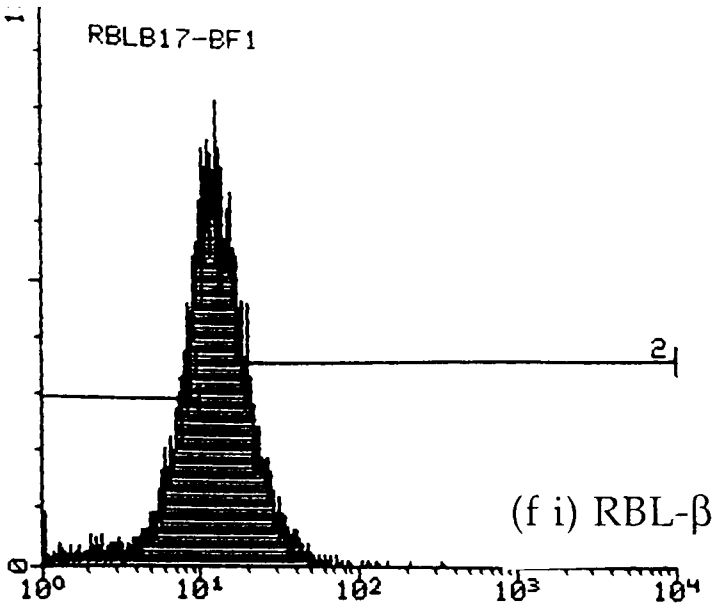
(e i) RBL-α1(AV1S2A2-CD3ζ only) stained with βF1



Parameter FL1-H	FL1-Height		Ungated
M Left,Right	Events	%	Peak
0 1.00, 9910	10000	100.00	107
1 1.00, 9.73	8813	88.12	107
2 9.30, 9910	1477	14.76	52

PkChl	Mean	Median	SD	CV
5.98	6.94	6.04	29.27	>100.0
5.98	5.78	5.64	1.79	31.07
10.09	14.38	10.94	75.66	>100.0

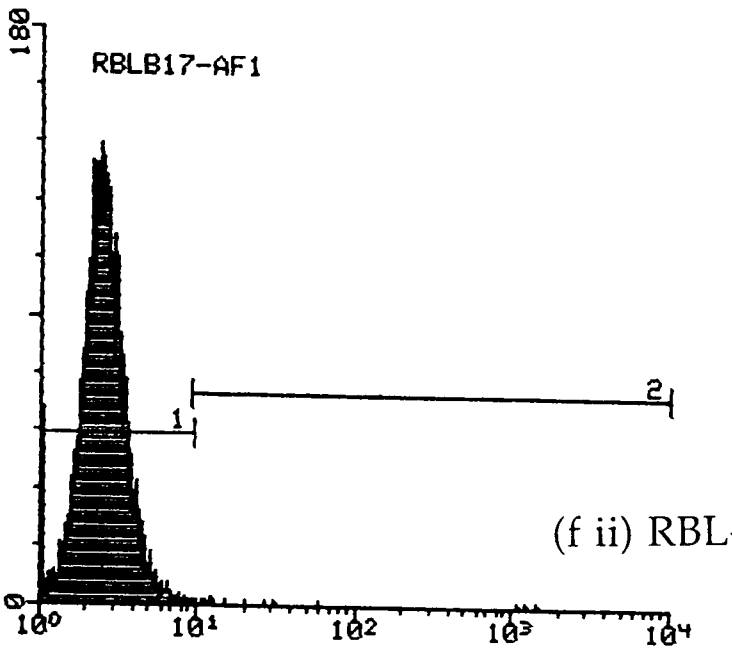
(e ii) RBL-α1(AV1S2A2-CD3ζ only) stained with αF1



Parameter	FL1-H	FL1-Height		Ungated
M	Left,Right	Events	%	Peak
0	1.00, 9910	10000	100.00	107
1	1.00, 9.73	2889	28.89	78
2	9.30, 9910	7521	75.20	107

PkChl	Mean	Median	SD	CV
12.63	15.83	12.30	115.23	>100.0
9.73	7.07	7.69	2.22	31.48
12.63	18.85	14.08	132.73	>100.0

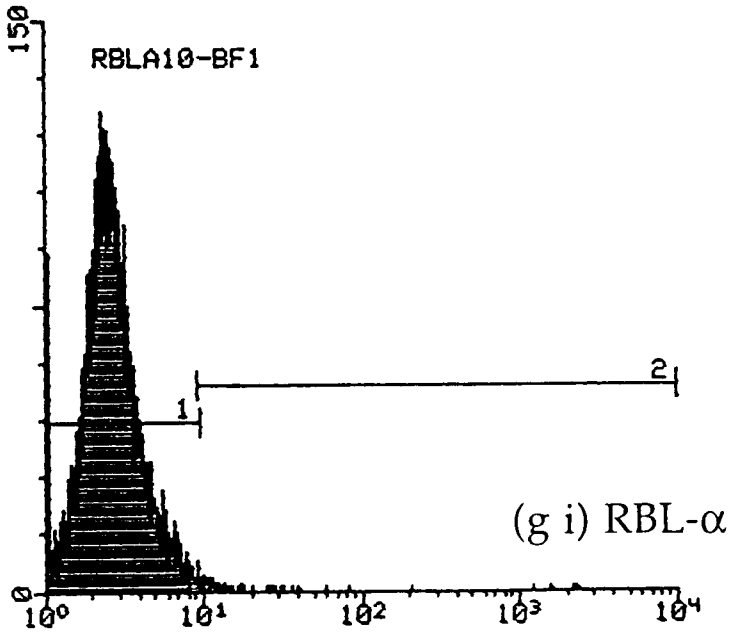
(f i) RBL-β17 (BV17S1-CD3ζ only) stained with βF1



Parameter	FL1-H	FL1-Height		Ungated
M	Left,Right	Events	%	Peak
0	1.00, 9910	10000	100.00	143
1	1.00, 9.73	9982	99.81	143
2	9.30, 9910	20	0.20	2

PkChl	Mean	Median	SD	CV
2.39	3.07	2.54	22.12	>100.0
2.39	2.67	2.54	0.90	33.88
12.40	204.56	12.46	463.09	>100.0

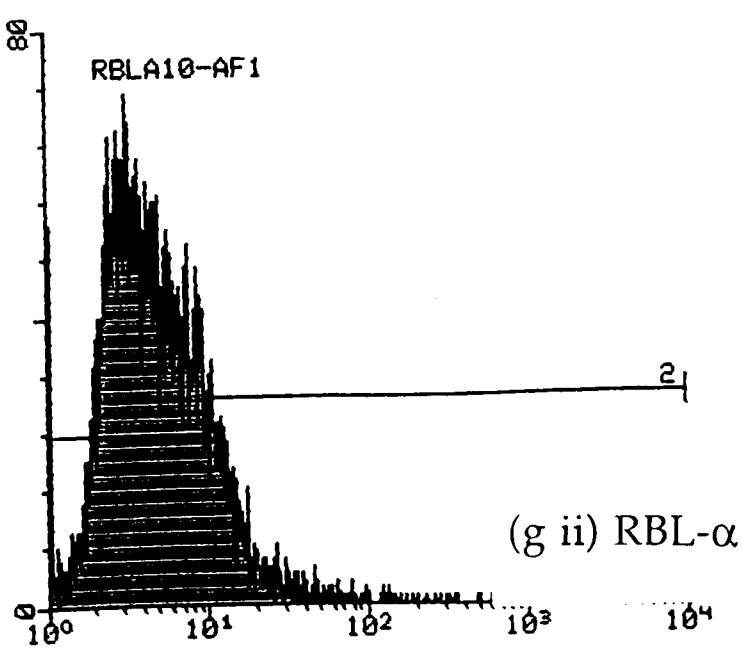
(f ii) RBL-β17 (BV17S1-CD3ζ only) stained with αF1



Parameter	FL1-H	FL1-Height		Ungated
M	Left,Right	Events	%	Peak
0	1.00, 9910	10000	100.00	126
1	1.00, 9.73	9917	99.17	126
2	9.30, 9910	99	0.99	6

PkChl	Mean	Median	SD	CV
2.37	3.73	2.63	37.46	>100.0
2.37	2.88	2.59	1.29	44.68
9.30	89.78	11.30	368.06	>100.0

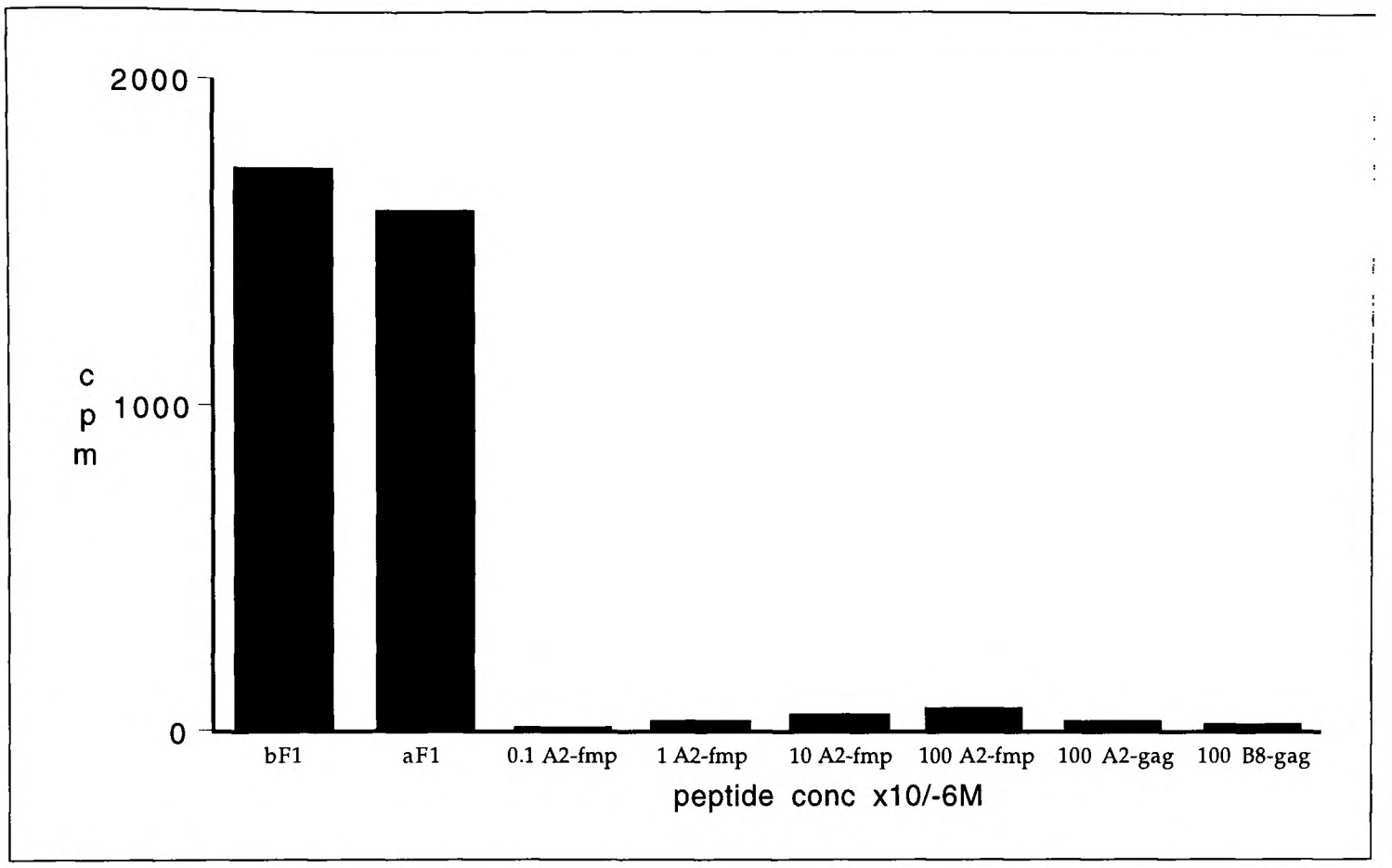
(g i) RBL-α10 (AV10S2A1-CD3ζ only) stained with βF1



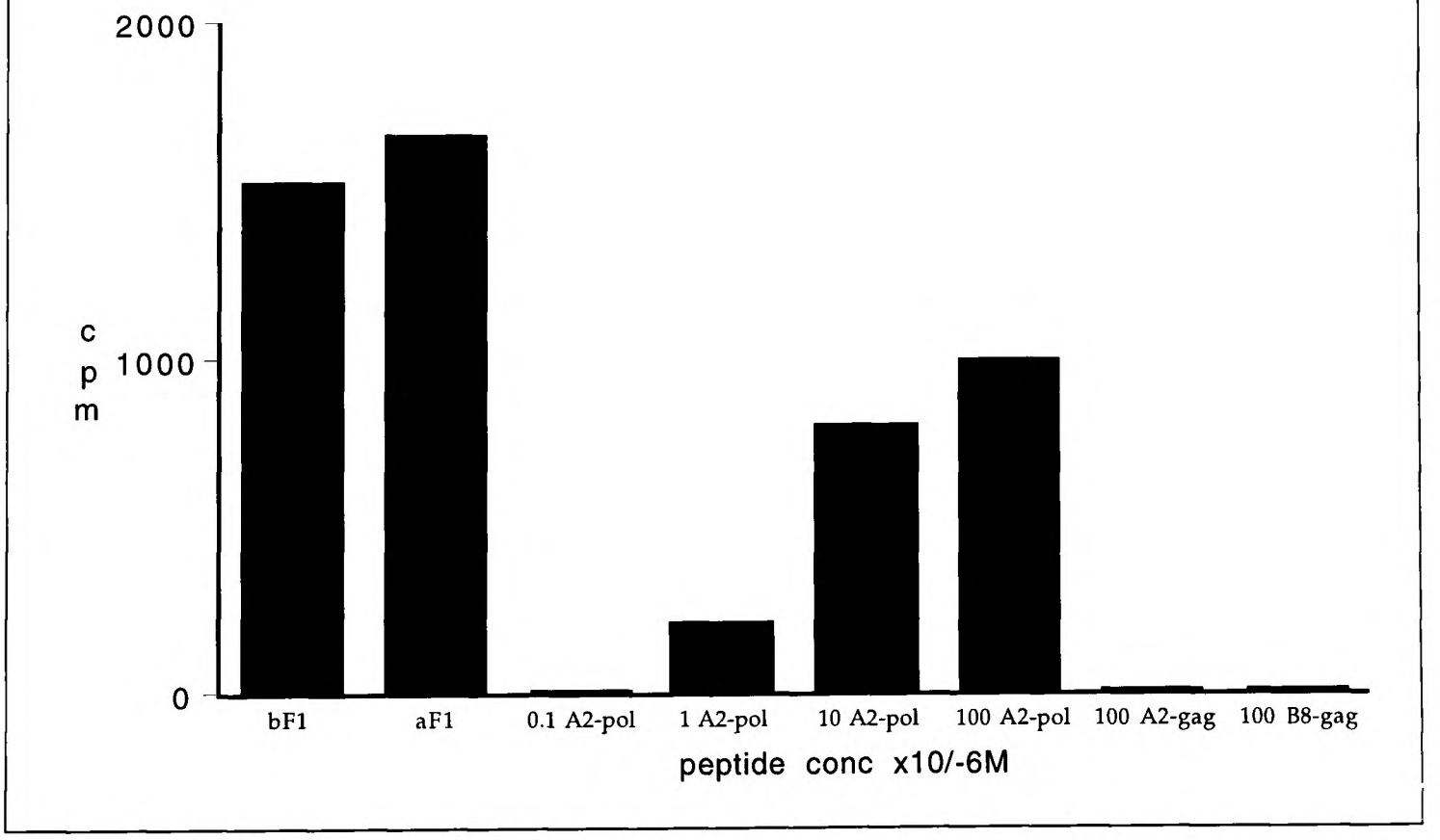
Parameter	FL1-H	FL1-Height		Ungated
M	Left,Right	Events	%	Peak
0	1.00, 9910	10000	100.00	71
1	1.00, 9.73	8453	84.53	71
2	9.30, 9910	1742	17.41	41

PkChl	Mean	Median	SD	CV
3.30	11.88	4.47	167.87	>100.0
3.30	4.42	3.88	2.16	48.90
9.47	47.80	12.70	400.35	>100.0

(g ii) RBL-α10 (AV10S2A1-CD3ζ only) stained with αF1



(a)



(b)

Fig 3.5a&b. Response of (a)RBL-38 and (b)RBL-008 to mAbs and peptide pulsed target cells.

RBL transfectants were labelled as described in chapter 2. They were then co-incubated with peptide pulsed target cells or mAbs (βF1-anti Cβ, αF1-anti Cα). Peptide concentration or mAb is shown on the horizontal axis and degranulation response on the vertical axis in counts per minute (cpm). Results are the mean values of triplicate measurements and these experiments were performed on at least 5 occasions.

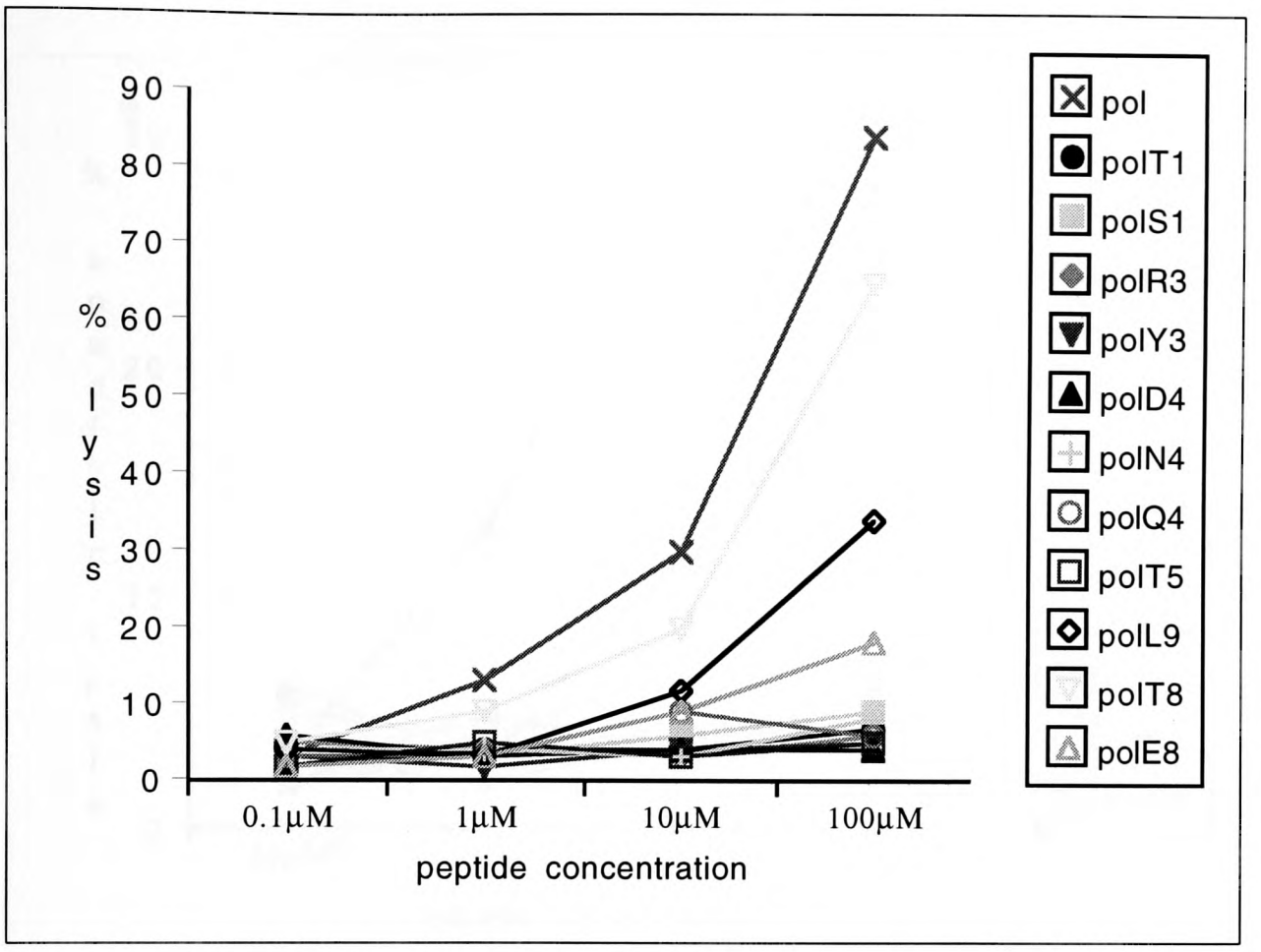


Fig 3.6a
Response of RBL-008 to target cells pulsed with pol peptide variants.
RBL transfectants were labelled as described in chapter 2 and were then co-incubated with peptide pulsed target cells. Peptide concentration is shown on the horizontal axis and degranulation response is shown on the vertical axis as a percentage of the response to 5μl of βF1 ascites. The peptides are named using the single letter code for aminoacids followed by the position of the substitution (see chapter 2 for aminoacid sequences). Results are the means of triplicates and these experiments were performed on at least 3 occasions (Sequence of pol ILKEPVHGV).

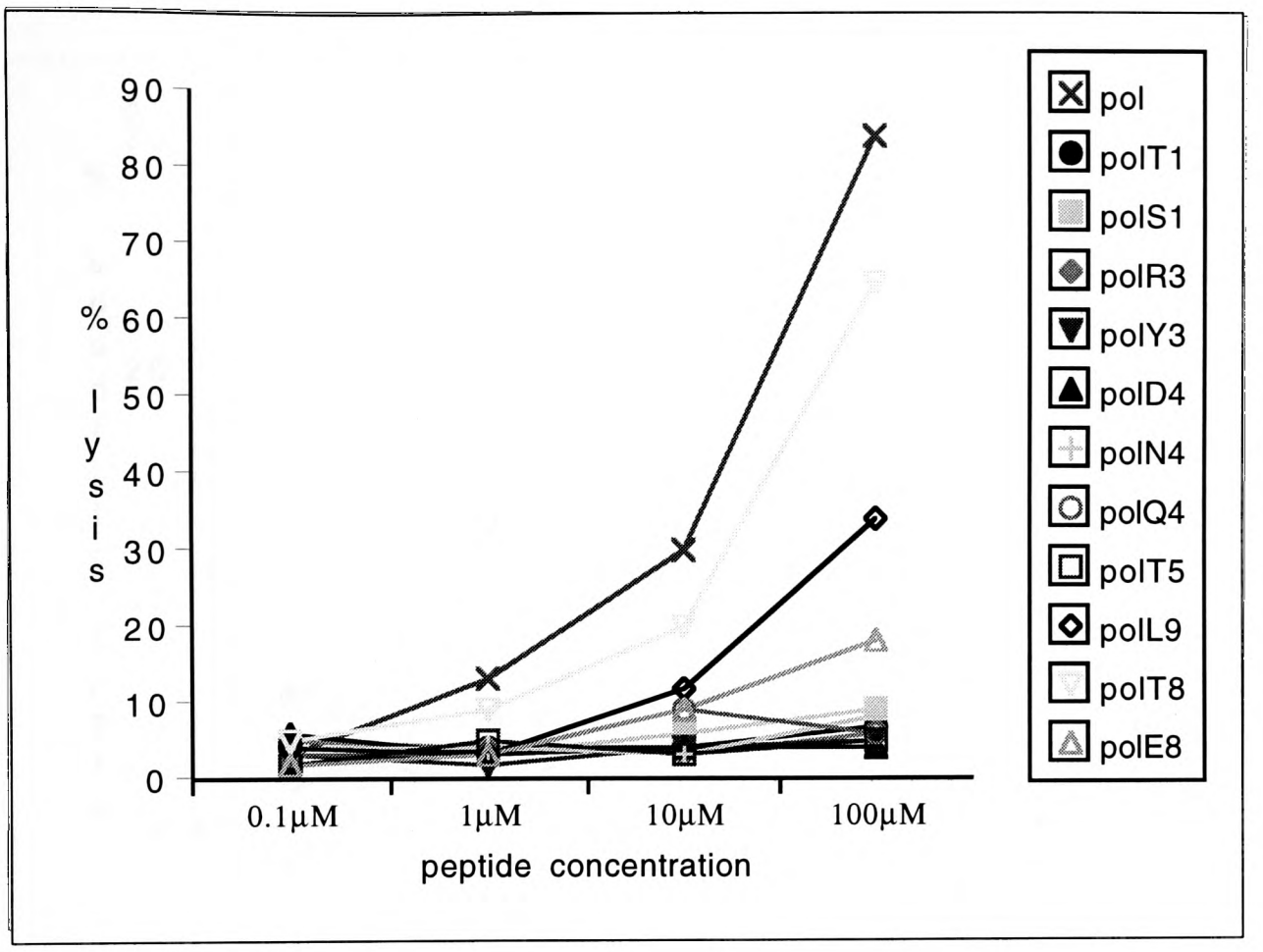
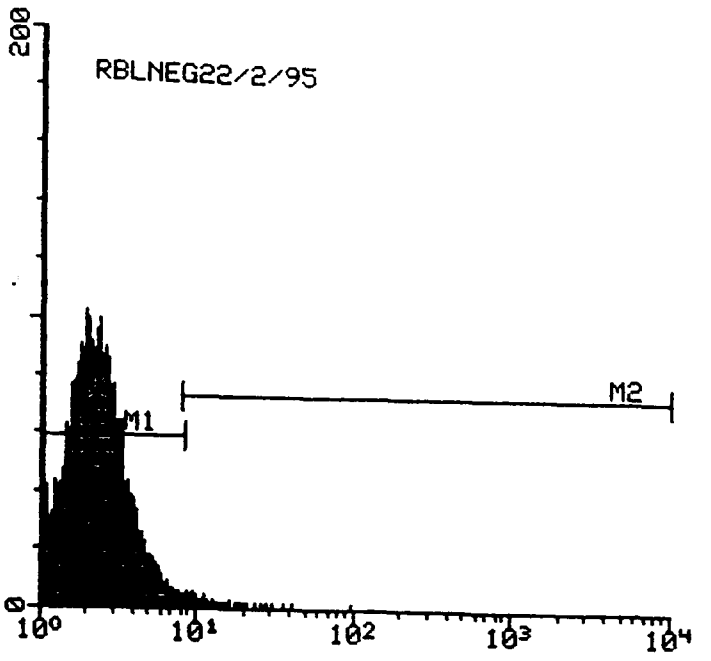
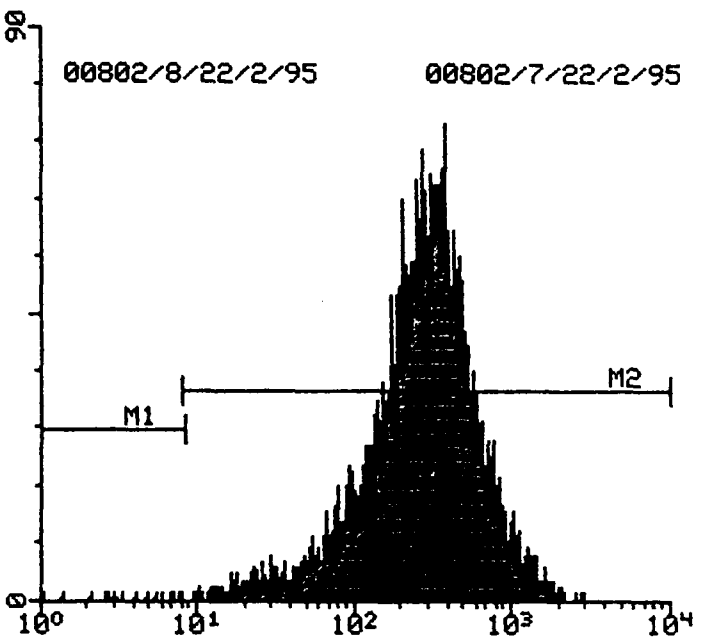


Fig 3.6b. Response of CTL clone 008A3 to target cells pulsed with pol peptide variants.
For CTL assays target cells were labelled with ^{51}Cr as described in chapter 2 and then pulsed with peptide before addition of CTL, the response is expressed on the vertical axis as % specific lysis calculated by the formula: (experimental release - media release) / (triton release - media release), peptide concentration is shown on the horizontal axis. The peptides are named using the single letter code for aminoacids followed by the position of the substitution (see chapter 2 for aminoacid sequences). Results are the means of triplicates and these experiments were performed on at least 3 occasions. (Sequence of pol ILKEPVHGV). Assays performed by S. McAdam.



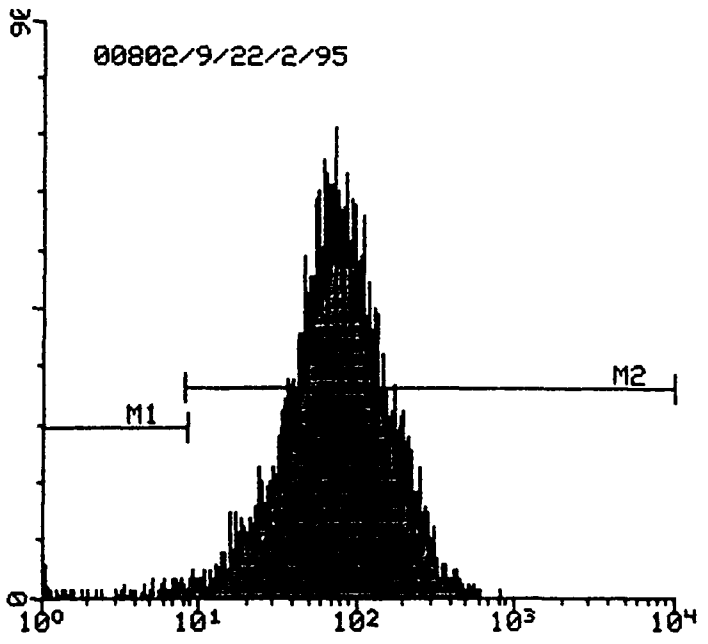
Parameter FL2-H FL2-Height				Gate G1= R1	
M	Left,Right	Events	%	Peak	PkChl
0	1.00, 9910	10000	100.00	209	1.07
1	1.00, 8.58	9907	99.07	209	1.07
2	8.28, 9910	106	1.06	5	8.98

(i)



Parameter FL2-H FL2-Height				Gate G1= R1	
M	Left,Right	Events	%	Peak	PkChl
0	1.00, 9910	10000	100.00	75	375.16
1	1.00, 8.58	17	0.17	1	1.06
2	8.28, 9910	9983	99.83	75	375.16

(ii)



Parameter FL2-H FL2-Height				Gate G1= R1	
M	Left,Right	Events	%	Peak	PkChl
0	1.00, 9910	10000	100.00	73	72.99
1	1.00, 8.58	85	0.85	5	1.07
2	8.28, 9910	9918	99.18	73	72.99

(iii)

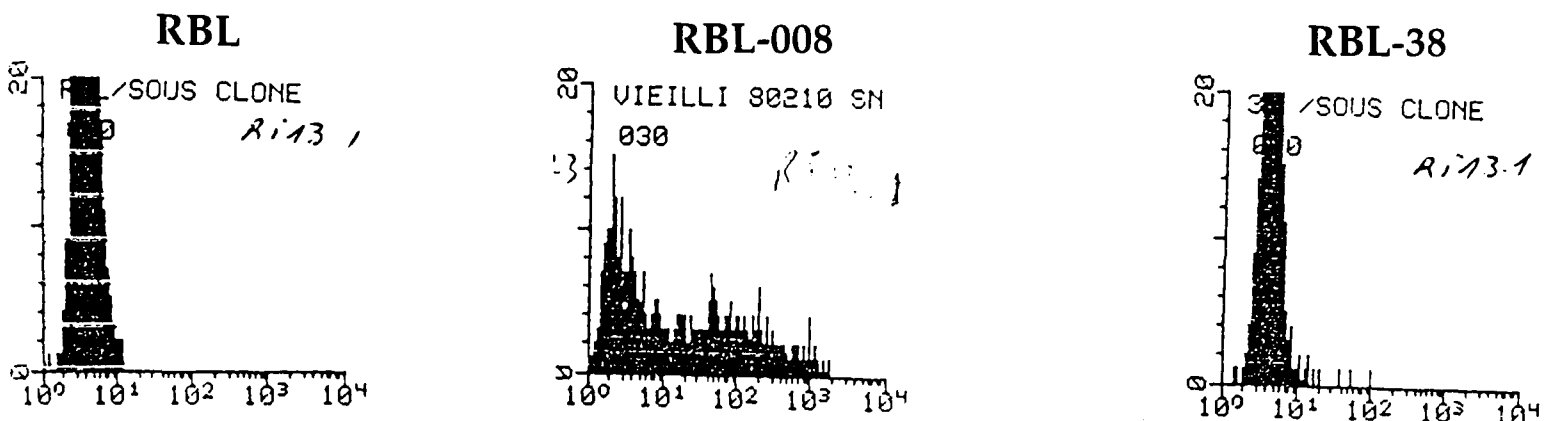
Fig 3.7 Expression of the A3 TCR-CD3ζ chains by clone RBL-00802

$2x10^5$ cells were stained with mAbs βF1 or αf1 according to methods described in Chapter 2.

(i) Untransfected RBLs stained with βF1 and αf1

(ii) RBL-00802 stained with αF1

(iii) RBL-00802 stained with βF1



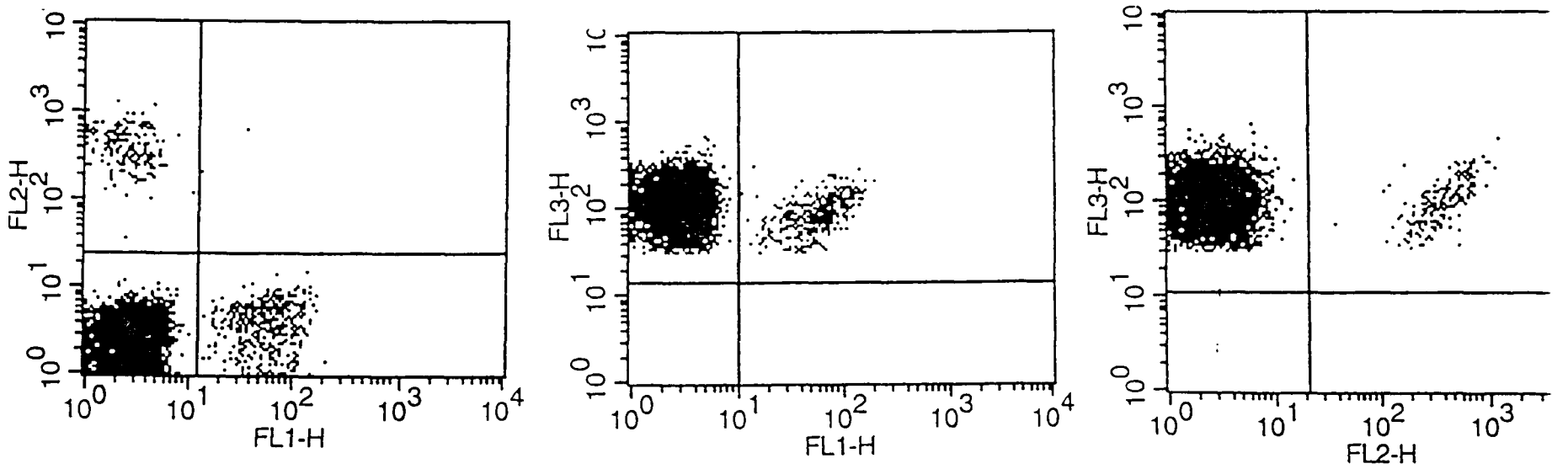


Fig 3.8a

Characterisation of candidate mAb against BV1S1A2

A representative set of flow cytometric histograms is shown for mAb R13.1 (anti-BV1S1A2). Details of staining by other mAbs is summarised in table 3.1. Histograms show staining of untransfected RBLs, RBL-008, RBL-38, RBL-α1, RBL-β1, RBL-α10 and RBL-β17. In addition triple staining of whole blood with anti-CD3 (FL3-H), anti-TCRBV2 (FL1-H) and R13.1 (FL2-H) is shown. For staining of transfected/untransfected RBLs $2x10^5$ cells were stained with mAb according to methods described in Chapter 2, for staining of whole blood 100ml of mAb was triple stained with candidiate supernatant-GAM PE, anti-CD3-PE cy5 and anti-TCRBV2 FITC.

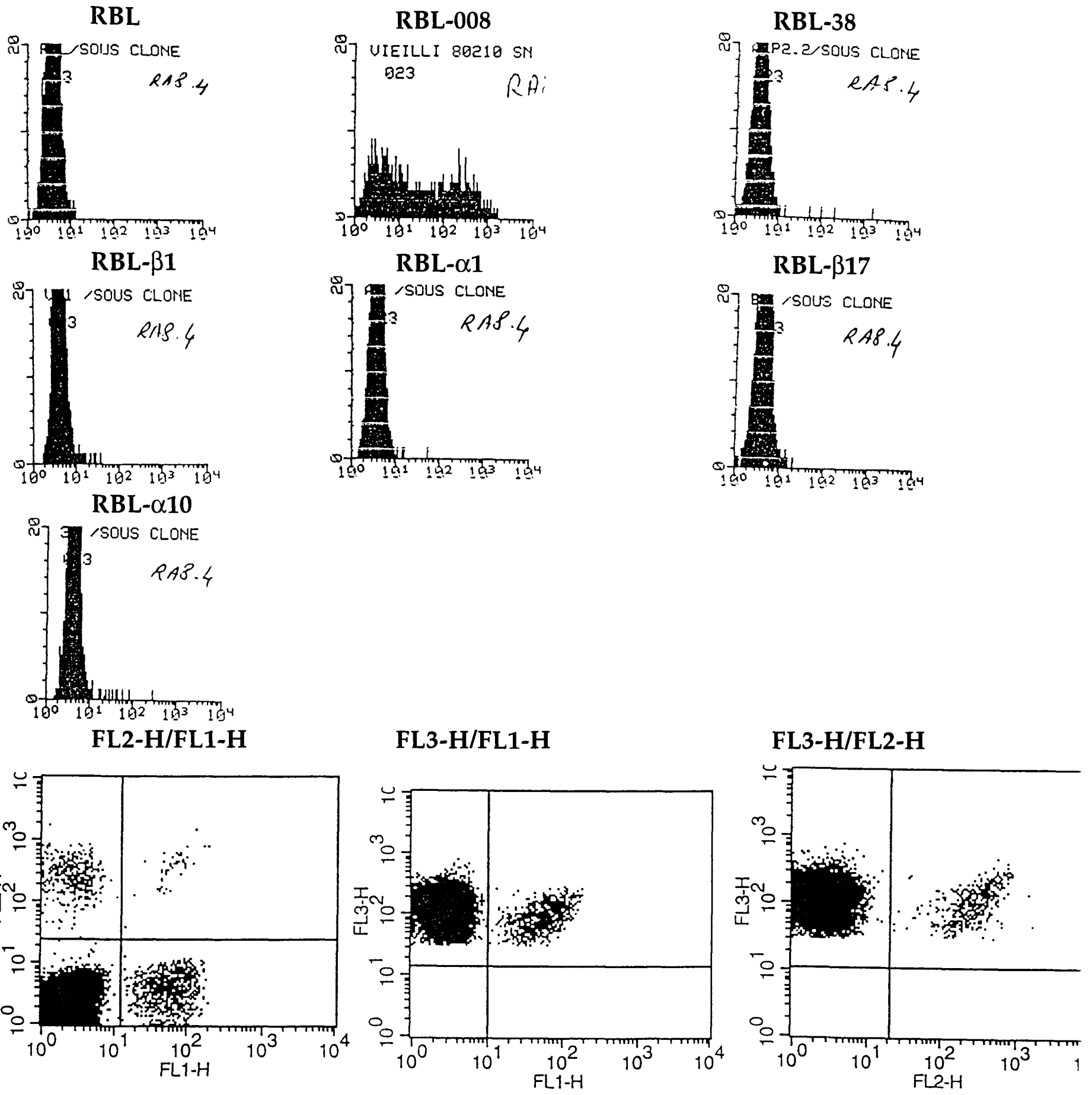


Fig 3.8b

Characterisation of candidate mAb against AV2S2A2

A representative set of flow cytometric histograms is shown for mAb RA8.4 (anti-AV2S2A2). Details of staining by other mAbs is summarised in table 3.1. Histograms show staining of untransfected RBLs, RBL-008, RBL-38, RBL-α1, RBL-β1, RBL-α10 and RBL-β17. In addition triple staining of whole blood with anti-CD3 (FL3-H), anti-TCRBV2 (FL1-H) and RA8.4 (FL2-H) is shown. For staining of transfected/untransfected RBLs $2x10^5$ cells were stained with mAb according to methods described in Chapter 2, for staining of whole blood 100ml of mAb was triple stained with candidiate supernatant-GAM PE, anti-CD3-PE cy5 and anti-TCRBV2 FITC.

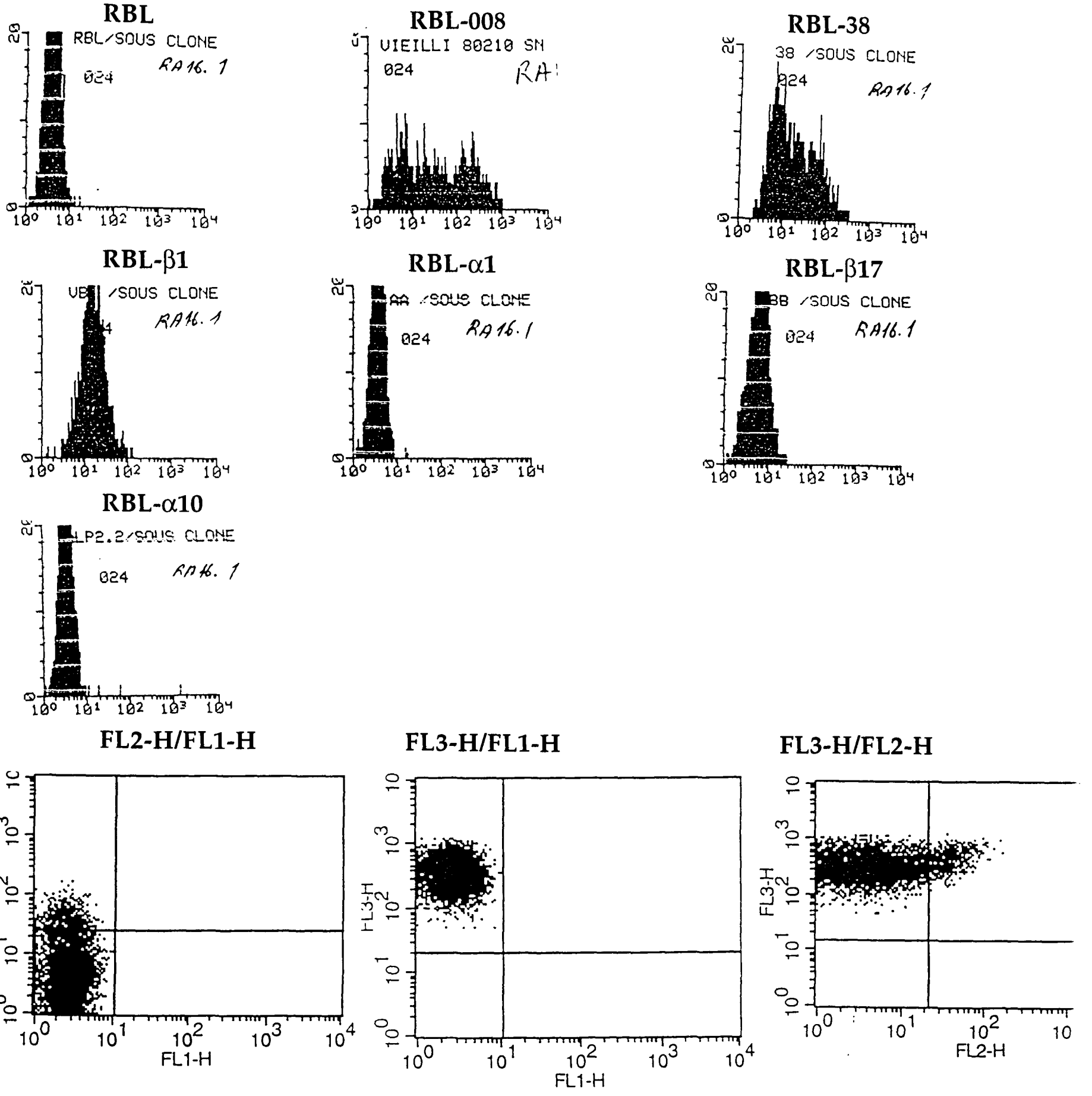


Fig 3.8 c

Characterisation of candidate mAb against Cβ.

A representative set of flow cytometric histograms is shown for mAb RA16.1 (anti-Cβ). Details of staining by other mAbs is summarised in table 3.1. Histograms show staining of untransfected RBLs, RBL-008, RBL-38, RBL-α1, RBL-β1, RBL-α10 and RBL-β17. In addition triple staining of whole blood with anti-CD3 (FL3-H), anti-TCRBV2 (FL1-H) and RA8.4 (FL2-H) is shown. For staining of transfected/untransfected RBLs $2x10^5$ cells were stained with mAb according to methods described in Chapter 2, for staining of whole blood 100ml of mAb was triple stained with candidiate supernatant-GAM PE, anti-CD3-PE cy5 and anti-TCRBV2 FITC.

Chapter 4
The Effect of Peptide on Contacts Between the TCR and MHC Molecules

4.1 Introduction

4.11 The Role of Peptides in Thymic Selection

The recently reported crystal structures of two TCR/MHC-peptide co-complexes (Garcia et al 1996a, Garbozci et al 1996) confirm the results of many earlier studies (Davis & Bjorkman 1988, Chothia et al 1988, Claverie et al 1989, Novotny et al 1986, Chien & Davis 1993, Jorgensen et al 1992, Sun et al 1995, Ehrich et al 1993) by demonstrating that the TCR forms contacts with both the MHC and peptide components of its ligand. The relative significance of contacts between the TCR and the MHC as opposed to those between the TCR and the peptide is probably related to the developmental status of the T cell. Thus the responses of fully differentiated T cells, whose function is to detect virally infected cells, are primarily determined by contacts between the TCR and the antigenic peptide. A fact highlighted by the discovery of altered peptide ligands which can act as partial agonists and antagonists for the T cell (Jameson & Bevan 1995, Sloan-Lancaster & Allen 1996). However the role of TCR-peptide interactions in determining the responses of developing thymocytes is less well established.

Because the MHC-peptide ligands of the TCR are a combination of self-MHC and foreign peptides, thymocytes have to undergo two selection processes. Positive selection promotes development of cells which can recognise self-MHC while negative selection eliminates the subpopulation of these cells

which recognise self-peptide and self-MHC and which are therefore potentially autoreactive (Jameson et al 1995). While the repertoire of self-peptides present in the thymus must influence negative selection it is less clear whether self peptides play a **specific** role in positive selection or whether they are merely required for the stable expression of MHC molecules. Recent experiments using fetal thymic organ culture (FTOC) from TCR transgenic $\beta 2M^-$ and TAP^- mice have suggested that self peptides may play a very specific part in positively selecting a particular TCR (Ashton-Rickart et al 1994, Hogquist et al 1994). In these studies the TCR transgenic mice do not constitutively express cell surface MHC class I molecules. However MHC class I expression can be rescued by the addition of exogenous peptide (and β2M in the case of the $\beta 2M^-$ mouse) thereby allowing the effect of individual peptides on TCR selection to be examined. The results of these investigations suggest that the transgenic TCR can only be positively selected in the presences of peptides which closely resemble the eventual agonist peptide. This has been interpreted as implying that TCR-peptide interactions are vital for positive selection. Further observations made in transgenic FTOC also indicate that peptide density is important in determining the balance between positive and negative selection (Ashton-Rickardt et al 1994, Allen 1994). Hence a positively selecting ligand at low density may be converted into a negatively selecting ligand at high density. This in turn has lead to the affinity-avidity model of thymocyte selection (Fig 4.1) in which very low avidity TCR/MHC-peptide interactions lead to programmed cell death, high avidity interactions lead to negative selection and those in

between result in positive selection. The affinity window for positive selection has even been defined for those peptides shown to be important in a transgenic FTOC system (Alam et al 1996). However, despite these advances it has been suggested that the studies of positive selection in TCR transgenic $\beta 2M^-$ and TAP^- mice are fundamentally flawed (Schumacher & Ploegh 1994). In both systems low levels of MHC in the FTOC, even after the addition of peptide, may mean that only a few high affinity peptides may reach the requisite avidity for positive selection thereby giving an unwarranted emphasis to the role of peptide. An alternative proposal is that positive selection proceeds largely independent of peptide. In this so called 'non-interference' model thymocytes are positively selected provided they express a TCR that has a low affinity interaction with self-MHC and the peptide becomes involved only when it interferes with these contacts (Fig 4.2). This hypothesis has found recent support from two independent experimental systems. Firstly it has been shown that transgenic mice expressing a single MHC-peptide complex can select a diverse T-cell repertoire. This implies that many TCRs can interact with a single MHC peptide complex which argues against the requirement for a highly specific TCR-peptide interaction for positive selection (Ignatowicz et al 1995, Miyazaki et al 1996). Secondly in invariant chain (Ii) knockout mice the introduction of a specific Ii-antigenic peptide fusion molecule directly into the thymus also leads to positive selection of a diverse repertoire of T cells (Nakano et al 1997). Furthermore some of these T cells are specific for peptides which show neither sequence homology nor cross reactivity

with the selecting peptide. Therefore these data also suggest that there is no necessity for highly specific TCR-peptide interactions in positive selection.

4.12 Competitive Inhibition Assays

If the TCR is positively selected primarily by specific contacts with peptide then one would predict that only a limited number of MHC-peptide complexes would be able to interact with the TCR. If however there is a significant interaction with the MHC molecule as suggested by the 'non-interference' model then one might expect multiple MHC-peptide complexes to engage the TCR irrespective of the peptide presented. The experiments described in this chapter analyse these predicitions by using a novel bioassay to estimate the numbers of HLA-A2 peptide complexes which can engage the A3-TCR.

In chapter 3 the generation of the A3-TCR-CD3ζ transfectant RBL-008 and its response to peptide pulsed target cells was described. This assay was modified by using multimerised recombinant HLA-A2 pol peptide complexes to elicit a fixed response from an RBL transfectant which can then be competitively inhibited by a range of monomeric recombinant MHC-peptide complexes. Comparison of the inhibition curves then provides an indication of the ability of MHC-peptide complexes to engage the TCR. Furthermore the combination of a transfected TCR and recombinant MHC-peptide complexes allows analysis of TCR-ligand interactions without the potentially confounding contribution of coreceptor molecules. The results suggest that the great majority of HLA-A2 binding peptides permit contact between the TCR and the MHC

molecule. The potential functional and structural implications of these interactions is discussed.

4.2 Results

4.21 RBL-008 Retains Specificity in Response to Multimerised MHC-peptide Complexes

The recombinant MHC-peptide complexes used in these experiments were prepared using well established methods (Garboczi et al 1992) in which the extracellular portion of the class I heavy chain and β2M are overexpressed separately in *E. coli*.. The inclusion bodies containing these proteins are then purified, denatured in urea, and refolded around a synthetic peptide by an *in vitro* peptide-dependent folding method. Finally the folded MHC-peptide complexes are exchanged into PBS, purified by HPLC and concentrated appropriately (Fig 4.3). The recognition of recombinant MHC-peptide complexes by the transfected A3-TCR-CD3ζ receptor was studied in degranulation assays using RBL-008. Since induction of responses from RBLs requires receptor cross-linking (Metzger et al 1986) multivalent MHC-peptide arrays were generated and used as targets in place of peptide pulsed cells. The multivalent arrays were formed by first coating sheep anti-mouse dynabeads with either the mAb W6/32 (Barnstable et al. 1978) or the mAb BB7.2 (Parham & Brodsky 1981) and then saturating the mAb coated beads with purified MHC-peptide complexes. Ten million beads were used in each well of the assay plate, giving an estimated final concentration of multivalent MHC of 0.1-0.2μM. The results of the degranulation assays show that RBL-008 responds to HLA-A2 pol and HLA-

A2 polE8 multimerised by W6/32 but not to other multimerised HLA-A2 peptide complexes and confirm that monovalent MHC-peptide complexes are non-stimulatory (Fig 4.4). The failure of RBL-008 to respond to HLA-A2 pol multimerised by BB7.2 is likely to reflect the fact that the epitope recognised by this mAb is on the MHC class I α-helices, therefore BB7.2 would be expected to prevent TCR recognition. In summary the response of RBL-008 to recombinant protein is MHC-restricted and peptide specific and mimics that of the CTL clone A3 to peptide pulsed target cells (see Chapter 3).

4.22 Degranulation of RBL-008 can be Competitively Inhibited by Monovalent Recombinant MHC-peptide Complexes

Because degranulation of RBL-008 is triggered by multimerised, but not monovalent, recombinant HLA-A2 pol complexes it was reasoned that this response might be differentially inhibited by distinct monovalent MHC-peptide complexes thereby providing information on their relative ability to engage the TCR. Initial experiments using HLA-A2 pol multimerised by linkage to W6/32 coated beads were uninformative because the monovalent competitor displaced HLA-A2 pol from the beads obscuring the effects of competition for TCR engagement (data not shown). Therefore an irreversibly crosslinked form of multimerised HLA-A2 pol was generated by attaching biotinylated HLA-A2 pol to streptavidin coated beads. In order to ensure that the MHC α-helices and antigenic peptide were accessible to the A3-TCR-CD3ζ receptor biotinylation was restricted to the β2M subunit of the MHC-

peptide complex. This was achieved by biotinylating solubilised β2M and then purifying the monobiotinylated species before adding it to a standard refolding reaction with heavy chain and peptide. The gel filtration trace of biotinylated β2M shows three peaks (Fig 4.5): the first peak ("1") is broad and is likely to represent multi-biotinylated β2M, the next two peaks are sharp and represent monobiotinylated ("2") and unbiotinylated ("1") β2M respectively. This interpretation is consistent with the notion that a sharp peak represents a species of a homogeneous hydrodynamic radius (which would be the case for mono- or non-biotinylated protein), and that a broad peak represents a mixture of different hydrodynamic radii (which would be the case for a multi-biotinylated protein). The final HLA-A2-peptide complex incorporating biotinylated β2 M showed the expected gel filtration profile (Fig 4.6) and was purified and concentrated as before. Dynabead immunoassays (DIA see Chapter 2) confirmed the conformational integrity of the biotinylated HLA-A2 peptide complexes by showing that this protein could be captured on streptavidin dynabeads and was specifically reactive with the mAb BB7.2 (Parham and Brodsky 1981) (Fig 4.7). In contrast, non-biotinylated soluble HLA-A2 peptide complexes, which were reactive to BB7.2 in an ELISA, were not reactive in the DIA since they are unable to bind streptavidin beads. When multimerised biotinylated MHC-peptide complexes were used in degranulation assays the results confirmed that MHC restricted peptide specific responses were maintained in the presence of the biotin group (Fig 4.8). Specific competitive inhibition of the response of RBL-008 to multimerised biotinylated HLA-A2 pol was then

confirmed by showing that monovalent HLA-A2 pol could competitively inhibit the response whereas monovalent HLA-B8 gag (Nixon et al 1988) could not (Fig 4.9).

Having established a system for examining TCR/MHC-peptide interactions by competitive inhibition the analysis was extended to include HLA-A2 complexed to peptides with widely differing sequences. HLA-A2 complexes with either the polE8 peptide (ILKEPVHEV) which is closely related to pol in sequence and a weak agonist or the gag peptide (SLYNTVATL) (McMichael & Walker 1995) which has an unrelated primary sequence were found to be effective competitors (Fig 4.9). In contrast a third HLA-A2 peptide complex, HLA-A2 TLW (TLWVDPYEV) (Hunt et al 1992) also with a primary peptide sequence unrelated to pol was unable to significantly inhibit degranulation (Fig 4.9).

The results of the competitive inhibition assays were confirmed by direct binding experiments in which RBL-008 was incubated with ^{125}I labelled monomeric HLA-A2 pol in the presence of unlabelled competing HLA-A2 peptide complexes. Specific binding of iodinated HLA-A2 pol to RBL-008 was inhibited by unlabelled HLA-A2 pol, HLA-A2 polE8 and HLA-A2 gag but not by HLA-A2 TLW (Fig 4.10).

4.23 Competitive Inhibition of Degranulation by Recombinant HLA-A2 Random Peptide Complexes Shows That Very Few HLA-A2 Binding Peptides Block TCR-MHC Interactions

An assessment of the proportions of peptides which either block or permit TCR-ligand interactions was obtained in

competition experiments using HLA-A2 complexed with a random peptide library (Flynn et al 1991). Significantly we observed effective inhibition of degranulation using these HLA-A2 random peptide complexes (Fig 4.9). The diversity of peptides bound to HLA-A2 was analysed by eluting the peptides from the recombinant protein (performed by D.Barouch). The HPLC profile of the eluted peptides suggested that a wide range of peptides were bound to HLA-A2 (Fig 4.11) and this was supported by Edman sequencing which showed that all amino acids were represented at all positions other than the anchor residues (see D Barouch D. Phil thesis 1995 for Edman sequencing data).

Although direct binding measurements (Khilko et al 1995, Cerundolo et al 1991) have shown that MHC-peptide complexes are remarkably stable, it was important to exclude exchange of either peptide or MHC heavy chain between multimerised HLA-A2 pol and monovalent competitor (Fig 4.12). Multimerised HLA-A2 pol was preincubated with 6μM monovalent HLA-A2 complexed with either polE8, gag or random peptides. The preincubation step was for one hour at 37°C and therefore employed the same conditions as the degranulation assays. Following preincubation, the beads were washed and then compared in degranulation assays with beads which had been preincubated in medium alone. No change in stimulatory capacity was noted implying that peptides are not exchanged between monovalent and multimerised MHC-peptide complexes under assay conditions.

Finally we excluded the possibility that the different competition curves observed were the consequence of either

RBL desensitisation (Metzger et al 1986) or other non-specific inhibition by some MHC-peptide complexes but not by others (Fig 4.13). In these experiments the presence of 6μM monovalent MHC-peptide complexes had no effect on degranulation stimulated by crosslinking the TCR-CD3ζ molecule with the anti-TCR β constant domain antibody βF1.

4.3 Discussion

A variety of methods have been used to examine TCR/MHC-peptide interactions including surface plasmon resonance (SPR) technology (Matsui et al 1994, Corr et al 1994, al-Ramadi et al 1995, Lyons et al 1996, Alam et al 1996) direct measurement of binding of iodinated MHC-peptide to T cells (Sykulev et al 1994a&b) and inhibition of cellular responses by soluble TCR or by soluble MHC-peptide complexes (Weber et al 1992, Schneck et al 1989). The most accurate of these methods is SPR, however the production of soluble TCR has proved difficult and only a few laboratories have been able to consistently produce sufficient quantities for analysis. Furthermore there is no reliable means of demonstrating functionality for the soluble proteins used in SPR analyses and lower limits of detection of these methods may prevent measurement of functionally significant TCR-MHC-peptide interactions (al-Ramadi et al 1995). Direct binding of radiolabelled MHC directly to whole T cells (Sykulev et al 1994b) circumvents the need for soluble TCR but the effects of CD8 on the TCR/MHC-peptide interaction are difficult to control for. Indeed the nature of the contribution of CD8 to the TCR/MHC-peptide interaction is disputed (Parnes et al. 1989; Sherman et al. 1992; Cai and

Sprent 1994) with some contending that it has only a signalling function (Sykulev et al. 1994a) while others argue that it significantly contributes to the affinity of the co-complex (Leuscher et al. 1995, Garcia et al 1996b). The system described here is novel and has a number of advantages. firstly the use of a transfected TCR and recombinant MHC-peptide complexes means that the interaction between the these molecules is examined in isolation from coreceptor molecules. Secondly the use of a bioassay means that the functional integrity of both the TCR and the MHC-peptide complex can be guaranteed. Finally it is clear that monovalent MHC-peptide complexes do not have an effect on degranulation of RBL-008, similar experiments performed in T cells would be harder to interpret since it has been shown that single MHC-peptide complexes may induce T cell responses including apoptosis (Zavazava & Kronke 1996).

Competitive inhibition by single peptide-MHC complexes identifies two groups of peptides. Those which allow engagement between the TCR and the MHC-peptide complex such as pol, polE8 and gag and those which prohibit contact such as TLW. Engagement of the TCR by HLA-A2 pol and HLA-A2 polE8 is predictable since both peptides are agonists for the CTL clone A3. More surprising is the competition observed with HLA-A2 gag since this peptide has a primary sequence unrelated to the pol peptide and does not elicit a response from the original CTL. The lack of sequence similarity between the gag and pol peptides suggests that engagement of the TCR by the HLA-A2 gag complex may be dependent on contact between TCR and MHC rather than TCR and peptide. However

since there are reports of TCRs which cross react with unrelated peptides it remains a possibility that this TCR could form significant contacts with the gag peptide (Evavold et al 1995, Bhardwaj et al 1993, Hagerty & Allen 1995, Nanda et al 1995, Udaka et al 1995).

The apparent failure of HLA-A2 TLW to contact the TCR has two possible explanations. First the TLW peptide may sterically hinder contact between the TCR and the MHC molecule. Alternatively the TLW peptide may change the conformation of HLA-A2 which is then no longer recognised by the TCR. This possibility is supported by observations of peptide induced conformational changes in the crystal structures of HLA-B35, B8, HLA-A2 and H-2K^{b} (Madden et al 1993, Madden 1995, Fremont et al 1992, Smith et al 1996, Reid et al 1996).

Examination of the competition curve obtained with the HLA-A2 random peptide complexes suggests that most of the complexes within this mixture are capable of engaging the TCR. Since it is difficult to envisage how such a diverse set of peptides could all form contacts with the TCR it seems likely that most of the peptides bound to HLA-A2 are permissive for contacts between the TCR and the MHC. Extensive TCR cross reactivity has now been observed a number of times (Evavold et al 1995, Udaka et al 1995) and in one instance a CTL clone has been reported to respond to a highly complex random peptide library (Udaka et al 1995). In addition TCR engagement by non-cognate MHC-peptide complexes has also been demonstrated for a soluble TCR molecule which was able to inhibit T cell hybridoma responses of shared MHC restriction but distinct peptide specificity (Weber et al 1992). Failure to

detect such cross reactivity in experiments using surface plasmon resonance (Matsui et al 1994, Corr et al 1994., al-Ramadi et al 1995, Lyons et al 1996, Alam et al 1996.) may reflect the relatively small number of soluble TCR molecules available and the limited number of MHC-peptide complexes for each TCR which have been tested to date. A structural basis for this cross-reactivity has been suggested by the recent publication of two TCR crystal structures (Garcia et al 1996, Garboczi et al 1996) which indicate that the TCR contacts a large area of MHC and that the TCR is likely to interact with MHC-peptide in a common orientation.

One technical problem arising in these studies was the inability to inhibit degranulation to background levels. Consequently the inhibition profiles represent only the upper part of the competition curve and are not suitable for estimating TCR ligand affinities. Having said this it appears that HLA-A2 pol does have a steeper curve than the other complexes giving the impression of a higher affinity which is what one would expect of a stimulatory ligand. Monovalent HLA-A2 polE8 and HLA-A2 gag appear to have very similar inhibtion profiles. However multimersied HLA-A2 polE8 represents only a very weak stimulus and multimerised HLA-A2 gag is non-stimulatory it is quite possible that these two ligands straddle the affinity theshold for stimulating both the CTL and the RBL-008 cells. This point is particularly relevant in view of the recent reports indicating that small diffferences in affinity can profoundly affect T cell responses (Lyons et al 1996, Alam et al 1996). A further important consideration relates to reports showing that low level aggregation of the IgE

receptor in RBLs can lead to desensitisation of these cells (Metzger et al 1986). Since the TCR-CD3ζ receptor is thought to stimulate RBLs by a pathway analogous to that of the IgE receptor it may be that mulitvalent HLA-A2 gag does productively bind the A3-TCR-CD3ζ receptor but that the outcome is desensitisation rather than activation of RBL-008. Finally it is worth emphasising that the competition curve obtained with the random peptide library represents the average of all the interactions taking place and that some peptides within this complex mixture may infact have a higher affinity for the TCR than the wild type peptide (see Chapter 5).

In view of the requirement for peptide specificity interactions between the TCR and the MHC obviously can not be of functional significance in the periphery, however their existence may be a reflection of the process of thymic selection. Thymocytes are prompted to mature by low affinity/avidity interactions with self MHC-self peptide complexes in the thymus (Jameson et al 1995, Sebzda et al 1994). However as discussed in the introduction the role of self peptides in this process remains an area of debate. In view of the great diversity of TCRs that must be generated it seems unlikely that each TCR molecule can only be selected by a small number of closely related peptides as has been implied by the results of experiments using FTOC from TCR transgenic $\beta 2M^-$ and TAP^- mice. The possibility that a TCR can make contact with the MHC largely irrespective of peptide is consistent with a model in which positive selection could arise as a result of TCR-MHC interactions however this can not be said with certainty in the

absence of functional data demonstrating that the interactions observed here are sufficient for positive selection to take place.

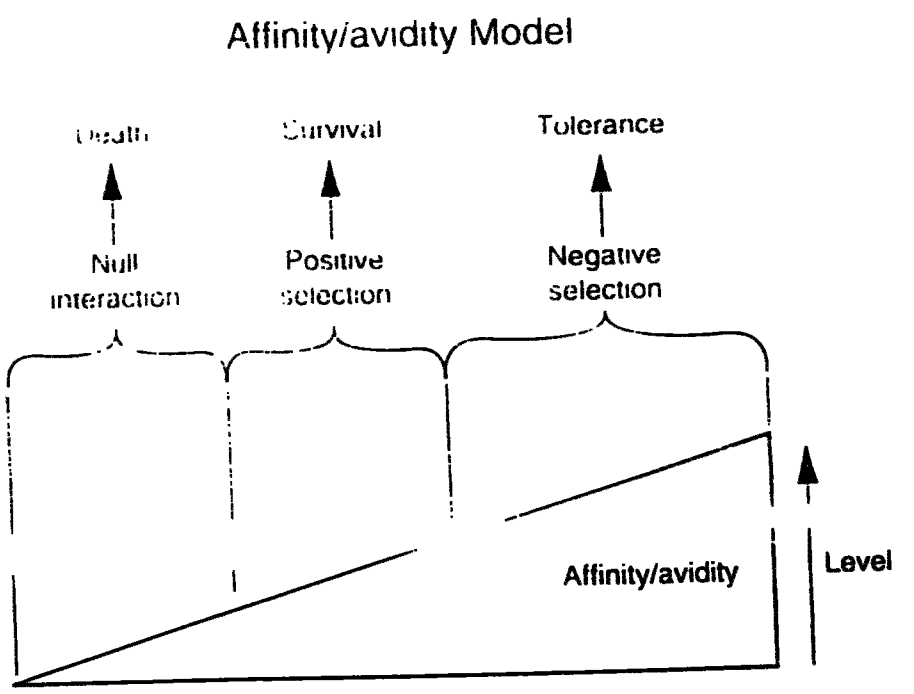


Figure 4.1
The affinity/avidity model of thymic selection
Positive and negative selection are governed by avidity thresholds. Thymocytes that can not interact with self peptide-MHC due to low affinity TCRs are subject to programmed cell death. Thymocytes expressing intermediate affinity receptors for self peptide-MHC surpass a positive selection threshold and are given a signal to continue maturation. High affinity TCRs are removed when the avidity level for self peptide-MHC surpasses the negative selection threshold (Adapted from Sebzda & Ohashi in T Cell Receptors 1995, Eds. Bell, Owen and Simpson)

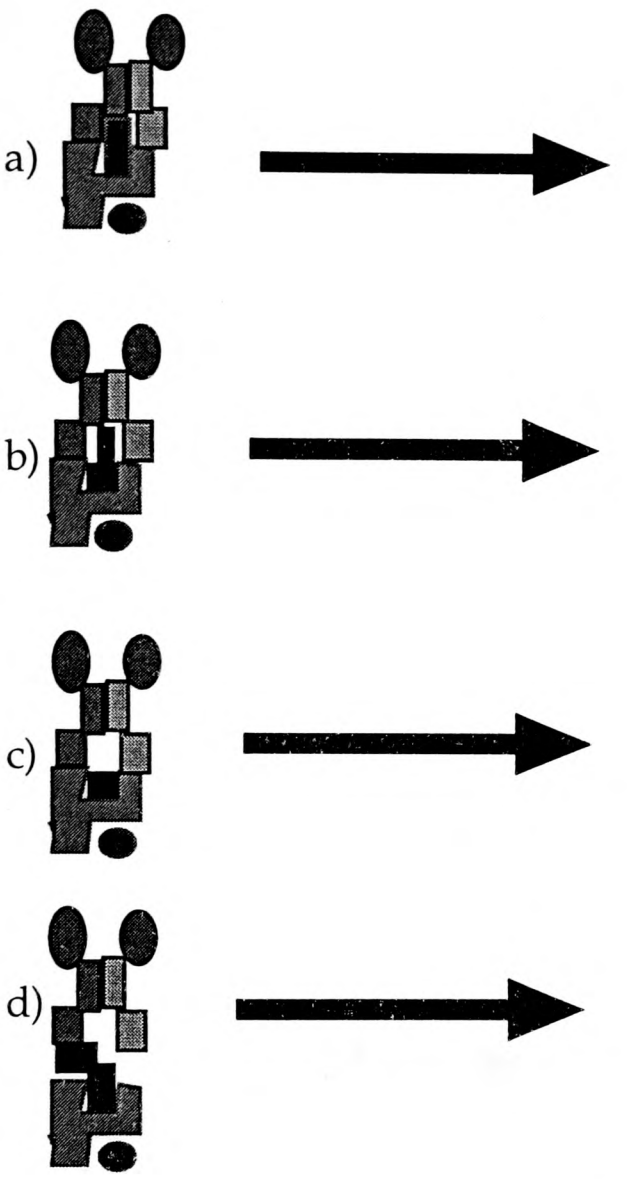
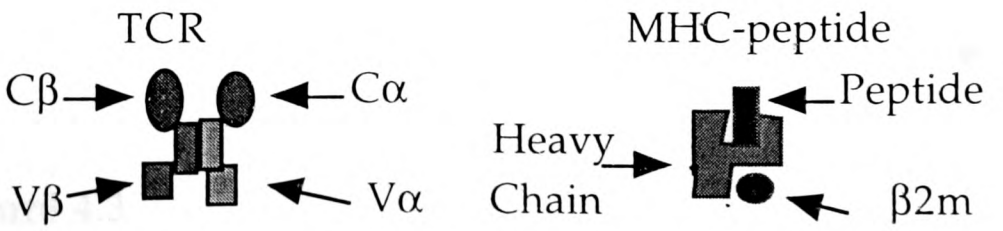


Figure 4.2 The Role of peptide in thymic selection.
Selection events are depicted according to both the altered peptide ligand (APL) model and the 'non-interference' model. (a) In both cases interaction of the TCR with the cognate peptide leads to negative selection. (b) In both cases interaction of the TCR with a closely related altered peptide ligand leads to positive selection. (c) In the APL model absence of specific TCR-peptide interaction means that positive selection is not initiated, however in the 'non-interference' model positive selection proceeds as a consequence of interactions between the TCR and the MHC molecule alone. (d) In both cases a peptide which is unrecognised and disrupts contacts between the TCR and the MHC molecule results in apoptosis.



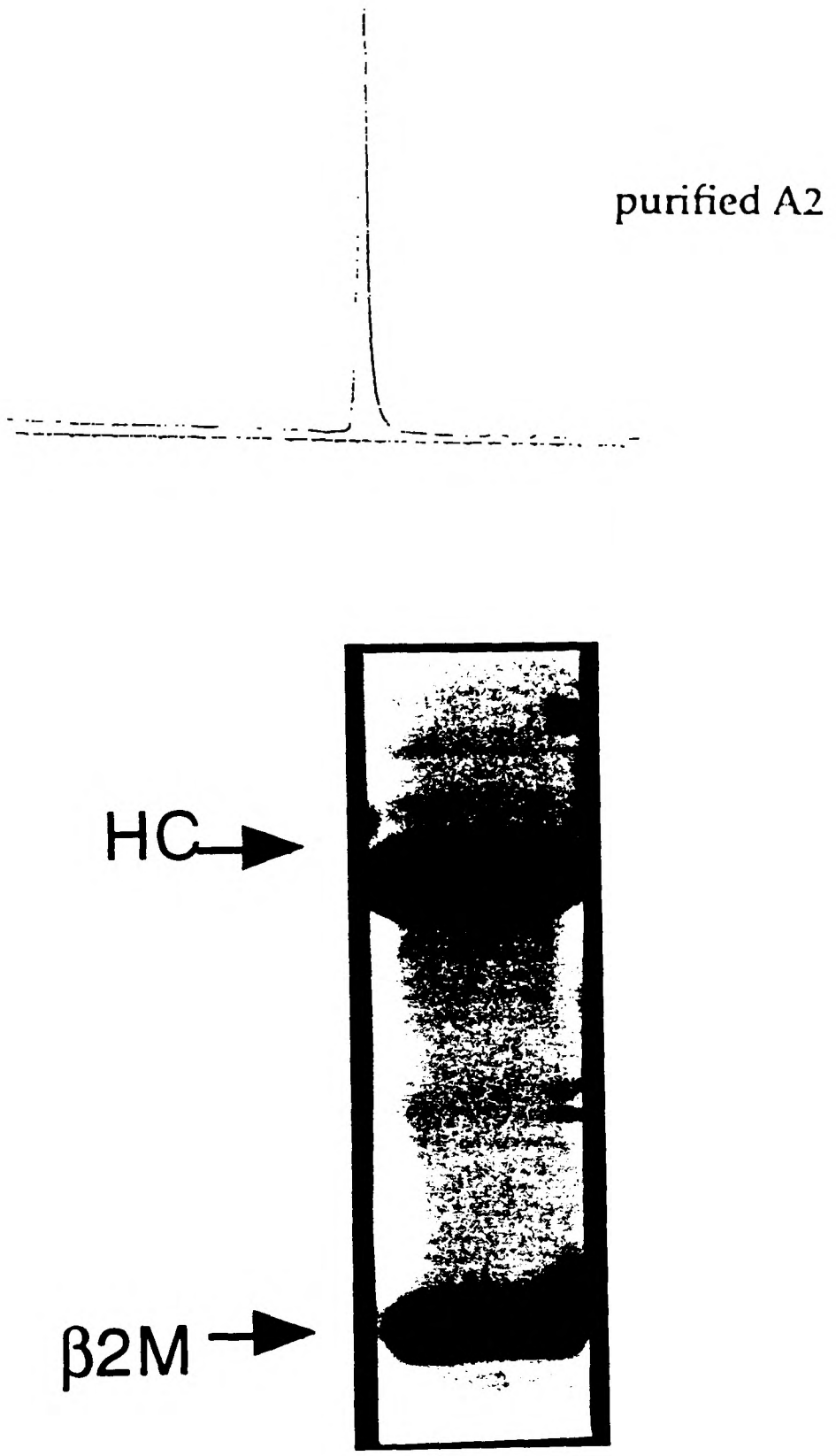


Figure 4.3
Gel filtration and SDS-PAGE analysis of HLA-A2.
The final purified HLA-A2 protein analysed by SDS-PAGE and gel filtration. Note that it is two clean bands on a gel and a single, symmetric peak on gel filtration.

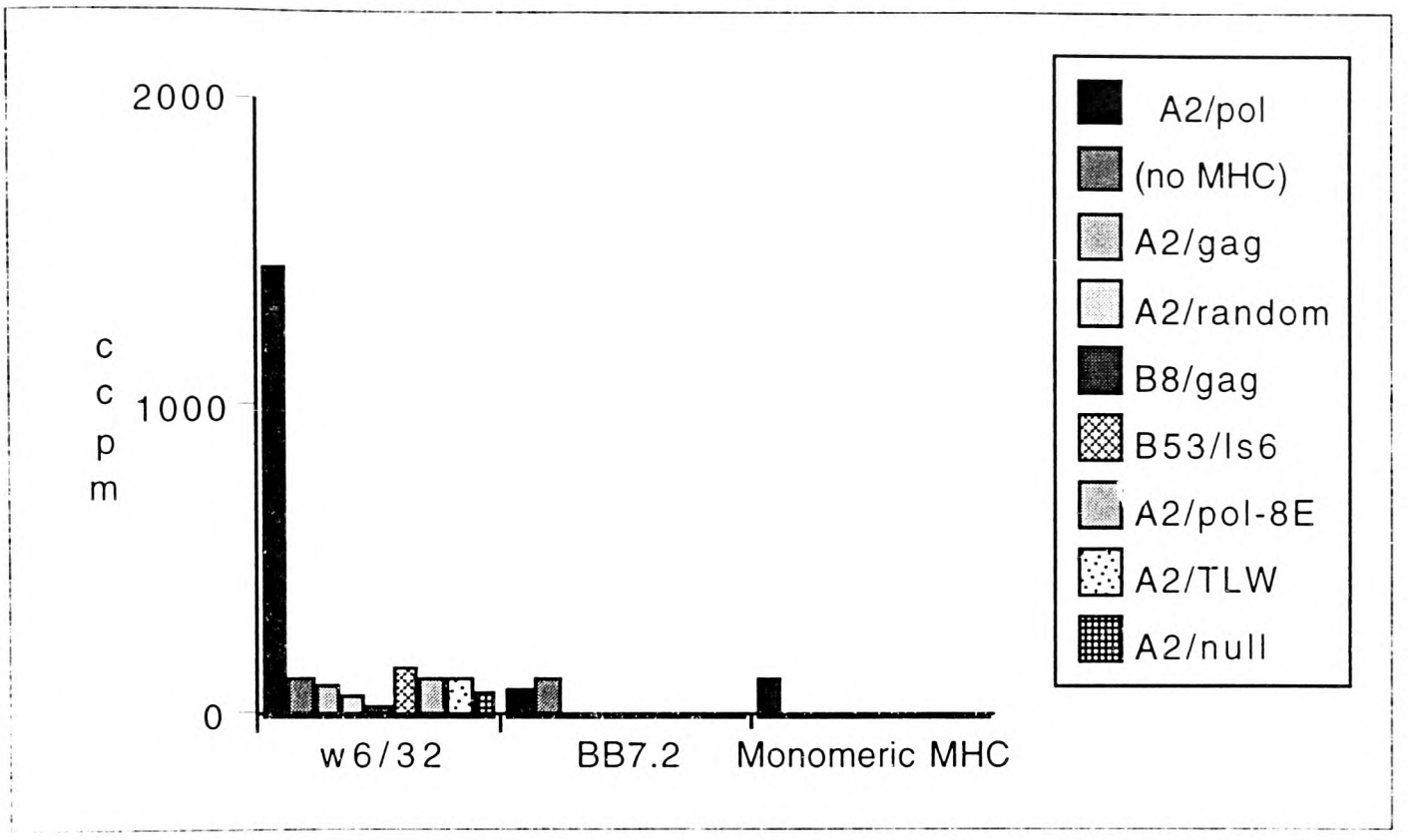
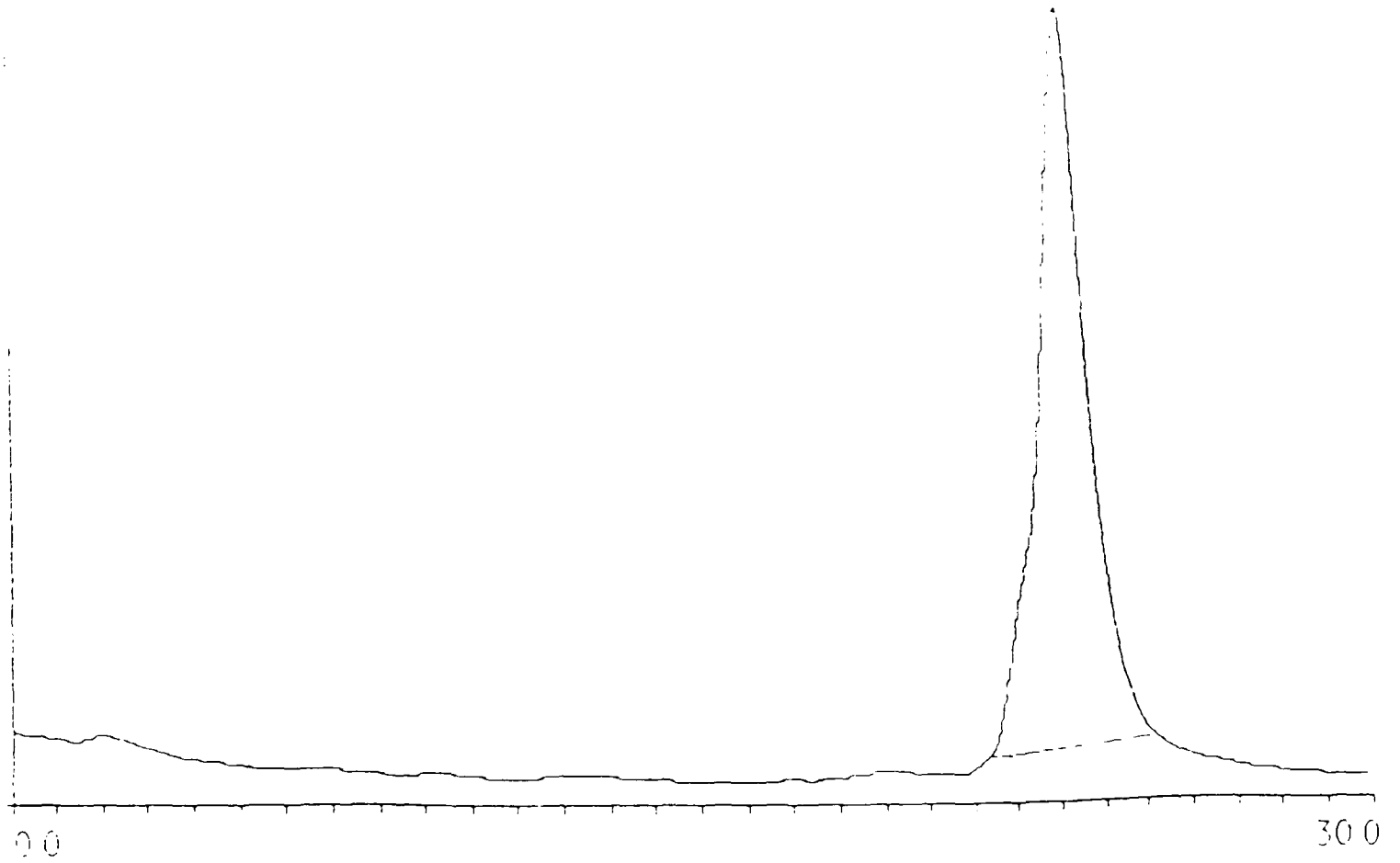


Figure 4.4
RBL-008 response to purified MHC-peptide complexes.
Specific degranulation response of RBL-008 transfectants to purified MHC-single peptide complexes. MHC-peptide complexes were added to the assay in monomeric form (final concentration 10μM) or in multivalent form on the surface of 10 million dynabeads (estimated final MHC concentration 0.1-0.2 μM). Multivalent MHC was prepared by linking monomeric MHC complexes to W6/32 or BB7.2-saturated dynabeads. RBL transfectants were labelled as described in chapter 2 and the degranulation response is shown on the vertical axis in counts per minute (cpm). Results are the means of triplicates and these experiments were performed on at least 3 occasions.



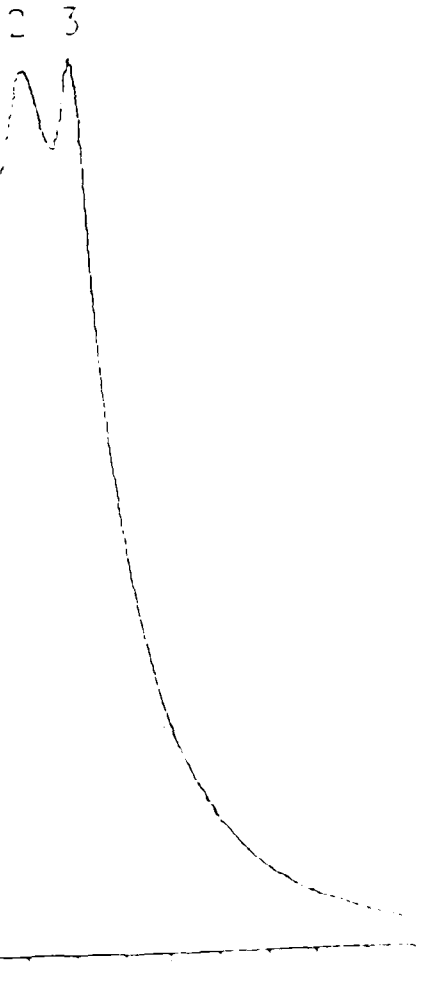


Figure 4.5

Biotinylation of β2m

HPLC gel filtration profiles of refolded β2m protein, before (top) and after (bottom) the biotinylation reaction. Note the appearance of two additional peaks representing the biotinylated forms of the protein, see text for further discussion.

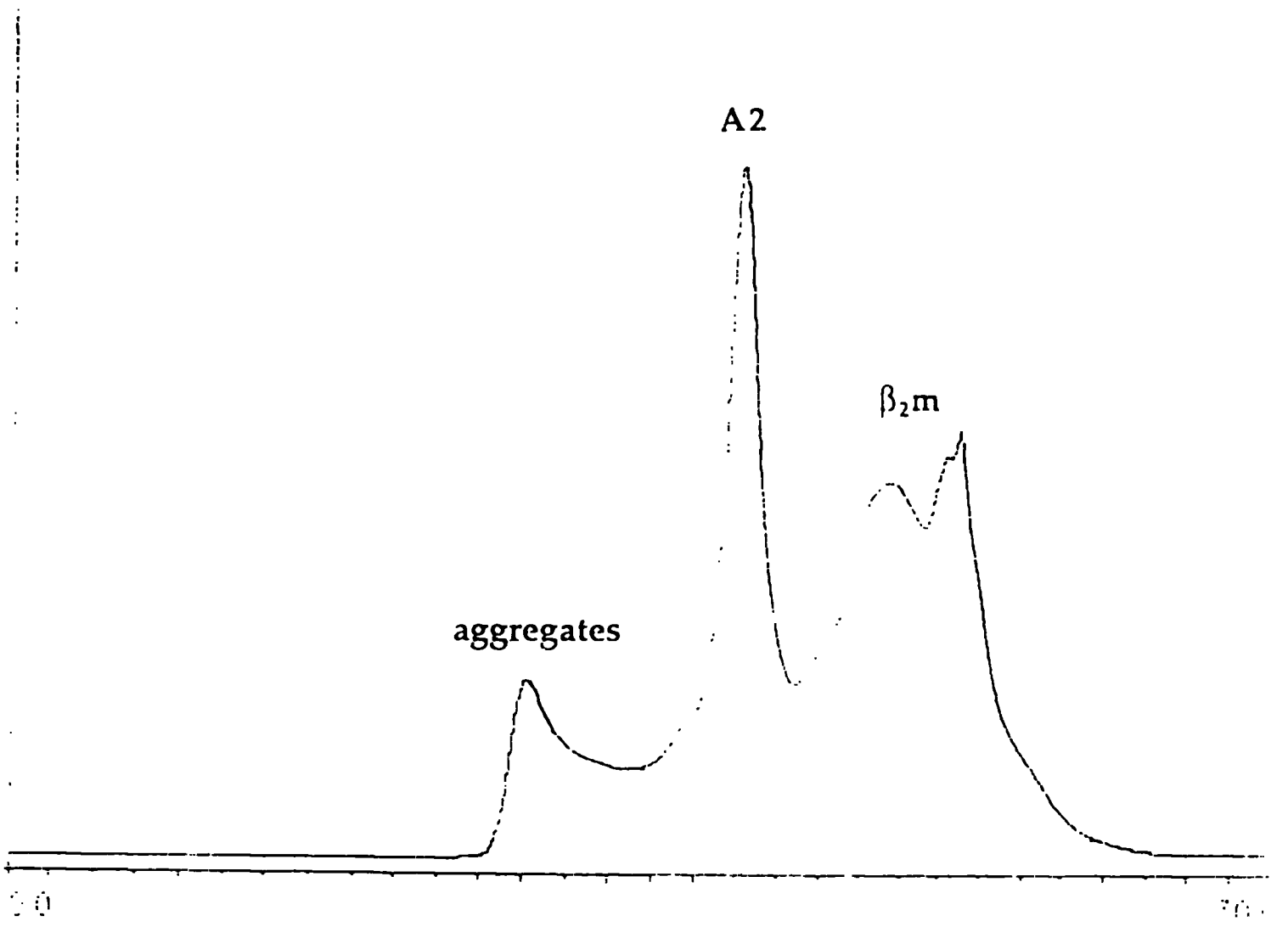


Figure 4.6

Refolding of A2 with biotinylated β2m

HPLC gel filtration profile of the refolding reaction of HLA-A2 around biotinylated β2m and a synthetic peptide. Note the appearance of an additional peak compared with the profile of biotinylated β2m alone which has a similar elution time to non-biotinylated refolded HLA-A2.

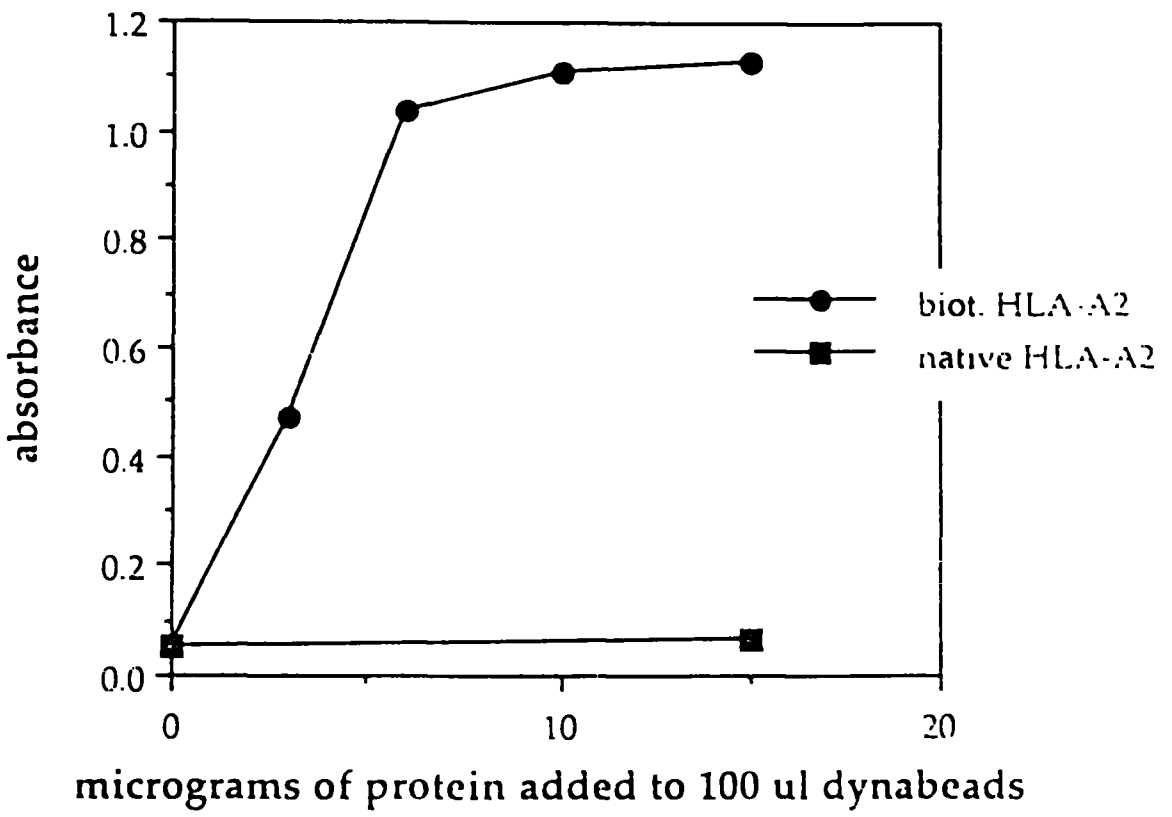


Figure 4.7

DIA of refolded A2 with biotinylated β2m

Dynabead immunoassay (DIA) of either biotinylated or non-biotinylated HLA-A2-pol protein captured on streptavidin dynabeads and detected by the mAb BB7.2, which recognizes the α2 helix. Note that both proteins were equally reactive by BB7.2 by an ELISA that did not involve capture on streptavidin (not shown).

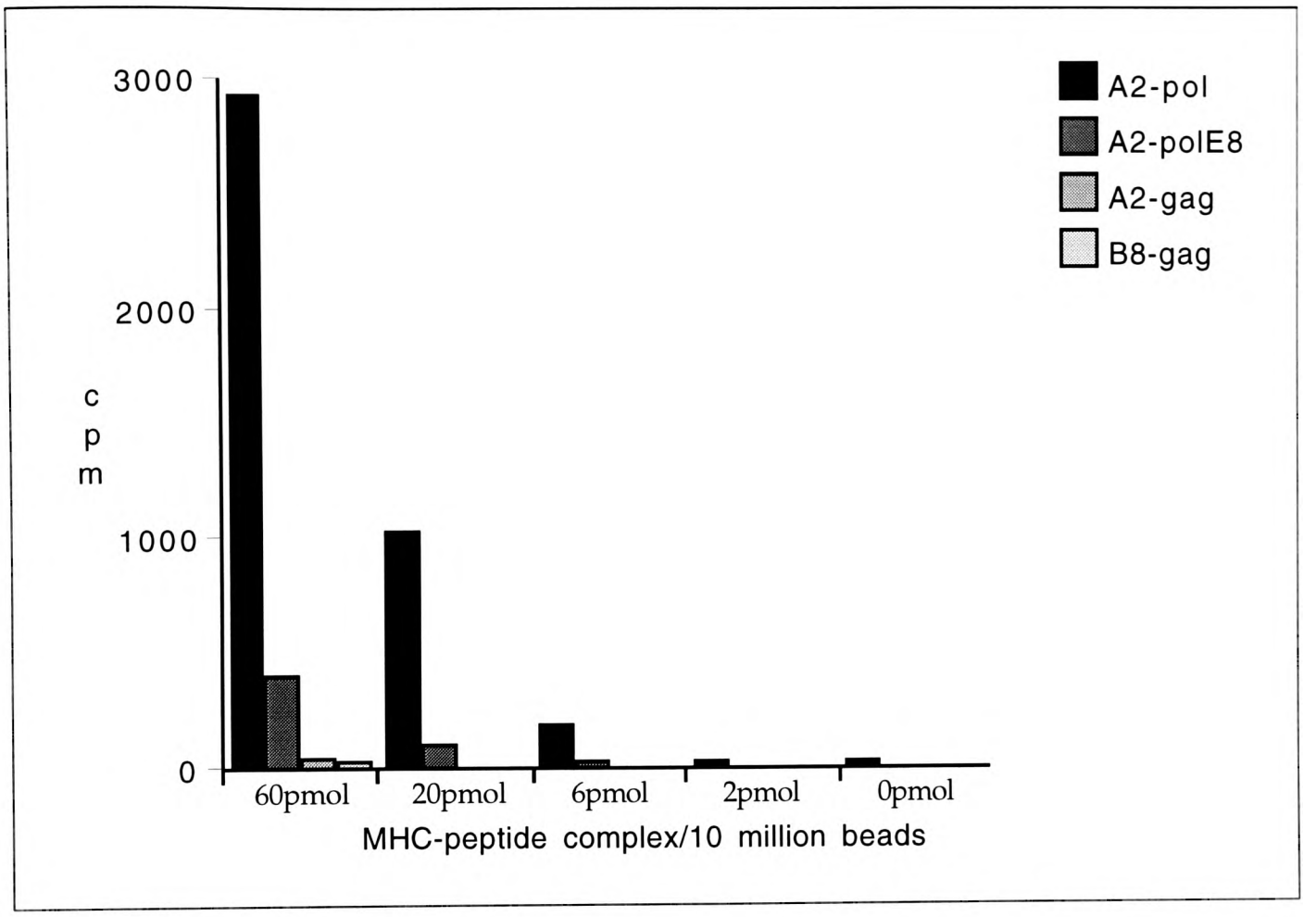


Figure 4.8
RBL-008 response to biotinylated MHC-pol complexes
Specific degranulation response of RBL-008 transfectants to purified MHC-single peptide complexes. MHC-peptide complexes were added to the assay in multivalent form on the surface of 10 million dynabeads. Multivalent MHC was prepared by linking monomeric A2-peptide complexes bearing biotinylated β2m to streptavidin dynabeads. RBL transfectants were labelled as described in chapter 2 and the degranulation response is shown on the vertical axis in counts per minute (cpm). Results are the means of triplicates and these experiments were performed on at least 3 occasions.

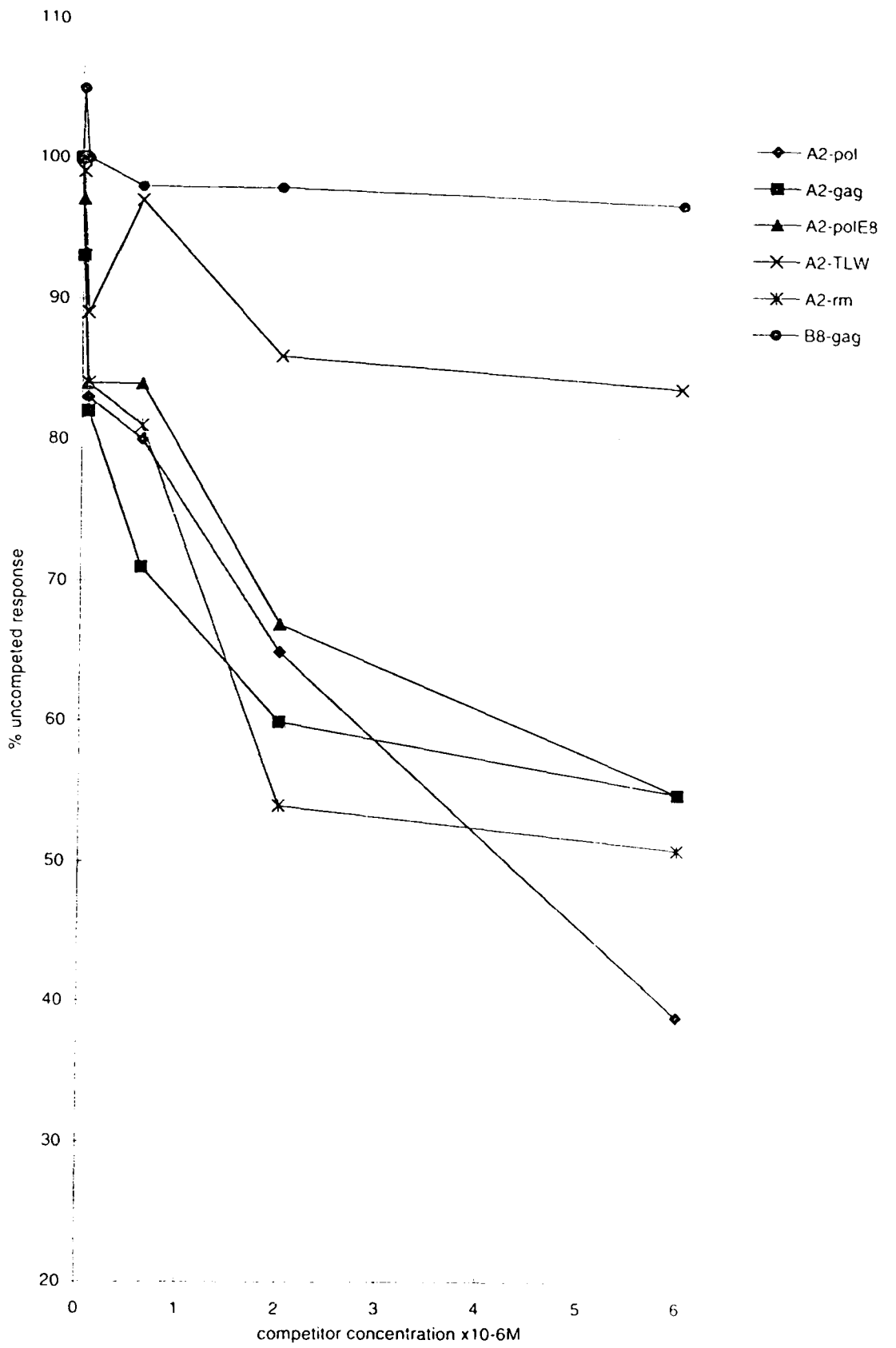


Figure 4.9
Competitive inhibition of the response of RBL-008 to multivalent HLA-A2 pol by a variety of MHC single peptide complexes.
A fixed multivalent HLA-A2 pol stimulus was used in each assay (20pmoles of protein/10^7) beads. Soluble, monomeric, non-biotinylated MHC peptide complexes were added as competitors in ascending concentrations as indicated on the horizontal axis. The vertical axis indicates the competed degranulation response as a percentage of the uncompeted response. The uncompeted response was approximately 1000 cpm. Results shown are means of between 3 and 8 independent experiments. The standard error of the mean was less than 5% for all results. (A2-rm = HLA-A2 random peptide complexes)

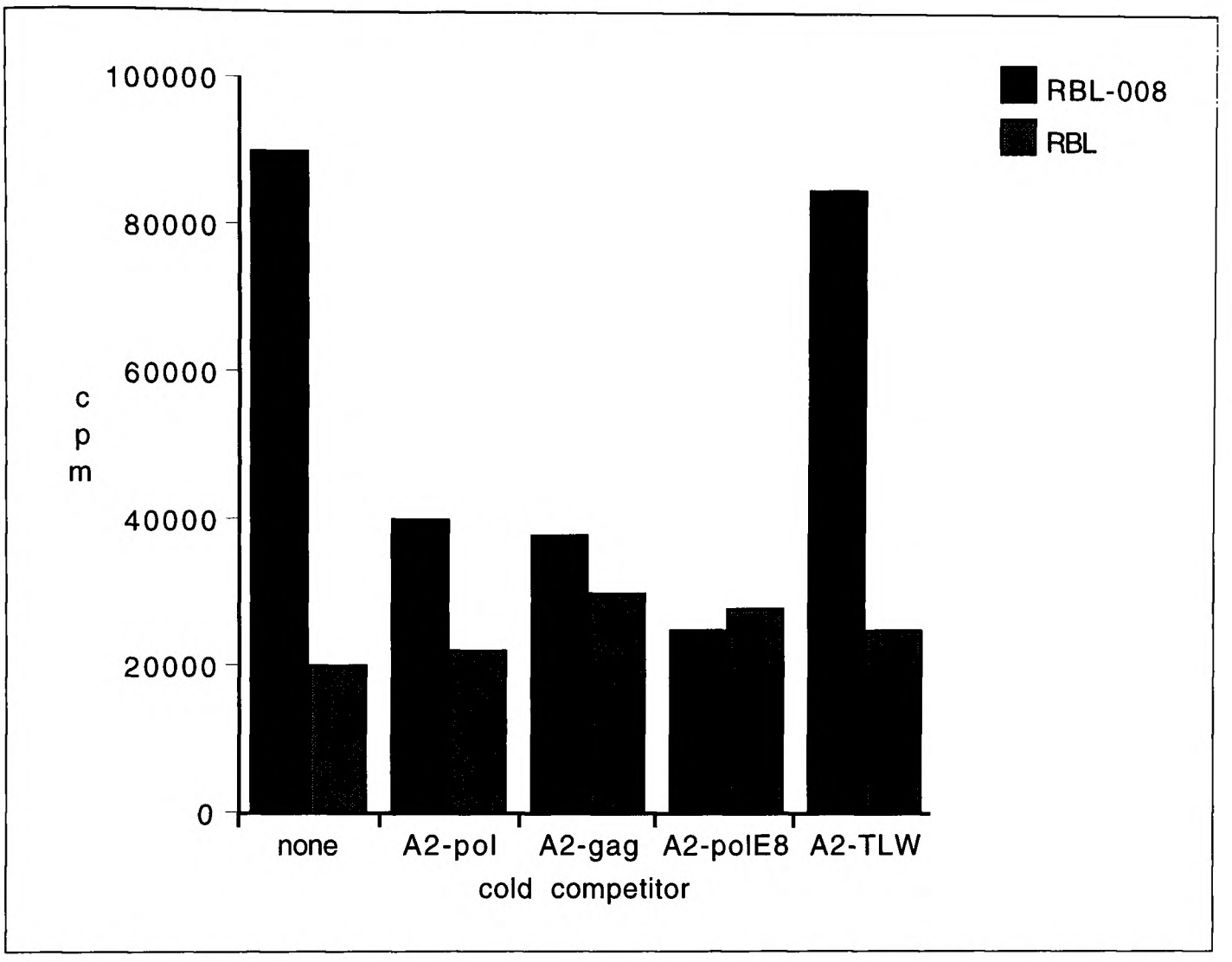


Figure 4.10
Competition for binding to untransfected RBL and RBL-008 between iodinated HLA-A2 pol and other unlabelled HLA-A2 peptide complexes.
Assays were performed in 96 well plates at a density of $5x10^5$ cells/well. The cells were blocked with BSA and washed. Next iodinated HLA-A2 pol at a concentration of 0.1μM was added to the cells in the presence or absence of unlabelled HLA-A2 peptide competitors (final concentration 10μM ie 100 fold excess). After a 30 minute incubation at room temperature the cells were washed to remove unbound counts and then the bound counts shown on the vertical axis were ascertained by gamma counter. Binding of labelled HLA-A2-pol was inhibited by unlabelled HLA-A2 complexed with pol,gag, or polE8 but not TLW.

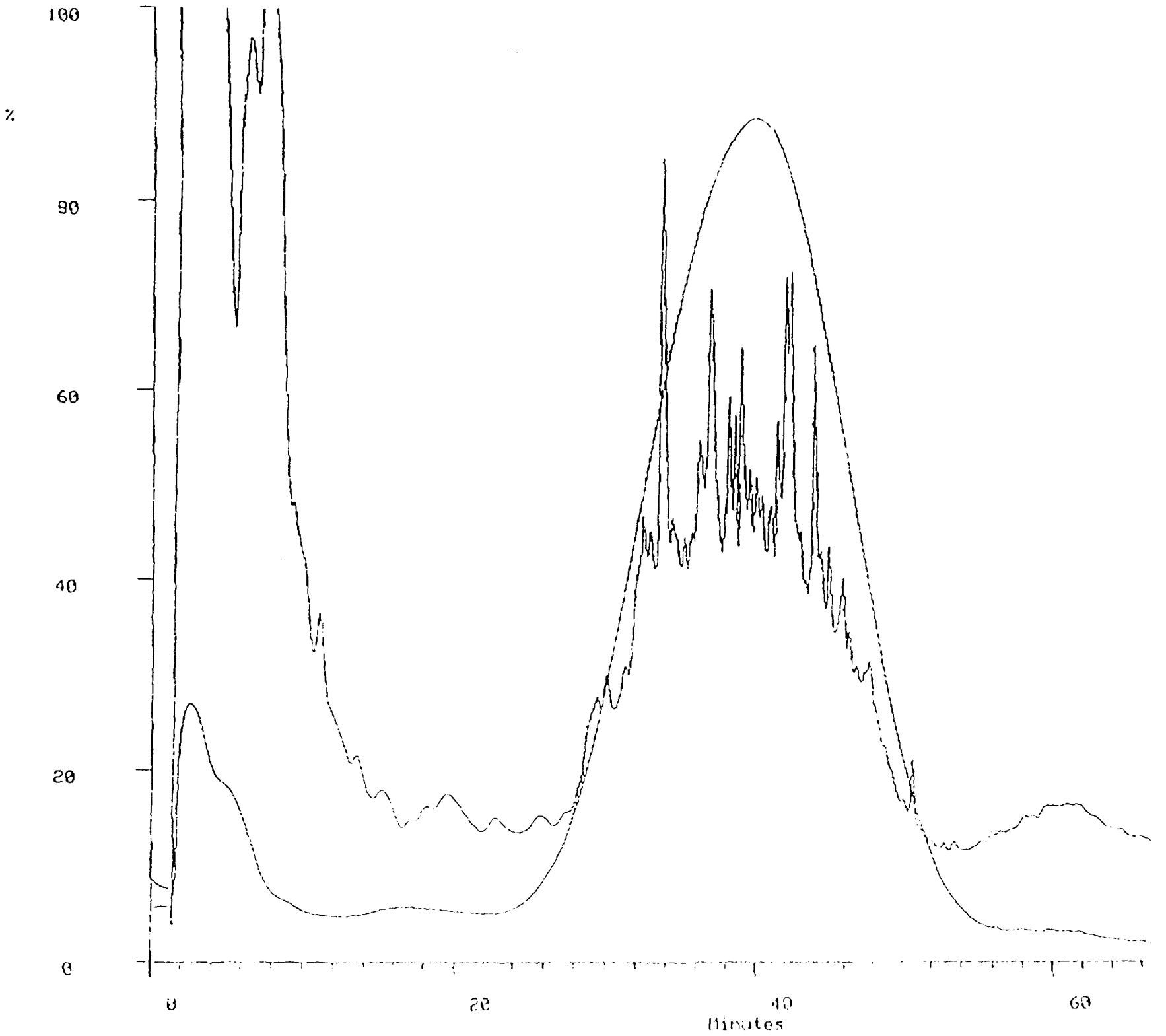


Figure 4.11.
Elution of random peptides from refolded A2.
HPLC reverse-phase analysis of the random peptides eluted from refolded HLA-A2 molecules. The smooth curve is a superimposed HPLC trace of the total random peptide library.

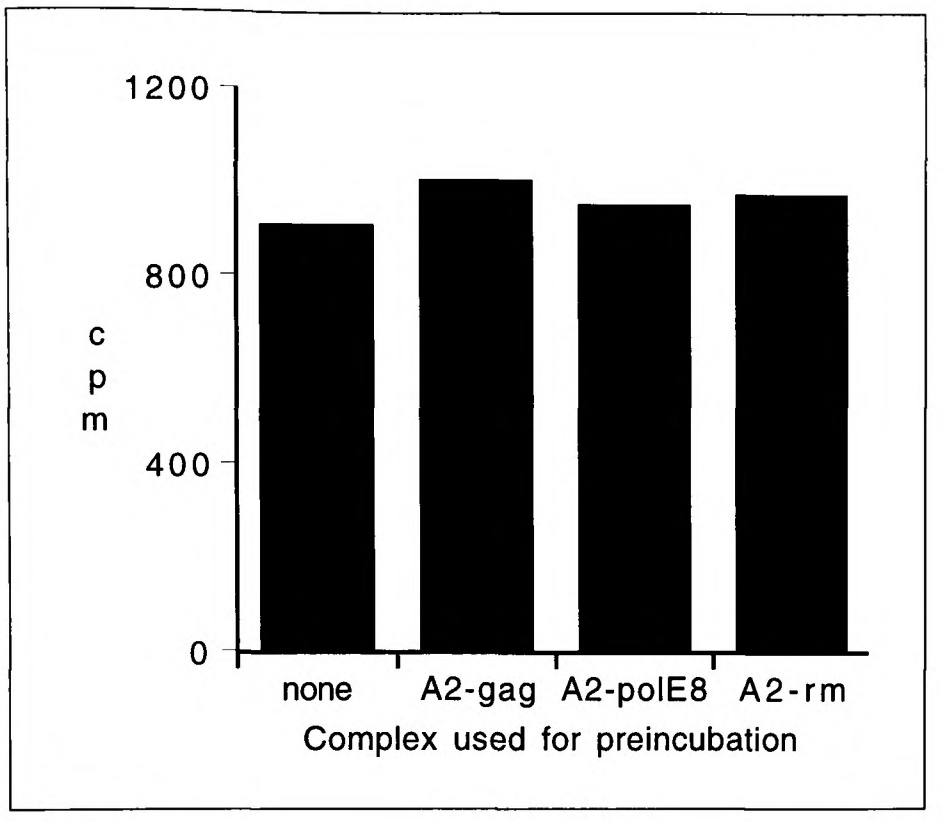


Figure 4.12
There is no exchange of peptide or MHC heavy chain between multimerised HLA-A2 peptide complexes and monvalent competitor.
Multivalent arrays of HLA-A2 pol (consisting of 20pmoles of protein/10^7 beads) were preincubated for one hour at 37°C with 6μM monovalent HLA-A2 complexed with either gag, polE8, or the random peptide library. The beads were then washed three times to remove excess monovalent competitor and used in uncompeted degranulation assays as described in materials and methods. Preincubation of HLA-A2 pol coated beads with monovalent competitor did not alter the stimulatory capacity of the beads indicating that there was no exchange of either peptide or heavy chain between multimerised and monovalent protein. Degranulation is represented in counts per minute (cpm) on the vertical axis and a background of 200 cpm was consistently observed. The results are means of triplicates. (A2-rm = HLA-A2 random peptide complexes)

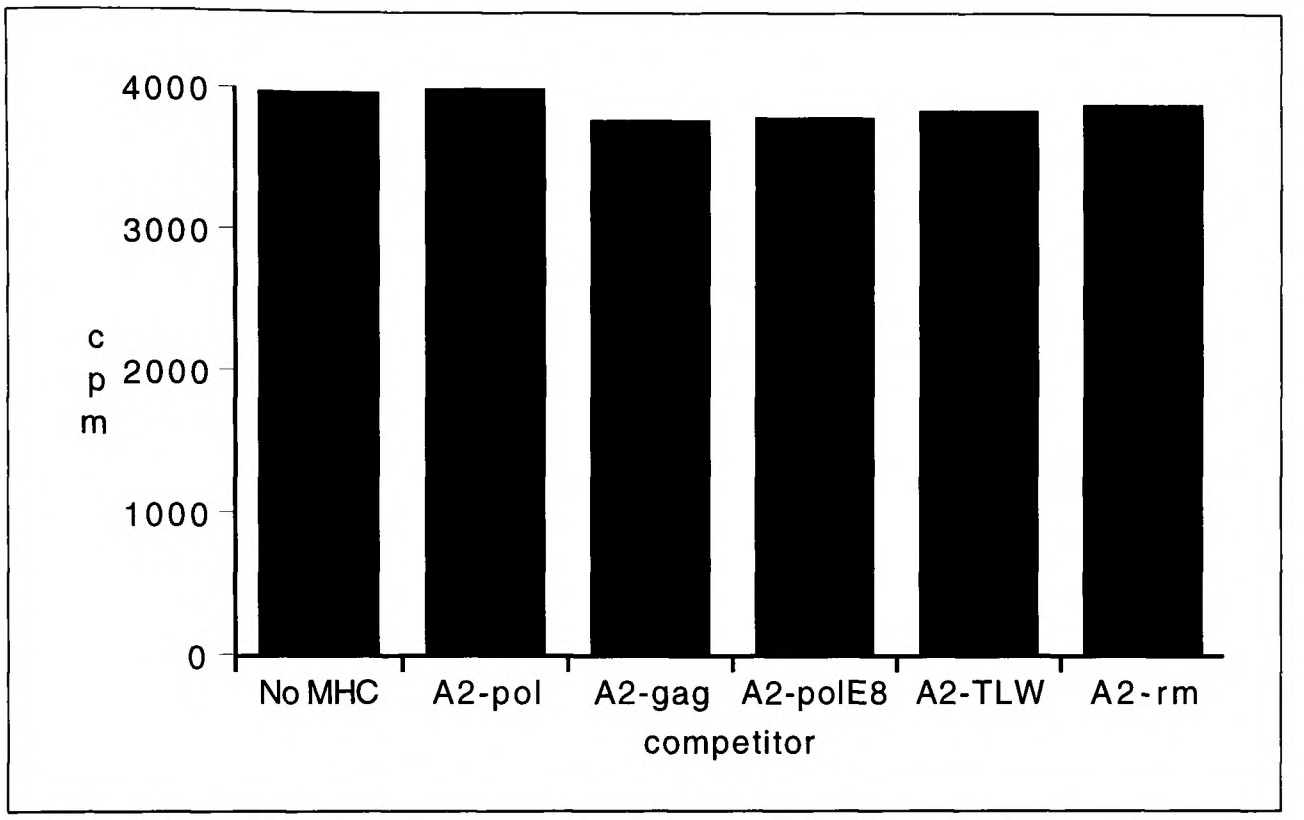


Figure 4.13
Monovalent competitor complexes do not exert a non-specific inhibitory effect on the RBL-008 transfectant.
Transfectants were labelled as described and stimulated to degranulate by the anti TCR-Cβ mAb βF1 in the presence or absence of 6μM monovalent competitor as shown. Competitor protein did not affect degranulation induced by antibody crosslinking indicating that recombinant HLA-A2 peptide complexes do not lead to a nonspecific inhibition of degranulation. Degranulation is represented in counts per minute (cpm) on the vertical axis and a background of 200 cpm was consistently observed. The results are means of triplicates. (A2-rm = HLA-A2 random peptide complexes)

Chapter 5
Flexibility in TCR-Peptide Interactions: An Amino Acid Substitution in an HIV pol Peptide Which Enhances TCR Recognition

5.1 Introduction

5.11 Flexibility in TCR-Peptide Interactions

Cell mediated immunity is dependent on the activation of antigen specific T cells which occurs when the TCR recognises a complex composed of a short peptide bound to an MHC molecule (see Chapter 1 for discussion of T cell activation). Despite the obvious need for specificity in T cell responses it is now well established that the TCR can exhibit considerable flexibility in its interaction with antigenic peptides (Evavold & Allen 1991, De Magistris et al 1992, Bhardwaj et al 1993, Evavold et al 1995). Multiple studies have shown TCR cross-reactivity between peptides with sequence homology (Wraith et al 1992, Luo et al 1993) and there are also several reports demonstrating that ostensibly unrelated peptides can interact with the same TCR (Hagerty & Allen 1995, Wucherpfennig & Strominger 1995). In addition a number of TCRs have now been shown to make sub-optimal contacts with subtle variants of the index peptide, termed altered peptide ligands (APLs), which can lead to dramatic functional consequences for the T cell (reviewed in Jameson & Bevan 1995, Sloan-Lancaster & Allen 1996).

Flexible TCR recognition of an APL was first reported following the identification of a T cell clone that produced IL-4 *and* proliferated in response to its wild-type ligand but

produced IL-4 in the *absence* of proliferation in response to a variant peptide containing a single amino acid change (Evavold & Allen 1991). APLs which uncouple T cell responses in this way are referred to as partial agonists and a range of partial responses have now been described including differential cytokine production and induction of anergy (Sloan-Lancaster et al 1993). Shortly after the discovery of partially agonistic APLs came the identification of antagonist peptides which can inhibit responses to stimulatory ligands (De Magistris et al 1992, Klenerman et al 1994, Bertoletti et al 1994).

Evidence suggests that flexibility in the recognition of ligand is an essential feature of T cell function. In particular the thymic development of some T cells may depend on the ability of the TCR to interact with a sub-optimal ligand (Allen 1994). Thus, fetal thymic organ culture experiments using thymi from mice expressing a transgenic TCR have shown that positive and negative selection of T cells can be mediated by peptides closely related to the cognate antigenic peptide (Hogquist et al 1994, Ashton Rickardt et al 1994). Furthermore the affinity of these peptides for the TCR has now been shown to correlate with their effect on selection such that high affinity ligands cause T cell deletion while lower affinity ligands promote cell survival (Alam et al 1996). In the periphery the ability of APLs to act as antagonists has raised the possibility of treatment of autoimmune diseases with specifically designed peptides.

5.12 Designing Altered Peptide Ligands Using MHC-peptide Crystal Structures

Crystallographic studies of MHC-peptide complexes have revealed a broadly similar structure in which the antigenic peptide is presented in a cleft in the MHC with the upward pointing residues of the peptide and the MHC α-helices forming the potential TCR binding site (Madden 1995, Bjorkman et al 1987a&b, Madden et al 1993, Fremont et al 1992, Smith et al 1996, Reid et al 1997). Most studies of flexible TCR-peptide interactions have taken an empirical approach by using this general structural pattern to focus amino acid substitutions on the likely TCR contact residues of the peptide (Evavold & Allen 1991, De Magistris et al 1992, Evavold et al 1995). However the increasing availability of crystal structures for individual MHC-peptide complexes has revealed significant variations in the structure of each complex (Bjorkman et al 1987a&b, Madden et al 1991, Madden et al 1992, Madden et al 1993, Fremont et al 1992, Smith et al 1996, Reid et al 1997). Therefore where a particular structure is available there is an opportunity to exercise more precision in the design of those variant peptides likely to influence TCR recognition. In this chapter flexible peptide recognition by the 008A3 CTL clone TCR is investigated by making a series of pol peptide variants based on the available stuctural data for both TCR/MHC-peptide co-complexes (Garcia et al 1996a, Garbozci et al 1996) and more specifically the HLA-A2 pol complex (Madden et al 1993).

The resolution of the crystal structure of two TCR/MHC-peptide co-complexes (Fig 1.4) has provided important new information about this trimolecular interaction (Garcia et al

1996a, Garbozci et al 1996). Firstly it has confirmed the results of earlier mutational analyses by showing that the TCR CDR loops contact upward pointing residues on both the MHC α-helices and the antigenic peptide (Fig 5.1, Chapter 6, Engel & Hedrick 1988, Nalefski et al 1990, Jaulin et al 1992, Sun et al 1995, Jorgensen et al 1992, Chien & Davis 1993). Secondly conventional ideas about the orientation of the TCR on the MHC-peptide have been challenged. The concentration of diversity in the CDR3 loop originally prompted predictions that the TCR would align with its MHC-peptide ligand such that the CDRsα1 and 2/β1 and 2 would contact the MHC α-helices while the CDRsα3/β3 would bind to the peptide (Novotny et al 1986, Chothia et al 1988, Davis & Bjorkman 1988, Bjorkman & Davis 1989). However more recent mutational studies suggested that the TCR might infact lie diagonally across the MHC-peptide complex (Sun et al 1995, Sant'Angelo et al 1996) and that many TCRs might bind in this orientation (Sun et al 1995). Interestingly the structures of both the murine 2C TCR/H-2Kb-dEV8 peptide (Garcia et al 1996a) and the human A6 TCR/HLA-A2-tax peptide (Garbozci et al 1996) co-complexes support these findings by showing that both TCRs adopt a roughly diagonal orientation on the MHC-peptide complex by lying between the N-terminal peaks of the MHC α-helices (Fig 5.2).

The crystal structure of the HLA-A2 pol peptide complex has shown that the glycine residue at position 8 (P8) makes no significant contact with HLA-A2 nor, in the absence of a side chain, would it be likely to protrude from the peptide binding groove such that it would contact the TCR (Madden et al 1993). However if the predicted common orientation for TCR binding is

superimposed on the HLA-A2 pol complex (Garcia et al 1996a & Garbozci et al 1996) P8 would be expected to be located in the TCR contact region. Therefore it was hypothesised that amino acid substitutions at P8 in the pol peptide might lead to altered and possibly improved TCR recognition. This proposition was tested by synthesising a series of pol peptide variants with substitutions for glycine at P8 which were used to stimulate degranulation of RBL-008. In contrast to other studies analysing the effect of variant peptides on TCR recognition (Udaka et al 1995, Hagerty & Allen 1995, Bhardwaj et al 1993, Evavold et al 1995, Wucherpfennig & Strominger 1995) a variant peptide was identified which was recognised better than the index peptide. This finding has implications for the promiscuity of TCR-peptide interactions and also for the design of functionally useful APLs.

5.2 Results

5.21 Response of RBL-008 to pol Variant Peptides

The response of RBL-008 to a panel of variants of the pol peptide incorporating amino acid substitutions for the glycine at peptide P8 (Fig 5.3) shows that the majority of substitutions at this position lead to a total loss of recognition. However three peptides, polT8, polN8 and polR8 are partially recognised while one peptide, polA8 is recognised better than index. Comparison of the peptide concentrations required to achieve a 50% response suggests that polA8 is about 100 times more potent than the index peptide.

5.22 Binding of pol and polA8 to HLA-A2

The binding of pol and polA8 to HLA-A2 was compared using two independent methods. In the first the antigen processing mutant 0.174 (Cerundolo et al 1990, Elvin et al 1993) was used to directly assess the binding of these peptides to HLA-A2 by immunoprecipitating ^{35}S-methionine labelled HLA-A2 heavy chain from cell lysates in the presence of varying concentrations of peptide using the conformation dependent mAb BB7.2 (Parham & Brodsky 1981). Comparison of the amount of heavy chain precipitated by pol and polA8 over the same range of concentrations indicates that HLA-A2 binds both of these peptides with a very similar affinity (Figs 5.4a&b). The second approach compared the ability of peptides pol and polA8 to inhibit the binding of a third peptide to HLA-A2 giving an indirect measure of relative binding (Sutton et al 1993). The results indicate that pol and polA8 were able to inhibit the binding of the flu matrix peptide (fmp) to HLA-A2 with equal efficacy as assessed by the degree to which lysis of fmp pulsed targets by a specific CTL line is suppressed by the presence of competing peptide (Fig 5.4c).

5.23 Competitive Inhibition of RBL-008 by Soluble Recombinant MHC-peptide Complexes

The studies in Chapter 4 showed that RBL-008 can be stimulated by multimerised recombinant HLA-A2 pol peptide complexes but not by the same complexes in monovalent form. These observations were repeated here using soluble recombinant HLA-A2-peptide complexes cross-linked on sheep anti-mouse coated magnetic beads via the mAb w6/32

(Barnstable et al 1978). The recombinant MHC-peptide complexes were prepared as described in Chapter 2 and a representative Coomassie stained gel and FPLC trace are shown in Figures 5.5a&b. Degranulation of RBL-008 stimulated by ten million beads coated with fixed concentrations of either recombinant HLA-A2 pol or HLA-A2 polA8 confirmed an increased response to polA8 compared with index (Fig 5.5c). The requirement for a multivalent ligand was then exploited in competition assays where the response of RBL-008 to a fixed stimulus was specifically inhibited by monovalent MHC-peptide complexes. In a modification of previous methods (see Chapter 4) RBL-008 cells were preincubated with the monovalent competitor, thereby allowing the TCR/MHC-peptide interaction to reach equlibrium before the addition of the stimulus in the form of pol peptide pulsed target cells. Comparison of the inhibition curves shows that HLA-A2 polA8 is a more potent inhibitor of RBL-008 than HLA-A2 pol, there was no inhibtion by HLA-B8 gag complexes (Fig 5.5d). Control experiments using the anti Cβ mAb βF1 to stimulate degranulation by cross-linking the TCR-CD3ζ receptor showed that recombinant HLA-A2 peptide complexes did not exert a non-specific inhibitory effect on RBL-008 (Fig 5.5e).

5.24 The Effect of Mutations in the α-helices of HLA-A2 on Recognition of Peptides pol and polA8

A panel of C1R cells expressing HLA-A2 molecules with amino acid changes in the upward pointing residues of the α-helices were used to present either the pol or polA8 peptides to RBL-008. Plasmids encoding the HLA-A2 mutant molecules were

obtained from M. Matsui and their subsequent transfection into C1R cells is described in Chapter 2. The positions and nature of the mutations together with the level of cell surface expression relative to wild-type are shown in table 5.1 and Figure 5.6. Lines are named according to the mutated position. this is preceded by the wild-type residue and followed by the mutant residue. All HLA-A2 mutant cell lines were shown to be capable of presenting the pol peptide to an HLA-A2 restricted HIV pol specific CTL line (Fig 5.7). The response of RBL-008 to pol peptide was totally disrupted by mutations at positions 65,72,154.155,158 and 163 and while those at positions 58, 82 and 149 did not significantly affect recognition (Fig 5.8a). In contrast when polA8 was presented to RBL-008 in the context of the HLA-A2 mutants recognition was maintained in the presence of a mutations at position 65 and to a certain extent 72 (Fig 5.8b). The possibility that mutants R65W and Q72H bind polA8 better than pol was excluded by direct binding assays (Figs 5.9a&b) (Tussey et al 1994). ^{125}I labelled peptide was incubated with lysates of metabolically labeled cells. Lysates were immunoprecipitated with BB7.2 (or W6/32 in the case of C1R-B8) and analysed by SDS-PAGE, representative autoradioraphs are shown in figure 5.9a. The intensities of the heavy chain and peptide bands were measured by scanning densitometry and the amount of peptide bound was normalised to the amount of heavy chain precipitated. The results show that neither mutant binds preferentially to polA8, but Q72H binds both peptides better than R65W. There was no peptide binding to HLA-B8.

5.3 Discussion

In this chapter the published structure of the HLA-A2 HIV pol peptide complex (Madden et al 1993) was used to direct the design of variant peptides which were considered likely to have significant effects on TCR recognition. The aminoacid substitutions were all at P8 because the wild-type residue is a glycine which lacks a side chain and is therefore unlikely to contact the TCR even though it lies within the likely TCR recognition site (Garcia et al 1996a, Garbozci et al 1996). The responses of RBL-008 show that all but one of the variants resulted in either lost or diminished recognition however the introduction of an alanine (polA8) lead to improved recognition. Enhanced binding of polA8 by HLA-A2 is excluded as the reason for this observation since both direct and indirect assessments of peptide-MHC interactions clearly show that HLA-A2 has a similar affinity for both pol and polA8. The alternative explanation for improved recognition of polA8 is that the alanine induces a favourable alteration in the binding of the A3-TCR to the MHC-peptide complex. This possibility was addressed in competitive inhibition assays comparing the ability of soluble recombinant HLA-A2 peptide complexes to engage the A3-TCR (see also Chapter 4, Vessey et al 1997). These assays make use of the fact that RBL-008 can only be triggered by multivalent ligands which means that the response to a fixed multivalent stimulus can be competitively inhibited by monovalent soluble recombinant MHC-peptide complexes. Comparison of the inhibition curves obtained with the HLA-A2 pol and HLA-A2 polA8 complexes suggests that HLA-A2 polA8 has a higher relative affinity for the A3-TCR

than HLA-A2 pol providing a potential mechanism for the improved recognition of the polA8 peptide. The finding that the introduction of an alanine at P8 in the pol peptide has a marked effect on TCR recognition but no effect on peptide binding to HLA-A2 mirrors earlier observations involving alanine substitutions in another HLA-A2 restricted epitope, the flu matrix peptide (fmp) (Parker et al 1992). Parker and colleagues reported that an alanine substitution at any position in the fmp had no effect on HLA-A2 binding but in each case lead to altered CTL reactivity. However, in contrast to the results reported here improved recognition of substituted peptides was not demonstrated.

Further evidence for the enhanced recognition of polA8 was provided by using target cells expressing HLA-A2 molecules with mutations at a number of upward pointing residues in the α-helices. Significantly the mutants R65W and Q72H were found to abolish recognition of pol while having only a partial effect on the recognition of polA8. As with wild-type HLA-A2 neither mutant showed preferential binding of polA8 which suggests that preserved recognition of this peptide when presented by R65W and Q72H is a result of the higher relative affinity that polA8 appears to have for the A3-TCR. It is also of note that R65W binds polA8 less well than Q72H despite eliciting a better response from RBL-008. Such a discrepancy between the binding and presentation of peptides has been observed with other HLA-A2 mutant molecules (Tussey et al 1994).

The minimal effect of HLA-A2 mutants E58R, R82S and A149G on the recognition of pol by the A3-TCR implies that

these positions may not be contact sites for this TCR. By contrast the crystal structure of the A6-TCR/HLA-A2-tax co-complex (Garbozci et al 1996) shows that HLA-A2 residues 58 and 149 do contact the A6-TCR. These differences suggest that even though the TCR may bind MHC-peptide complexes with a common orientation there are likely to be subtle differences in the precise points of contact. Variation in TCR contact residues could explain why target cell lysis by a pol specific CTL *line* was unaffected by the mutations in HLA-A2 (Fig 5.7) since a range of TCRs are likely to be represented within a T cell line. Support for this interpretation is provided by an earlier report showing that target lysis by several H-2Kd restricted *Plasmodium berghei* peptide specific T cell clones was affected differently by a series of mutations in the MHC α-helices (Jaulin et al 1992).

There are a number of possible ways in which polA8 could enhance recognition by the A3-TCR ranging from the localised effects of altered amino acid side chains to secondary shifts in both MHC and peptide TCR contact residues. Recent structural data for HLA-B8 complexed with five different single amino acid variants of an HIV gag peptide indicate that all manner of structural changes may be induced by altering peptide residues and that these can lead to alterations in TCR recognition and T cell function (Reid et al 1996). Consequently, in the absence of structures for the A3-TCR co-complexed with both HLA-A2 polA8 and HLA-A2 pol it is impossible to be certain of the nature of the change in the interaction between the A3-TCR and the HLA-A2-peptide complex. However in view of the recently published TCR/MHC class I-peptide co-complexes

(Garcia et al 1996a & Garbozci et al 1996), which suggest the P8 residue may lie within a generally applicable TCR contact region it is tempting to speculate that the addition of the small methyl side chain in the alanine residue might enhance TCR binding through a direct interaction.

The demonstration that polA8 has a higher relative affinity for the A3-TCR than pol has implications for the results of Chapter 4 in which RBL-008 was effectively inhibited by monovalent recombinant HLA-A2 complexed to a random peptide library. This result implied that many peptides in the random library permit engagement of the TCR by the HLA-A2 molecule, however it was speculated that the presence of high affinity peptides within the random mix could help explain the degree of inhibition observed. Similar conclusions were drawn by Udaka and colleagues who identified a CTL clone capable of responding to a random peptide library (Udaka et al 1995). It was estimated that the T cell clone might recognise up to 10,000 different peptides, although the point was made that this Figure could be revised down if there were a number of high affinity peptides in the random peptide library which were recognised at very low concentrations.

The results presented in this Chapter support and extend those other reports of flexible TCR-peptide interactions, however with one exception (Sykulev et al 1994a), those studies found that altering the sequence of the index peptide resulted in diminished TCR recognition (Udaka et al 1995, Hagerty & Allen 1995, Bhardwaj et al 1993, Evavold et al 1995, Wucherpfennig & Strominger 1995). This may well reflect the approach taken to designing such variant peptides which has

generally involved the substitution of dominant TCR contact residues. In contrast, knowledge of the structure of HLA-A2 pol has directed substitutions to a position which is essentially 'functionally neutral' in the index peptide with the result that an extra TCR contact site may have been added to the peptide. Much has been made of the potential immunotherapeutic value of the ability of variant peptides to partially activate (Evavold & Allen 1991), anergise (Sloan-Lancaster et al 1993) or antagonise (DeMagistris et al 1992) T cells. However, variant peptides exhibiting improved TCR recognition could also be of potential value in modulating the immune response, firstly, through super-efficient stimulation of immune responses by peptide vaccines and secondly by inducing negative thymic selection of potentially harmful autoreactive T cell clones. The latter application is made all the more intriguing by recent data which challenges established theories of thymic selection by demonstrating that moth cytochrome C (MCC) specific T cell clones can be positively selected on the index peptide (Nakano et al 1997). Thus, assuming the affinity/avidity model of thymic selection to be correct (Chapter 4, Hogquist et al 1994, Ashton-Rickardt et al 1994, Jameson et al 1995) the use of thymus targeted self index peptides to try and delete autoreactive T cell clones might be ineffective requiring instead the design of APLs with a higher affinity for the TCR.

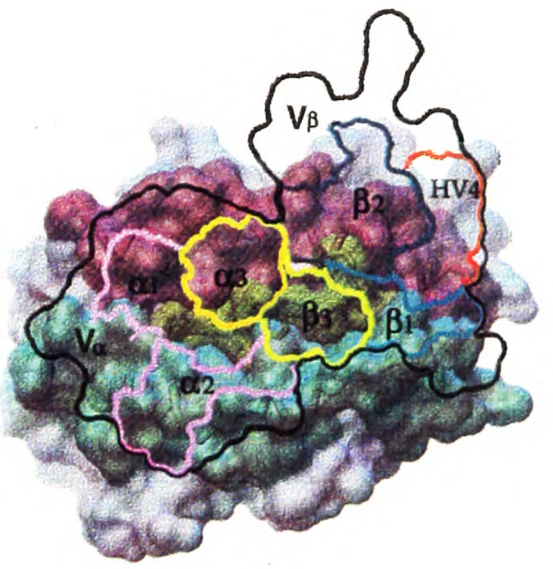


Fig 5.1
Footprint of the 2C TCR binding site on the surface of the H-2kb-dEV8 peptide molecular surface.
This shows that the TCR lies diagonally across the MHC-peptide and that contacts are made with both MHC and peptide. The outline of the TCR is shown in black, the borders between the CDRα1 and 2 in pink, the borders between the CDRsα3 and β3 are shown in yellow and the borders between the CDRsβ1 and 2 in blue. The α1 helix of the MHC is in magenta, the peptide in olive green and the α2 helix of the MHC in dark green. (Adapted from Garcia et al 1996a).

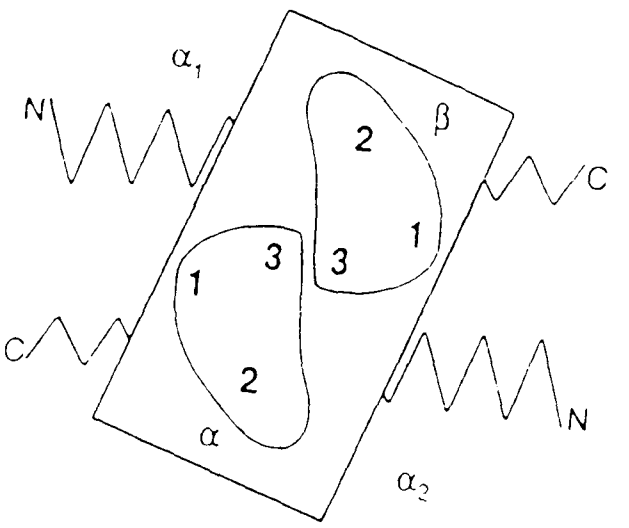


Fig 5.2
Schematic representation of the position of the A6-TCR on the HLA-A2 tax peptide
This shows that this TCR also has a diagonal orientation between the N-terminal peaks of the MHC α-helices. Numbers refer to TCR CDR loops. (Adapted from Garbozci 1996).

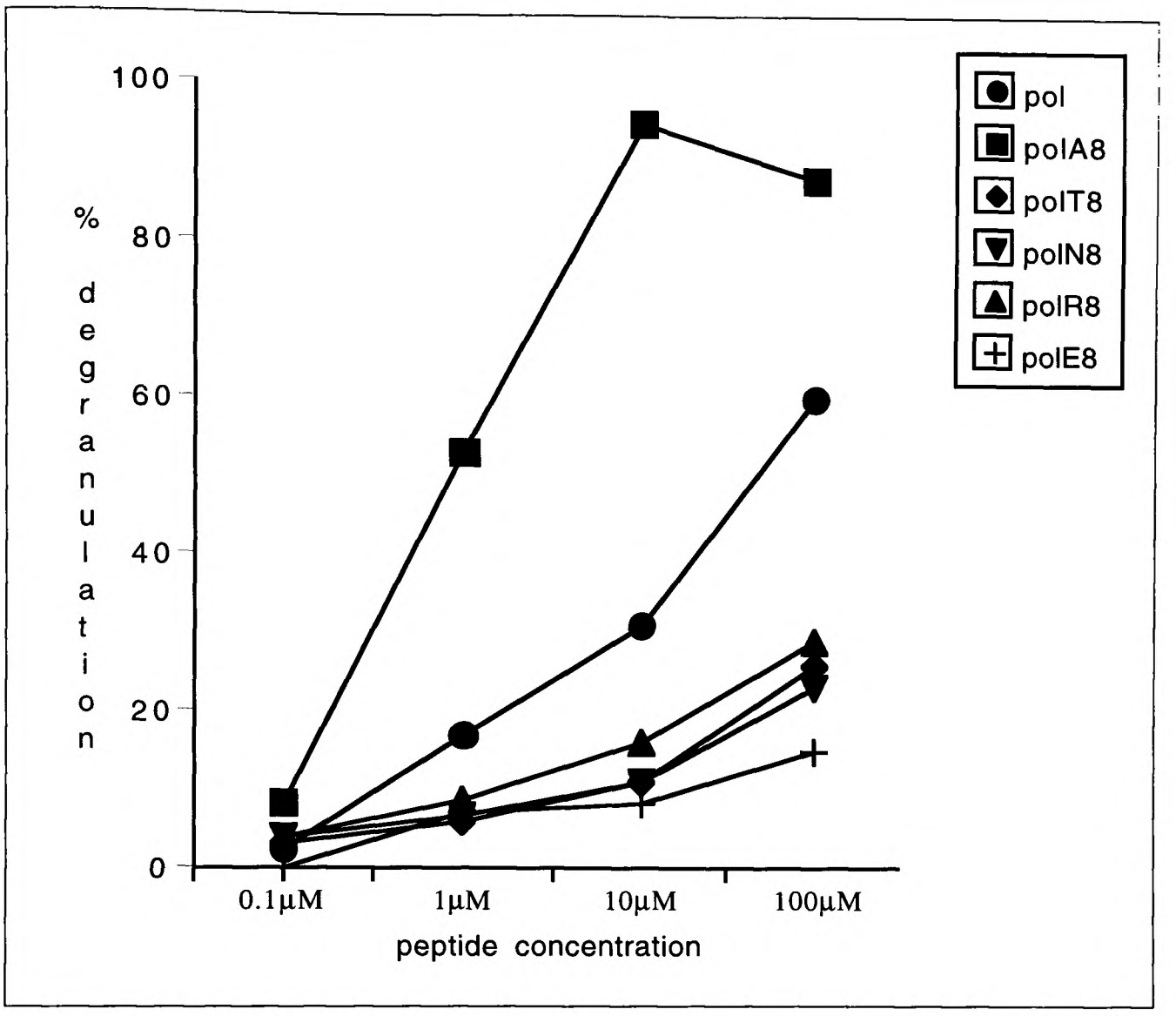


Figure 5.3
The effect of amino acid substitutions at P8 in the HIV pol peptide on recognition by the A3-TCR-CD3ζ chimeric receptor.
HLA-A2 C1R cells were pulsed with various concentrations of peptide and then added to labelled RBL-008 cells. The degranulation response is expressed on the vertical axis as a percentage of the response to a fixed concentration of the βF1 mAb. The response to βF1 in this assay was approximately 1000cpm and a background release of approximately 100cpm was subtracted from the results. The results shown are the mean values of triplicate measurements and the experiment was repeated on three occasions. The following peptide variants did not elicit a response of greater than 100cpm above background:polF8, polI8, polM8, polP8, polQ8, polS8, polV8, polW8 and polY8.

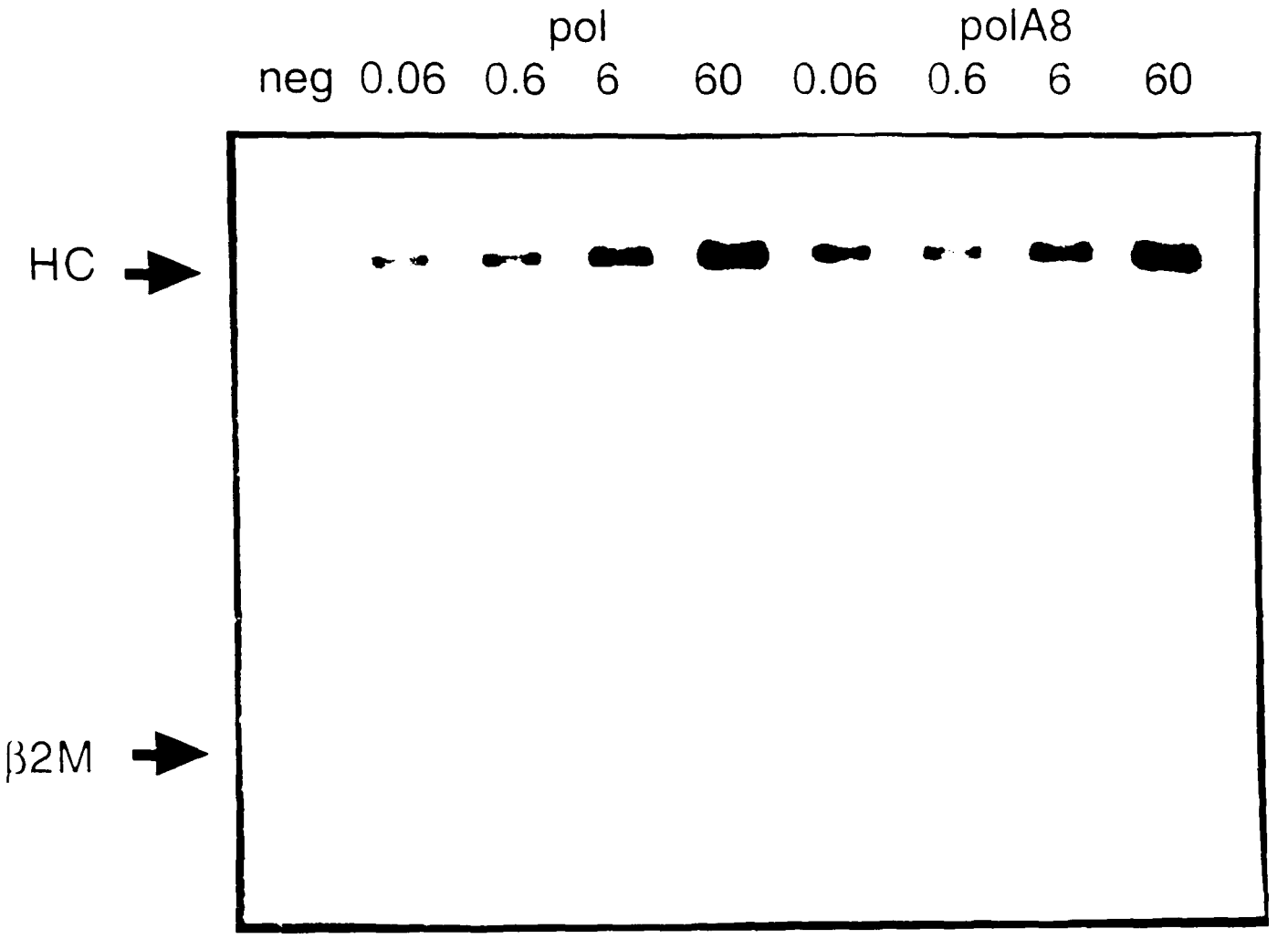


Figure 5.4a&b.
Binding of pol and polA8 to HLA-A2.
0.174 cells were labelled with ^{35}S-methionine and then lysed. Heavy chain was immunoprecipitated in the presence of ascending concentrations of peptide (0.06-60μM). Immunoprecipitates were analysed on a 15% polyacrylamide gel under reducing conditions. The gels were fixed , dried and exposed to radiographic film.
(a) A representative autoradiograph showing immunoprecipitated heavy chain over a range of peptide concentrations. The concentration of peptide (μM) used is shown across the top of the figure (HC= heavy chain)

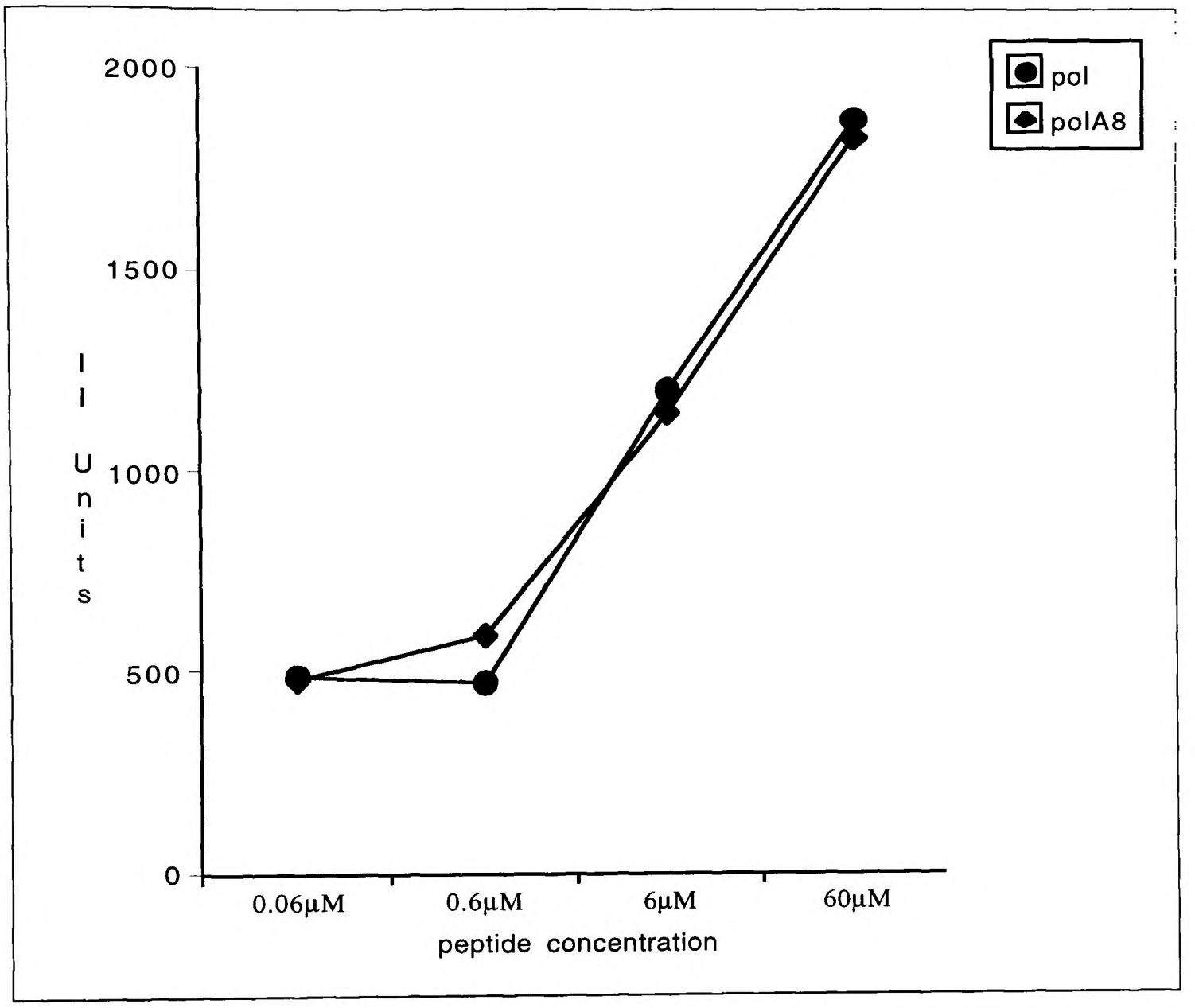


Figure 5.4a&b.
Binding of pol and polA8 to HLA-A2.
(b) Graphical representation of the amount of heavy chain immunoprecipitated over a range of peptide concentrations. The quantity of heavy chain immunoprecipitated was determined by scanning densitometry of autoradiographs and is reported in image intensity units (IIU) shown on the vertical axis. The peptide concentration is shown on the horizontal axis. A background of 200 IIU was observed when heavy chain was immunoprecipitated in the absence of peptide and this was subtracted from the figures shown. The results are representative of a set of experiments that were repeated on two occasions.

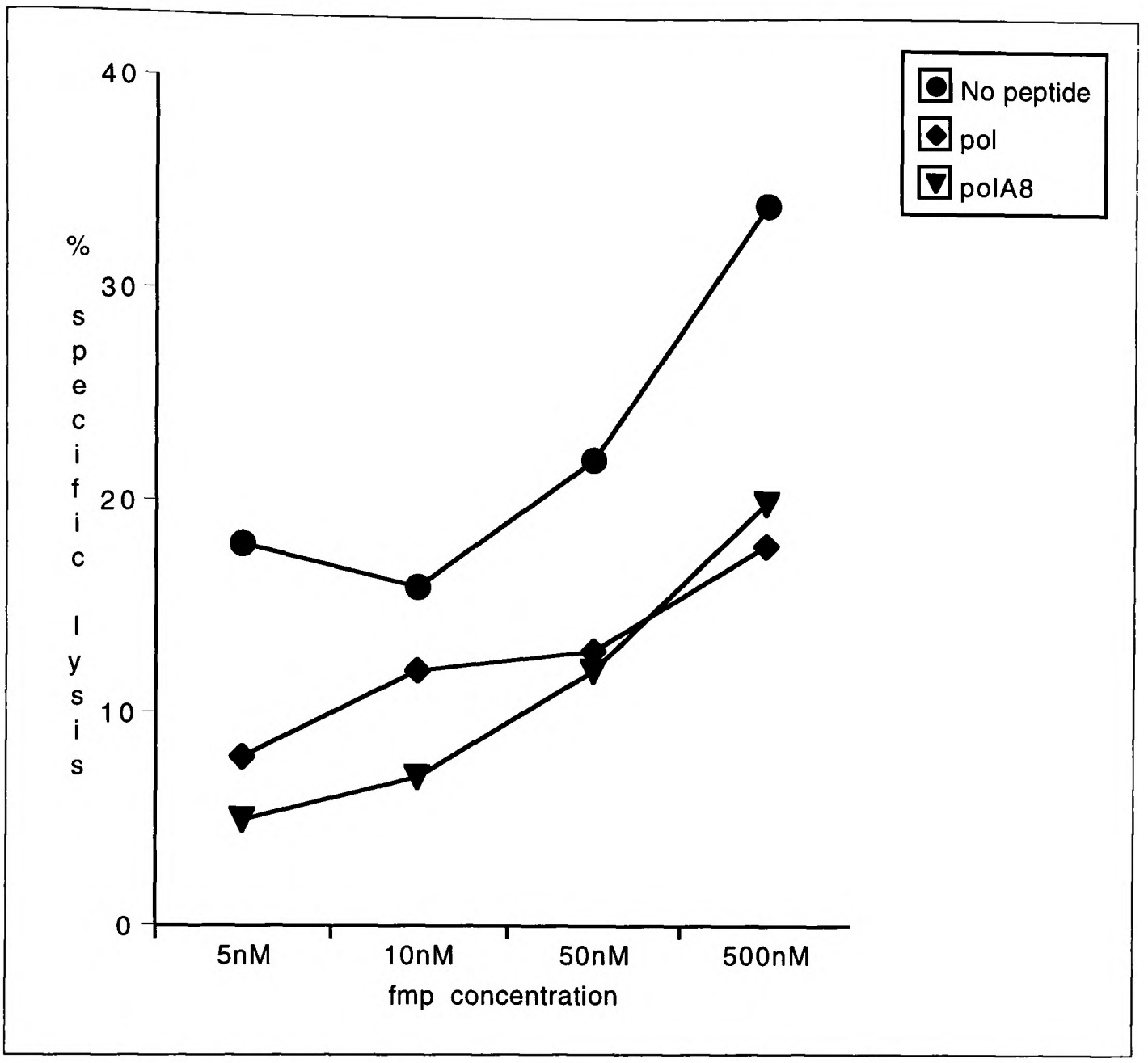


Figure 5.4c.
Binding of pol and polA8 to HLA-A2.

Autologous B cell targets were labelled with ^{51}Cr and pulsed with 60μM pol, polA8 or gag p17 peptide. Target cells were then plated out in 96 well plates containing either 60μM pol, polA8 or gag p17 (control peptide) together with various concentrations of flu matrix peptide. Finally flu matrix specific CTL were added to each well. Supernatants were collected and responses measured by liquid scintillation counting. Maximum release was measured by lysing target cells with 5% Triton X-100. Specific lysis is calculated as follows: (experimental release - media release) / (maximum release - medium release) x 100. The data shown are the mean values of triplicate measurements and are representative of three separate experiments.

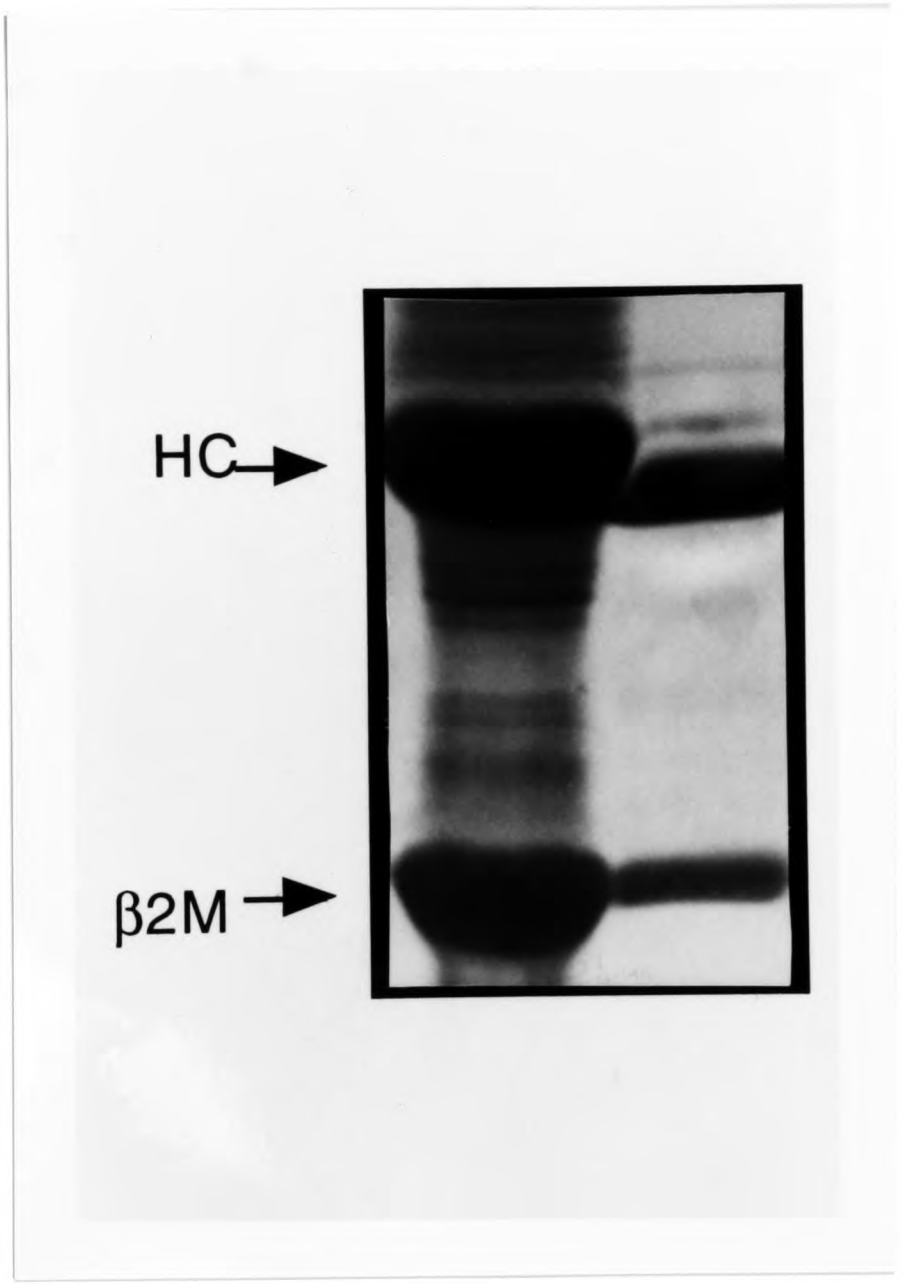


Figure 5.5a
SDS-PAGE analysis of bacterially expressed HLA-A2 heavy chain and β2m before. The left lane shows unpurified protein and the right lane purified protein on a 15% polyacrylamide gel stained with Coomassie blue.
(HC = heavy chain)

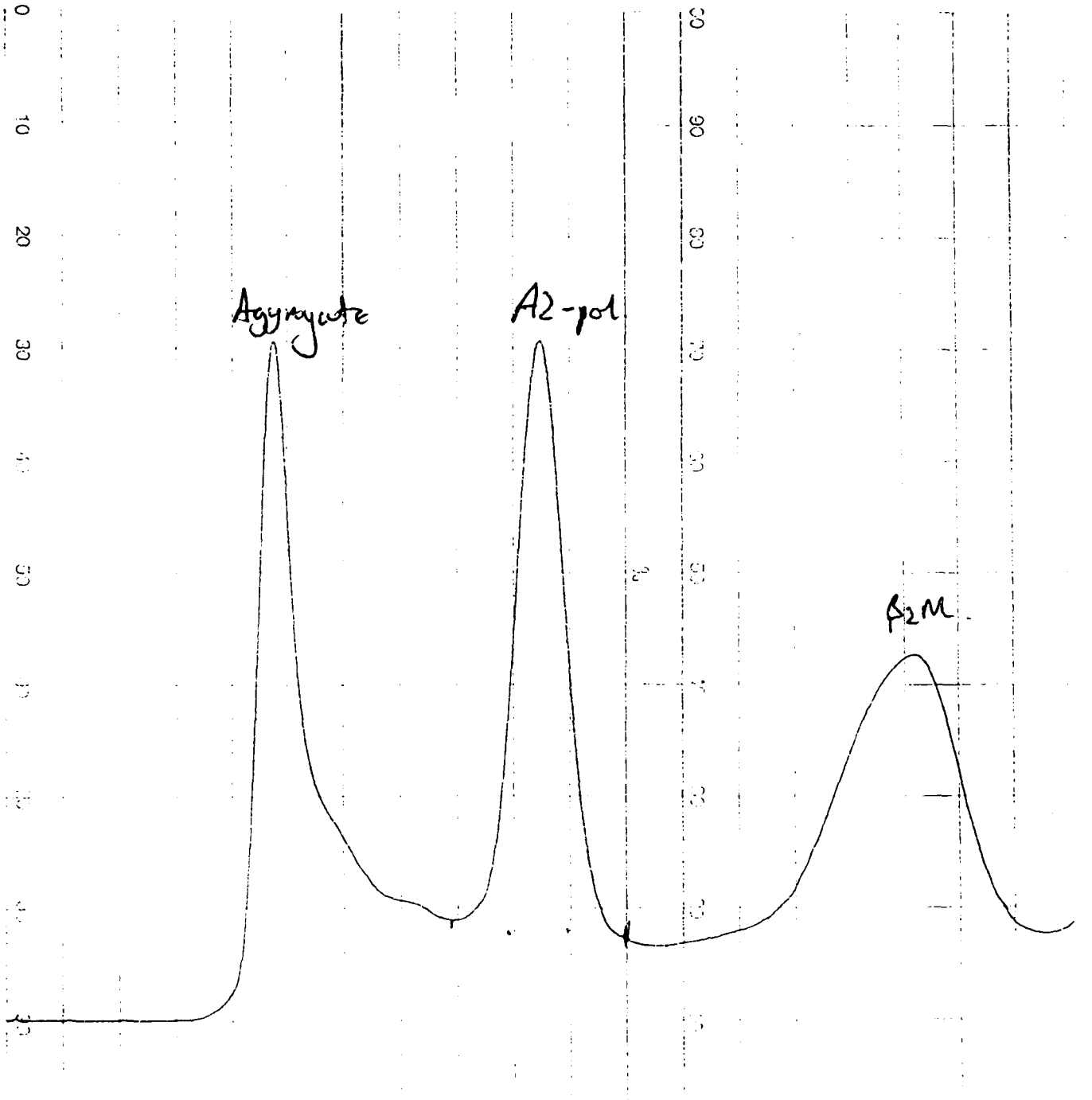


Figure 5.5b
FPLC trace of refolded HLA-A2 pol.Two separate peaks are seen one representing refolded MHC-peptide complex and the other representing excess β2m.

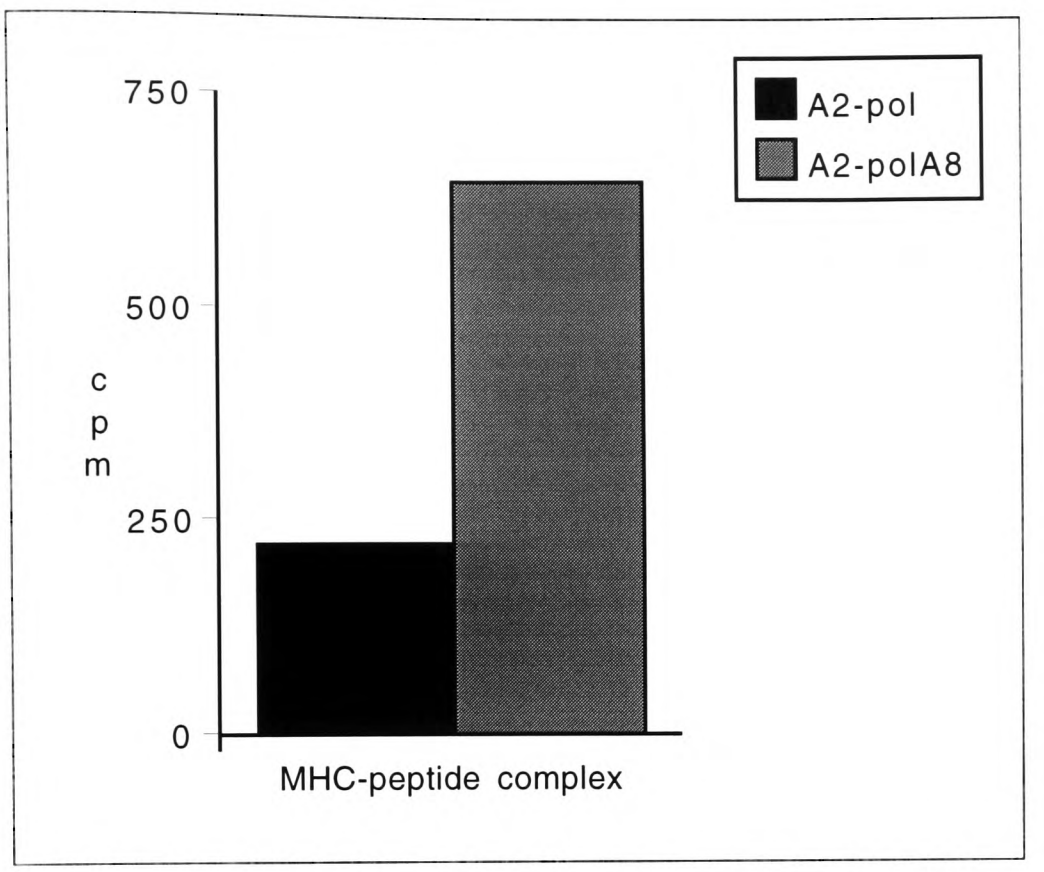


Figure 5.5c
Enhanced stimulation of RBL-008 by recombinant HLA-A2 polA8 complexes
Recombinant HLA-A2 molecules complexed with either pol or polA8 were multimerised on magnetic beads (5µg of protein/10 million beads) and then added to labelled RBL-008 cells. The cellular response was measured by degranulation which is represented as counts per minute (cpm) on the vertical axis, background release was approximately 100cpm and this was subtracted from the results. The results shown are the mean values of triplicate measurements and the experiment was repeated on three occasions.

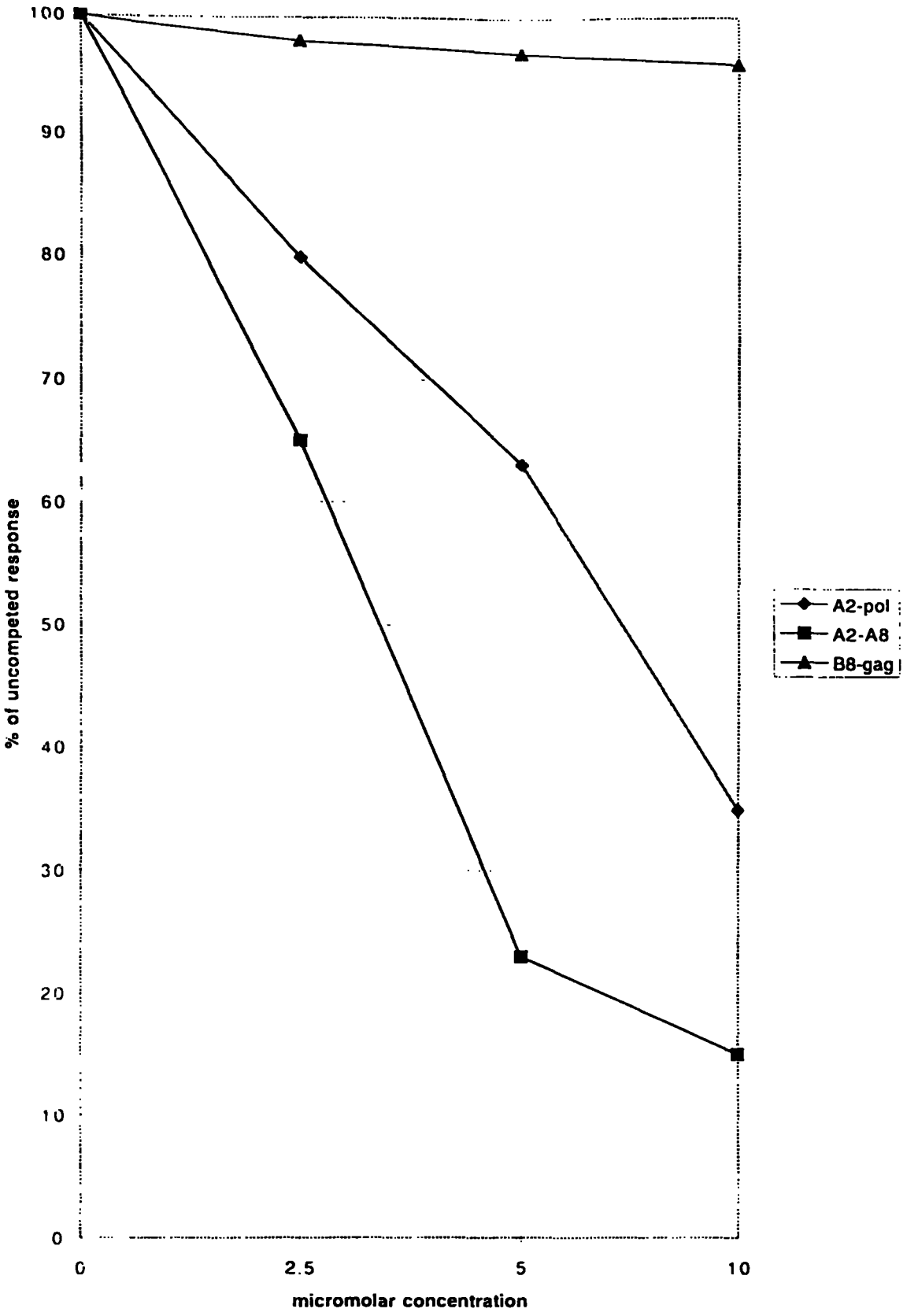


Figure 5.5d
Competitive inhibition of the response of RBL-008 to pol peptide pulsed C1R A2 cells by a variety of MHC single peptide complexes.

A fixed stimulus of $5x10^5$ C1R-A2 cells pulsed with 50μM pol peptide was used in each assay. Competing soluble, monomeric MHC peptide complexes were added in ascending concentrations as indicated on the horizontal axis. The vertical axis indicates the competed response as a percentage of the uncompeted response. The uncompeted response was approximately 600 cpm. Results shown are the mean values of duplicate measurements and are representative of 3 independent experiments.

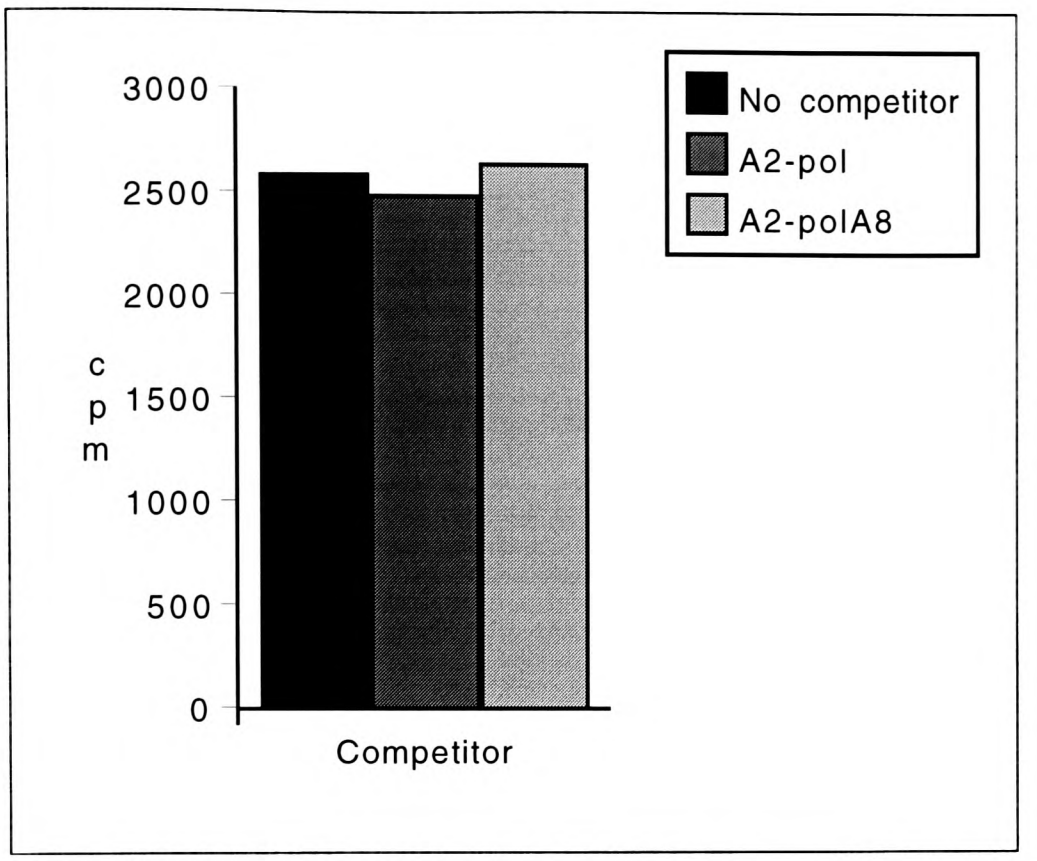


Figure 5.5e
Monovalent competitor complexes do not exert a non-specific inhibitory effect on the RBL-008 transfectant.
Transfectants were labelled as described and stimulated to degranulate by a fixed quantitiy of the anti TCR-Cβ mAb βF1 in the presence or absence of 10μM monovalent competitor as shown. The cellular response was measured by degranulation which is represented as counts per minute (cpm) on the vertical axis, background release was approximately 100cpm and this was subtracted from the results. The results are the mean values of triplicate measurements.

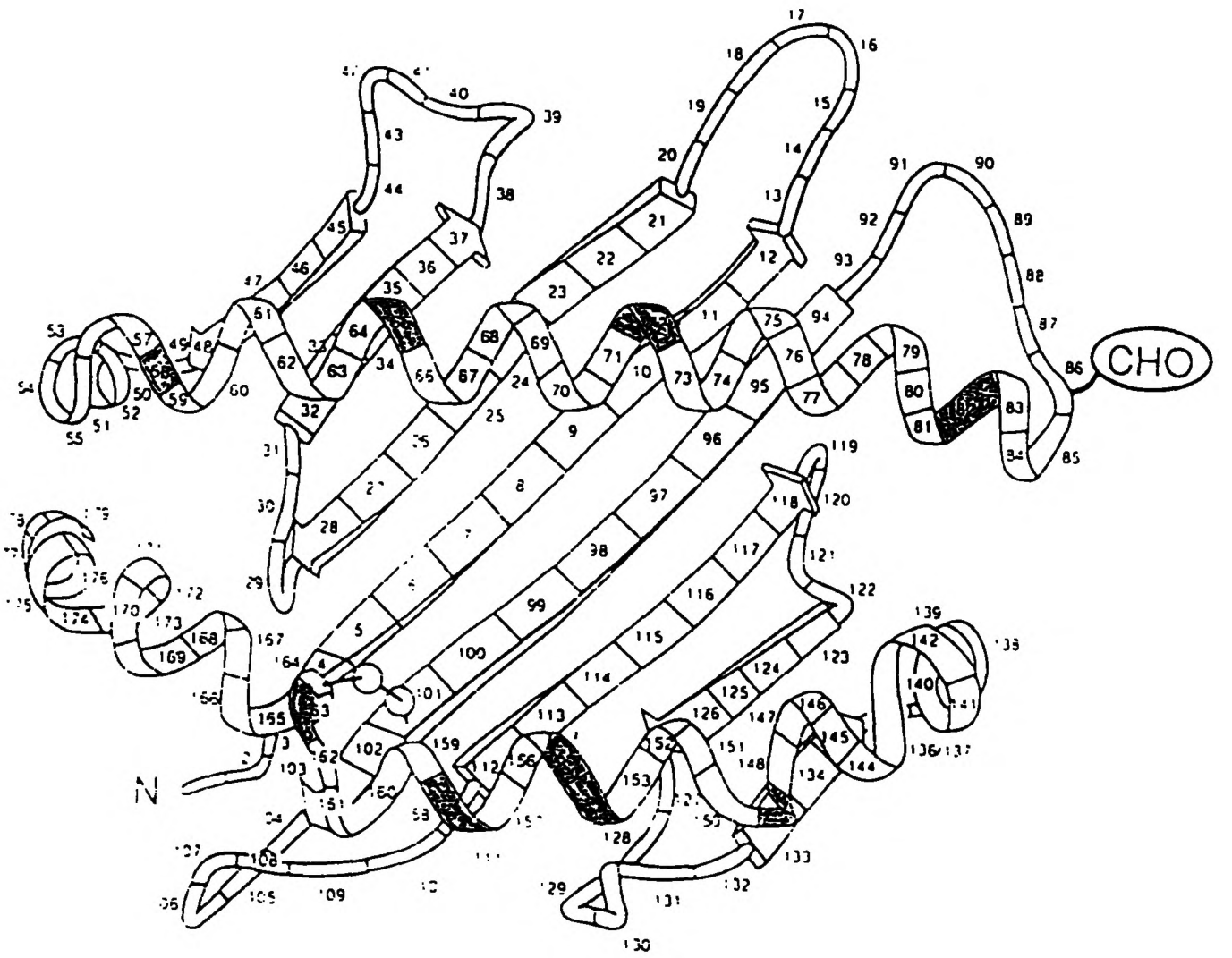


Fig 5.6
Schematic representation of HLA-A2
This shows the positions of amino acid changes in the mutant molecules used in this study.

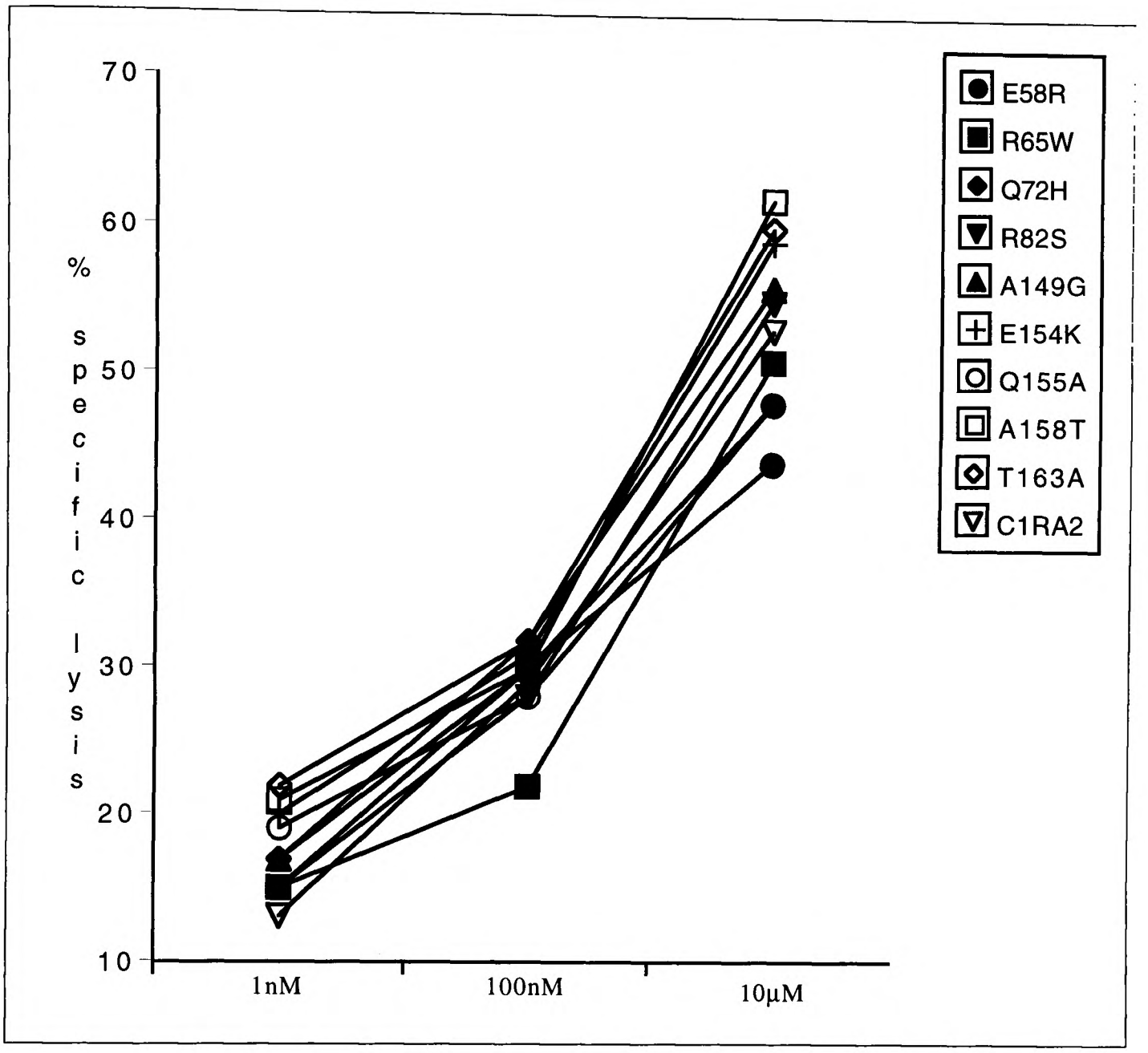


Figure 5.7
Presentation of pol peptide by HLA-A2 mutants
C1R transfecctants were labelled with ^{51}Cr and pulsed with various concentrations of pol peptide. Target cells were then plated out in 96 well plates and HIV pol specific CTL were added to each well. Supernatants were collected and responses measured by liquid scintillation counting. Maximum release was measured by lysing target cells with 5% Triton X-100. Specific lysis is calculated as follows: (experimental release - media release) / (maximum release - medium release) x 100. The data shown are the mean values of triplicate measurements and are representative of three seperate experiments.

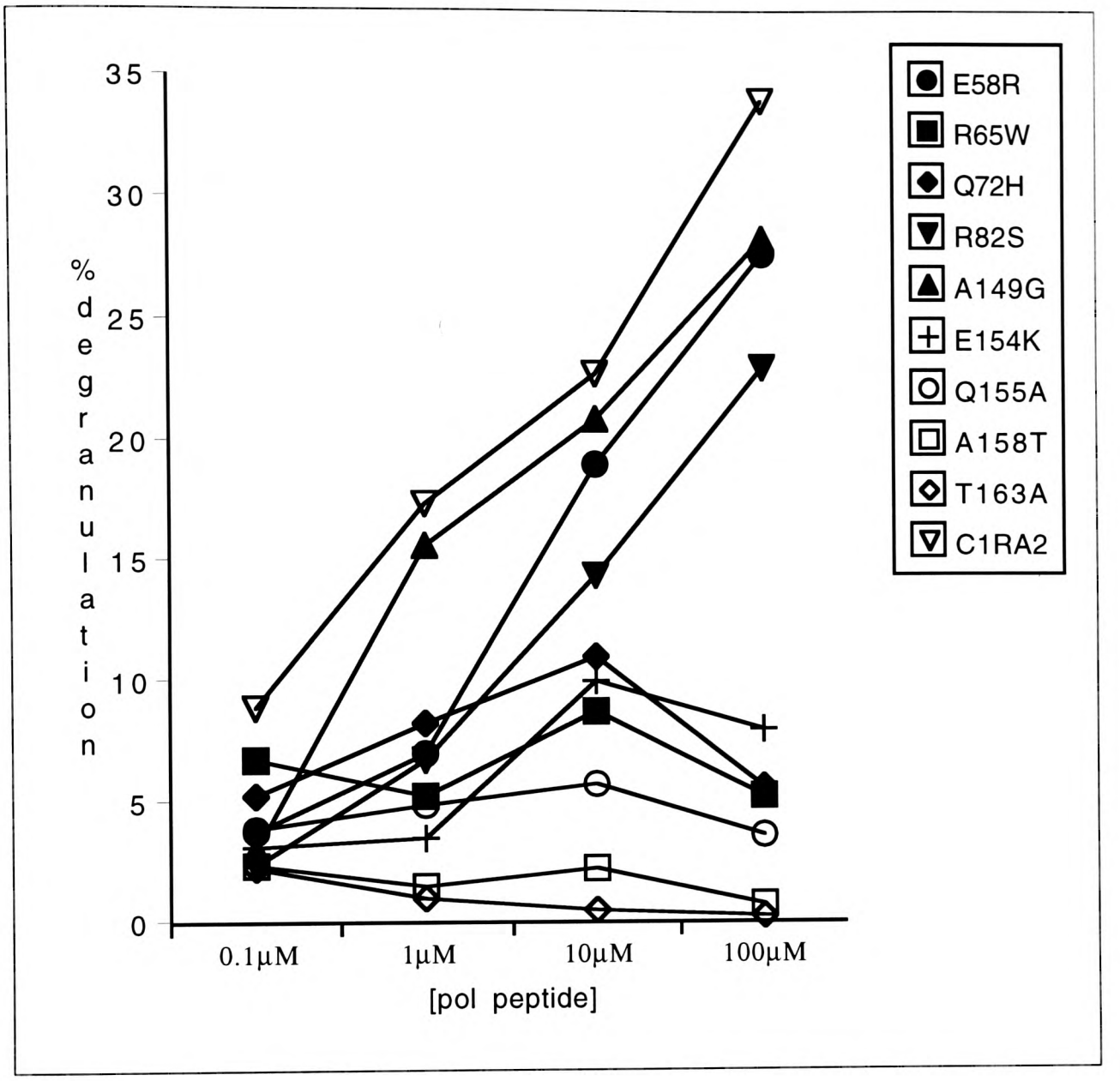


Figure 5.8a

The effect of mutations in the HLA-A2 α-helices on recognition of the pol peptide by the A3-TCR-CD3ζ chimeric receptor.

HLA-A2 C1R cells expressing mutant HLA-A2 molecules were pulsed with various concentrations of peptide and then added to labelled RBL-008 cells. The degranulation response is expressed on the vertical axis as a percentage of the response to a fixed concentration of the βF1 mAb. The response to βF1 in this assay was approximately 1000cpm and a background release of approximately 100cpm was subtracted from the results. The results shown are the mean values of triplicate measurements and the experiment was repeated on three occasions. Pol peptide is ILKEPVHGV.

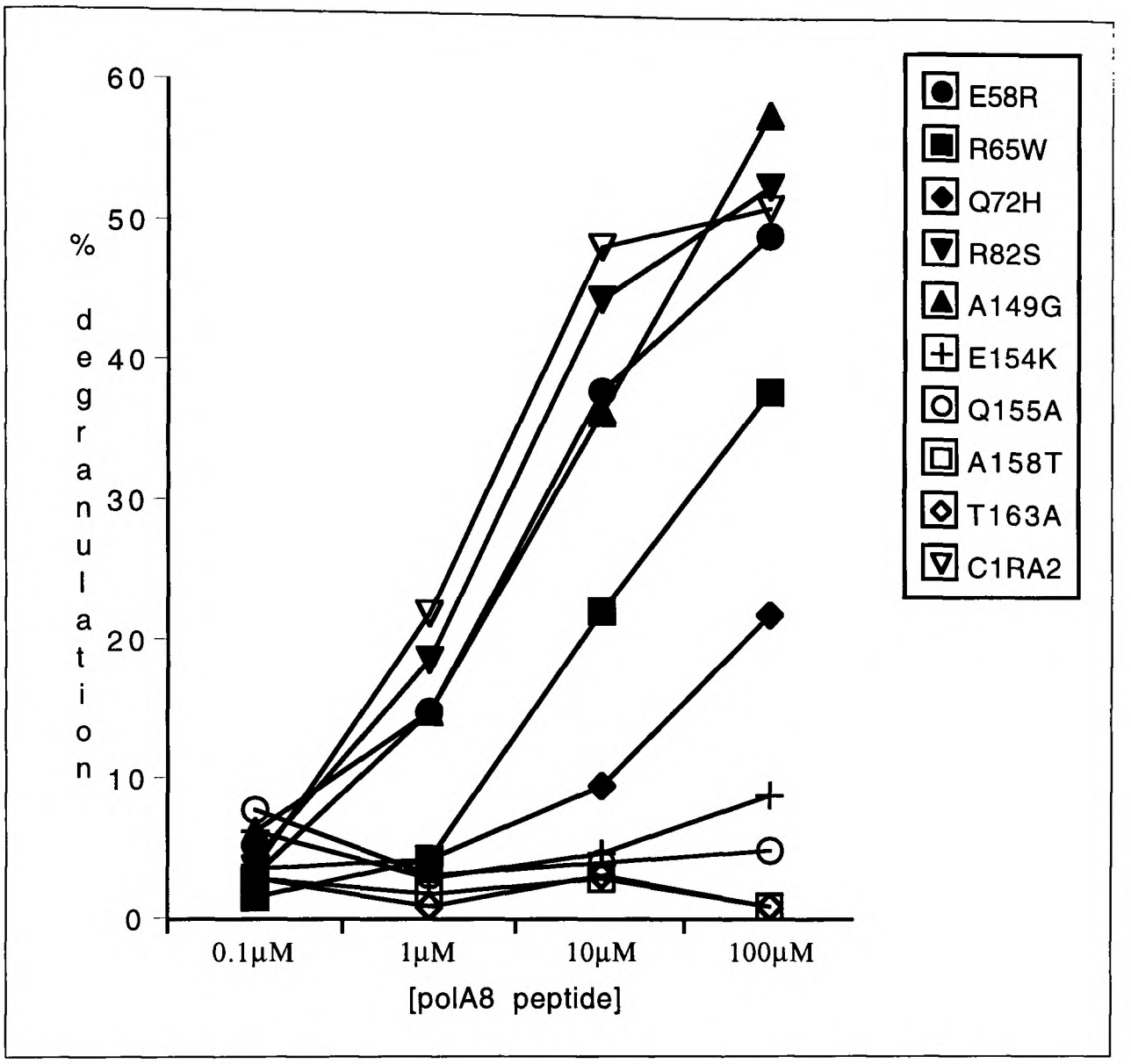
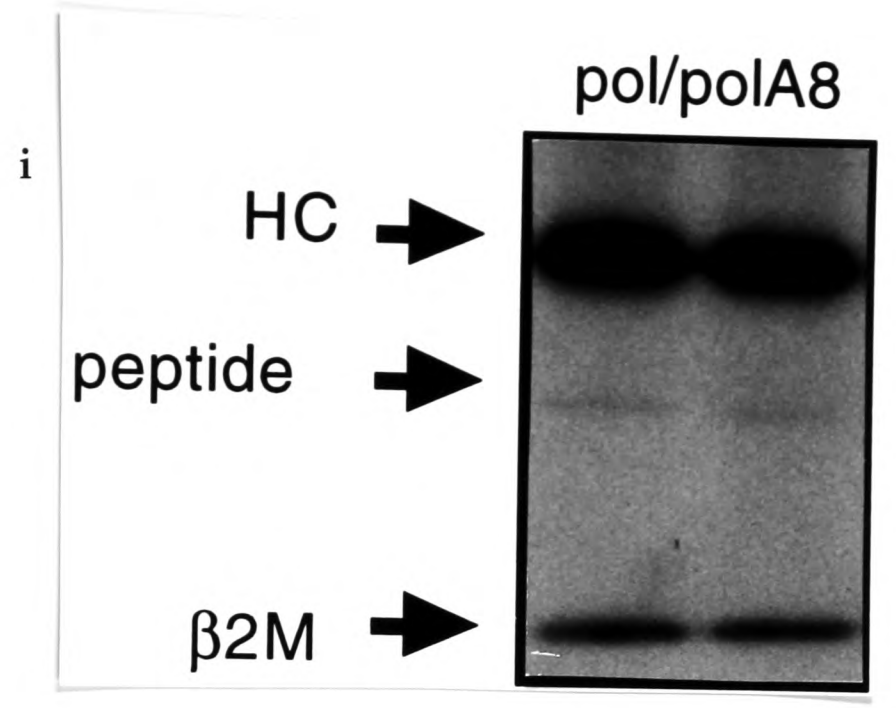
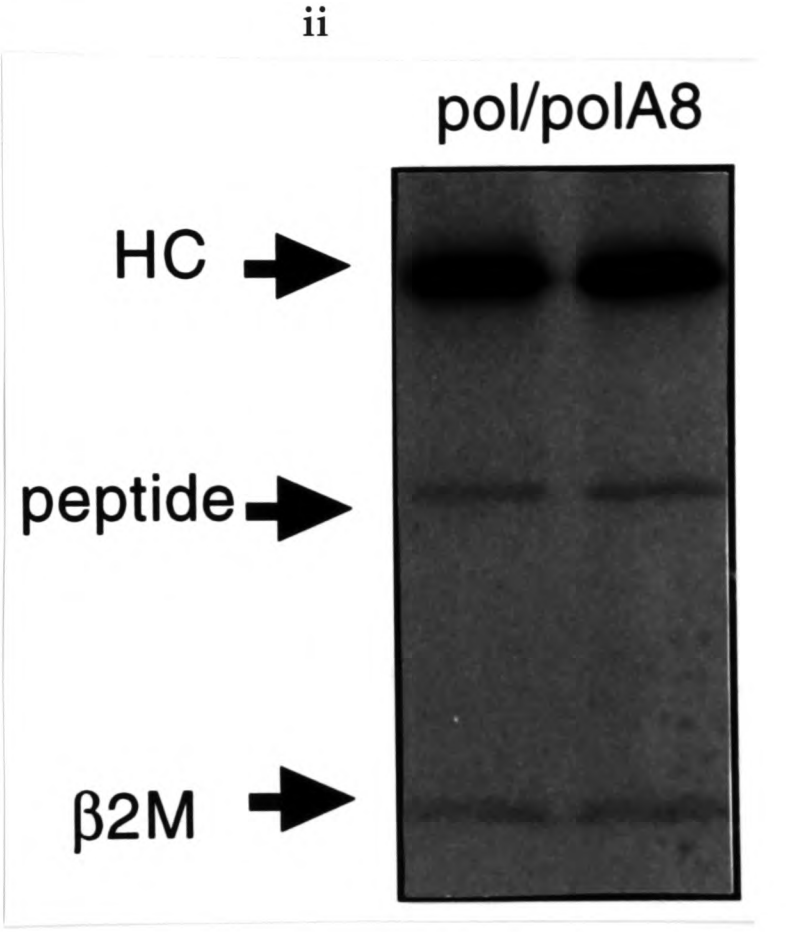


Figure 5.8b

The effect of mutations in the HLA-A2 α-helices on recognition of the polA8 peptide by the A3-TCR-CD3ζ chimeric receptor.

HLA-A2 C1R cells expressing mutant HLA-A2 molecules were pulsed with various concentrations of peptide and then added to labelled RBL-008 cells. The degranulation response is expressed on the vertical axis as a percentage of the response to a fixed concentration of the βF1 mAb. The response to βF1 in this assay was approximately 1000cpm and a background release of approximately 100cpm was subtracted from the results. The results shown are the mean values of triplicate measurements and the experiment was repeated on three occasions. PolA8 is ILKEPVHAV.





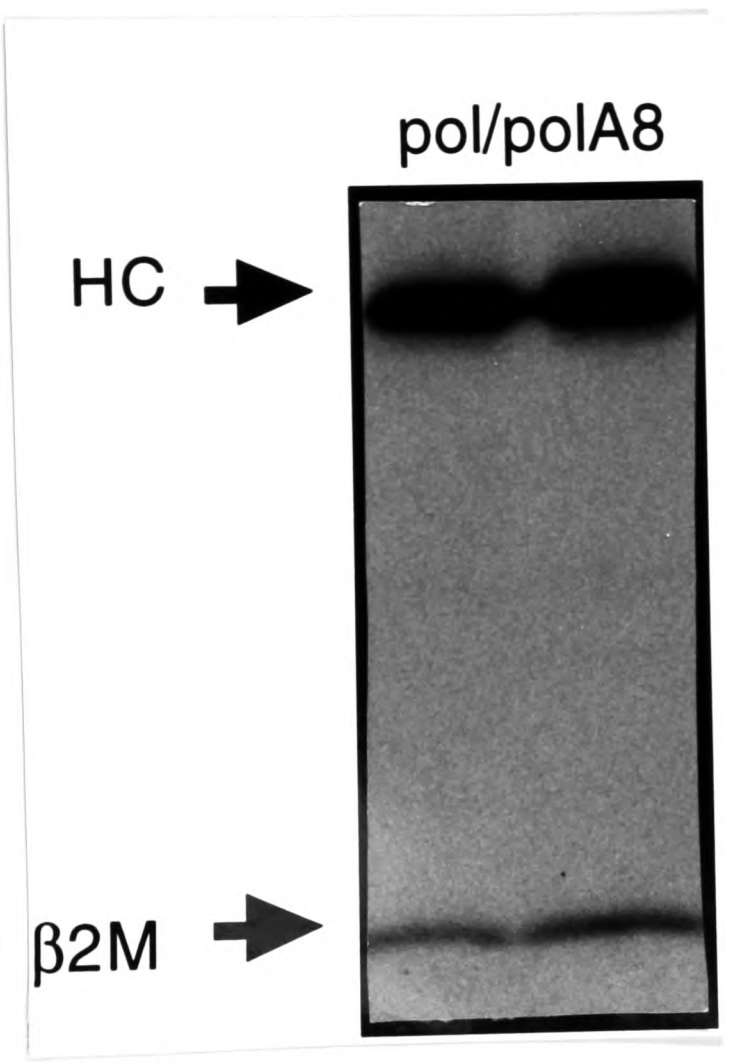


Figure 5.9a&b
Binding of pol and polA8 to HLA-A2 mutants R65W and Q72H.
C1R cells expressing either HLA-B8 or one of the HLA-A2 mutants R65W or Q72H were labelled with ^{35}S-methionine and then lysed. Heavy chain was immunoprecipitated in the presence of a fixed amount of iodinated peptide. Immunoprecipitates were analysed on a 15% polyacrylamide gel under reducing conditions. The gels were fixed, dried and exposed to radiographic film.
(a) Representative autoradiographs of immunoprecipitations from (i) C1R-R65W (ii) C1R-Q72H and (iii) C1R-B8 (HC = heavy chain).

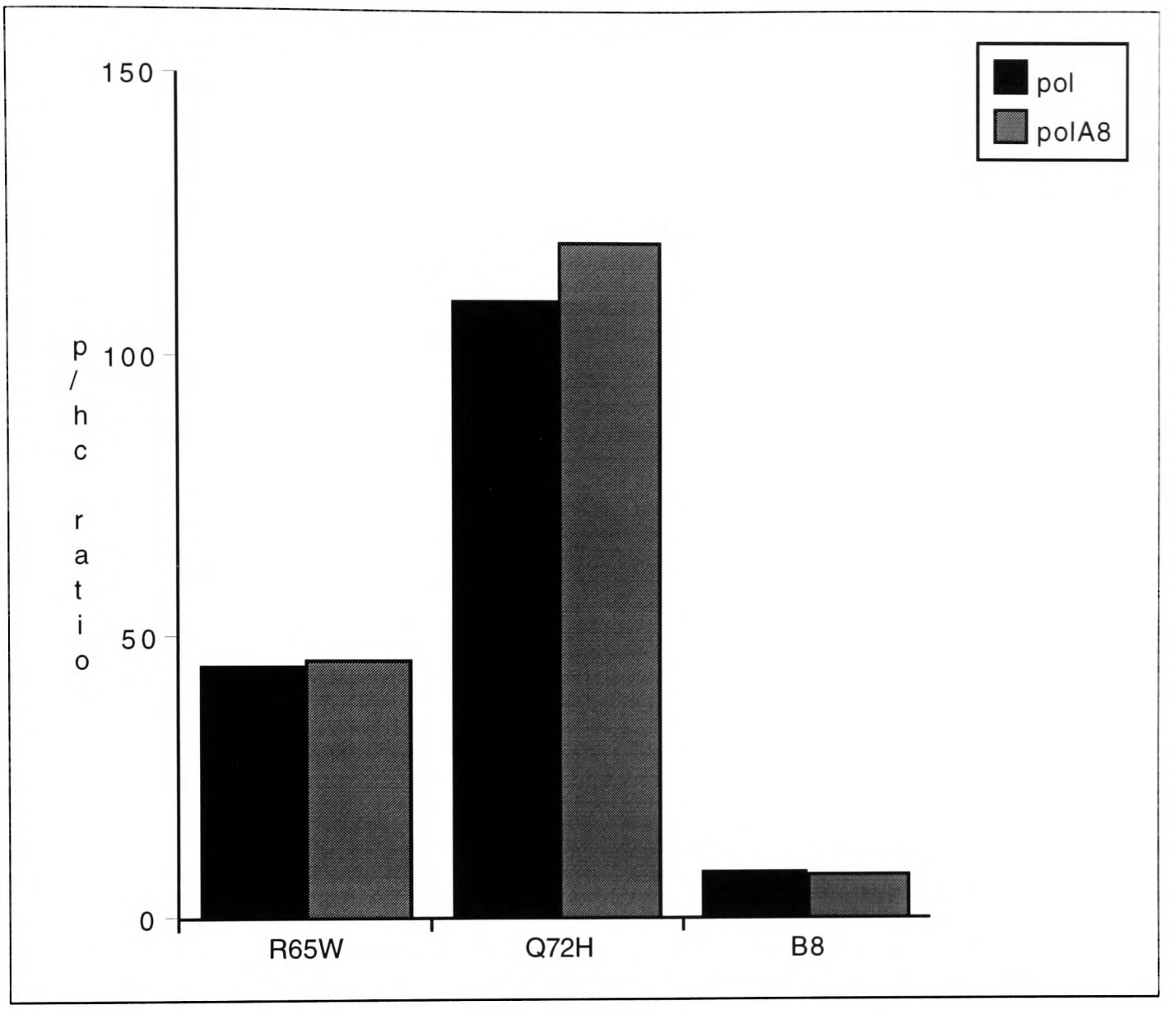


Figure 5.9a&b
Binding of pol and polA8 to HLA-A2 mutants R65W and Q72H.
(b) Graphical representation of the amount of pol or polA8 binding to MHC molecules, R65W, Q72H, or B8. Peptide and heavy chain bands on autoradiographs were quantitated by scanning densitometry. The amount of peptide bound by each of the three MHC molecules was normalized to the amount of heavy chain immunoprecipitated by the formula (peptide intensity - background) / (heavy chain intensity - background). The peptide/heavy chain ratio is shown on the vertical axis. The results are representative of a set of experiments that were repeated on two occasions.

Mutant	Expression Level
E58R	75%
R65W	132%
Q72H	61%
R82S	82%
A149G	69%
E154K	76%
Q155A	79%
A158T	126%
T163A	153%

Table 5.1
HLA-A2 mutants.
C1R cell lines are named according to mutated position in the HLA-A2 molecule this is preceded by the one letter code for the wild type residue and followed by that of the mutant residue. Expression levels were determined by FACS analysis using the mAb w6/32 and are expressed as a percentage of wild-type expression level.

Chapter 6
A Functionally Significant Allelic Polymorphism in a T Cell Receptor V β Gene Segment

6.1 Introduction

6.11 Organisation of the Human TCR Genes

The genes of the α and β chains of the human TCR are located on chromosomes 14q11-12 (Croce et al 1985) and 7q35 (Isobe et al 1985) respectively. Like the immunoglobulins, the TCR proteins have variable and constant domains and the variable domain gene is generated by the somatic recombination of variable gene segments (V), diversity segments (D except in the case of α and γ chains) and joining segments (J). The C domain which does not undergo rearrangement is encoded by constant region genes (C) consisting of four separate exons (Fig 6.1).

DNA and protein sequence alignments have estimated the numbers of functional α and β chain V gene segments (designated TCRAVand TCRBV genes) at 42 and 46 respectively (Arden et al 1995, this includes information on subfamilies and nomenclature). The Jα gene segments which encode 16-25 amino acids of the final TCR variable domain lie between the Vα and Cα domain gene segments (Fig 6.1). By contrast the Jβ and Dβ gene segments fall into two clusters. The first lies between the Vβ gene segments and the first of two C region genes (termed Cβ1 and Cβ2) and contains six functional Jβ gene segments and a single Dβ1 while the second, located between Cβ1 and Cβ2 comprises a single Dβ2 gene segment and seven functional Jβ2 gene segments (Fig 6.1). The Jβ gene segments encode 15-17 amino acids of the variable region of the TCR

while the Dβ gene segments contribute a further 2-5 amino acids.

6.12 Generation of TCR Diversity

As with Igs, the principle diversity in the TCR is concentrated into three hypervariable loops or complementarity determing regions (CDRs). While CDR1 and 2 are encoded in the V gene segment CDR3 is generated by recombination of the V,J and D region gene segments (Fig 6.2). Gene segment rearrangement is directed by highly conserved recombination sequences comprised of heptameric or nonameric motifs spaced by 12 or 23 nucleotides, corresponding to one or two turns of the double helix respectively (Akira et al 1987, Yancopoulos et al 1986). In the recombination of gene segments with the same transcriptional orientation pairs of heptamer and nonamer sequences are juxtaposed and the intervening DNA is looped out and excised as a closed circle, the two gene segments are then joined (Fig 6.2). Occasionally two gene segments with opposing transcriptional orientation are joined. In this event the recombination sequences are again juxtaposed but rather than excising the looped out DNA it is inverted and retained in the genome (Fig 6.2) (Fujimoto & Yamagishi 1987, Okazaki et al 1987, Lai et al 1987, Malissen et al 1986). Although the full details of the enzymatic control of the recombination process remain unclear an important role has been assigned to the recombinase activator gene products (RAG1 and 2) since mice lacking these genes are unable to rearrrange Ig or TCR gene segments.

The nature of gene segment recombination events in both Igs and TCRs lead to greater diversity in CDR3 than either CDR1 or CDR2. However several features of TCR gene rearrangement mean that the variability is skewed even further toward CDR3, reflecting the importance of this region in contacting antigenic peptide (Garbozci et al 1996, Garcia et al 1996a). Firstly N region diversification leads to the random addition of nucleotides between the V and J (and D in β chains) segments in both TCR polypeptide chains as compared with only the heavy chain in Igs (Elliot et al 1988, Siu et al 1984, Luria et al 1987, Quetermous et al 1986). Secondly there are a large number of J region gene segments compared with Ig genes. Thirdly several V region genes are flexible with respect to their 3' joining points, a phenomenon not seen in Igs (Chien et al 1987) and finally D regions are frequently translated in all three possible reading frames in TCR β chains an event which happens only rarely in Ig heavy chains. Estimated differences in the number of potential amino acid sequences that can occur at CDR3 are $\sim 10^{15}$ for the TCR compared with $\sim 10^{11}$ for Igs (Davis & Bjorkman 1988).

6.13 Allelic Variation in the TCRV Gene Segments

A further potential source of TCR diversity is allelic variation which has been reported in both the coding and non-coding sequences of the TCRAV and TCRBV gene segments (Cornelis et al 1993, Deulofeut et al 1995, Grier et al 1990, Hansen et al 1992, Posnett et al 1994, Reyburn et al 1993, Robinson et al 1989) (table 6.1). The full extent of this variation remains undefined although it is clearly limited when compared with

the high degree of polymorphism in MHC genes. In the TCRBV gene segments 24 allelic polymorphisms have been identified of which 17 are productive and the remainder are silent. In the TCRAV gene segments there are 13 allelic polymorphisms 9 of which are productive. Although a number of the productive polymorphisms give rise to non-functional TCR chains (Luyrink et al 1993, Charmley et al 1993, Malhotra et al 1992, Wilson et al 1990) analysis of the remainder shows that they are predominantly in CDR1, CDR2, the fourth hypervariable region or at sites that are predicted to influence the conformation of the hypervariable loops (Foote & Winter 1992). This suggests that a significant number of the allelic variants identified so far may have an effect on antigen recognition and could therefore alter the TCR repertoire and represent candidate genes for disease association. In this chapter direct evidence is provided for a functional effect of an allelic polymorphism in a TCRBV gene segment

The allelic polymorphism studied was first identified by direct sequence analysis of the BV1S1 gene in seven unrelated individuals (Robinson 1989). A single base pair change (C/G) was located in the predicted CDR2 loop of the TCR β chain antigen binding site. The change encodes a substitution of a neutral glutamine by a positively charged histidine and is therefore likely to be of structural significance. The glutamine containing allele is referred to as BV1S1A1N1 and the histidine containing allele as BV1S1A2.

6.2 Results

6.21 Generation and Expression of the BV1S1A1N1 Allelic Variant

The HLA-A2 restricted HIV pol-specific CTL clone A3 expresses a TCR utilizing the BV1S1A2 gene in combination with the alpha gene segment AV2S1A2 (chapter 3, Vessey et al 1996). The generation of A3-TCR-CD3ζ chimeric receptor molecules from the clone A3 TCR and their expression in RBL cells to produce the transfectant RBL-008 is described in detail in chapter 3. In order to create the beta chain variant BV1S1A1N1 the sequence of the BV1S1A2 gene was altered by site directed mutagenesis according to the method of Kunkel (Kunkel 1985) using the oligonucleotide BV1S1A1N1 (Fig 6.3, see Chapter 2 for oligonucleotide sequence). The template for the mutagenesis was generated from construct RV001 which encodes a BV1S1A2-Cβ fragment truncated at the interchain disulphide bond (see Chapter 3, Fig 3.3). Screening for succesful mutagenesis was carried out by standard dideoxy sequencing on a series of 4 clones one of which was positive for the base change (data not shown). The BV1S1A1N1-Cβ gene segment was then subcloned into the expression vector BJ043 (see Chapter 3, Fig 3.1) to create a novel TCRβ-CD3ζ chimera (Fig 6.3). Transfection of this gene together with the original AV2S1A2-CD3ζ gene into RBL cells generated the transfectant RBL-008glut (see Chapter 2 for details of transfection conditions and selection of stable transfectants). Expression of both chains of the TCR-CD3ζ chimeric receptor was demonstrated using monoclonal antibodies to the constant domains of the alpha and beta chains (Fig 6.4ab&c). Although

these studies do not conclusively prove that the BV1S1A1N1-CD3ζ and AV2S1A2-CD3ζ molecules form heterodimers they are highly supportive since several investigators have previously noted that RBLs transfected with both alpha and beta constructs tend to express higher levels of both chains than RBLs transfected with one chain only (Engel et al 1992, see also chapter 3). The proposed explanation for this is that heterodimers are more readily formed than alpha or beta homodimers.

6.22 The Effect of the BV1S1A1N1 Gene Segment on Recognition of HLA-A2 pol Targets

The ability of the chimeric receptor molecules expressed by RBL-008glut to transmit the signals necessary for degranulation was assessed by cross-linking either the alpha or beta chain with mAbs αF1 (anti Cα) and βF1 (anti Cβ) respectively (Fig 6.5). The results clearly demonstrate that cross-linking of either chain can elicit degranulation of labelled RBL-008glut and that the response is comparable to that seen in the transfectant RBL-008. However when the two cell lines are compared with respect to their response to pol peptide pulsed C1R-A2 target cells only RBL-008 undergoes degranulation (Fig 6.6) indicating that the change from histidine to glutamine in the CDR2 loop of this TCR has a profound effect on the recognition of the cognate antigen. The human BV1S1 gene product is also reported to bind to the streptococcal superantigen (sAg) SPE-C (Tomai et al 1992). However presentation of this toxin to RBL-008 and RBL-008glut by the high MHC-class II expressing Raji cell line at

concentrations of up to 100µg/ml did not elicit a response (data not shown).

6.23 Attempts to Restore Antigen Recognition by RBL-008glut

Since the allelic polymorphism in BV1S1A1N1 is in the putative antigen binding site of the TCR attempts were made to try and restore antigen recognition by the receptor expressed by RBL-008glut. It was reasoned that by mutating the predicted TCR contact residues on both the antigenic peptide and the the HLA-A2 molecule and using these as targets both individually and in combination that a compensating mutation might be identified. Such a mutation would then give insight into the topology of the interaction between the TCR-CD3ζ receptor and the MHC-peptide ligand.

The predicted TCR contact residues on the HLA-A2 molecule are thought to be those which lie along the α-helices and have upward pointing side-chains (Bjorkman et al Nature 1989 329 506), and this has now been confirmed for one HLA-A2 restricted TCR (Garbozci et al 1996). Accordingly a representative panel of mutant HLA-A2 genes was obtained and transfected into C1R cells. Chapter 5 contains the details of the HLA-A2 mutant cell lines. The position and the nature of the changes together with the expression levels are shown in table 5.1 and a diagrammatic representation of the HLA-A2 molecule with the position of the mutations is shown in Fig 5.6. All of the HLA-A2 mutants were shown to be functional molecules capable of presenting pol peptide to a CTL line, as shown in Fig 5.7.

A number of pol peptide variants with mutations at various positions in the sequence were also available (table 6.2) and these were tested for recognition by RBL-008glut both in the context of HLA-A2 and the other HLA-A2 mutants. The results of degranulation assays indicated that there was no combination of mutation in either HLA-A2 or the peptide which compensated for the histidine to glutamine change in the TCR-CD3ζ beta chain.

6.3 Discussion

In this study an allelic polymorphism in the BV1S1 gene segment was shown to have a significant effect on recognition of a specific MHC-peptide complex by a T cell receptor. This observation has implications both for the topology of the interaction between this TCR and the HLA-A2 pol peptide complex and also for the generation of the TCR repertoire.

The recent publication of the crystal structures of both murine and human TCRs complexed with an MHC class I peptide complexes (Garcia et al 1996, Garbozci et al 1996) confirms earlier predictions which proposed that the TCR would resemble an Ig Fab fragment with the three hypervariable CDR loops providing the antigen combining site (Chothia, et al 1988, Novotny et al 1986, Claverie et al 1989, Davis & Bjorkman 1988). The pivotal role of the CDR loops in TCR function is supported both by experiments in which specific antigen recognition can be moved from one TCR to another by transfer of the CDR loops (Katayama et al 1995) and by changes in antigen recognition on mutagenesis of the CDR loops (Nalefski et al 1992, White et al 1993, Engel & Hedrick 1988). The

observations in this chapter are consistent with these findings since the substitution of a neutral glutamine by a positively charged, basic histidine within the CDR2 loop clearly changes antigen recognition. In the absence of detailed structural information about the A3-TCR it is difficult to be certain whether the amino acid change directly disrupts an MHC-peptide contact residue or has secondary effects on distant parts of the antigen binding site. However the failure to restore recognition with MHC and peptide mutants tends to suggest that the disruption of the binding site is not restricted to a single contact site. Interestingly the structure of the A6-TCR/HLA-A2-tax peptide co-complex suggests that the CDRβ2 loop of that receptor does not make any significant contact with the MHC-peptide complex (Garbozci et al 1996). Consequently if HLA-A2 restricted TCRs bind to the MHC with a very similar orientation then it may be that the CDRβ2 loop of the A3-TCR also fails to contact HLA-A2 supporting the conclusion that the histidine for glutamine substitution has a secondary, rather than direct, effect on antigen recognition. However, data relating to the topology of the TCR/MHC-peptide interaction is conflicting since studies using MHC mutant molecules have suggested both significant variation between TCRs of the same specificity (Jaulin et al 1992, Chien & Davis 1993) and a restricted pattern of TCR/MHC-peptide interaction (Sun et al 1995). The model which best reconciles the data is that TCRs adopt roughly the same orientation on the MHC-peptide complex by lying between the N-terminal peaks of the α-helices but that there are subtle differences in the precise contact points. This is certainly the case for the two TCR/MHC-

peptide co-complex structures currently available since, in contrast to the human A3-TCR, the CDRβ2 of the murine 2C-TCR does make contact with MHC and peptide (Garcia 1996a).

Bacterial and viral superantigens are capable of stimulating subpopulations of T cells expressing specific Vβ elements. T cell responses require the presence of MHC-class II which is required for binding and presentation of these antigens. However since the sAgs bind to an invariable part of the MHC-class II rather than the peptide binding groove T cell responses are not MHC restricted (reviewed in Gasciogne 1993). The failure of both RBL-008 and RBL-008glut to respond to SPE-C contrasts with earlier data implying that at least one of the two BV1S1 alleles encodes a TCR Vβ domain that interacts with this sAg (Tomai et al 1992). There are two possible explanations for this, either the original data was erroneous or the RBL/TCR-CD3ζ system is not sensitive enough to detect this interaction. The reported affinities for sAg-TCR interactions range from $1.4x10^{-4}$-$8x10^{-7}$ (Fremont et al 1996) therefore it is possible that some of the lower affinity interactions could fall below the detection level of the RBL/TCR-CD3ζ system. Having said this the original report of a BV1S1-SPE-C interaction was based on a PCR analysis of T cell expansion rather than the use of specific mAbs, which is a more reliable approach. The availability of the BV1S1 specific mAbs reported in Chapter 3 should allow clarification of this position.

Obviously allelic variation that alters antigen recognition could potentially affect TCR usage in response to a specific epitope and, in cases where the response is oligoclonal, might even result in a failure to respond at all. A logical extension of

this is that some TCR V gene polymorphisms may confer an increased susceptibility to autoimmune disease by influencing the response to self antigens. Alteration in the TCR repertoire as a result of TCRV gene segment polymorphism has already been demonstrated in the mouse where a substitution in the CDR1 loop of Vβ3 gave rise to a total shift in TCR usage in response to a specific MHC-peptide ligand (Gahm et al 1991). Studies in the human suggest that they could also be susceptible to changes in TCR usage as a result of TCRV gene segment polymorphism. For instance 11% of a sample population were found to be homozygous for a polymorphism in BV20S1 which creates a null allele (Malhotra et al 1992, Charmley et al 1993), a result which has clear implications for the generation of a full TCR repertoire.

Although the results presented in this chapter show that allelic variation alters the *in vitro* recognition profile of the clone A3 TCR it is unclear how homozygous inheritance of the BV1S1A1N1 allele could effect the response to HLA-A2 HIV pol in vivo. One possibility is a shift in the TCR repertoire with either alternative BV-gene usage or possibly coupling of the BV1S1A1N1 segment with an alternative AV-gene chain to achieve recognition. Analysis of TCRBV gene segment usage in the response to HLA-A2 pol targets is currently underway (J. Wilson personal communication) and this will help to establish whether there is a correlation between BV1S1 usage and genotype.

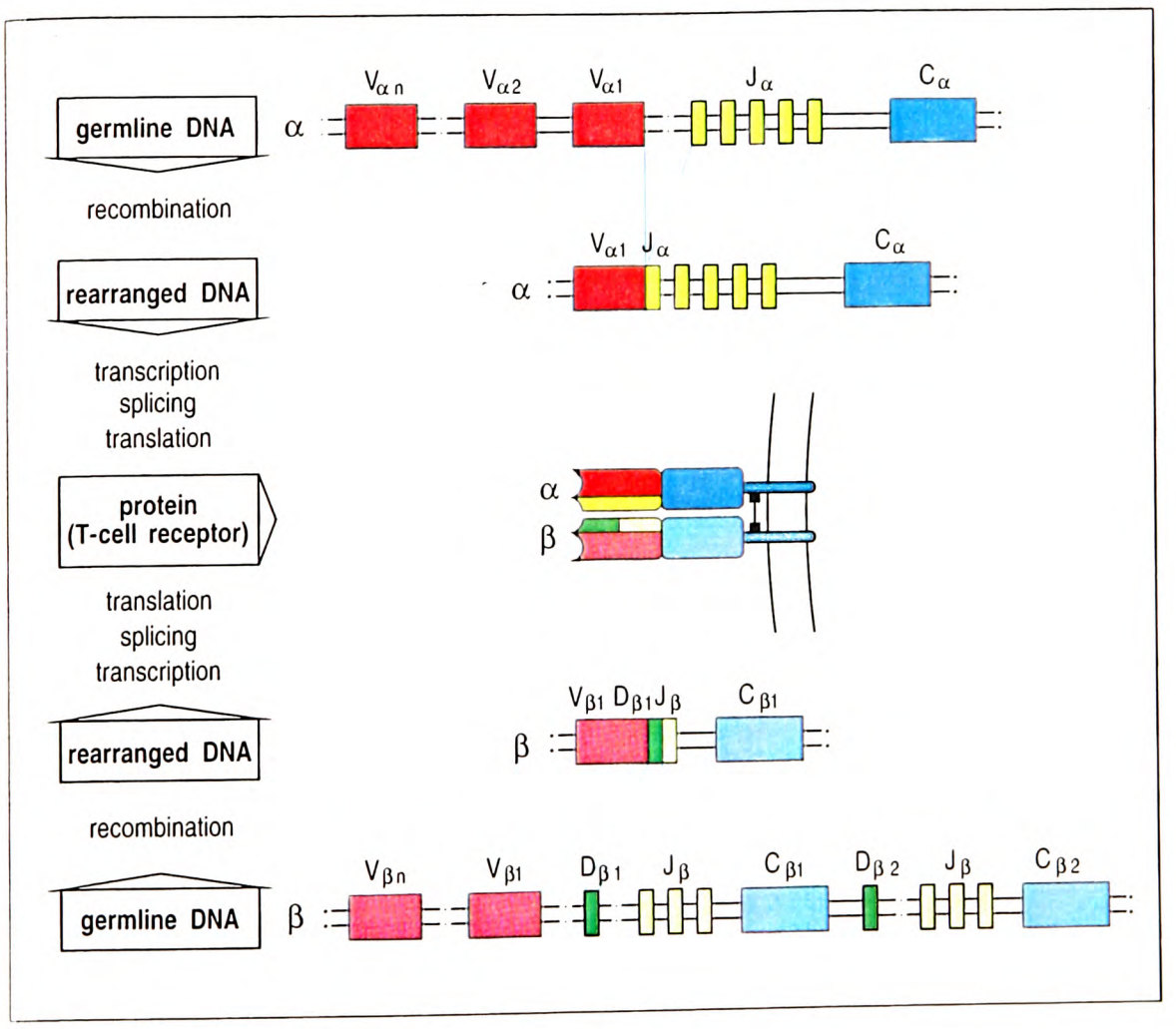


Figure 6.1

Organisation of the TCR α and β genes

The TCR α and β genes are composed of discrete segments that are joined by somatic recombination during development of the T cell. (Adapted from Janeway and Travers).

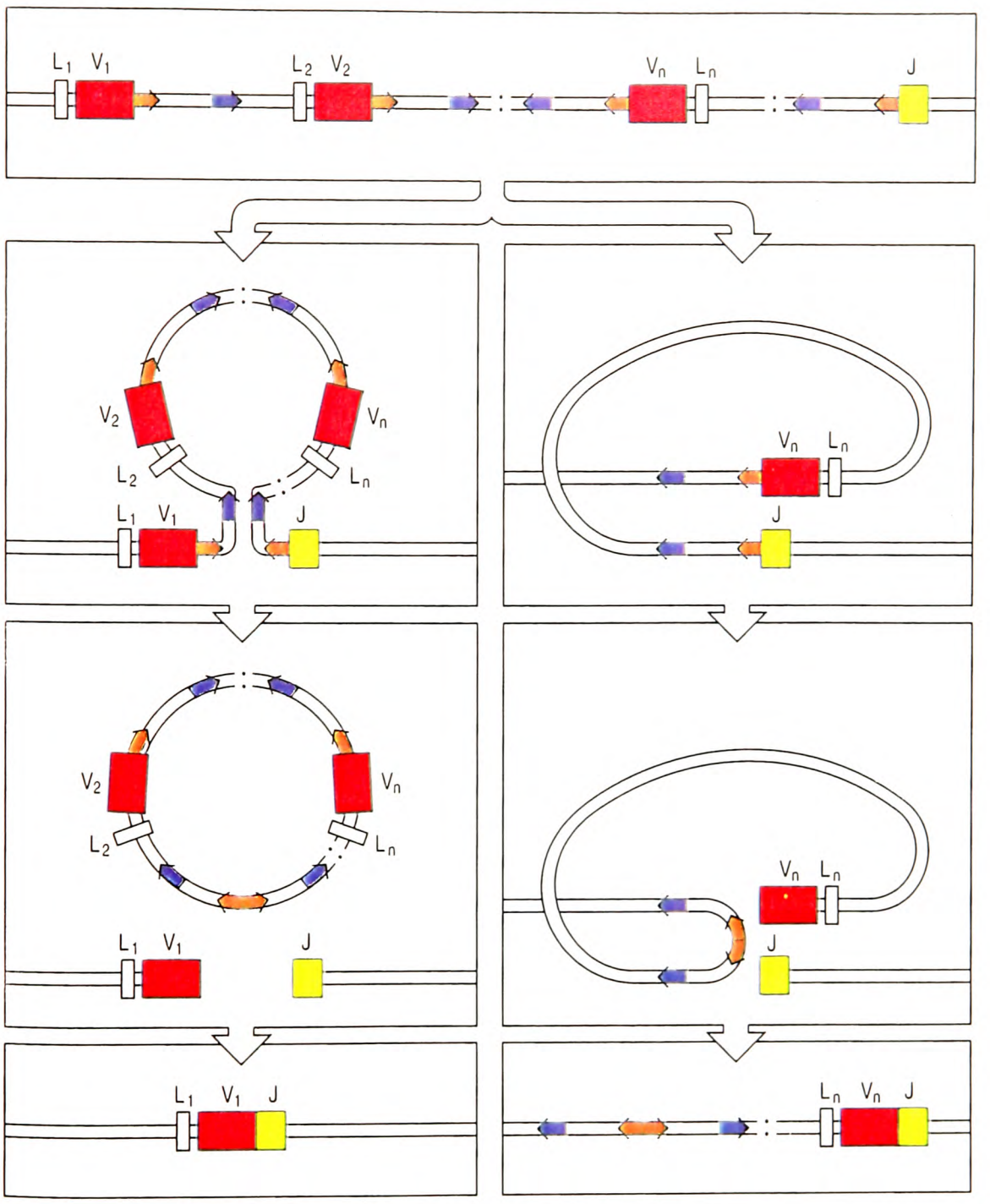


Figure 6.2
Mechanism of TCR gene rearrangement
In every V region recombination event the signals flanking the gene segments are brought together, heptamers are shown in orange, nonamers are shown in purple. In most cases the V and J segments are in the same transcriptional orientation as shown in the left-hand panels. Juxtaposition of the recombination signals results in looping out of the intervening DNA which is then excised as a complete circle. The V and J segments are then joined. Where V and J segments are in the opposite transcriptional orientation the looped DNA is inverted and maintained in the genome shown in the right-hand panels. (Adapted from Janeway and Travers).

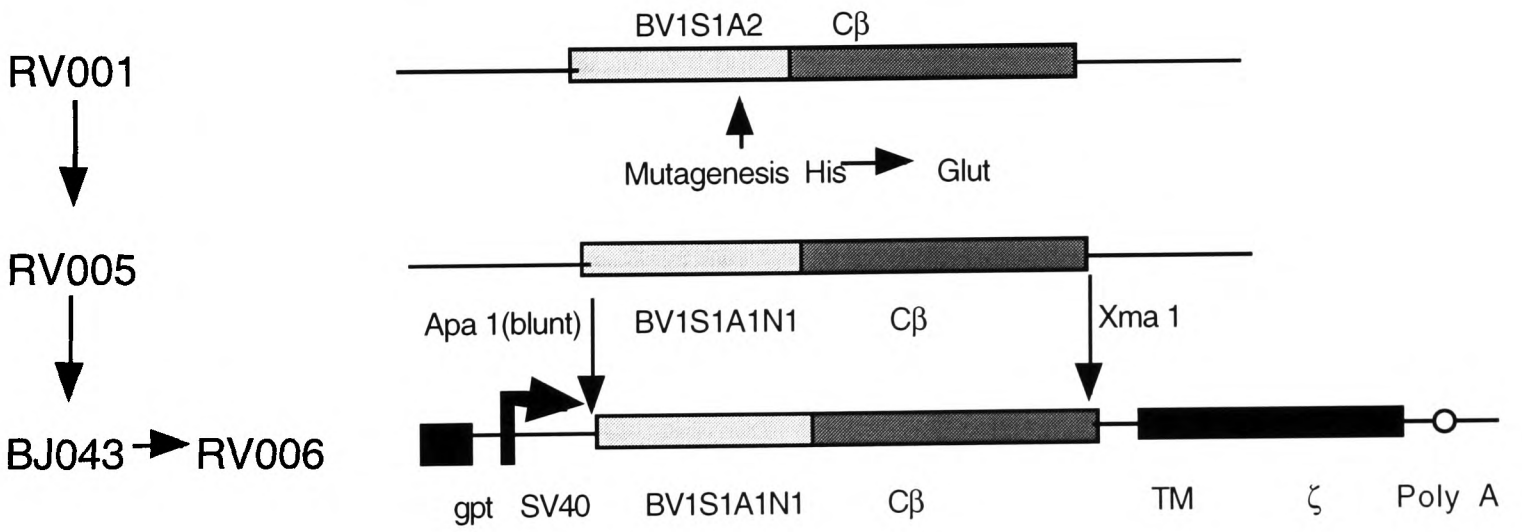
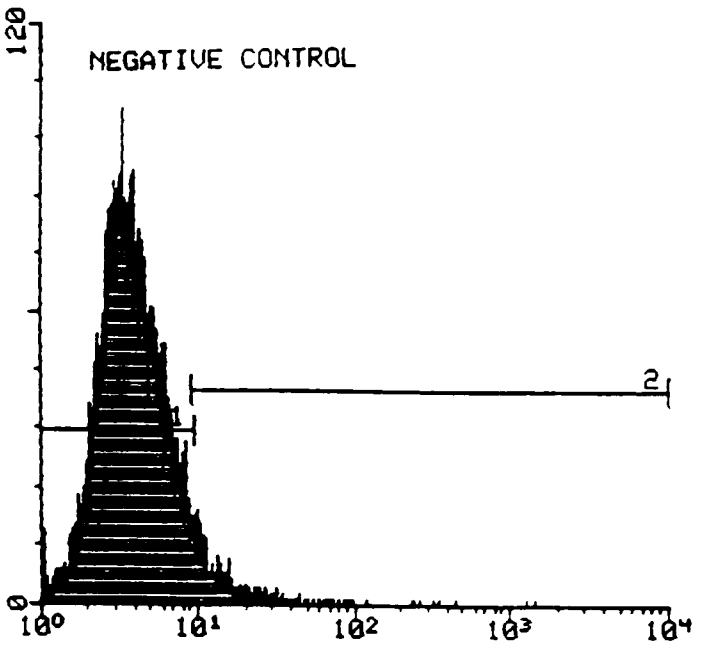


Figure 6.3

Generation and cloning of BV1S1A1N1-CD3ζ chimera

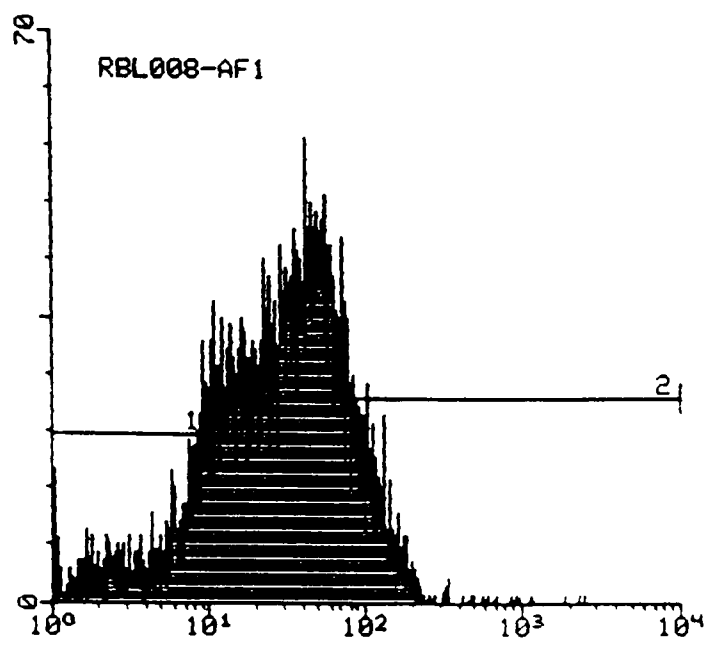
Construct RV001, which consists of a BV1S1A2-Cβ fragment truncated at the interchain disulphide bond cloned in pBluescript KS- (see Fig 3.3), was mutagensised with oligonucleotide BV1S1A1N1 to give a BV1S1A1N1-Cβ fragment (construct RV005) which was then subcloned into BJ043 to create a BV1S1A1N1-CD3ζ chimera (Construct RV006).



Parameter	FL2-H	FL2-Height		Ungated
M	Left,Right	Events	%	Peak
0	1.00, 9910	10000	100.00	102
1	1.00, 9.73	9434	94.34	102
2	9.30, 9910	650	6.50	19

PkChl	Mean	Median	SD	CV
3.39	5.26	3.82	21.30	>100.0
3.39	4.11	3.74	1.77	43.08
9.82	22.54	12.28	81.43	>100.0

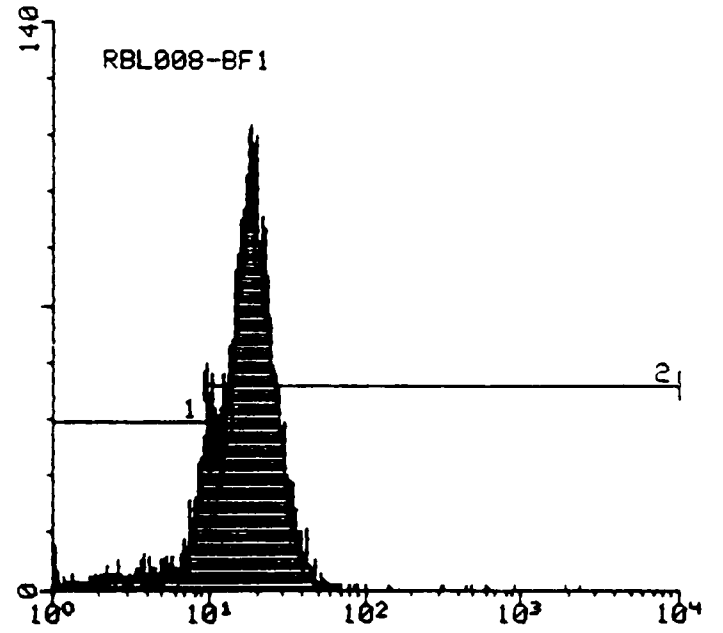
(a) Untransfected RBL cells.



Parameter	FL2-H	FL2-Height		Ungated
M	Left,Right	Events	%	Peak
0	1.00, 9910	10000	100.00	57
1	1.00, 9.73	1452	14.51	32
2	9.30, 9910	8662	86.61	57

PkChl	Mean	Median	SD	CV
41.41	42.59	31.79	57.45	>100.0
9.13	5.84	6.26	2.70	46.35
41.41	48.32	37.23	59.70	>100.0

(b) AV2S1A2 expression by RBL-008, primary mAb αF1.



Parameter	FL2-H	FL2-Height		Ungated
M	Left,Right	Events	%	Peak
0	1.00, 9910	10000	100.00	96
1	1.00, 9.73	946	9.46	33
2	9.30, 9910	9219	92.19	96

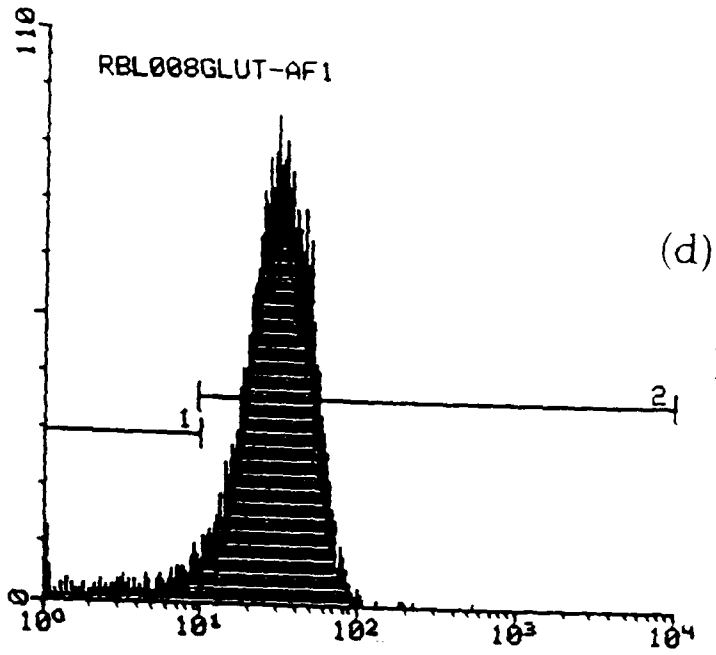
PkChl	Mean	Median	SD	CV
14.33	20.61	17.36	53.29	>100.0
9.64	7.09	7.80	2.33	32.84
14.33	21.80	18.16	55.33	>100.0

(c) BV1S1A2 expression by RBL-008, primary mAb βF1.

Figure 6.4abcd&e

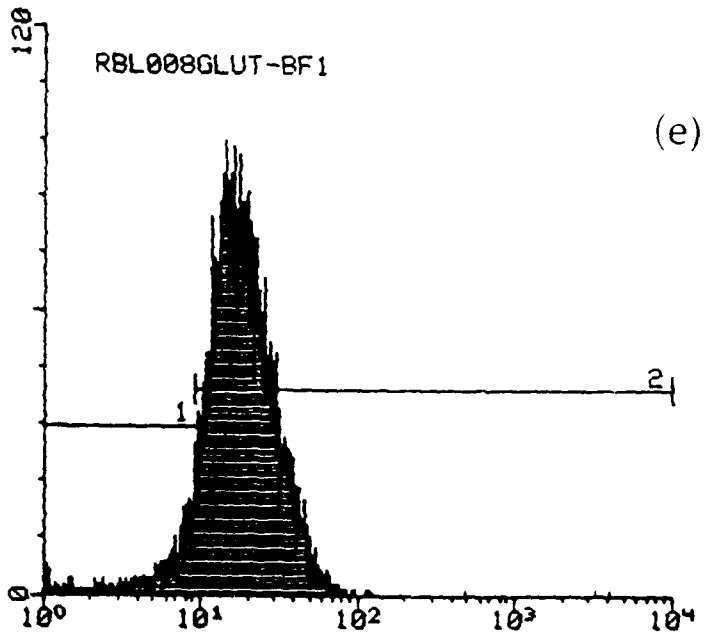
Expression of TCR-CD3ζ chimeric molecules by RBL-008glut

$2x10^5$ cells were stained with the primary monoclonal antibody followed by a PE-conjugated anti-mouse secondary antibody (DAKO). The negative control was stained with all mAbs.



(d) AV2S1A2 expression by RBL-008glut, primary mAb αF1

Parameter FL2-H FL2-Height M	Left,Right	Events	%	Ungated Peak	PkChl	Mean	Median	SD	CV
0	1.00, 9910	10000	100.00	94	30.23	32.16	30.02	16.70	51.94
1	1.00, 9.73	545	5.45	14	1.07	5.32	5.44	2.83	53.19
2	9.30, 9910	9492	94.92	94	30.23	33.61	31.31	15.88	47.23



(e) BV1S1A1N1 expression by RBL-008glut primary mAb βF1

Parameter FL2-H FL2-Height M	Left,Right	Events	%	Ungated Peak	PkChl	Mean	Median	SD	CV
0	1.00, 9910	10000	100.00	115	18.26	18.85	17.75	33.89	>100.0
1	1.00, 9.73	1399	13.99	56	9.56	6.70	7.98	2.78	41.52
2	9.30, 9910	8869	88.68	115	18.26	20.48	18.47	35.64	>100.0

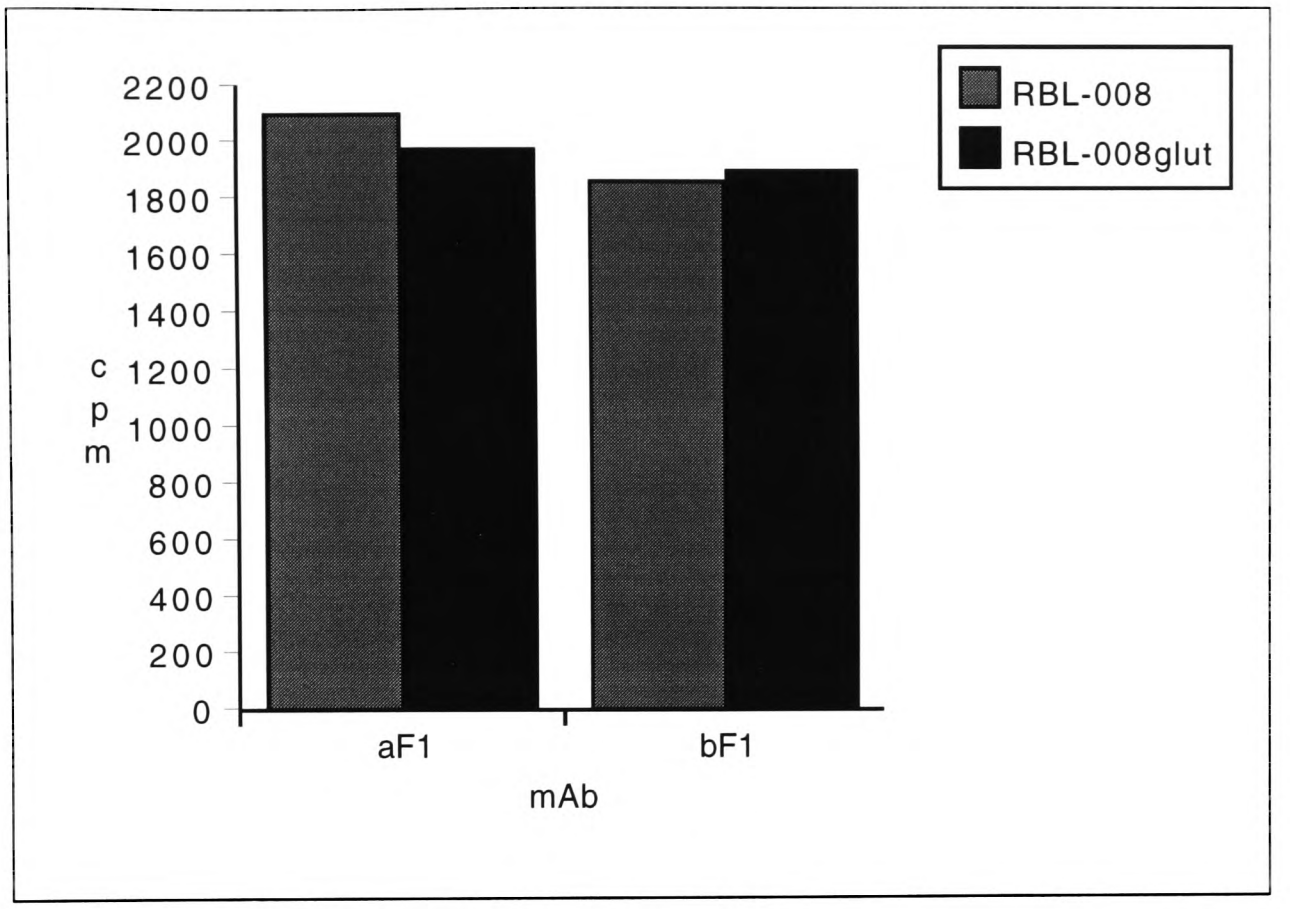


Figure 6.5
Degranulation of RBL-008glut provoked by mAbs to the alpha and beta chain constant regions

Transfectants were labelled as described and stimulated to degranulate by either the anti TCR-Cβ mAb βF1 or the anti-TCR-Cα mAb αF1. The cellular response was measured by degranulation which is represented as counts per minute (cpm) on the vertical axis, background release was approximately 100cpm and this was subtracted from the results. The results are means of triplicates.

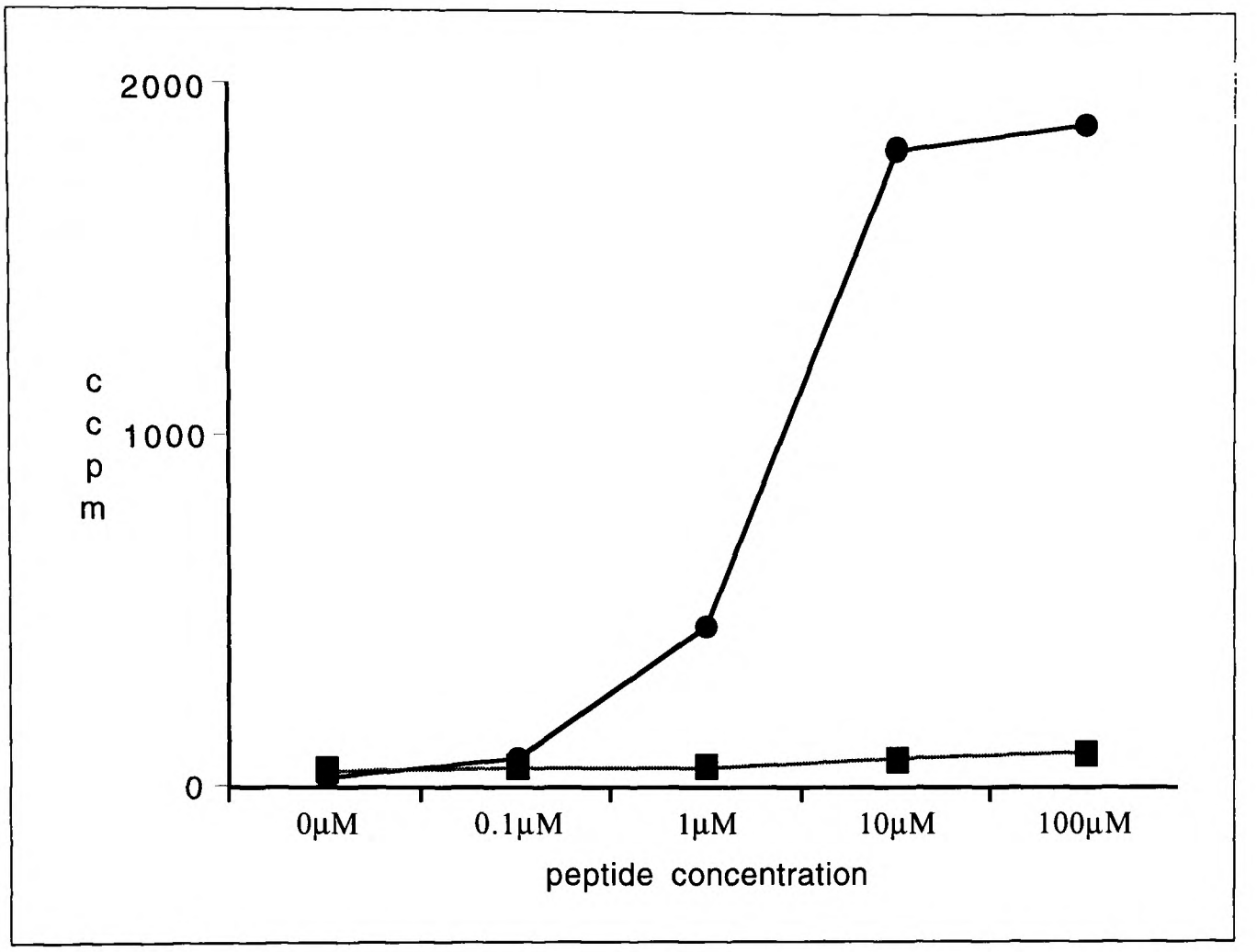


Figure 6.6
Response of RBL-008glut/RBL-008 to pol peptide pulsed HLA-A2 C1R target cells
Degranulation assays were performed in triplicate and are representative of at least four independent experiments. A background media release of 100-200 cpm was consistently observed and subtracted from the experimental data.

RBL-008

RBL-008glut

Allele	CDR1		CDR2		
			48		
AV2S1A1			F TTC		
AV2S1A2			S TCC		
	28	32 (FRB)	54	88 (FRB)	
ADV6S1A1N1	P CCA	L CTA	Q CAG	Y TAC	
AV6S1A2N1	Q CAA	L CTC	E GAG	Y TAC	
AV6S1A2N2	Q CAA	L CTA	E GAG	Y TAT	
			50		
AV7S1A1			A GCT		
AV7S1A2			G GGT		
		42 (FRO)			
AV8S1A1		R AGA			
AV8S1A2		G GGA			
				82 (loop)	
AV10S1A1				P CCT	
AV10S1A2				T ACT	
				64 (FRB)	
AV21S1A1N1				V GTT	
ADV21S1A1N2				V GTC	
			46		
BV1S1A1N1			Q CAG		
BV1S1A2			H CAC		
	8 (FRO)	39 (FRO)			
BV2S1A1	W TGG	Q CAG			
BV2S1A2	R AGG	Q CAG			
BV2S1A3N1	R AGG	K AAG			
			46	79 (loop)	
BV2S2A1O			A GCT	C TGT	
BV2S2A2O			T ACT	S AGT	
	16 (loop)		60	82 (loop)	90 (FRB)
BV6S1A1N1	Y TAT		N AAC	R CGG	C TGT
BV61A2P	D GAT		K AAA	Q CAG	R CGT
	5 (loop)				
BV6S4A1	N AAC				
BV6S4A2	D GAC				
		36 (FRO)			70 (HV4)
BV6S5A1N1		S AGC			G GGA
BV6S5A2		R AGG			E GAA
	22 (FRO)	29	43 (FRI)		
BV12S1A1N1	H CAT	Y TAT	L CTG		
BV12S1A1N2	H CAC	Y TAT	L CTG		
BV12S1A1N3	H CAC	Y TAC	L CTA		
BV12S1A1N4	H CAC	Y TAC	L CTG		
			55		
BV16S1A1N1			E GAG		
BV16S1A1N2			E GAA		
		54 (FRB)		68 (FRO)	81 (loop)
BV20S1A1N1		R CGA		P CCC	L CTC
BV20S1A1N2		R CGA		P CCC	L CTT
BV20S1A2P		Z TGA		S TCC	L CTT
		34 (FRB)			
BV21S2A1N1		R CGG			
BV21S2A2		L CTG			

Table 6.1
Allelic variation in the TCRAV and TCRBV gene segments
The position and nature of the allelic variants described in the TCRAV and TCRBV gene segments to date. FRB=Buried framework regions, FRO=Outward facing framework regions, HV4=Hypervariable region 4. See text for discussion. (Adapted from Arden et al 1995)

WILD TYPE SEQUENCE								
I	L	K	E	P	V	H	G	V
I1S		K3Y	E4D	P5T			G8A	V9L
I1T		K3R	E4N	P5F			G8T	
			E4Q	P5G			G8E	
				P5M			G8F	
				P5Q			G8I	
				P5S			G8L	
				P5V			G8M	
				P5Y			G8N	
							G8P	
							G8Q	
							G8R	
							G8S	
							G8V	
							G8W	

Table 6.2
Pol peptide variants
This table gives a complete list of the pol peptide variants used in restoration experiments. Nomenclature shows the wild-type residue followed by the position of the amino acid change and then the new amino acid.

Chapter 7
Functional Aspects of the Structural Relationship between the V and C Domains of the TCR

7.1 Introduction

7.11 The Immunoglobulin Superfamily

The TCR is a member of the Immunoglobulin superfamily (Ig-SF) of molecules which includes a wide variety of other immunologically important proteins such as MHC class I and II molecules, the chains of the CD3 complex and the CD4 and CD8 coreceptors (reviewed in Williams & Barclay 1988). The principal criterion for inclusion in the Ig-SF is the conservation of sequences which predict the presence of Ig-related domains. These domains have key structural features which result in the formation of a characteristic tertiary structural unit termed the Ig-fold. The basic structure of the Ig fold comprises two β sheets consisting of anti-parallel β-strands of about 5-10 amino acids. Between the sheets a hydrophobic interior is formed from inward-pointing hydrophobic amino acids, the interaction between the sheets is further stabilised by a conserved disulphide bond. Ig folds can be broadly subdivided into V-like or C-like groups depending on whether they resemble an Ig V-domain or C-domain. V-like domains have about 65-70 amino acids between the conserved disulphide bond and there are four β strands in each β sheet. In C-like domains the sequence between the disulphide bond is shorter at 55-60 residues giving rise to β sheets with 4 and 3 β strands respectively (Fig 7.1).

7.12 Structural Variations Between the TCR and Other Ig-SF Members

Following the cloning of the TCR genes (Hedrick at al 1984a&b, Chien et al 1984) sequence analyses predicted that the TCR heterodimer would resemble an Ig Fab fragment with each chain consisting of one V-like Ig domain and one C-like Ig domain (Bjorkman & Davis 1988, Davis & Bjorkman 1989, Chothia et al 1988). The recent resolution of the crystal structures of both the murine and human TCRs has confirmed that the TCR does broadly resemble an Ig Fab fragment although there are differences both in the structure of Cα and also in the size of the contact area between the V and C domains (Garcia et al 1996a, Garbozci et al 1996).

The structure of the Cα domain shows that one β sheet does consist of the predicted four β strands (see Fig 7.1) however the other β sheet is much more loosely constructed than is the case in a conventional C-like Ig domain (Garcia st al 1996a). This loose arrangement means that there are significant gaps between the β strands which expose the hydrophobic core of the domain and it has been suggested that this may account for the reported susceptibility of the TCR α chain to degradation in the endoplasmic reticulum (Kearse et al 1994). Currently there is no functional explanation for the unusual structure of Cα although there is some mutational data to suggest that this is the binding site for CD3ζ (Geisler et al 1994).

This chapter describes experiments which examine the structural relationship between the TCR V and C domains. These studies were originally commenced as a means of

directing the efforts of others in our group in the design of appropriate constructs for the expression of soluble TCR molecules in mammalian transfection systems. In particular there was interest in designing a TCR VαVβ fragment which would be structurally homologous to functional Ab Fv fragments. However before investing considerable effort into this project it was thought prudent to establish whether the TCR V-domains were able to fold independently of the C-domains when expressed in mammalian cells. One previous study had addressed this question by attempting to express a TCR consisting of VαCβ and VβCα chimeric chains in a T cell hybridoma (Casorati et al 1993). Failure to express this chimeric molecule on the cell surface was interpreted as demonstrating that V and C domain folding is interdependent. However there is an alternative explanation since the domain swapping might actually result in failure to assemble the TCR rather than failure to fold the V and C domains. TCR assembly is a highly ordered process in which the TCR α and β chains are added to the components of the CD3 complex, transfer to the cell suface is eventually driven by the addition of the ζ chains (reviewed in Exley et al 1991). Failure to assemble the TCR in this way results in degradation in the ER and clearly this could explain the results obtained by Casorati and colleagues (Casorati et al 1993). In the RBL system cell surface expression of TCR-CD3ζ fusions is not dependent on a complex assembly process. Therefore the effect of mutations which disrupt the interaction between the V and C domains can be more accurately tested by assessing transfectants for intracellular and extracellular expression of the various constructs.

7.2 Results

7.21 Design and Generation of Constructs

As described in Chapter 3 the first available TCR for expression as a CD3ζ chimera was the HLA-A2 restricted flu-matrix peptide specific JM22 receptor which utilises the V segment genes BV17S1 and AV10S1A1. Unfortunately the transfectant RBL-38 which expresses this chimeric receptor did not respond reliably in degranulation assays despite clear cell surface expression of both the TCRα and TCRβ CD3ζ chains (see Chapter 3, Fig 3.4). Despite the unreliability of the bioassay it was decided to observe the effect on cell surface expression of deleting the entire Cα and Cβ-domains and fusing the Vα and Vβ domains directly to the transmembrane and intracellular portions of CD3ζ. These constructs were created by mutagenesis of the JM22 TCR genes (Fig 7.2a) which had been cloned into pBluescriptKS- as fragments truncated at the C domain interchain disulphide bond (template plasmids were termed BJ008 and BJ011 for the α and β chain respectively, see Chapter 3, Fig 3.3. Oligonucleotides were AV10S2DELC, BV17S1DELC for α and β chain respectively, see Chapter 2 for sequences).

The subsequent cloning of the 008A3 TCR (see Chapter 3) and its expression to give the transfectant RBL-008 meant that there was a reliable bioassay which could be used to test the effect of mutating the V/C domain interface and therefore two further constructs were designed. In the first the Vα and Vβ domains were paired with the Cβ and Cα domains respectively ina reiteration of the approach of Casorati and colleagues (Casorati et al 1993). As discussed above it was hoped that the

expression of these constant domain swap (CDS) constructs as CD3ζ chimeras would circumvent potential problems with TCR assembly. The CDS constructs were generated by amplifying the V domain of each chain and then cloning this into a plasmid encoding the C-domain of the reciprocal chain (Fig 7.2b. Primer pairs for amplifying the V-domains were AV2S1A2F/AV2S1A2CB and BV1S1F/BV1S1CA for the α and β chains respectively. The sequences are given in Chapter 2). The second pair of constructs generated also sought to separate the TCR V and C-domains. however in this case a single glycine residue was placed between the two domains on each chain. Glycine was chosen because the lack of a side chain means that it is likely to be minimally disruptive and it may also impart some flexibility to the junction between the V and C domains. Site directed mutagenesis was used to add a codon for glycine between the Vα and Cα and Vβ and Cβ domains of the A3-TCR (Fig 7.2c. Oligonucleotides were AV2S1A2GLY and BV1S1GLY for the α and β chains respectively. Sequences are given in Chapter 2). All the constructs described in this section were checked either by standard DNA sequencing techniques or by automated sequencing (performed by J. Wyer) before being used to stably transfect RBL cells as described in chapter 2. Transfectants expressing the C-domain deletion, the C-domain swap and the glycine linker were designated RBL-CDEL, RBL-CDS and RBL-GLY respectively.

7.22 The Effect of Disrupting the TCR V-C Domain Interface on Expression and Function of TCR-CD3ζ Chimeric Molecules.

Transfectants RBL-CDEL, RBL-CDS and RBL-GLY were analysed for cell surface expression of TCR-CD3ζ chimeric receptor molecules by FACS analysis using a panel of mAbs (Fig 7.3abcd&e). RBL-CDEL showed no cell surface expression of the BV17S1-CD3ζ construct when tested with an anti- BV17S1 mAb (Fig 7.3c). In addition there was no response in repeated degranulation assays (data not shown) confirming that no functional TCR molecules were expressed on the cell surface. A wider panel of mAbs was available for testing transfectants RBL-CDS and RBL-GLY including the commercially available mAbs βF1 and αF1 which recognise the Cα and Cβ respectively and also mAbs which were generated in conjunction with Immunotech and are directed towards the AV2S1 and BV1S1 gene products (see Chapter 3, Fig 3.8 for details of these mAbs). In all instances for both transfectants no cell surface expression was detected by FACS analysis (fig 7.3d&e). Furthermore neither transfectant could be stimulated to degranulate in response to either crosslinking antibodies or pol peptide pulsed C1R cells transfected with HLA-A2 (data not shown).

In view of the failure of all these constructs to express on the RBL cell surface it was clearly important to analyse intracellular expression. In the case of RBL-CDEL a rabbit polyclonal serum to a peptide epitope in the CD3ζ chain was used to detect the presence of TCR-CD3ζ chimeric proteins in western blots of cell lysates (Fig 7.4a). The results show that two bands representing the TCRVα-CD3ζ and TCRVβ-CD3ζ

fusions were identified. Comparison with a western blot of RBL-38 lysate shows that the bands identified from RBL-CDEL are of the expected size for proteins lacking the TCR C-domains. In the case of transfectants RBL-CDS and RBL-GLY western blots of cell lysates were carried out using the anti-Cα and anti-Cβ mAbs αF1 and βF1 respectively. Both cell lines showed intracellular expression of Cα and Cβ containing proteins (Fig 7.4b&c).

7.3 Discussion

The failure of the TCR VαCD3ζ and VβCD3ζ chimeras to reach the surface of the RBL-CDEL cell line suggests that the interaction between the TCR V and C domains plays an essential part in the expression of functionally competent TCR fragments in *mammalian* cells. This conclusion is supported by an earlier demonstration that TCRV-IgCκ chimeras can only be expressed intracellullarly in myeloma cells, a finding that also indicates that the TCR C-domains are not interchangeable with other Ig C-like domains (Traunecker et al 1989). The specific nature of the requirement for the TCR C domains is illustrated further by the failure of the TCR VαCβCD3ζ and VβCαCD3ζ proteins to be expressed on the surface of RBL-CDS which demonstrates that the TCR Vα and Vβ domains must associate with their respective C-domains. The result obtained with RBL-CDS also confirms that the earlier report of Casorati and colleagues showing that Cα and Cβ were not interchangeable. was not due to solely to a failure of TCR-CD3 complex assembly (Casorati et al 1993).

The requirement to correctly pair Vα with Cα and Vβ with Cβ for expression of the TCR in mammalian cells is in contrast to

the situation with regard to Igs where there does not appear to be such interdependence between the V and C domains. Comparison of the 2C TCR crystal structure (Garcia et al 1996a) with that of an Ig offers a structural, but not functional, explanation for these differences. In the TCRβ chain there is an extensive area of contact between Vβ and Cβ which is about twice that of a V_H-C_{H1} in an Fab with a similar elbow angle between the V and C domains. Not only is this area larger but there are also 8 interdomain hydrogen bonds between conserved residues indicating that the association of these two domains is very much tighter than in an Ig molecule. The interface between Vα and Cα is less extensive than that between Vβ and Cβ with only a solitary hydrogen bond. Nonetheless it is significantly larger than the contact surface between a corresponding V_L-C_L pair. The close relationship between the V and C domains of the TCR probably explains why deletion of the C region has such a marked effect on expression of the TCRVα- and Vβ-CD3ζ constructs since this will result in the exposure of large hydrophobic surfaces, especially in the β chain. The requirement for a specific association between Vα and Vβ and their respective C domains is likely to reflect the marked structural differences between Cα and Cβ (see section 7.1, Garcia et al 1996a).

The importance of the proximity of the V and C domains for TCR expression was examined further by introducing a glycine residue between the V and C domains of each chain of the A3-TCR-CD3ζ chimera. This change was designed to separate the V and C domains with minimal disruption to the overall structure of the receptor. However even this slight distortion of the V-C

interface prevented cell surface expression emphasising the close structural relationship between the V and C domains.

In contrast to the observations reported here two pieces of data have emerged to suggest that the Vα-Cα association may in fact be less critical than that between Vβ and Cβ. Firstly a functional three domain TCR-CD3ζ chimera lacking Cα has been expressed in RBL cells (Chung et al 1994) and secondly the crystal structure of an isolated Vα domain expressed in *E. coli* has been solved (Fields et al 1995). This latter result suggests that there may be a difference between mammalian and bacterial expression systems with respect to the requirement for the TCR constant domains. Indeed, several studies have reported expression soluble TCRVαVβ fragments in *E. coli* (Novotny et al 1991, Ward 1992, Hilyard et al 1994, Strong et al 1994, Reiter et al 1995). However only two of these have been shown to have any ligand binding activity (Hilyard et al 1994, Reiter et al 1995) and in neither case has the TCR fragment been of value in determining the kinetics of the TCR/MHC-peptide interaction, which casts some doubt over the functional integrity of the soluble molecule. Furthermore, despite the publication of the structure of the Vα domain (Fields et al 1995), there have been no reports of VαVβ structures and both the TCR heterodimer (Garbozci et al 1996, Garcia et al 1996a) and the isolated β chain structures (Bentley et al 1995) have included the respective C domains. Therefore, regardless of the production of Vα-Vβ fragments in *E. coli* the balance of evidence would suggest the TCR C domains are required for the expression of a functional TCR molecules. Although there is now a structural explanation for this

observation the functional reasons for the evolution of this close relationship between V and C domains remain obscure. Possibilities include a role in TCR aggregation or perhaps the formation of contacts with co-receptor molecules or the components of CD3, a possibility that may be confirmed with the eventual resolution of complex multicomponent crystal structures.

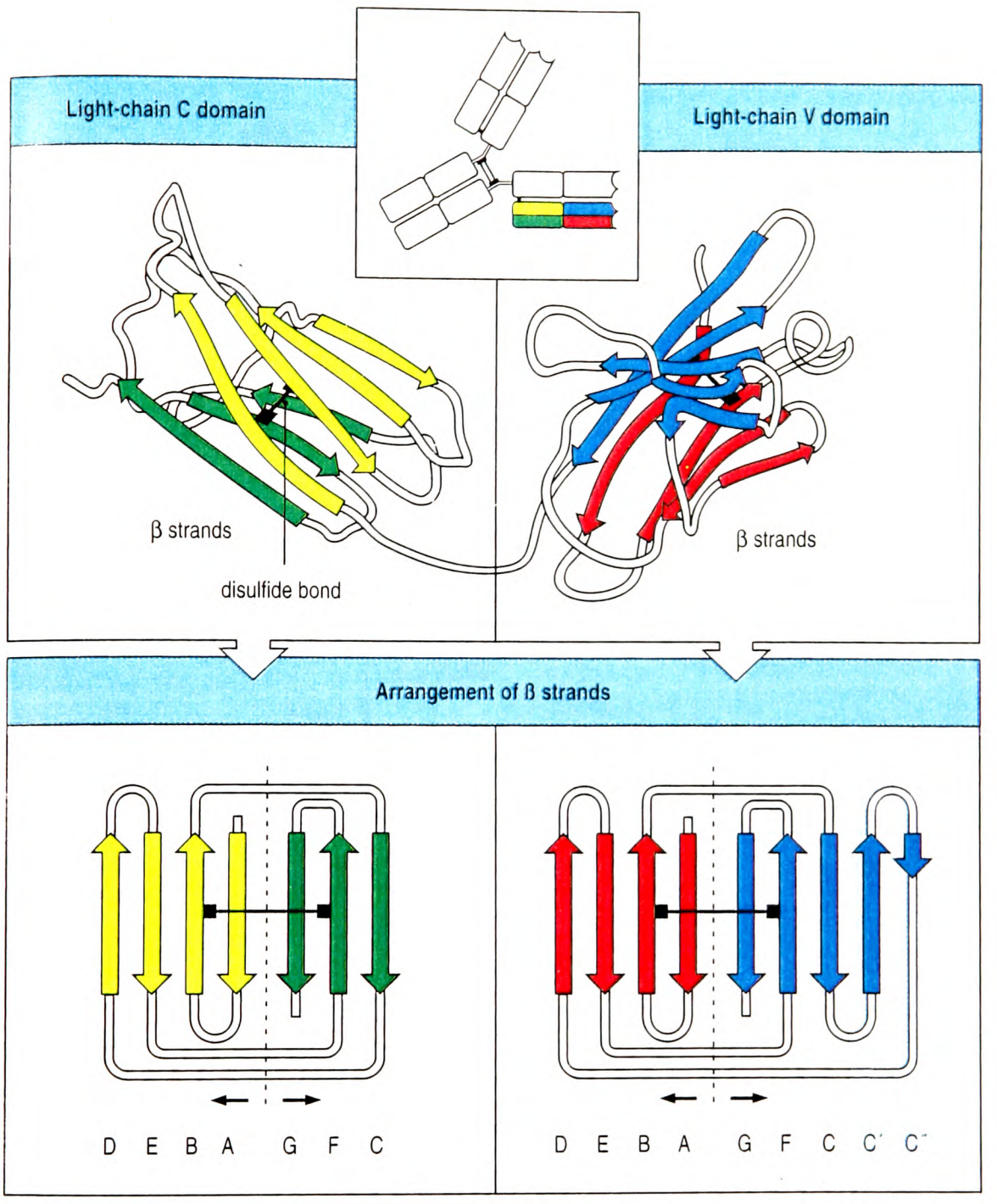


Figure 7.1.
The Immunoglobulin Fold.
The folding patterns of V-like and C-like immunoglobulin folds. Note that in each case the two β sheets are comprised of β strands of the same colour. The β sheets in the V-like domainare comprised of four β strands while in the C-like domain they are composed of 3 and 4 β strands respectively. The conserved disulphide bond is shown in black. (Adapted from Janeway & Travers).

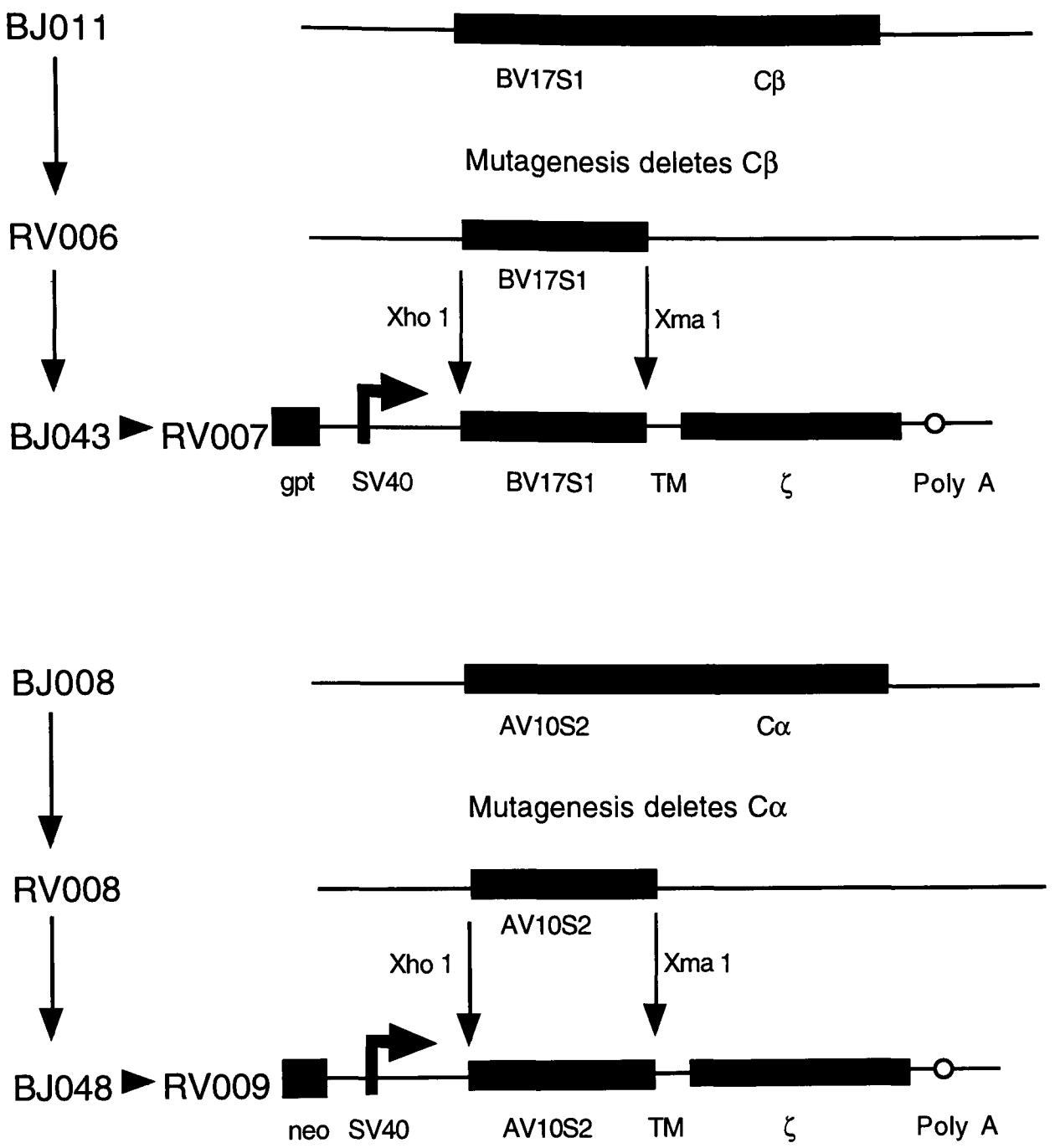


Fig 7.2ab&c
Strategies for generating V/C domain mutants

(a) JM 22 CDEL beta and alpha constructs. The Cβ domain was deleted from BJ011 (see Fig 3.3) by mutagenesis creating construct RV006. The BV17S1 V domain fragment was then cloned into the expression vector BJ043 (see Fig 3.2) to give construct RV007 encoding a BV17S1-CD3ζ fusion. The Cα domain was deleted from BJ008 (see Fig 3.3) by mutagenesis creating construct RV008. The AV10S2 V domain fragment was then cloned into the expression vector BJ048 (see Fig 3.2) to give construct RV009 encoding an AV10S2-CD3ζ fusion.

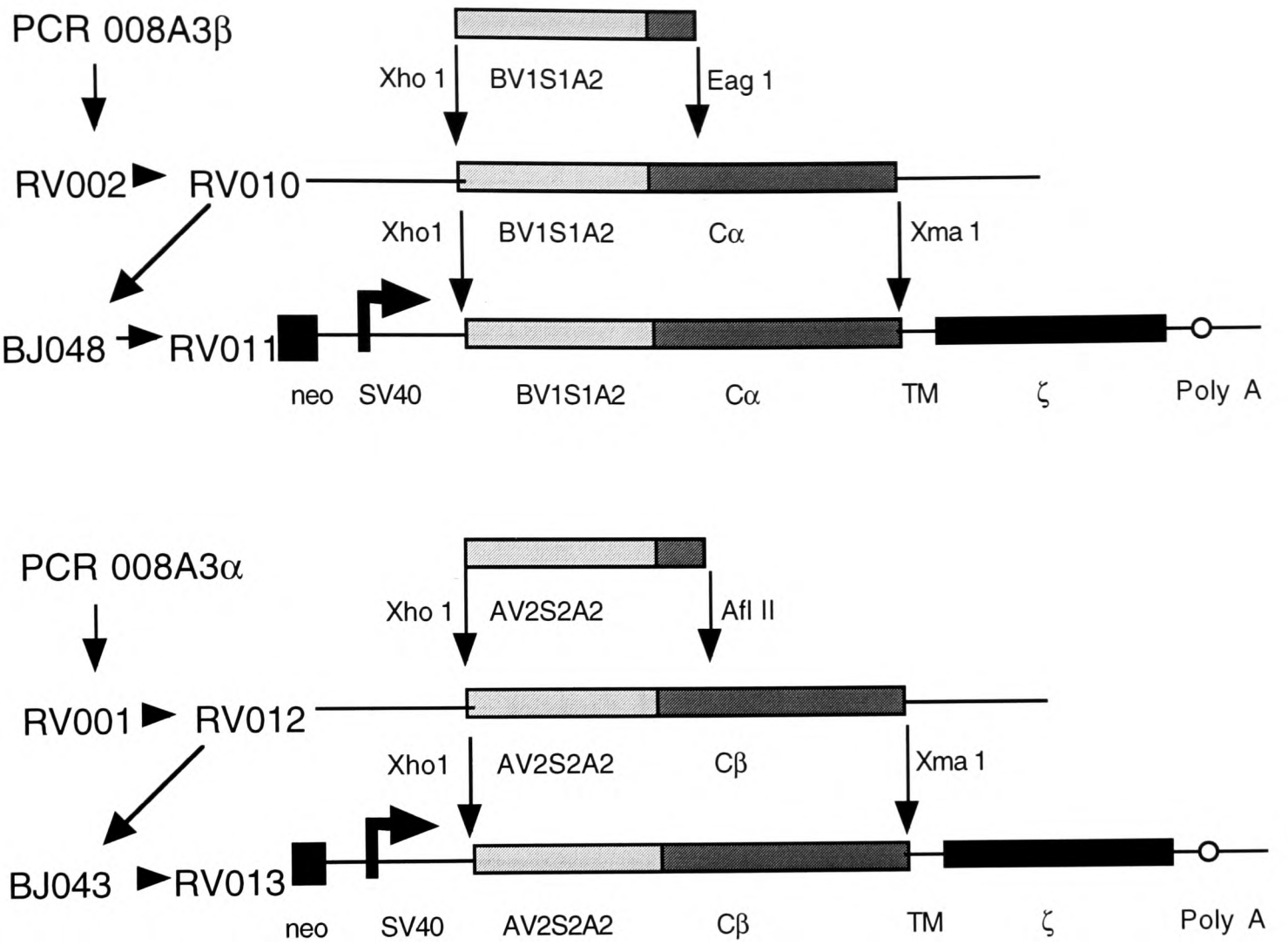


Fig 7.2ab&c

Strategies for generating V/C domain mutants

(b) A3-CDS beta and alpha constructs. A PCR fragment encoding the BV1S1A2 V domain and a small part of the Cα domain was generated by PCR. This was then cloned into vector RV002 (see Fig 3.3) to give construct RV010 encoding a BV1S1A2-Cα fusion. This V-C fragment was then subcloned into expression vector BJ048 (see Fig 3.2) to give construct RV011 encoding a BV1S1A2-Cα-CD3ζ fusion. A PCR fragment encoding the AV2S2A2 V domain and a small part of the Cβ domain was generated by PCR. This was then cloned into vector RV001 (see Fig 3.3) to give construct RV012 encoding anAV2S2A2-Cβ fusion. This V-C fragment was then subcloned into expression vector BJ043 (see Fig 3.2) to give construct RV013 encoding an AV2S2A2-Cβ-CD3ζ fusion.

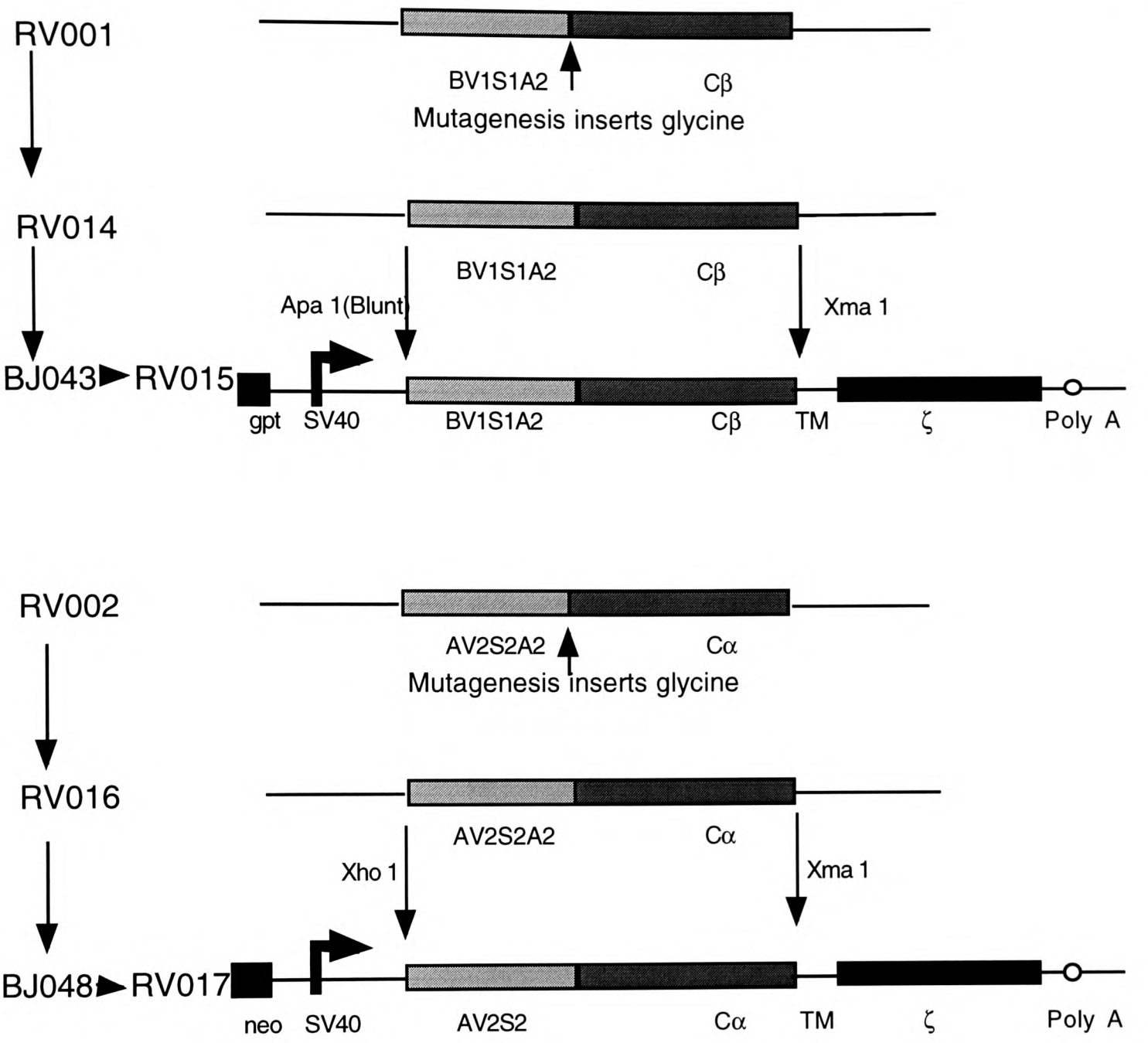
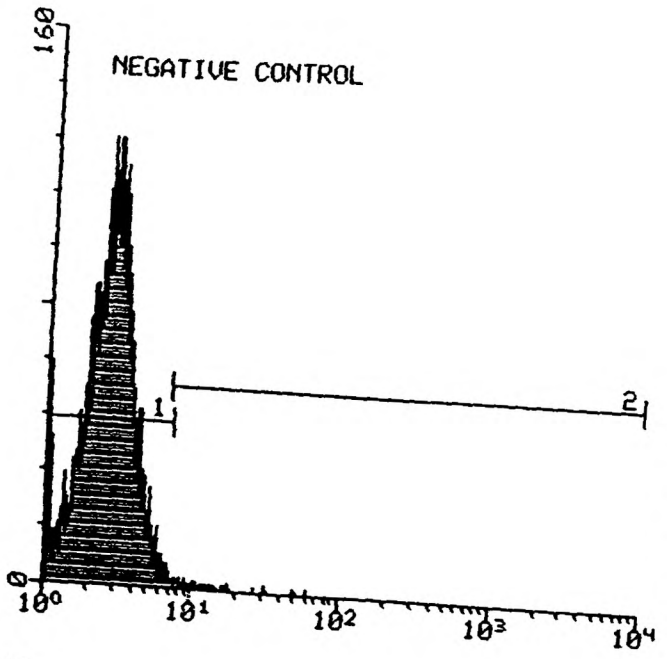


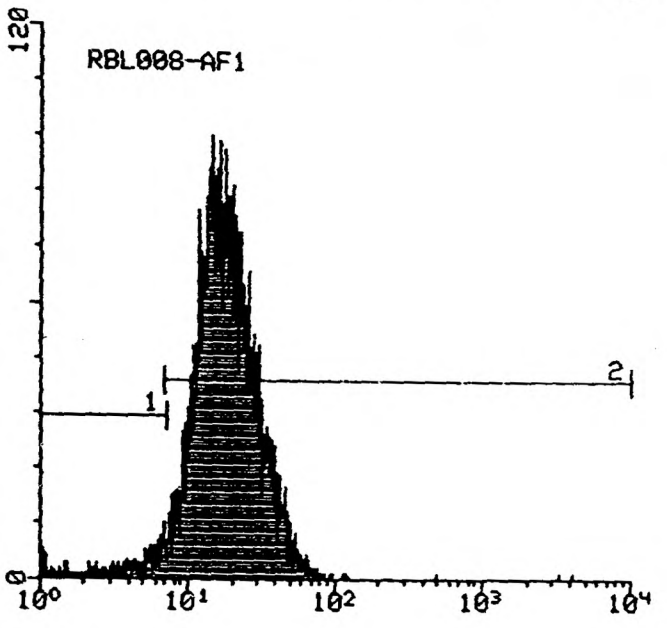
Fig 7.2ab&c
Strategies for generating V/C domain mutants
(c) A3-GLY beta and alpha constructs. Construct RV001 (see Fig 3.3) was mutagenised so that a glycine residue was inserted between the V and C domains (construct RV014). This V-gly-C fragment was then cloned into the expression vector BJ043 (see Fig 3.2) to create vector RV015 encoding a beta V-gly-C-CD3ζ fusion. Construct RV002 (see Fig 3.3) was mutagenised so that a glycine residue was inserted between the V and C domains (construct RV016). This V-gly-C fragment was then cloned into the expression vector BJ048 (see Fig 3.2) to create vector RV017 encoding an alpha V-gly-C-CD3ζ fusion.



Parameter FL2-H	FL2-Height		Ungated
M Left,Right	Events	%	Peak
0 1.00, 9910	10000	100.00	129
1 1.00, 7.23	9937	99.37	129
2 6.91, 9910	81	0.81	4

PkChl	Mean	Median	SD	CV
2.45	2.84	2.64	1.48	52.02
2.45	2.78	2.65	1.00	36.24
7.10	11.60	8.53	8.58	73.99

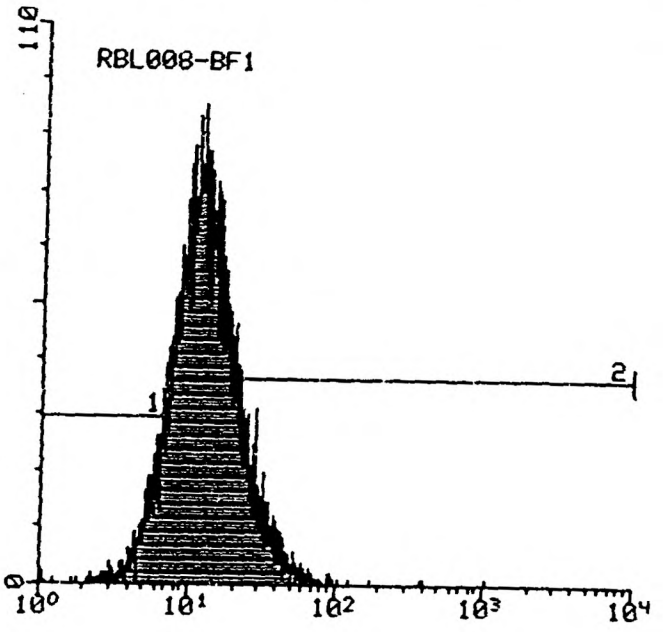
(a) Untransfected RBLs stained with aF1 and bF1, a similar result was obtained with mAbs RA8.4 (anti AV2S2) and R13.1 (anti BV1S1).



Parameter FL2-H	FL2-Height		Ungated
M Left,Right	Events	%	Peak
0 1.00, 9910	10000	100.00	96
1 1.00, 7.23	390	3.89	12
2 6.91, 9910	9655	96.54	96

PkChl	Mean	Median	SD	CV
14.33	20.61	17.36	53.29	>100.0
6.91	4.80	5.36	1.86	38.84
14.33	21.19	17.93	54.14	>100.0

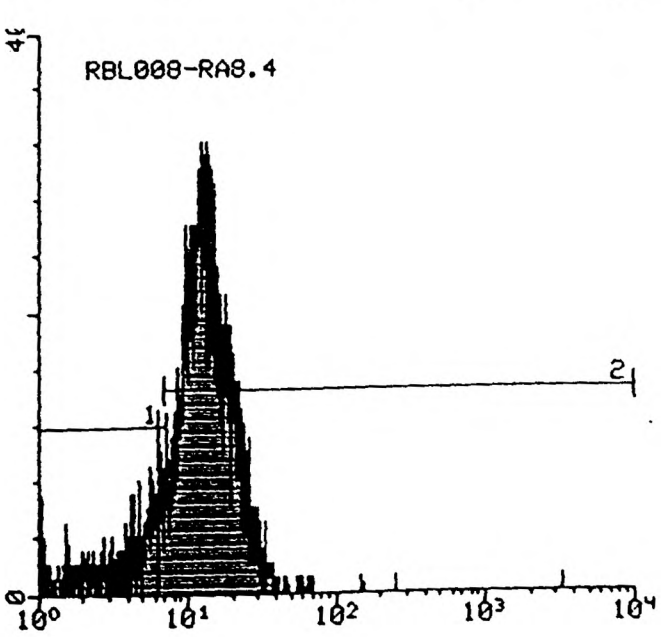
(b i) RBL-008 stained with αF1



Parameter FL2-H	FL2-Height		Ungated
M Left,Right	Events	%	Peak
0 1.00, 9910	10000	100.00	94
1 1.00, 7.23	1193	11.92	46
2 6.91, 9910	9009	90.09	94

PkChl	Mean	Median	SD	CV
11.86	14.78	12.19	14.51	98.14
7.16	5.79	6.09	1.13	19.59
11.86	15.80	13.21	14.93	94.51

(b ii) RBL-008 stained with βF1



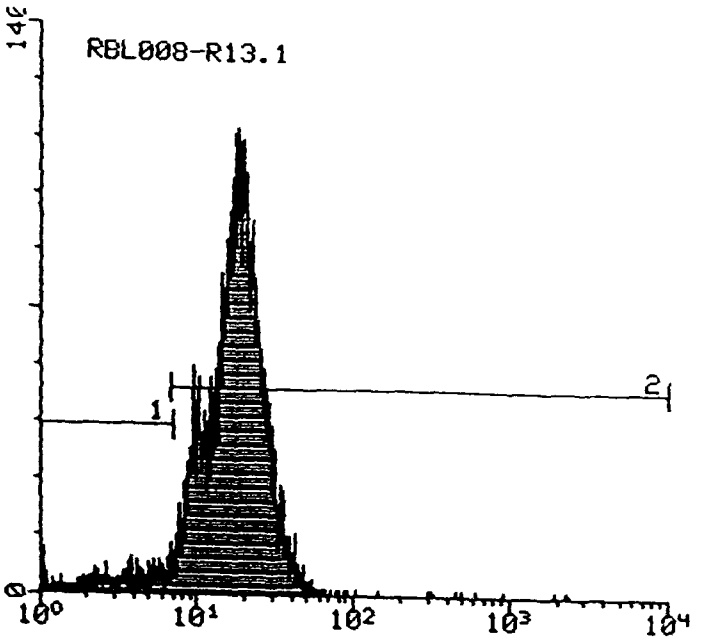
Parameter FL2-H	FL2-Height		Ungated
M Left,Right	Events	%	Peak
0 1.00, 9910	2990	100.00	32
1 1.00, 7.23	502	16.78	13
2 6.91, 9910	2528	84.54	32

PkChl	Mean	Median	SD	CV
12.18	14.49	12.48	60.84	>100.0
6.37	4.54	4.96	1.88	41.49
12.18	16.35	13.50	66.00	>100.0

(b iii) RBL-008 stained with RA8.4

Fig 7.3 abcd&e
Cell surface expression of V/C domain mutants

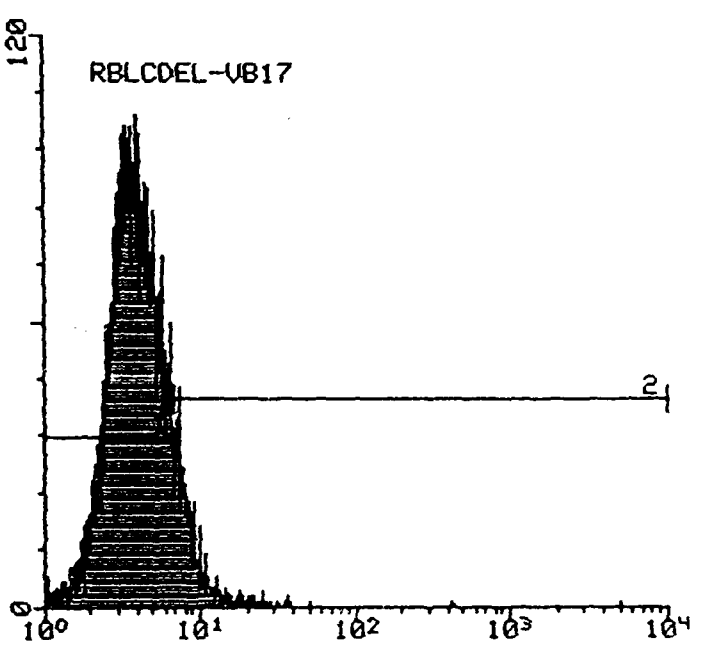
2x10^5 cells were stained with mAbs according to methods described in Chapter 2.



M	Parameter FL2-H Left,Right	FL2-Height Events	%	Ungated Peak
0	1.00, 9910	10000	100.00	115
1	1.00, 7.23	602	6.01	12
2	6.91, 9910	9438	94.37	115

PkChl	Mean	Median	SD	CV
18.26	18.85	17.75	33.89	>100.0
6.79	3.89	3.65	1.87	48.18
18.26	19.75	17.95	34.67	>100.0

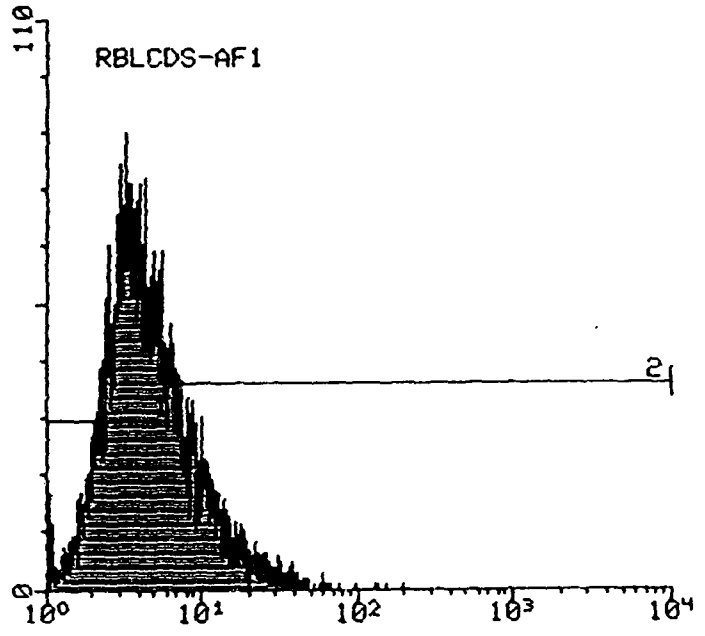
(b iv) RBL-008 stained with R13.1



M	Parameter FL2-H Left,Right	FL2-Height Events	%	Ungated Peak
0	1.00, 9910	10000	100.00	103
1	1.00, 7.23	9083	90.82	103
2	6.91, 9910	1126	11.25	46

PkChl	Mean	Median	SD	CV
4.03	5.04	4.02	50.21	>100.0
4.03	3.98	3.78	1.33	33.49
7.43	13.95	8.18	149.35	>100.0

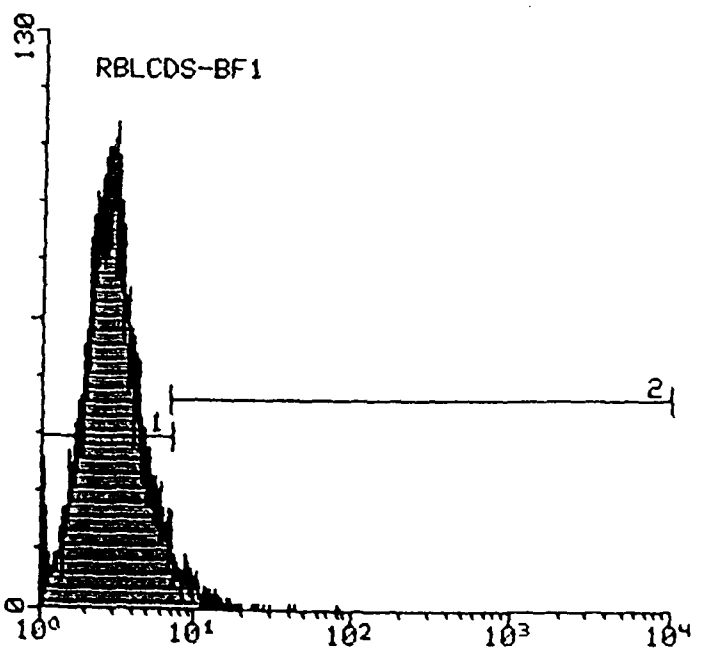
(c i) RBL-CDEL stained with antiBV17S1



M	Parameter FL2-H Left,Right	FL2-Height Events	%	Ungated Peak
0	1.00, 9910	10000	100.00	88
1	1.00, 7.23	7894	78.93	88
2	6.91, 9910	2319	23.18	41

PkChl	Mean	Median	SD	CV
3.30	5.84	4.18	6.08	>100.0
3.30	3.84	3.63	1.45	37.71
7.16	12.77	10.14	9.53	74.63

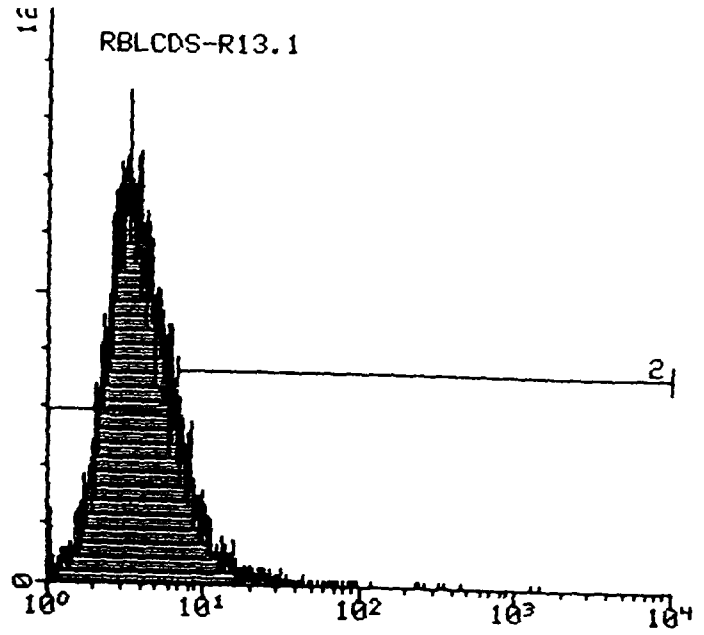
(d i) RBL-CDS stained with αF1



M	Parameter FL2-H Left,Right	FL2-Height Events	%	Ungated Peak
0	1.00, 9910	10000	100.00	109
1	1.00, 7.23	9638	96.37	109
2	6.91, 9910	451	4.50	22

PkChl	Mean	Median	SD	CV
3.02	3.33	2.87	2.09	62.97
3.02	3.06	2.82	1.24	40.56
6.91	9.69	8.59	5.03	51.98

(d ii) RBL-CDS stained with βF1



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M	Left,Right	Events	%	Peak
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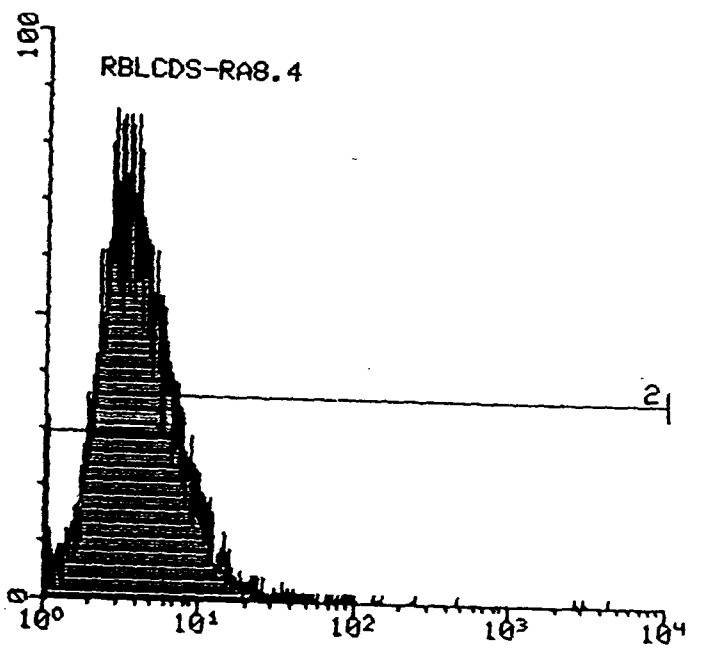
1	1.00, 7.23	8758	87.57	102
2	6.91, 9910	1424	14.24	35

PkChl	Mean	Median	SD	CV
3.39	5.26	3.82	21.30	>100.0
3.39	3.79	3.56	1.38	36.40
6.97	14.56	8.93	55.48	>100.0

(d iii) RBL-CDS stained with R13.1



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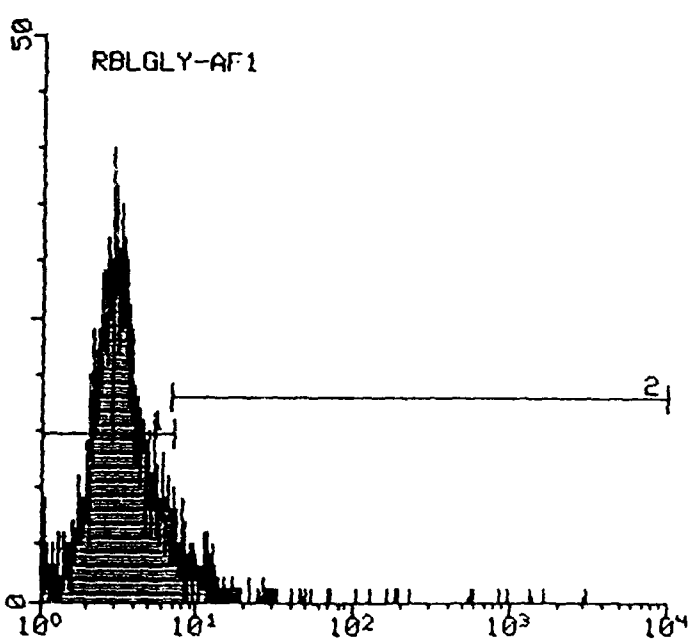
0	1.00, 9910	10000	100.00	86
1	1.00, 7.23	8491	84.90	86
2	6.91, 9910	1720	17.19	38

PkChl	Mean	Median	SD	CV
2.71	6.04	3.79	61.00	>100.0
2.71	3.68	3.45	1.45	39.39
6.97	17.83	9.24	146.52	>100.0

(d iv) RBL-CDS stained with RA8.4



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M	Left,Right	Events	%	Peak
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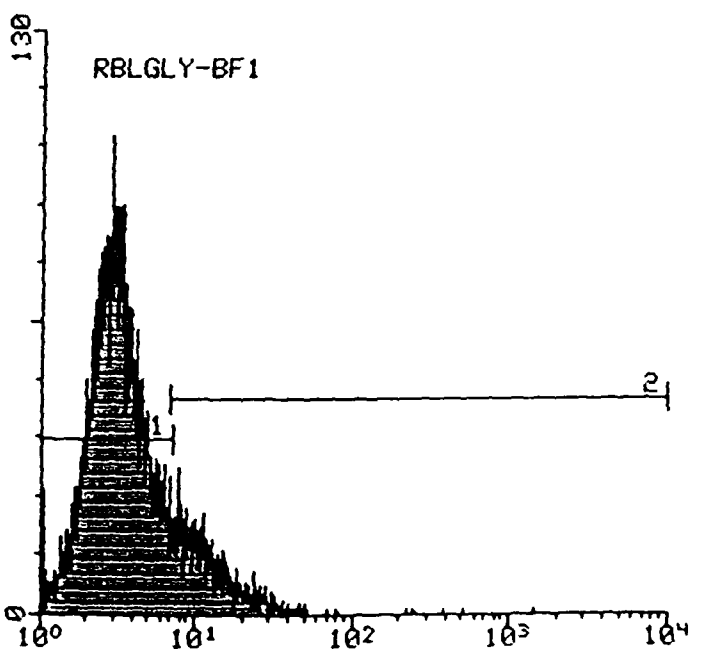
1	1.00, 7.23	2735	92.08	40
2	6.91, 9910	266	8.95	10

PkChl	Mean	Median	SD	CV
2.91	7.33	3.19	73.28	>100.0
2.91	3.33	3.09	1.27	38.33
7.04	48.45	9.61	241.42	>100.0

(e i) RBL-GLY stained with αF1



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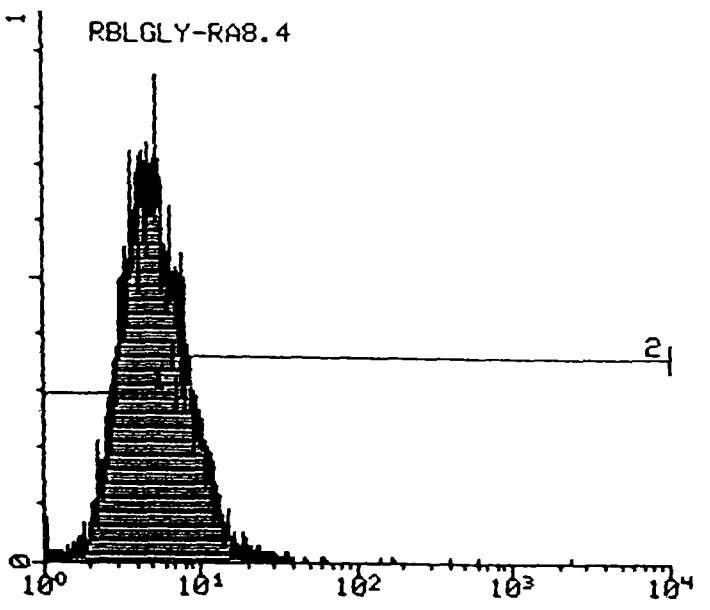
1	1.00, 7.23	8424	84.24	106
2	6.91, 9910	1690	16.90	32

PkChl	Mean	Median	SD	CV
2.91	5.15	3.36	16.46	>100.0
2.91	3.32	3.06	1.33	39.97
7.91	14.38	10.65	38.66	>100.0

(e ii) RBL-GLY stained with βF1



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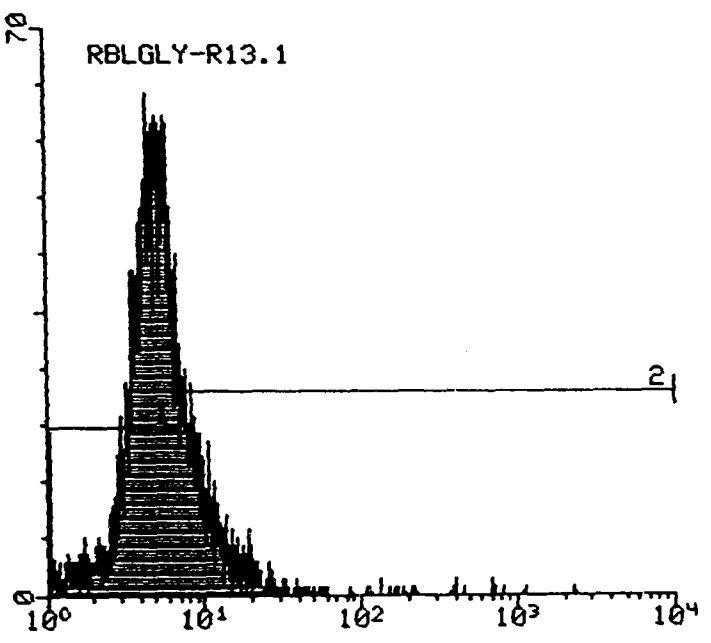
1 1.00, 7.23	7737	77.37	103
2 6.91, 9910	2603	26.02	65

PkChl	Mean	Median	SD	CV
5.37	6.19	5.05	25.21	>100.0
5.37	4.51	4.49	1.35	30.09
7.63	11.29	8.74	49.02	>100.0

(e iii) RBL-GLY stained with RA8.4



Parameter FL2-H	FL2-Height		Ungated
M Left,Right	Events	%	Peak
0 1.00, 9910	5430	100.00	62
1 1.00, 7.23	4209	77.51	62
2 6.91, 9910	1382	25.45	31

PkChl	Mean	Median	SD	CV
4.57	8.04	5.26	44.11	>100.0
4.57	4.63	4.72	1.37	29.59
6.97	18.31	9.28	86.62	>100.0

(e iv) RBL-GLY stained with R13.1

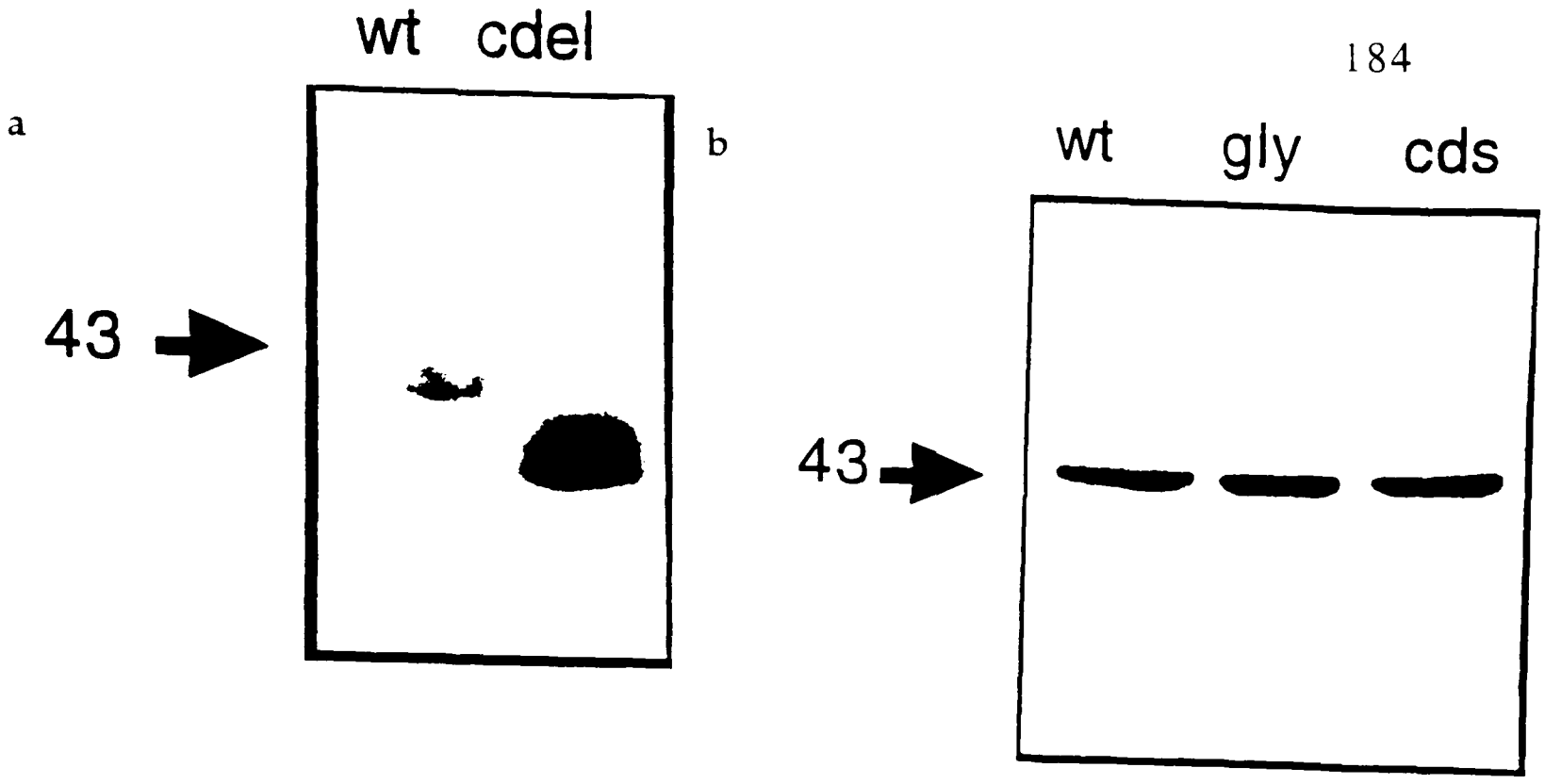


Fig 7.4ab&c
Intracellular expression of V/C domain mutants
SDS-PAGE of cell lysates was performed using 15% acrylamide gels. For Western blotting protein was transferred to a nitrocellulose membrane in a semi-dry blotter. After blocking the primary Ab was then added to a final concentration of 5μg/ml). The membrane was then washed 3 times in PBS and a 1:2000 dilution of secondary antibody was added (either horse radish peroxidase conjugated goat anti-mouse of mouse anti-rabbit antibody (both Dako)). After three further washes in PBS the blot was developed with an electro-chemical luminescence kit (Amersham) and exposed to autoradiographic film. (43 is mol wt in KD)

(a) Lysate of RBL-CDEL stained with anti-CD3ζ rabbit polyclonal antibody. Note that the bands are doublets showing both alpha and beta-CD3ζ fusions.

(b) Lysate of RBL-CDS and RBL-GLY stained with mAb αF1

(c) Lysate of RBL-CDS and RBL-GLY stained with mAb βF1

Chapter 8
General Discussion

This section summarises the results presented in this thesis and attempts to assess the significance of these findings with respect to the field of TCR biology, for in depth discussions reference should be made to the final section of each chapter.

The work presented in this thesis provides a detailed characterisation of a convenient and reliable bioassay for the analysis of TCR/MHC-peptide interactions. Although the system uses RBL cells transfected with TCR-CD3ζ chimeric receptors and is therefore based on the work of others (Engel et al 1992) several novel developments are described. Firstly the use of selectable markers linked to both chains of the TCR-CD3ζ receptor (Chapter 3) results in an increase in the stability of expression and consequently improves the reliability of the bioassay. Secondly the requirement for TCR-CD3ζ cross-linking in eliciting a response from RBL transfectants has been exploited in competitive inhibition assays using non-stimulatory monovalent recombinant MHC-peptide complexes (Chapters 4 and 5). The development of the competitive inhibition assays represents a significant addition to the range of methods available for examining TCR/MHC-peptide interactions (see Introduction to Chapter 3). Although similar approaches have been taken using T cells (Schneck et al 1989, Symer et al 1992, Weber et al 1992) these are potentially flawed because of functional effects of soluble MHC-peptide on the cells (Zavazava & Kronke 1996) and the presence of multiple TCRs (Padovan et al 1993, Valitutti et al 1995) and

other coreceptors (Parnes 1989) on the T cell surface. In contrast competitive inhibition of RBL/TCR-CD3ζ transfectants with soluble MHC-peptide ensures that the TCR/MHC-peptide interaction is being studied in isolation. Furthermore the fact that both binding partners can be tested in a bioassay is an advantage over systems that rely on measuring the interaction between two soluble molecules whose function can not be guaranteed (Fremont et al 1996, Novotny et al 1991, Ward 1992, Strong et al 1994). Once the competitive inhibition assay was established it was used to contribute to two significant observations. In Chapter 4 the A3-TCR was shown to be capable of engaging the HLA-A2 molecule virtually irrespective of peptide. This demonstrated that this receptor makes significant contacts with both MHC and peptide, an observation subsequently confirmed by the solution of crystal structures of other TCR/MHC-peptide complexes (Garica et al 1996a & Garbozci et al 1996). In addition this finding also has implications for the part played by peptide in positive selection. Two models currently exist to explain the role of peptide in positive selection, one suggesting that peptide has a highly specific role (Allen 1994) and the other that peptide only affects positive selection by blocking TCR-MHC interactions and preventing the stimulation and selection of thymocytes (Schumacher & Ploegh 1994). The results of the experiments in Chapter 4 suggest that the if TCR-MHC interactions are sufficient to select the TCR very few peptides would block this process, i.e the A3-TCR would be positively selected in the presence of many unrelated peptides. Since this result was produced a number of reports have emerged confirming that

TCRs can infact be selected by totally unrelated peptides (Miyazaki et al 1995, Nakano et al 1997). In Chapter 5 the competitive inhibition assay was used to show that improved recognition of a pol peptide variant was due to increased affinity for the TCR rather than increased stability of the HLA-A2-peptide complex. The identification of an altered peptide ligand which is recognised better than the index peptide is significant for two reasons. Firstly the design of the peptide relied on a knowledge of the crystal structure of HLA-A2 pol (Madden et al 1993), an approach which should become increasingly viable with the publication of more MHC-peptide structures. Secondly there are potential therapeutic implications for 'better recognised' epitopes, not least in negatively selecting potentially autoreactive T cell clones.

Besides the development of competitive inhibition assays the RBL/TCR-CD3ζ system also proved valuable because it allows the effect of mutations in the TCR to be tested. This facility was exploited in Chapters 6 and 7. The results in chapter 6 provide the first *in vitro* confirmation of the functional effect of allelic variation in the TCRV genes. This implies that allelic variation represents a small but significance source of potential diversity in the TCR repertoire and also that these variants are potential candidates for disease association genes. In Chapter 7 the effect of disrupting the association between the V and C domains of the TCR was examined. The results supported data from other groups (Casorati et al 1993, Chung et al 1994) by indicating that there is an important structural relationship between the respective V and C domains of the TCR α and β chains, which seems to have a critical effect

on TCR expression and function. The nature of the interface between the V and C domains has subsequently been revealed by the solution of the TCR/MHC-peptide co-complex structure (Garcia et al 1996a, Garbozci et al 1996).

Although these studies have provided novel information about some of aspects of the TCR/MHC-peptide interaction there are, naturally, many unresolved questions. Among the most significant are; the intrinsic affinity of the A3-TCR for the pol and polA8 peptides, the relative contributions of HLA-A2 and peptide to the A3-TCR binding site, the difference between the structures of the A3-TCR/HLA-A2-pol and the A3-TCR/HLA-A2 polA8 complexes and the effect of allelic variation in the BV1S1 gene on A3-TCR structure. A common requirement for all these future experiments is the production of soluble A3-TCR and at the time of writing the outlook for producing this reagent is good (B.Willcox personal communication). The data obtained using the RBL/TCR-CD3ζ system is of particular value since it confirms that the α and β chains of the A3-TCR do indeed represent a functional heterodimer, data of this type has not been available for the two TCRs whose structures have been solved to date (Garcia et al 1996a & Garbozci et al 1996). In addition it should be possible to use the bioassay to prove the functional integrity of the soluble form of the A3-TCR by showing that the soluble molecule is able to specifically compete out the response of RBL-008 to HLA-A2-pol.

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