The isolation and identification of MHC-bound CTL peptide epitopes expressed by tumour cells.

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Declaration

I hereby declare that no part of this thesis has previously been submitted in support of an application for any degree or qualifications to this or any other University or Institute of Learning.

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To Jim, for being there, for believing in me and for making me laugh.

Finally, to Mum, I know you won't see this, but I hope you are proud of me.

"Hard students are commonly troubled with gowts, catarrhs, rheums, cachexia, bradypepsia, bad eyes, stone and collick, crudites, oppilations, vertigo, winds, consumptions and all such diseases as come by over-much sitting; they are most part lean, dry, ill-coloured.....and all through immoderate pains and extraordinary studies." *Burton's Anatomy of Melancholy*

"Our deeds still travel with us from afar
And what we have been makes us what we are." *George Eliot, Middlemarch*

"Such wilt thou be to mee, who must
Like th'other foot, obliquely runne
Thy firmness makes my circle just
And makes me end, where I begunne." *John Donne*

Abstract

Cytotoxic T lymphocytes are an essential component of the anti-tumour immune response and they are activated by binding of the T cell receptor to a peptide-MHC complex on the tumour cell surface. These surface-bound peptides originate from intracellular proteins that have been degraded into peptides by the proteasome and presented by MHC class I.

Many tumours inappropriately express mutated or overexpressed self-proteins (p53, ras), tissue specific antigens (MAGE, MelanA) or tumour-specific fusion proteins (bcr-abl) which, due to their tumour association can be used as targets for anti-tumour immunotherapy.

This study presents a technique to isolate and identify surface-MHC bound tumour antigen peptides from tumour cells. Peptide isolation was achieved using a mild acid buffer to destabilise surface MHC class I molecules; concentration and purification of the peptides was facilitated by ion-exchange-HPLC and subsequent peptide sequence information was generated by electrospray mass spectrometry. The sequence data was then entered into a database to determine the protein origin.

Peptides were isolated from an HLA-A3 transfected K562 cell line model, which naturally expresses bcr-abl but no other HLA class I molecules, to determine whether peptides from the fusion region were naturally presented by HLA-A3. PBMC from HLA-A3 positive CML patients was then analysed in the same way. The identification of bcr-abl fusion region peptides from the K562 transfectants and HLA-A3 positive CML patient material is, to the best of my knowledge, the first conclusive demonstration that the bcr-abl fusion protein is processed intracellularly resulting in the surface expression of peptides from the fusion region. These peptides represent an entirely tumour specific antigen and there is potential for their use as a target in anti-tumour immunotherapeutic strategies.

Abbreviations

A3	HLA-A3 restricted peptide originating from bcr-abl
abl	proto-oncogene located on chromosome 9
ALL	acute lymphocytic leukaemia
APC	antigen presenting cell
b2a2	splicing variant of bcr-abl oncogenic fusion protein
β_2M	beta-2-microglobulin
b3a2	splicing variant of bcr-abl oncogenic fusion protein
B8	HLA-B8 restricted peptide originating from bcr-abl
BAGE	Breast carcinoma antigen
B cell	B lymphocyte
BCG	Bacille Calmette-Guerin
bcr	breakpoint cluster region (proto-oncogene located on chromosome 22)
β-gal.	beta-galactosidase
b ion	peptide fragment ion occurring when peptide is cleaved from the amino terminal end during mass spectrometry
BSA	bovine serum albumin
CD	cluster of differentiation antigen
CEA	carcinoembryonic antigen
CFU	colony forming unit
CLIP	class I peptide associated with antigen processing
CMI	cell mediated immunity
CML	chronic myelogenous/myeloid leukaemia
CMV	cytomegalovirus
C/T	cancer-testis antigen
CTL	cytotoxic T lymphocyte
Cx	calnexin
Da	dalton
DC	dendritic cell
ddH_2O	distilled, deionised water

DC	dendritic cell
ddH_2O	distilled, deionised water
DISC-HSV	disabled single cycle-herpes simplex virus
DNA	deoxyribonucleic acid
ELISPOT	enzyme-linked immunospot
ER	endoplasmic reticulum
ESI	electrospray ionisation
EUCAPS	European Concerted Action in Peptide Sensitisation
FACS	Fluorescence Activated Cell Sorting
FCS	foetal calf serum
fmol	femtomoles
FITC	fluorescein isothiocyanate
GAGE	gastrointestinal antigen
GM-CSF	granulocyte-macrophage colony stimulating factor
gp70	glycoprotein 70 protein
H_2O	water
HCl	hydrochloric acid
Hep.B.	MHC class II restricted peptide originating from hepatitis B viral protein
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HPLC	high performance liquid chromatography
HPV	human papilloma virus
HSP	heat shock protein
HSV	herpes simplex virus
ICAM	intercellular adhesion molecule
IFA	incomplete Freunds adjuvant
IFN	interferon
Ig	immunoglobulin

IL	interleukin
IU	international units
kDa	kilodalton
KIR	killer inhibitory receptor
Ii	invariant chain
LPS	lipopolysaccharide
M	mole/molar
MAGE	melanoma antigen
MHC	major histocompatibility complex
ml	millilitre
mM	millimole/molar
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass to charge ratio
NH_3	amino terminal
NK	natural killer cell
nmol	nanomole/molar
p53	tumour suppressor protein
p73	tumour suppressor protein
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
Ph^+	Philadelphia chromosome
pmol	picomole
PSA	prostate specific antigen
PSMA	prostate specific membrane antigen
RAG	recombination activating gene
RAGE	renal antigen

RP-HPLC	reverse-phase high performance liquid chromatography
RT	retention time/room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SEREX	serological screening of cDNA expression libraries
SCF	stem cell factor
SD	standard deviation
S/N	signal to noise ratio
TAP	transporters associated with antigen processing
TCA	trichloroacetic acid
T cell	T lymphocyte
TCR	T cell receptor
TGF	transforming growth factor
T_h	T helper (CD4+) cell
TIL	tumour infiltrating lymphocyte
TNF	tumour necrosis factor
WinMDI	Multiple Document Interface for Windows (flow cytometric analysis)
y ion	peptide fragment ion occurring when peptide is cleaved from the amino terminal end during mass spectrometry

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Chapter 1: Introduction

1.0 Introduction

A wide range of potentially dangerous organisms constantly bombards the human body and the immune system has several mechanisms to cope with pathogens thus ensuring our survival. These mechanisms can be divided into two types, innate and acquired, each of which have specialised cells and molecules associated with their functioning. Innate immunity is non-specific, in that it tackles all pathogens in a similar way and is "antigen unrestricted" (Medzhitov and JanewayJr 1998; Suffredini, et al. 1999). Acquired immunity is generated towards antigens associated with a specific pathogen and cells activated after pathogenic exposure are only able to recognise antigens through a response which is genetically restricted and antigen specific (Robertson 1998).

For many years research has concentrated on the manipulation of the adaptive, or acquired, immune response to treat infectious diseases and cancer. The acquired immune system is responsible for generating lasting immunity after exposure to a pathogen (Robertson 1998), a property which forms the basis of direct vaccine strategies. However, while the development of an acquired immune response takes several days, most infectious organisms are cleared from the body within a few hours by the components of the innate immune system irrespective of whether prior exposure to the pathogen has occurred (Janeway and Travers 1994)

1.1 Innate Immunity

The innate immune system is comprised of three main effector arms; phagocytic cells, activation of the complement cascade and natural killer cells. Recognition of invading organisms is dependent upon receptors for common bacterial constituents such as mannose, lipopolysaccharide (LPS) and glycans found on the surface of the cells of the innate system (Stahl and Ezekowitz 1998; Naito et al. 1999; Suffredini, et al. 1999). Thus, micro-organisms or cells infected with the pathogen can be recognised, an increasingly important concept in general immunology which will be discussed further in a later section. It is important to note, however, that the ability to distinguish infected cells from non-infected cells is an essential aspect of the innate immune system (Robertson 1998).

1.1.1 Phagocytic response

Phagocytes (neutrophils, macrophages and also dendritic cells; (Medzhitov and Janeway 1997) (Fearon 1997; Colaco 1999) express the pattern recognition receptors for mannose, LPS, glycans and complement. Binding to these receptors induces ingestion of the pathogen and secretion of cytokines (IL-1, -6, -8, -12 and TNFα) inducing an inflammatory response and cellular recruitment (Fearon 1997). The cytokines released have effects both in the innate and acquired responses, with IL-1, –6, -8 and –12 being important in activation of B and T lymphocytes as well as phagocyte recruitment and inflammation (Robertson 1998).

1.1.2 Complement Activation

Complement is a group of blood proteins activated following infection by three distinct pathways and forms part of both innate and acquired immunity. The induction of each of these three pathways leads to the cleavage (and ensuing activation) of a complement protein, C3 convertase, the products of which are responsible for inflammatory reactions, phagocyte recruitment and lysis of pathogens. The classical (acquired immune system) pathway is dependent on antibody binding to a pathogen (opsonisation) and the subsequent binding of complement to the antibody Fc receptor leading to C3 cleavage. (Robertson 1998). The lectin pathway of complement activation is mediated by mannose binding proteins (MBP) and C reactive protein which recognise and bind bacterial cell wall components leading to cleavage of C3. The alternative pathway of complement activation is facilitated by the binding of C3b proteins, (resulting from spontaneous cleavage of C3 in the blood stream) to pathogen surfaces, such as bacterial cell walls. The central complement component, C3, is cleaved into two fragments C3a and b. C3a acts as an inflammatory agent, recruiting other phagocytes to the site of infection while C3b binds to the infectious agent permitting phagocyte recognition (via complement receptors) and ingestion, it is also able to bind other complement components to form a 'membrane attack complex' capable of forming pores in the pathogen membrane leading to its lysis (Staines et al. 1994, Janeway and Travers 1994, Fearon 1998).

1.1.3 Natural Killer (NK) cells

NK cells are important in the innate response to intracellular pathogens such as viruses which cannot be dealt with by phagocytes and complement. Viral infection can only be stemmed by the death of infected cells and is mediated, in the main, by cytotoxic T lymphocytes (CTL) through an acquired response recognising viral peptides on the cell surface in the context of major histocompatibility molecules (MHC) (see section 1.3). Since the acquired response takes time to develop, the innate immune system, in the form of NK cells, combats infection instantaneously (Robertson 1998; Tormo et al. 1999).

NK cells express the phenotypic markers CD56, CD16, are CD3 negative and can be activated by IFN α, β, IL-2 and IL-12. TNF-α released from phagocytes together with IL-12 act in synergy to cause NK cells to secrete IFNγ which subsequently activates macrophages and upregulates MHC expression (Janeway and Travers 1994).

As with other cells of the innate immune system, NK cells can distinguish between infected and uninfected cells. It is thought that inhibitory receptors (killer inhibitory receptors; KIRs) are expressed on healthy cells preventing their lysis by NK. KIRs are a diverse group of receptors including MHC class I and class II molecules (Colonna and Samaridis 1995; Jiang et al. 1996; Lanier and Phillips 1996; Moretta et al. 1996) and CD94, all of which are also expressed on T lymphocytes (Janeway and Travers 1994; Rees and Mian 1999).

NK cells bind carbohydrates on cell surfaces causing release of cytotoxic granules and thus lysis of the infected cell (Kos 1998; Robertson 1998). This action is inhibited by healthy, uninfected cells by the simultaneous binding of KIRs (Robertson 1998), or other NK receptors such as members of the C-type lectin family, to MHC I molecules on the surface of the healthy cell (Tormo et al. 1999). The crystal structure of a murine receptor, L49A, bound to MHC class I molecules (H-2KD^d) both on the target cell and the NK cell itself, has recently been solved and it is likely that the interactions seen are similar in humans (Tormo et al. 1999).

Virally infected cells, eg EBV, (Zeidler et al. 1997), and tumour cells (Restifo et al. 1993; Maeurer et al. 1996) often show MHC antigen loss in an attempt to avoid recognition by CTLs. However, this ablates NK cell inhibition by KIRs causing these cells to be more sensitive to NK cell lysis. To support this view, a study by Jiang et al (1996) showed that non-MHC expressing K562 tumour cells were less sensitive to NK lysis when transfected with MHC class II molecules. Cells with an increased cell surface expression of HSP-72 are also more sensitive to NK lysis (Botzler et al. 1996). Other viruses, such as CMV (Karre and Welsh 1997) and some tumours (Ikeda et al. 1997) may express MHC encoding genes (with KIR activity) causing inhibition of the NK response thus preventing immediate killing of infected cells by the innate response.

It has been intimated above that cells of the innate immune system trigger inflammatory reactions and cytokine production in response to infectious agents, subsequently recruiting further cells of both the innate and acquired systems to the site of infection, (Matzinger 1994; Fearon 1997; Medzhitov and Janeway 1997; Medzhitov and JanewayJr 1998; Colaco 1999; Suffredini et al. 1999). This, together with the suggestion that dendritic cells (DCs) are important in the initial induction of the immune response, (via pathogen ingestion and cytokine production - (Colaco 1998; Colaco 1999; Rescigno et al. 1999) supports the view that innate and adaptive immunity are inextricably linked. Innate immunity is therefore essential for the induction of acquired immunity, an important consideration when attempting to direct acquired immunity against tumours using immunotherapy. Indeed, the emerging 'danger theory' (Matzinger 1994), responsible for the *"shift"* in immunological thinking away from the self/non-self discrimination (Burnet 1970; Janeway 1992) towards the discrimination between "dangerous" and "non-dangerous" antigens, considers the partnership between innate and acquired immunity essential to successful anti-tumour therapy (Fuchs and Matzinger 1996; Todryk et al. 2000)

1.2 Acquired Immunity

Acquired immunity develops in response to the presence of a pathogen and the resulting immunity is specific for that pathogen. The response is mediated by either B- or T-lymphocytes as a result of "signals" from dendritic cells, macrophages and each other; helper T cells activate B cell prolieration and B cells can act as antigen presenting cells to T cells (Lanzavecchia 1993; Linsley et al. 1991; Parker 1993).

The following section will discuss the generation of antigen-specific immunity by these cells and their participation in the immune response.

1.2.1 B lymphocyte development

B cell development (figure 1.1) occurs in the bone marrow which eventually results in the production of antibody secreting B cells or memory B cells throughout life in gradually decreasing numbers (Forster and Rajewski 1990). Antibody mediated protection is known as humoral immunity. Each mature B cell expresses a unique receptor resulting from the rearrangement of immunoglobulin genes; two chains, one light and one heavy, are produced and combine to form surface receptors of a single specificity. When mature, B cells can secrete antibody of the same specificity as the surface immunoglobulin or can become long-lived memory cells lying dormant until the pathogen triggering their activation is encountered again (Chen and Alt 1993).

B cell development passes through, and is dependent upon, several stages which are characterised by multiple steps in gene rearrangement and the ensuing expression of cell surface markers. The B cell matures from a pluripotent stem cell through early and late pro B cell stages during which CD45R, MHCII, CD 19, CD38, CD40 and CD44 are expressed and gene rearrangement is ongoing (Chen and Alt 1993). During these early stages, the B cell precursors are dependent on the stromal cells in the bone marrow (Hayashi et al. 1990). The precursor cells are bound to hyaluronic acid on the stromal cells via CD44 allowing activation of the tyrosine kinase c-kit and intracellular signalling results in precursor cell proliferation. At this stage, the precursors have successfully rearranged the heavy chain gene and thus the first stage of development is complete.

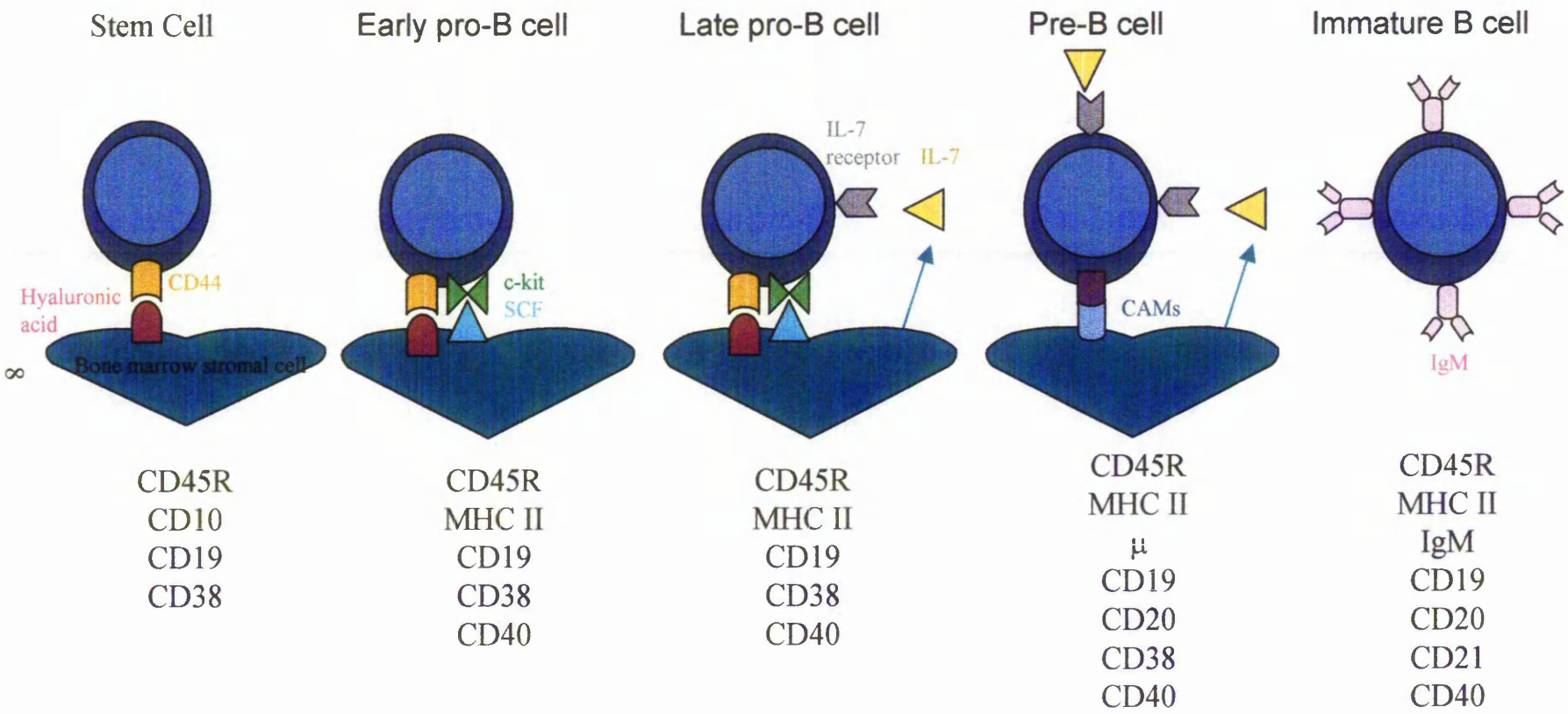


Figure 1.1: B lymphocyte ontogeny and surface marker expression

Modified from Chapter 5, Janeway and Travers, Immunobiology

The proliferation of cells at this stage generates a large number of cells with successfully rearranged heavy chain genes which then undergo light chain gene rearrangement (Decker, Boyle et al. 1991). The B cells express the heavy chain on the surface and enter the pre-B cell stage which is dependent on IL-7 secreted by the stromal cells to allow their continued development (Hayashi, et al. 1990; Kincade et al. 1993). Light chain gene rearrangement continues until intact IgM moleules are manufactured and expressed on the surface. The B cell is now termed 'immature' and is IL-7 independent (Hayashi et al. 1990; Kincade et al. 1993).

The first level of selection (positive selection) occurs when the B cells express functional immunoglobulin receptors and cells unable to rearrange the heavy chain gene are deleted (Liu et al. 1997). There are two known mechanisms of B cell selection enabling the deletion of self- reactive B cells. Following expression of the immunoglobulin receptor on the cell surface, B cells undergo negative selection, during which any cells expressing self-reactive immunoglobulin receptors undergo apoptotic cell death (Russell et al. 1991; Nossal 1994; Nemazee et al. 2000). The genes responsible for gene rearrangement (RAG-1 and –2) are downregulated in surviving B cells which then leave the bone marrow to enter the peripheral circulation. A small population of autoreactive B cells do not downregulate RAG-1 and –2 and are able to rearrange the light chain genes thus escaping deletion (Oettinger et al. 1990). This phenomenon is not clearly understood. Negative selection also occurs if mature (circulating) B cells demonstrate autoreactivity or binding to high concentrations of antigen. These cells are rendered anergic following binding and migrate to the follicular mantle of the lymph node (Russell et al. 1991).

1.2.2 B cell activation and differentiation

B cells are activated following antigen binding to the B cell receptor (or surface immunoglobulin). The cells divide rapidly (clonal expansion) and differentiate into antibody secreting plasma cells or memory cells, a response which is dependent on T cell help. Th2 type CD4+ T cells bind B cell MHC-peptide complexes causing B cell activating cytokines (IL-4, -5, -6, -10) to be produced by the CD4+ cell. The cytokine released depends on the antigen encountered and in turn determine the immunoglobulin isotype produced (table 1.1).

Plasma cells migrate to the bone marrow or medullary cords of the lymph nodes. The lymph node is the site of the majority of antibody production, where the plasma cells form germinal centres consisting of highly proliferating, antigen unresponsive cells generating large amounts of specific antibody (Liu et al. 1992). The secreted antibody is of an identical specificity to that expressed on the surface of the B cell. However, following activation, B cells undergo isotype switching resulting in the secretion of different antibody types depending on the location of the original antigen stimulation, type of antigen and cytokines present (Harriman et al. 1993). During this process, cytokines act to decondense the chromatin within the B cells allowing further gene recombination and transcription resulting in the expression of a new antibody isotype (Chen and Alt 1993). Table 1.1 shows the cytokines involved in this process.

B cells are short-lived and continually replaced throughout life thus ensuring a large diverse pool of antigen receptors (Forster and Rajewski 1990). Following clearance of the antigen from the body, plasma cells die by apoptosis (Liu et al. 1991), but memory cells, which have lain dormant throughout this primary antibody response, continue to circulate around the body. Memory cells are activated to produce antibody in the event of a repeat infection with the same antigen responsible for their original generation. The process governing the original differentiation into plasma or memory cells is not completely understood, but it is thought that plasma cell differentiation depends on CD23 and memory cell differentiation on CD40 (Liu et al. 1997).

In addition to antibody secretion, B cells can act as antigen presenting cells to T lymphocytes in the same manner as dendritic cells (see section 1.4). High levels of surface MHC class II allow presentation of antigen, particularly soluble antigen such as bacterial toxins, to T lymphocytes in

Table 1.1 Cytokines affecting antibody isotype production

Cytokine	Promotes	Inhibits	Clinical Manifestation	Reference
IL-4	IgG_1, IgE	IgM, G_3, $G_{2\alpha}$	Allergic Inflammation Parasitic Response	(Rankin 2000) (Wang et al. 2000)
IL-5	IgA	-	Food Allergy	(Barnes 1995)
IFN-γ	IgG_3, $G_{2\alpha}$	IgM, G_1, E	Bacterial Response	(Kashino et al. 2000)
TGF-β	$IgG_{2\beta}$, IgA	IgM, G_3	Food Allergy (IgA) Oral Tolerance	(Barnes 1995) (Kalliomaki et al. 1999)

the lymphoid organs. The co-operation between the innate and acquired arms of the immune system is echoed here with B and T cell co-operation essential to their activation.

1.2.3 T lymphocyte development

T lymphocytes, responsible for cell mediated immunity (CMI) derive from stem cells in the bone marrow, and migrate to the thymus (a lymphoid organ located in the thorax) early in their development to continue the maturation process (Ewiyk 1991). The stages of T lymphocyte maturation can be characterised by the appearance of various cell surface markers at each stage (figure 1.2). The earliest T cell precursors present in the thymus are CD3 (associated with the T cell receptor; TCR), CD4 and CD8 (molecules responsible for cellular activation upon antigen stimulation) negative and, upon arrival from the bone marrow, undergo an intense period of proliferation and differentiation (Petrie et al. 1990). Studies in mice have shown that only 2% of these cells go on to become part of the peripheral T cell population due to the stringent selection processes occurring later in development (Shortman et al. 1990).

Once the T cells have migrated to the thymus they interact with the cortical thymic stroma leading to their rapid proliferation (Winoto and Baltimore 1991). The stromal cells express high levels of MHC class I and class II which are involved in selection. At this stage, the T cell precursors express CD2 and are undergoing TCR β chain rearrangement, following which the cells become large, double positive cells, expressing both CD4 and CD8. The TCR α chain rearranges and the cells express low levels of the rearranged TCR, while the α chain continues to rearrange until either the cell is positively selected or dies (Winoto and Baltimore 1991).

1.2.4 Positive selection of T cells

More than 90% of the double positive T cells die in the thymus as they are unable to recognise self-MHC (positive selection) bound to the stromal cells within the thymic cortex (Anderson et al. 1999). Cells not binding the MHC do not receive a signal and are deleted by programmed cell death,

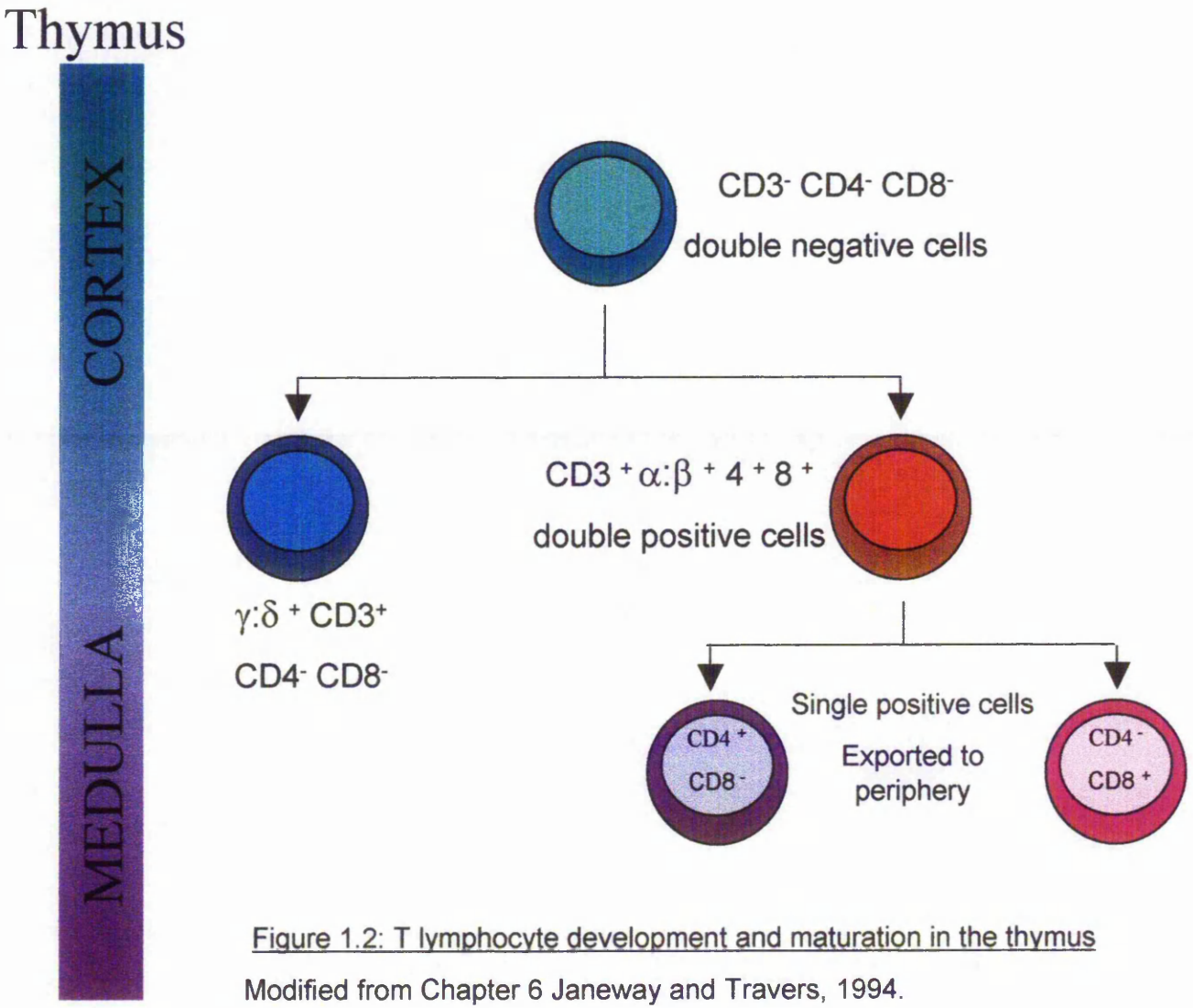


Figure 1.2: T lymphocyte development and maturation in the thymus

Modified from Chapter 6 Janeway and Travers, 1994.

apoptosis (Mariathasan et al. 1999). Those cells which are able to bind self-MHC are prevented from apoptotic death, possibly by anti-apoptotic gene (bcl-2) transcription induced by appropriate intracellular signalling (Nakajima et al. 2000). These then become single positive cells, losing either CD4 or CD8 expression, depending on whether they were able to recognise MHC class II or class I respectively (Anderson et al. 1999). The recombination genes (RAG-1 and –2) are then switched off and the cells then undergo negative selection (Nossal 1994).

1.2.5 Negative selection of T cells

Single (CD4 or CD8) positive T cells expressing high levels of CD3 are exposed to self - MHC expressing a variety of self peptides derived from proteins within the thymus. Any cells binding these peptides are deleted as they are autoreactive and potentially harmful (Nossal 1994). T cells recognising tissue specific proteins, or proteins that appear later in life, ie after puberty, cannot be deleted in the thymus and therefore must be inactivated in the periphery (anergy) following binding to such an antigen, most likely due to the lack of a second activation (costimulatory) signal (Gallucci et al. 1999).

Thymocytes not recognising self-peptides survive and are exported into the periphery to circulate around the lymphoid organs until specific antigen is encountered (Anderson et al. 1999).

Following puberty the thymus shrinks and less T cells are produced (Hirokawa et al. 1994). It is thought that peripherally located T cells must undergo self-renewal and that our entire supply of T cells is present before puberty (Hirokawa et al. 1994).

1.2.6 T cell activation

MHC: peptide complexes on antigen presenting cells (APC) bind to the T cell receptor (TCR) (figure 1.3a) which consists of two disulphide bound chains, most commonly $\alpha\beta$, and CD3. CD3 consists of two ε chains, and δ, γ, ξ chains. These are associated with transmembrane kinase signalling to the interior of the cell when antigen binds to the TCR thus promoting activation (Figure 1.3b). Two tyrosine kinases, lck and fyn are the first signalling molecules in the cascade, fyn is associated with the ξ chains of the TCR:CD3, and lck with the CD4 and 8 molecules on the T cell surface (CD4 on T helper cells and CD8 on cytotoxic T cells). Once MHC: peptide binds to the TCR a signalling

cascade begins with these two molecules and a CD45-linked kinase (Janeway 1992). The cascade leads to the activation of protein kinase C and increases in intracellular calcium activating DNA binding proteins which in turn activate transcription of the IL-2 receptor (Minami et al. 1993). Following this initial signal, T cells require an additional second signal to cause clonal expansion and production of antigen specific T cells. This second signal occurs due to the co-stimulatory molecule, B7, on the APC binding to CD28 on the T cell (figure 1.3b), generating another phase of intracellular signalling leading to transcription of the IL-2 gene which is important for the activation of T cells (Fraser et al. 1991; Minami et al. 1993). Helper-T cells produce IL-2 to activate CTL expressing the IL-2 receptor, i.e. the co-stimulation is provided by the APC via T cell help. Recent studies (Bennett and Carbone 1998; Ridge et al. 1998; Schoenberger et al. 1998) suggests that the helper T cell is activated by the APC which, in turn, is activated due to signals received from the T-helper cells (mediated by CD40), allowing the APC to directly co-stimulate the killer T cells. This would circumvent the problem of requiring three cell clusters for activation. T cells are activated to proliferate and generate large numbers of antigen specific T cells which recognise and kill any cells expressing the 'specific' peptide presented by MHC class I molecules. Without the co-stimulatory signal T cells become anergic, i.e. they are unable to respond to any antigen. In the absence of CD4 or CD8 molecules (co-receptor molecules initiating ick kinase signalling on antigen binding), more than 10,000 identical MHC: peptide complexes would be required to activate the T cell (Janeway 1992); thus, the importance of each of the cell surface markers in generating immune responses against antigen is paramount.

Following activation, CD8+ CTL are able to induce apoptosis in other cells as a physiological response in pathological conditions such as wound healing, or necrosis which is essential in the host response to pathogens. Secretory granules containing perforin are released from the CTL following intracellular signalling upon CTL binding to an infected target cell. Perforin monomers insert into the target cell membrane and polymerise, forming pores in the membrane allowing inward passage of a

Figure 1.3a The T cell receptor

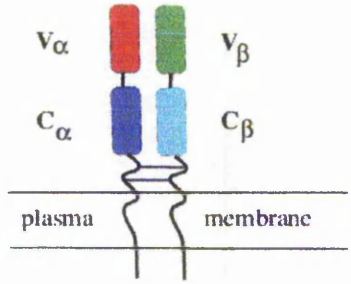


Figure 1.3b Antigen binding to the T cell receptor

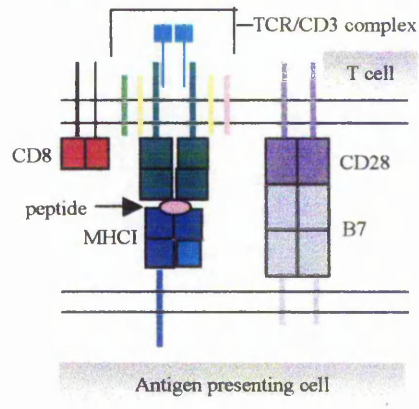


Figure 1.3c Intracellular signalling following antigen binding to the T cell receptor.

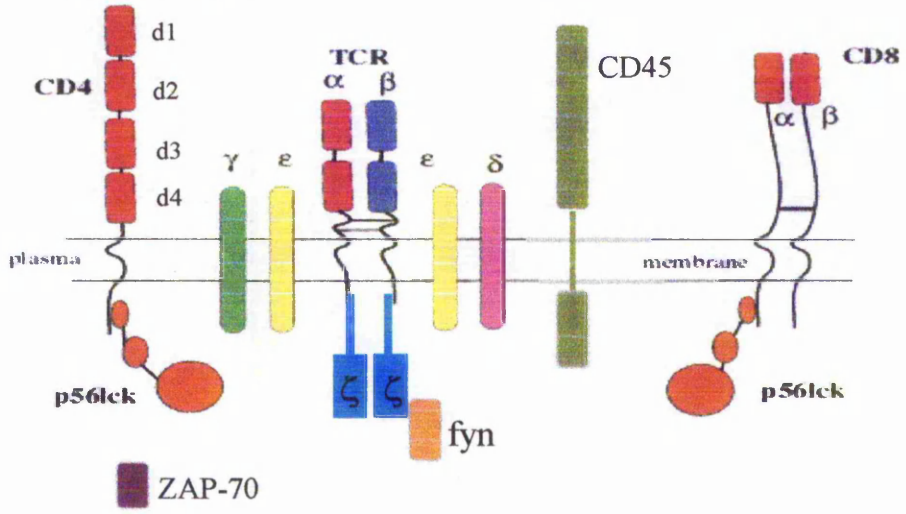


Figure 1.3 The T cell receptor, represented in (a), consists of four domains, two constant (C) and two variable (V), the variable domains allowing for heterogeneity of antigen specificity between T cell receptor (TCR) types. Activation of the T cell resulting in production of antigen specific T cells requires two signals, (b), generated by the binding of MHC-peptide complex to the TCR complex and the binding of B7 and CD28. Intracellular signalling resulting from the two signals is represented in (c). CD45 activates lck and fyn leading to ZAP-70 activation and cleavage of intracellular phosphatases. The cleaved phosphatases cause an increase in intracellular calcium ultimately activating DNA binding proteins and initiating IL-2 transcription resulting in T cell activation.

Modified from Janeway and Travers, 1994

serine protease, granzyme. Granzyme mediates DNA fragmentation leading to cell death, whilst osmotic cell lysis occurs due to the perforin pores (Stepp et al. 2000). The production of cytokines by the CTL, for example, IFNγ, (Romagnani 1997) and leakage of intracellular proteins from the target cell such as cytochrome C and heat shock proteins (Todryk et al. 2000) activates and recruits other cells of the immune system including macrophages, phagocytes, mast cells and dendritic cells (Romagnani 1997), (Abraham and Arock 1998), (Todryk et al. 2000), (Fuchs and Matzinger 1996). Presentation of fragments of intracellular pathogens by macrophages to CD4+ Th1 (inflammatory) T cells activates the T cell and causes the production of inflammatory cytokines. IFNγ induces the macrophage to further lyse intracellular pathogens and lymphotoxins enable lysis of infected macrophages thus recruiting further macrophages to engulf the pathogen and T cells to continue the immune response. The mechanisms mediating target cell lysis by T cells are not clear cut and ongoing research continues to facilitate our understanding of this increasingly complex phenomenon (Todryk et al. 2000).

1.3 MHC Processing

Acquired immunity is dependent on the presentation of antigen to the effector cells via the Major Histocompatibility Complex (MHC). Effector cells recognise antigen presented on the cell surface as peptides derived from the degradation of proteins, either foreign or self, processed via distinct pathways within the cell (Figure 1.5). The MHC display the resulting peptides on the surface of the cells causing effector cells to proliferate and subsequently to recognise and kill cells expressing the antigen.

The MHC antigens are polymorphic transmembrane glycoproteins that can be classified into two types by virtue of their structure; both types are coded on chromosome 6. MHC class I molecules are constitutively expressed on almost all cells, excepting only red blood cells and those within immune privileged sites, whereas MHC class II are only constitutively expressed on antigen presenting cells (APCs) and within the thymus. Their expression is inducible on macrophages and are only expressed on T cells following activation.

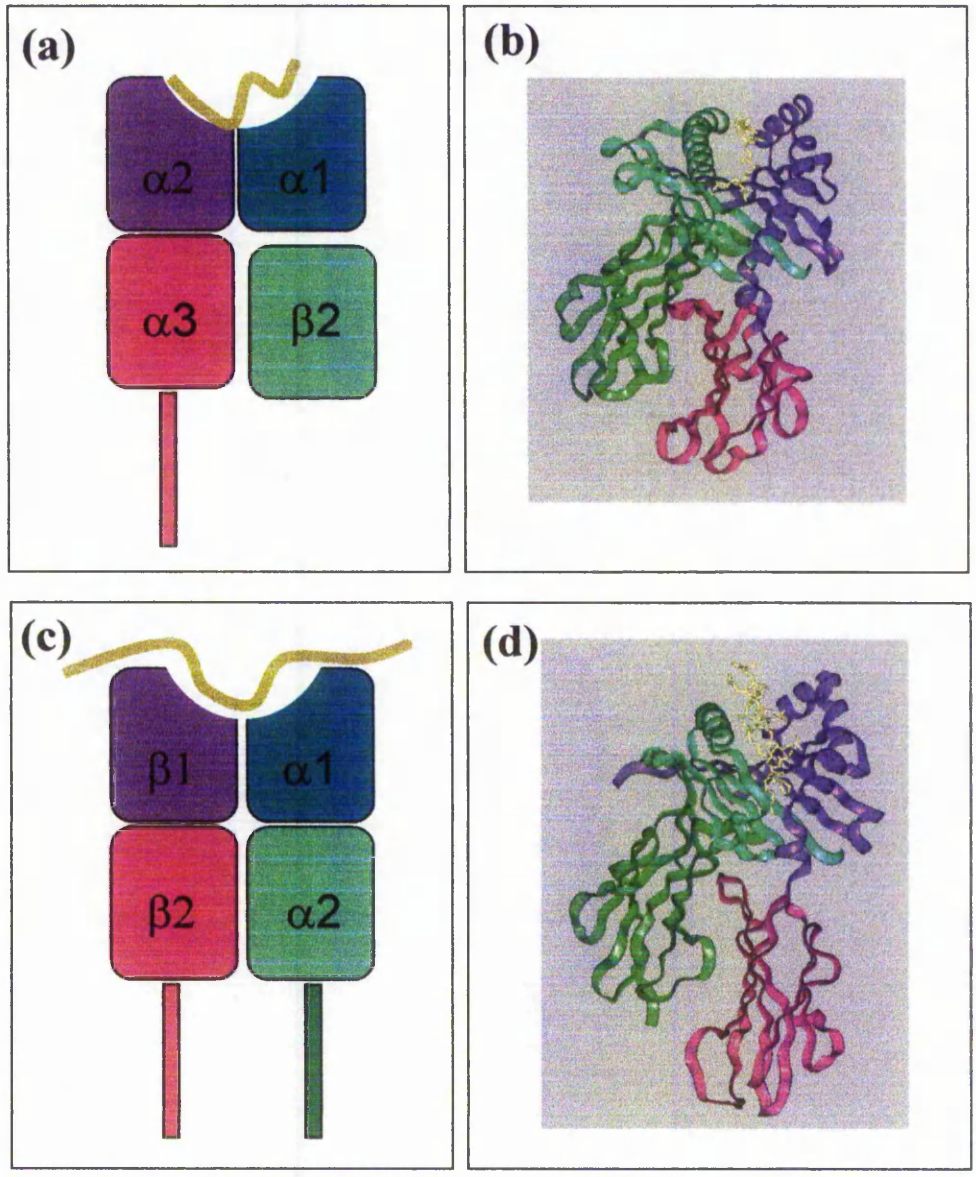


Figure 1.4 Structure of MHC class I and class II

MHC class I (a and b) is comprised of two chains, the α and β. The α chain consists of three domains, coded on chromosome 6, and is non-covalently bound to the β2-microglobulin, coded on chromosome 15. The peptide (mustard yellow) is bound within the peptide binding groove and is typically 8-10 amino acids in length. MHC class II consists of two chains, each of two domains, both coded on chromosome 6. The ends of the peptide binding groove are open allowing the ends of the peptide, usually 15-18 amino acids long, to protrude at either end of the groove.

Panels a and c are a schematic representation of the molecules (adapted from Janeway and Travers 1994), whilst b (HLA-B27) and d (HLA-DR1) are ribbon diagrams of the structures (domains coloured as schematic) as determined by X-ray crystallography (from http://depts.washington.edu).

1.3.1 MHC class I molecules

MHC class I molecules are divided into three loci, HLA-A, -B, and –C. Multiple alleles are now recognised for each locus, 59 alleles for the A locus, 118 for B and 36 for C (Maffei and Harris 1998). MHC class I antigens are 56-61kDa in size and are composed of two protein chains (Bjorkman and Parham 1990) (Figure 1.4a and b). The α chain consists of three domains, (α1, 2 and 3), and a smaller chain β_2 microglobulin (β_2M) coded on chromosome 15. Studies by Gooding and O'Connell (1983) showed that MHC class I molecules bind mainly peptides derived from cytosolic proteins (figure 1.5). Peptide fragments are chaperoned to the transporters associated with antigen processing, (TAP-1 and TAP-2), by heat shock proteins (Udono et al. 1994) and are then, in turn, transported to the endoplasmic reticulum, (ER), by the TAP 1 and 2 where they are able to bind MHC class I molecules. MHC class I molecules are bound by a protein called calnexin in the ER which allows the association of the α and β domains of the MHC (Williams and Watts 1995). The two domains then bind to calreticulin, homologous to calnexin, which then binds to the TAP proteins whereupon peptide binding to MHC occurs (Suh et al. 1994). The TAP/calreticulin is then removed and the MHC complex trafficks to the cell surface to display the peptide antigen (Neefjes et al. 1993). The importance of the TAP proteins in antigen presentation is demonstrated by the T2 lymphoblastoid cell line. T2 is TAP deficient (chromosome 6 part deletion), thus is defective in peptide transport (Steinle and Schendel 1994) and a restricted peptide set from the ER lumen is bound to the MHC which are expressed at only 20-50% of the wild-type level (Maffei and Harris 1998), (Steinle and Schendel 1994).

1.3.2 MHC class II molecules

MHC class II molecules are 63 kDa and are formed by two glycoprotein chains (figure 1.4c and d). Each has two extracellular domains, (α1, 2and β1, 2), both of which are coded on chromosome 6. MHC class II has three major subtypes; HLA-DP, DQ and DR which are further subdivided into several alleles. MHC II mainly bind peptides that have originated from extracellular proteins (figure 1.5), which are phagocytosed and degraded in an acidified intracellular vesicle generating peptides (Bennett et al. 1992).

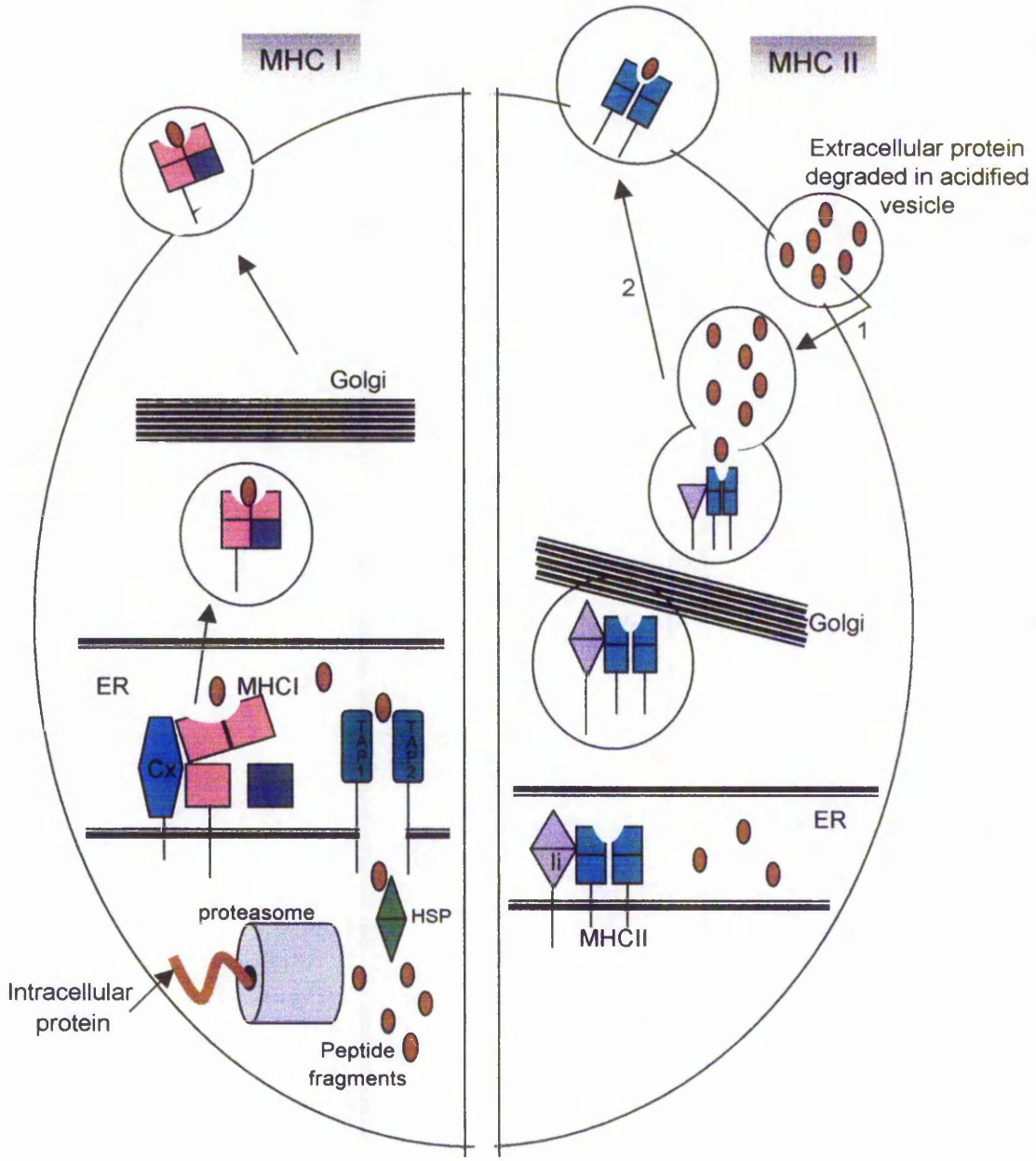


Figure 1.5 Processing of proteins for presentation by MHC molecules

Intracellular proteins derived from the cytosol are degraded by the proteasome for presentation by MHC class I to CD8+ T cells (left). Extracellular proteins are degraded in acidified vesicles which join with the MHC class II-invariant chain (Ii) complex enabling presentation of peptides to CD4+ T cells (right). See text for full explanation. Modified from Janeway and Travers, 1994 and P. Parham, 2000,

Cx=calnexin; HSP=heat shock protein; ER=endoplasmic reticulum; Ii=invariant chain.

The assembly of class II molecules is aided by the invariant chain (Ii), the α and β subunits dimerise and form a trimer with Ii (Bakke and Dobberstein 1990). The complex allows MHC passage through the Golgi and into an endocytic compartment where proteases within the endosome cause the invariant chain to dissociate from the MHC molecule. CLIP (class II associated invariant peptide) is left in occupation of the peptide binding groove until the endosome fuses with acidified peptide - containing vesicles (Bennett et al. 1992; Freisewinkel et al. 1993). The acidified vesicle fuses with the endosome allowing peptide to bind to the class II displacing CLIP and the complex then trafficks to the cell surface (Germain and Rinker 1993)

1.3.3 Peptide Binding to MHC

MHC class I bound peptides are generally 8-11 amino acids in length (Falk and Rotzschke 1994) and are held in the peptide binding groove (figure 1.6a) formed by the α1 and α2 domains (Maffei and Harris 1998). The groove consists of two alpha helices upon a sheet of 8 beta strands. Within the groove pockets of amino acid residues contribute to peptide specific binding (Madden 1995). The structure of these pockets determines which amino acids are able to bind to the binding groove. The B pocket on HLA-A2.1 is hydrophobic in nature due to its amino acid complement, showing preference for binding large aliphatic amino acids (Saper et al. 1991). The pockets are located at various amino acid positions along the peptide (A-F in class I) and this knowledge allows prediction of the peptides most likely to bind to the class I molecule. In the case of HLA-A2.1 the 2^{nd}, 3^{rd} and terminal residues have been predicted (anchor residues); the 2^{nd} residue binds to the B pocket and the amino acids leucine and methionine have been predicted to bind there (Falk 1991) and studies have shown that this is indeed the case (Gnjatic 1995).

Anchor residues have also been characterised for MHC class II molecules which are more polymorphic due to structural differences among the class II alleles and the length of peptides binding which range from 10-24 amino acids (Chicz et al. 1993). The MHC class II binding groove (figure 1.6b) is formed by the α1 and β1 domains and is composed of 2 alpha helices on an anti-parallel β-pleated sheet floor. The ends of the groove are open allowing the ends of the peptide to

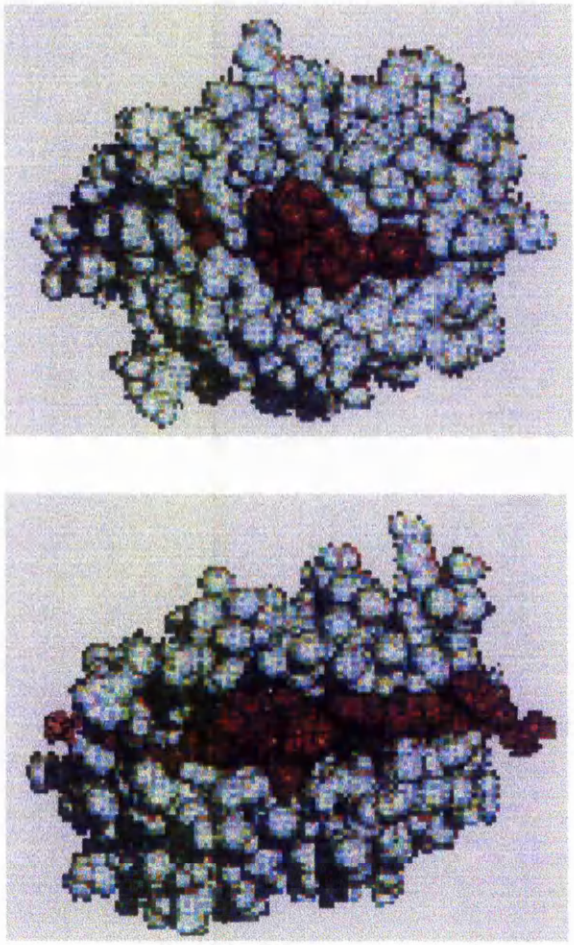


Figure 1.6 Peptide binding groove of MHC molecules

The crystal structure of the peptide binding groove of MHC class I and II molecules is shown above, the peptide is red and the groove in blue-green.

MHC class I, (top), binds peptides of 8-10 amino acid length completely within the peptide groove. Anchor residues have been well characterised for MHC class I and certain amino acids are known to preferentially bind at certain positions. Amino acids at positions 2, 3 and terminal are crucial to peptide binding. MHC class II are less well characterised, the peptide (15-18 amino acids long) is able to protrude from each end of the peptide groove as shown (bottom), however, less is known about important anchor residues, and whether the protruding residues contribute.

From Janeway and Travers 1994.

protrude and thus enabling longer peptides to be accommodated. There are also pockets within the binding groove, but many of the alleles have different characteristics which are not well understood (Maffei and Harris 1998).

1.4 Dendritic Cells

A specific immune response to a 'new' or previously unseen antigen, requires activation of naïve T cells which is most efficiently mediated by a professional antigen presenting cell, the dendritic cell (Banchereau and Steinman 1998). Dendritic cells are unique antigen presenting cells (APC) in that they are able to deliver both activation and co-stimulation signals to cause activation of naïve T cells, thus illustrating the critical role they play in the immune response.

The term dendritic cell (DC) is applied to a collection of cells with morphological similarities including Langerhans cells in the epidermis, veiled DC (afferent lymphatics), interstitial DC (connective tissue and solid organs), blood DC, interdigitating DC (lymphoid organs) and germinal centre DC (Morse and Lyerly 1998). The Langerhans cell was the first described dendritic cell (Banchereau and Steinman 1998) and, in the main, DCs are thought to originate from a $CD34^+$ progenitor cell, but depending on the location of the DC could differentiate along different pathways (Morse and Lyerly 1998). The $CD34^+$ dendritic cell-colony forming unit (DC-CFU) is part of the myeloid lineage which can differentiate into DCs or macrophages depending on the cytokines present. The proof that DCs can be produced from these cells is demonstrated by the ability to generate DC from blood PBMC in the presence of certain cytokines (GM-CSF, IL-4 and TNF-α). (Peters et al. 1996; Hart 1997)

Alternatively, thymic and interdigitating DC are generated via the lymphoid pathway from $CD34^+$ bone marrow cells which have the potential to develop into DCs, T cells, B cells or NK cells. Myeloid derived DCs are the easiest to obtain (from human blood) and are used in experimental and clinical cancer immunotherapy procedures (Banchereau and Steinman 1998; Morse and Lyerly 1998).

1.4.1 Antigen uptake by dendritic cells

Dendritic cells engulf antigen using various means including macropinocytosis (approximately 2400fl/hr can be taken up by the DC - Personal communication; M.Muthana), mannose receptor and FCγRII receptor mediated endocytosis or phagocytosis. Following the internalisation of antigen

from infected or necrotic cells, the DC are then able to process and present the antigen on the surface enabling T cells to be activated (Gallucci et al. 1999) and to respond to the internalised antigen in the presence of appropriate cytokine signals. Each stage of T cell activation via dendritic cells is explained in detail below, beginning with the maturation of DCs in response to a potentially 'dangerous' antigenic insult.

1.4.2 Dendritic cell activation

Immature DCs (those not yet exposed to activation signals from dangerous antigen), are efficient at internalising and processing antigen from infected or necrotic cells but are poor activators of T cells. They are phenotypically characterised as having low levels of HLA-class I and class II, costimulatory molecules CD80 and CD86 and CD40 (Gallucci et al. 1999). Dendritic cells progress through several maturation stages following internalisation of antigen. The first stage is initiated by infectious agents such as lipopolysaccharide from bacteria, cytokines such as TNF -α, IL-1β or by cellular necrosis. This stage of maturation is characterised by an upregulation of surface MHC class I and class II, CD18, CD54, CD58 and a decreased ability to capture and process antigen. The maturation of DCs is completed following CD40-CD40 ligand interaction with T cells and the presence of Th1 type cytokines (Mackey et al. 1998). This stage is characterised by an upregulation of CD80 and CD86 (B7.1, B7.2 costimulatory molecules), secretion of TNF-α, IL-1β, IL-12 and a potent T cell activating ability via antigen presentation on the surface in the context of MHC molecules (Girolomoni and Ricciardi-Castagnoli 1997).

Recent work has suggested that this second stage of maturation is mediated by T helper cells. It was originally thought that a three cell interaction between DCs, T killer and T helper cells was required to activate DCs (Mitchison and O'Malley 1987; Keene and Forman 1982). However, more recently, it has been found that CD40L expressed on the surface of activated T helper cells can bind CD40 on the DC thus causing an alteration within the DC which allows it to activate cytotoxic T lymphocytes (Ridge et al. 1998; Bennett et al. 1998; Schoenberger et al. 1998). Other research suggests that the alteration of the dendritic cell is characterised by the upregulation of the costimulatory molecules B7.1 and B7.2, (Cella et al. 1996; Schoenberger et al. 1998; Girolomoni

and Ricciardi-Castagnoli 1997; Morse and Lyerly 1998), an observation not supported in the work by Ridge et al. (1998).

Following final stage activation, the DCs are motile and can migrate from the peripheral tissues to the lymphoid organs where they activate circulating lymphocytes which undergo clonal expansion and in turn migrate from the lymphoid organs to the periphery to kill the infected cells originally responsible for their activation (Peters et al. 1996).

Recent studies on dendritic cells have concluded that they may be responsible for inducing tolerance to self-antigens in the periphery and that self-tolerance is not entirely pre-determined in the thymus. DCs are able to internalise both necrotic and apoptotic cells during maturation (Gallucci et al. 1999), however, the capture of apoptotic cells does not lead to the upregulation of co-stimulatory molecules on the DCs, unlike the capture of necrotic cells (Sauter et al. 2000; Steinman et al. 2000; Gallucci et al. 1999). Since apoptotic cells are the by-product of physiological, programmed cell death, the internalisation and processing of antigens from apoptotic cells would result in the presentation of self-antigens (Gallucci et al. 1999). The lack of co-stimulation induces tolerance in CTL responding to the self-antigens thus preventing autoimmunity (Sauter et al. 2000; Steinman et al. 2000; Gallucci et al. 1999). Internalisation of apoptotic cells resulting from viral infection or tumour environments may activate the DCs to upregulate co-stimulatory molecules in response to IFN-α released from CTL or heat-shock protein from the tumour environment. Following the activation of DCs, an immune response can be initiated against the captured antigens and prevent these antigens evading the immune system (Albert et al. 1998; Steinman et al. 2000). These observations form part of the emerging 'danger theory', first postulated by Polly Matzinger in 1994. The theory suggests that immune responses are only formed against "dangerous antigens", that is, those stimulating cytokine production (IFN-α) or heat shock proteins, in response to necrosis, hypoxia or other trauma (Fuchs and Matzinger 1996; Todryk et al. 2000). These findings are being further investigated and the theory refined as knowledge increases, however it is likely that currently held opinions concerning tumour immunotherapy will be influenced by these findings.

1.5 Tumour Antigens

Many tumours aberrantly express proteins which are not expressed in normal cells (tumour specific) or are expressed at lower levels in normal cells (tumour-associated). These tumour antigens, as they are known, are generated by the activation of oncogenes by mutation, rearrangement, translocation or glycosylation of amino acids or sequences of amino acids. Some tumour antigens result from the upregulation of self-proteins. The normal intracellular protein processing pathway acts to degrade the tumour antigen proteins into peptides which are then presented on the surface in the context of MHC (as described in section 1.3) and thus have the potential to induce T cell reactivity against tissues expressing the protein.

Since the identification of the first tumour antigen from melanoma patient tissue using gene cloning (Bruggen et al. 1991), and the demonstration that antigen-specific CTL could be induced to lyse tumours expressing the protein, (MAGE-1), many more tumour antigens have been identified and characterised in a similar manner. Other methods are now being used to identify tumour antigens including biochemical purification of tumour antigen peptides from tumours and their subsequent sequencing by HPLC-mass spectrometry (Slingluff et al. 1993) and use of a serological approach (SEREX) based upon screening tumour recombinant cDNA libraries with patient antibody-containing serum (Sahin et al. 1995; Old and Chen 1998).
Tumour antigens are grouped according to their origin and expression pattern, and many types of each group have now been identified and shown to be immunogenic by in-vitro CTL assays.

Cancer Testis antigens (CT), are those not expressed in normal adult tissue, except in male germline tissue and other immunoprivileged sites such as placenta and eye (Eynde et al. 1995; Jager et al. 2000). The first tumour antigen, MAGE, falls into this category and a family of approximately 12 MAGE genes are now known to exist with MAGE 1-4, 6 and 12 known to be expressed by a wide range of tumours (Mulcahy et al. 1996). A number of MAGE related genes, denoted BAGE, GAGE and RAGE (Boel et al. 1995; Eynde et al. 1995; Gaugler et al. 1996) have also been identified in melanoma, breast and renal carcinomas. Many of these CT antigens have been shown to induce CTL with the ability to lyse tumour cells expressing them (Mulcahy et al.

1996; Gaugler et al. 1996). One of the more recently discovered CT antigens, NY-ESO-1, was identified in oesophageal cancer using the SEREX technique (Chen et al. 1997). HLA-A2 binding peptides derived from NY-ESO-1 have been shown to induce CTL and humoral responses in-vitro (Jager et al. 1998), in-vivo responses have also been observed (Jager et al. 2000). More recently, MHC class II epitopes have been identified and recognised by CD4+ cells from melanoma patients (Jager et al. 2000). NY-ESO-1 has been shown to be expressed in a wide variety of tumours particularly melanoma, but also, breast, non-small cell lung, ovarian and prostate cancers (Jager et al. 2000).

A second group of tumour antigens are the differentiation antigens. These tissue-specific antigens have mainly been identified in melanoma as CTL directed against melanomas are also able to recognise normal melanocytes. Peptides originating from tyrosinase, gp100 and MelanA/MART-1, also known as the melanoma differentiation proteins (MDP), have been identified as being able to induce CTL in-vitro (Kittlesen et al. 1998; Wolfel et al. 1994; Rivoltini et al. 1999). Prostate-specific antigen and prostate-specific membrane antigen (PSA and PSMA) are also examples of tissue specific differentiation antigens, as they are also expressed both on normal and tumour tissue. CTL induced in-vitro against HLA-A2 restricted peptides from prostate-specific antigen were able to lyse both peptide-pulsed HLA-A2 expressing targets and HLA-A2+ prostate tumour cells (Correale et al. 1997). Carcinoembyonic antigen (CEA), an oncofoetal antigen normally expressed in gut epithelium and found to be expressed in some gastro-intestinal carcinomas has also been investigated for its potential as an immunotherapeutic tumour antigen (Tsang et al. 1995) and reviewed by Hodge (1996).

Many tumours express peptide antigens derived from normal proteins that are mutated in the tumour cells. The best studied of these is p53, the tumour suppressor protein known to control the cell cycle (Lane 1992) in combination with a large, and ever increasing, number of accessory proteins (Agarwal et al. 1998; El-Deiry 1998; McArdle et al. 2000). Approximately 60% of human tumours contain mutations in the p53 gene which lead to its cell cycle control function being compromised (Mowat 1998; Lane 1994; Soussi 1994; Hollstein et al. 1991). Few researchers have

been able to demonstrate immunogenicity of mutated p53 epitopes in-vivo as point mutations can occur at peptide anchor residues thus preventing MHC presentation of those peptide sequences, often these are immunodominant antigens (Theobald et al. 1998; McArdle et al. 2000). However, synthetic analogues of p53 peptides with point mutations have been used to induce CTL able to lyse tumour cells in-vitro (McCarty 1998; Bertholet et al. 1997; Ciernik et al. 1995; Yanuck and Carbone 1993). Significant CTL responses against mutant p53 (Gabrilovich et al. 1995; Ciernik et al. 1996), and anti-p53 antibodies (Soussi 1996) have been demonstrated in breast cancer patients, suggesting that it is possible for an immune response to be generated against mutant p53. However, the appearance of anti-p53 antibodies has been associated with a poor prognosis for patient survival (Schlichtholtz et al. 1992; Peyrat et al. 1995). Studies investigating immunogenicity of wild-type p53 showed that CTL generated to wild type p53 sequences were capable of lysing cell lines expressing the mutant p53 gene. This suggests that the immune response to mutant p53 may depend on the similarity of the mutant and the wild-type proteins (Vierboom and Nijman 1997; Gnjatic 1998; Barfoed et al. 2000).

Other antigens arising from mutations which have been identified as tumour antigens include the cell cycle control proteins ras (Gjertsen et al. 1995) and CDK4 (Wolfel et al. 1995), the apoptosis regulator caspase-8 (Mandruzzato et al. 1997), MUM-1 (Coulie et al. 1995), β-catenin (Robbins et al. 1995), elongation factor-2 (Hogan et al. 1998) and bcr-abl (Bosch et al. 1996)

The bcr-abl antigen arises from the translocation between chromosomes 9 and 22 (Philadelphia chromosome) which results in the production of a fusion protein comprising parts of both the bcr and abl oncogenes. The fusion region of the protein encompasses a novel amino acid residue and thus represents a potential target for tumour-directed immunotherapy. Indeed, it has been demonstrated that peptides derived from this region can generate CTL in-vitro which are capable of lysing tumour cells expressing the bcr-abl protein (Berke et al. 2000; Norbury et al. 2000; Nieda et al. 1998; Osman et al. 1999).

Overexpressed tumour antigens are derived from unmutated (wild-type) proteins which are normally expressed at low levels in many tissues. The two main proteins within this group are HER2/neu and p53. HER2/neu is a transmembrane protein with tyrosine kinase activity which is overexpressed in a number of tumours including, breast, lung and ovarian cancers (Peoples et al. 1995; Yoshino et al.

1994). HER2/neu peptides able to generate CTL responses against ovarian, breast and colon carcinomas have been identified (Fisk et al. 1995; Peoples et al. 1995). Overexpression of wild-type p53 is a characteristic of some human cancers and this phenomenon is thought to be due to inactivation of accessory proteins responsible for the degradation of p53 (Smith 1999; Olivier et al. 1998). Some researchers have documented the generation of anti-wtp53 CTL in-vitro as discussed above. (Gnjatic 1998; Ropke et al. 1996; McArdleet al. 2000; Barfoed et al. 2000; Hurpin et al. 1998). A protein with approximately 50% homology to p53, named p73, has also been described as being overexpressed in some tumours and tumour cell lines (Zaika et al. 1999). Recently, peptide epitopes from the overexpressed tumour antigen mucin (MUC-1), a highly glycosylated transmembrane protein, have been demonstrated as suitable candidates for immunotherapy in-vitro (Brossart et al. 1999).

The aetiological agent of some human tumours are viral, such as Epstein-Barr virus (EBV) in Burkitt Lymphoma, hepatitis B and C virus in liver cancers, human papilloma virus (HPV) in cervical cancers and human T lymphotrophic virus (HTLV) in T cell leukaemias. EBV peptides were shown to be capable of generating specific T cells in healthy patients (Herr et al. 1999) and human papilloma virus E7 antigens have been shown to induce CTL in-vitro (Ressing et al. 1995). Few useful clinical responses have been demonstrated in-vivo (Borysiewicz et al. 1996) suggesting that this area of tumour antigen therapy is still experimental.

The understanding and continued identification of tumour antigens is crucial for the development of vaccine technologies to treat cancer. Researchers have not yet been able to identify tumour antigens for every tumour type, and it is possible that ubiquitous or shared tumour antigens such as p53, HER2/neu and MAGE gene family may be useful in immunotherapy of tumours with as yet uncharacterised antigens.

1.6 Tumour Escape

Numerous studies have reported tumour regression using tumour vaccines in in-vitro and in-vivo models, however responses in clinical patients are variable and vaccine induced CTL are present at low levels, if at all (Kawakami et al. 1994; Colaco 1999). Cytotoxic T lymphocytes able to lyse tumours and antibodies specific to tumour antigens (Soussi 1996) have been detected in some cancer patients thus suggesting that some tumours can be recognised by the immune system. Also, CTL specific for MelanA peptides have been discovered in some non-tumour bearing patients (Chen et al. 1998). However, many tumours grow uninhibited by the patients' immune system and there are several postulated mechanisms which may act in isolation or in combination to allow the tumour to escape recognition by the immune system.

Downregulation of MHC molecules and/or tumour antigens.

Many tumours are known to express low levels of MHC class I molecules (Chouaib 1997; Hicklin et al. 1999; Pawelec et al. 1997) and are therefore less immunogenic, expressing fewer peptides on the cell surface and thus fewer targets for T cells. Some form of HLA loss has been estimated to occur in 90% of tumour cells in established tumours, but each tumour displays a different level of downregulation (Nawrocki and Mackiewicz, 1999). The HLA downregulation in primary tumours is highly variable (Hicklin et al. 1999) ranging from 16% (melanoma) to 50% (prostate). This relatively low level of MHC downregulation in melanoma may explain why it is one of the most immunogenic tumours in humans (Schreurs et al.1998).

Several distinct phenotypes of HLA downregulation exist and have been characterised in both cell lines and fresh tumours. Total loss of HLA (Giacomini 1999), allelic HLA loss, e.g. HLA A*0201(Marincola et al. 1994) or locus downregulation, for example loss of all HLA-B molecules (Marincola et al. 1994) have been seen in tumours, with some demonstrating multiple phenotypes. This suggests that it is a complex phenomenon resulting from a series of mutations occurring over the course of disease progression (Hicklin et al. 1999). Deletion of specific HLA alleles can result in loss of the immunodominant tumour antigen and thus the tumour may escape immune surveillance. This could, however, lead to the presentation of less dominant or hitherto silent antigens (Chen

1998) and the potential for tumour recognition as shown by Lehmann et al (1995). Some studies have suggested that these HLA loss variants may be more susceptible to natural killer cells as inhibitory HLAs (killer inhibitory receptors; KIRs) have been removed (Ikeda et al. 1997; Rees and Mian 1999).

The molecular basis of HLA downregulation is attributed to mutations or abnormal regulation of the HLA genes located on chromosome 6 (Maeurer et al. 1996) leading to defective antigen processing. (Restifo et al. 1993) described the downregulation of proteins required for antigen processing. The proteosome subunits, LMP2 and LMP7, and the peptide transport proteins TAP1 and TAP2 are all essential for processing and presentation of peptide antigens. Their loss results in a low level of surface HLA due to the reduction in the supply of peptides (Chen et al. 1996). However, the most common molecular basis for total HLA class I loss is mutations in the β_2-microglobulin gene leading to loss of β_2-microglobulin expression and thus stable MHC cannot be expressed on the surface (Chen et al. 1996; Hicklin et al. 1999).

Tumour antigen downregulation, as shown in melanoma (SlingluffJr et al. 2000), is a distinct phenomenon from HLA downregulation. Melanoma cells not senstitive to lysis by T cells specific for the melanocytic differentiation proteins (MDP; MelanA, tyrosinase, gp100) were demonstrated to have HLA expression comparable to sensitive cell lines. Antigen processing was also seen to be functional, however immunohistochemical staining showed that the MDP were not expressed by these cell lines despite being originally obtained from metastatic melanomas which had previously expressed MDP.

Downregulation of surface molecules is not restricted to tumour cells. Tumour infiltrating DCs have been found to lack costimulatory molecules such as B7 (Boxhorn et al. 1998; Farzeneh et al. 1998) thus rendering them unable to provide the second signal of activation and stimulate anti-tumour immunity. Through the use of engineered APCs containing costimulatory molecules, successful T cell responses can be induced against certain antigens thus demonstrating that costimulation is an important component of the anti-tumour immune response (Bellone 1997; Boxhorn et al. 1998; Farzeneh et al. 1998; Schoenberger et al. 1998).

Tumours themselves are able to produce certain immunosuppressive factors such as the cytokines IL-10 (Czarniecki 1988; Chouaib 1997) and TGFβ (Whiteside and Rabinowich 1998). These cytokines are responsible for diverting the immune response away from a CTL to an antibody response (Th1 to Th2) by suppressing the CTL/Th1 mediators. IL-10 in particular is able to prevent DC differentiation and maturation thus preventing CTL activation (Girolomoni and Ricciardi-Castagnoli 1997).

Direct inhibition of T cells can also occur by the binding of tumour cell surface CD58 (LFA-3) to CD2 on the T cell thus interfering with signal transduction. Tumour secreted CD54 (ICAM-1) can also interfere with T cell-tumour cell interactions (Becker et al. 1993) and has been found to correlate with disease progression (Grothey et al. 1998). Lauritzsen et al (1998) demonstrated that T cells responding to tumour-secreted or shed antigens were deleted as if they were reacting against self proteins.

Anergic T cells – resulting from lack of secondary signal following antigen specific (primary signal) activation - are present in the tumour cell environment. These cells are able to prevent proliferation of non-anergic T cells specific for the same antigen by binding to the APC expressing their common tumour antigen (Taams et al. 1998). In addition, the Fas/Fas ligand interaction has received much attention in the study of tumour escape mechanisms. Fas receptor is widely expressed on immune cells enabling programmed cell death (apoptosis) following Fas ligand binding. This interaction is important in immune homeostatic circumstances such as positive/negative selection, control of autoimmunity and in failure of DNA repair (section 1.2, Machius et al. 1999; Nawrocki and Mackiewicz 1999). Since it is a normal physiological response, the immune system is not alerted to the apoptotic cell, thus it is a form of 'silent' cell death. Expression of Fas ligand (FasL) on tumour cells has been demonstrated in a number of tumours (Strand et al. 1996; Niehans et al. 1997; O'Connell 1997) and therefore the tumour cell has the ability to silently kill the T cell and thus escape immune surveillance (Chappel and Restifo 1998; Whiteside and Rabinowich 1998)

Original observations by Mizoguchi et al in tumour bearing mice (1992) showed that some tumours also displayed a downregulation of proteins involved in the T cell activation cascade (see section

1.2). Studies have shown that tumour infiltrating lymphocytes (TIL) have reduced or undetectable levels of two intracellular domains of the TCR; $p56^{lck}$ and $p59^{fyn}$. Absence or low expression of these proteins would prevent signalling through the TCR and thus affect T cell activation. A significantly lower five year survival rate is seen in head and neck carcinoma patients with loss of the TCR ξ chain from their TIL (Whiteside 1999). Research in this area has demonstrated these findings in a number of other tumours; renal cell carcinoma (Finke et al. 1993), colorectal carcinoma (Nakagomi et al. 1993), lung (Niehans et al. 1997), melanoma, ovarian and gastric (Pawelec et al. 1997; Nawrocki and Mackiewicz 1999).

Immunological "ignorance" has been suggested as another explanation of tumour escape, particularly in the context of solid tumours. Zinkernagel's group (Ochsenbein et al. 1999) showed that injection of single cell suspensions of tumour cells were able to induce an immune response in-vivo, but implantation of the same tumour cells as a solid tumour did not. This was correlated with the failure of the solid tumour cells to reach the draining lymph nodes and thus not being recognised by T cells. The tumour is thus "ignored" and is able to grow unchecked. Immunisation or natural in-vivo responses occurring at a late stage in tumour development, ie when the tumour is fairly large and cells are proliferating rapidly, are inefficient at mediating tumour rejection. The researchers showed, however, that a sustained T cell response, as induced by several immunisations, was able to reduce tumour size.

Tumour escape mechanisms are considered as a major barrier against successful immunotherapy (Pawelec et al. In Press). The understanding of the molecular basis underlying these escape mechanisms may make possible the design of vaccine strategies able to circumvent them thus allowing immune recognition of tumours.

1.7 Vaccine Technology

The theory of vaccination was first proven in the 18th century by Edward Jenner. Through inoculation of humans with vaccinia, he was able to protect them against smallpox, often a fatal disease at that time. He named this treatment vaccination, and it is still used today to prophylactically treat many diseases. The potential to inject exogenous proteins into patients as a form of tumour therapy was first shown by William Coley in the 19th century when he used streptococcal cultures (Coley's toxins – now thought to act as adjuvant) to treat tumours (Coley 1894). Research still continues today to enable vaccination strategies to be applied to the active specific immunotherapy of cancer.

There has been much study into an appropriate medium to present such a vaccine to the immune system. The development of immunisation protocols using tumour lysate, whole tumour cells, DNA encoding tumour antigens, HLA-restricted peptides originating from a tumour antigen and more recently, dendritic cells pulsed with peptides or expressing tumour antigens has gained much momentum.

1.7.1 Whole cell vaccines

Whole tumour cells, injected alone, with adjuvant or transduced with viruses, costimulatory molecules or cytokines have been investigated for their ability to act as an anti-tumour vaccine.

Early work by Morton (1992) using allogeneic melanoma cell lines as a vaccine (polyvalent vaccine) provided a foundation upon which recent whole cell vaccine strategies have been based.

Several clinical trials evaluating the survival advantage following treatment with cellular based vaccines are currently being carried out. Allogeneic tumour cell lines or autologous tumour cells are administered with a variety of adjuvants such as BCG (Hsueh et al. 1998; Hayashi et al. 1993; (Hoover et al. 1993), RIBI (Melacine®), Melacine® with TNFα, dinitrophenol (Mitchell and Eschen 1997; Herlyn and Birebent 1999) DETOX® (Reddish 1998), vaccinia (Sivanandham et al. 1998; Wallack et al. 1998) or, most recently, hapten modified autologous tumour cells (Avax Technologies) (Reuters 2000). Many are in clinical trials (Featherstone 1996; Ollila 1998), (Sivanandham et al. 1998; Reuters 2000), but so far despite some vaccines providing prolonged

relapse-free survival (Mitchell and Eschen 1997), no vaccine has demonstrated a definite overall survival rate in Phase III randomised clinical trials (Weber 2000).

1.7.2 Cytokine transduced whole cell vaccines

Difficulties in obtaining sufficient autologous tumour cells for treatment and in establishing cell lines from tumour material have meant that research has been ongoing into improving the immunogenicity of freshly isolated tumour cells or existing tumour cell lines. Several approaches using tumour cells or antigen presenting cells engineered to express cytokine genes, co-stimulatory molecules or tumour antigens are in various stages of pre-clinical or clinical trials.

Transduction of cytokine genes such as IL-2 (Gansbacher et al. 1990), IFNγ (Wantanabe et al. 1989), IL-4 (Li et al. 1990), TNFα (Asher et al. 1991)), GM-CSF (Dranoff et al. 1993; Ali et al. 2000; Nagai et al. 1998; Chiodoni et al. 1999), and IL-6 (Porgador et al. 1992), into tumour cells and evaluation of these vaccines in animal models has shown that tumours resulting from these engineered tumour cells can be rejected and that protection against subsequent challenge with the parental, unmodified tumour can be achieved (Dranoff et al. 1993).

Following original work by Dranoff et al, (1993), Ali et al (2000) demonstrated the use of a viral vector (disabled single cycle – herpes simplex virus - DISC-HSV) to transduce various cytokine genes to tumour cells and the application of these cells in prophylactic immunisation in a murine model. Tumour progression was prevented in approximately 80% of mice immunised with DISC-HSV-GM-CSF vaccines and the response was found to be reliant upon both CD4 and CD8-dependent immunity. Nagai et al, (Nagai et al. 1998), via immunisation of mice with GM-CSF expressing tumour cells were able to show 100% tumour free survival upon challenge with the parental cell line and rejection of a pre-existing tumour in 60% of animals. The studies discussed above have shown GM-CSF as the cytokine most able to demonstrate immunogenicity in murine models. Whether this finding can be extrapolated to human patients is still under investigation. Some studies (Soiffer et al. 1998; Simons et al. 1997; Qin and Chatterjee 1996) showed that treatment of human patients with GM-CSF gave some clinical responses, also Palmer et al (1999) demonstrated that IL-2 treatment enabled some anti-tumour responses to develop. However, these treatments are still experimental in human models.

1.7.3 Whole cell vaccines incorporating co-stimulatory genes.

It is generally accepted that a costimulatory signal delivered by the interaction between B7 on the target cell (or antigen presenting cell) and CD28 on the T cell is essential for induction of immunity. Many tumour cells express little or no B7 and this is thought to be one of the main ways in which a tumour can evade the immune response (see tumour escape section 1.6).

Investigations into the importance of the B7/CD28 interaction in anti-tumour immunity have been carried out and some researchers have developed strategies to improve cellular vaccine potency by transducing tumour cells or antigen presenting cells with co-stimulatory molecules (Chamberlain et al. 1996; Hodge et al. 1994; Sivanandham et al. 1998). Sivanandham et al (1998), using a vaccinia virus construct, transduced the B7-1 gene into CC-36 (colon carcinoma) cells and used these cells to vaccinate mice prior to tumour challenge. The vaccinated mice had significantly less tumour growth than control animals or those vaccinated with IL-2 transduced CC-36 cells. However, when used in therapy, against pre-implanted tumours, the B7-1 vaccine did not significantly improve survival when compared to untreated animals. Augmentation of this vaccine with cytokines such as IL-2 or IL-12 was suggested by the authors and other researchers (Rao et al. 1996; Putzer et al. 1997) as indicated by preliminary work mentioned in this study. Boxhorn et al (1998), in a similar study, did not find that administration of IL-12 improved the response to the B7-1 vaccine despite obtaining similar results with the efficacy of the B7-1 treatment.

The difficulties in obtaining allogeneic or autologous tumour cells for each patient has caused researchers to investigate ways in which engineered whole cell vaccines can be applied to treat any tumour. Bellone et al (1994), using engineered tumour cells as APCs (B7-1 transfected RMA-S cells) were able to induce antigen specific CTLs against synthetic peptides pulsed onto the surface of the RMA-S cells. The RMA-S cells are defective in antigen processing and presentation. When exogenous peptide is added and the cells cultured at low temperatures, MHC molecules are expressed and the peptides can bind thus causing the MHC to be stably expressed on the surface (Bellone et al. 1994; Mandelboim et al. 1994). In this study, ovalbumin peptides were used as a model antigen and antigen specific CTLs induced against synthetic peptides were able to lyse target cells pulsed with the ovalbumin peptide. Furthermore, CTLs induced against natural peptides acid

extracted from B16F1 melanoma cells and pulsed onto RMA-S cells were able to specifically lyse B16F1 cells but not RMA-S cells.

This technique represents a "midway" point between whole cell and peptide specific vaccines, encompassing the heterogeneity and immunogenicity of whole cell vaccines whilst enabling application to a specific tumour. However, this approach is still as labour intensive as whole cell vaccine technology. Research is now progressing further towards tumour-antigen peptide-specific vaccination to allow more easily applicable and practical forms of immunotherapy to be developed.

1.7.4 Tumour antigen-specific vaccines

Prediction of MHC-binding peptides from tumour antigen proteins is facilitated by computer algorithms such as BIMAS (http://bimas.dcrt.nih.gov/molbio/hla-bind/, Parker et al. 1994) and Syfpeithi (www.uni-tuebingen.de/kxi, Rammensee et al. 1997). The prediction is based upon the preference for certain amino acids to bind at anchor residues within the peptide binding groove of MHC molecules (see section 1.3). The algorithm scans a protein sequence for, in MHC class I, 9 amino acid length peptides conforming to the preferences for a given HLA type. Peptides are ranked in order of those most likely to bind to the MHC, thus enabling the prediction of the peptides most likely to be responsible for induction of an antigen specific CTL response. However, in practice it is not always the high affinity binding peptides which will be most able to induce an immune response. Indeed, high affinity binding peptides may induce anergy in the T cells they bind to (Maffei and Harris 1998) and thus moderate or lower affinity peptides may be more relevant in inducing active specific CTL. Research into CML and the bcr-abl fusion protein has shown a moderate-low affinity peptide (ranked 7 of 20 with intermediate binding (Bocchia et al. 1995) is able to induce specific CTL able to lyse CML patient cells in-vitro (Bocchia et al. 1996; Nieda et al. 1998; Osman et al. 1999; Norbury et al. 2000). In contrast, the top ranked peptide for p53 (HLA-A2 restricted) has been shown to induce CTL in in-vitro mouse and human models, (Theobald et al. 1997; Berke et al. 2000; Gnjatic 1998; McArdle et al.2000) and in-vivo mouse models (Theobald et al. 1997). Following prediction, the peptide epitopes can then be used as immunogenic targets in

vaccination strategies either using synthetic peptides or by gene delivery of the epitope (Toes et al. 1997; Chen et al. 1998; Iwasaki and Barber 1998).

The main drawback with tumour antigen-specific vaccines is in designing a delivery method to optimise the immunogenicity of the antigen/peptide and many approaches have been used with varying success. Perhaps the most applicable tumour-specific antigen vaccines would be to use synthetic analogues of MHC-restricted peptide epitopes. One of the earliest studies using synthetic peptides involved intradermal vaccination of melanoma patients with a mixture of melanoma-associated class-I peptide epitopes (Jager et al. 1996a). Despite inducing immunogenicity as DTH reactions and peptide-specific CTL, no clinical tumour regression occurred. This study highlighted the problems with peptide vaccination, in that peptides are poor immunogens (Bona et al. 1998; Herlyn and Birebent 1999; Pawelec et al. 1999). A follow up study by the same group (Jager et al. 1996b) administering GM-CSF as an adjuvant to the peptides demonstrated that tumour regression could be achieved using peptide immunisation. Schmidt et al (1997), vaccinating mice using peptide alone were unable to protect mice from tumour growth upon tumour challenge or demonstrate tumour regression. However, upon addition of poly-L-lysine as an adjuvant achieved a level of protection and approximating that achieved by use of GM-CSF secreting vaccines. Rivoltini et al (1999) investigated a method to increase the immunogenicity of naturally restricted peptides. Substitution of certain amino acids within a 9mer MHC-restricted peptide did not alter the binding affinity to the MHC, but seemed to act as a superagonist, generating high avidity CTL. Superagonist-stimulated CTL were able to recognise both the unmutated and mutated forms of the peptide at lower concentrations than CTL stimulated by the original, unaltered peptide. Use of similar methodology for Ras peptides has shown that it is possible to obtain clinical regression (both CD4 and CD8 mediated in this case) when treating patients with these mutated peptides (Gjertsen et al. 1997). These results were, however, greatly improved upon use of GM-CSF (Gjertsen 1998), and Abrams et al (1997) found ras peptides administered with DETOX adjuvant were able to generate both CD4 and CD8 ras peptide-specific T cells. Further investigation into immunisation with peptide/adjuvant combinations such as gp100 with IFA (Salgaller et al. 1996), IL-2 (Rosenberg 1998) and IL-12 (Noguchi et al. 1996), is continuing to generate interest.

The use of synthetic peptides in vaccines has also been associated with difficulties. Documentation of autoimmune reactions (Nestle et al. 1998) has meant that some researchers have moved towards the use of natural peptides, extracted from tumour cells. Tumour cells, engineered APC or dendritic cells have been pulsed with total tumour cell lysate (Nestle et al. 1998; Protti et al. 1996; Nair et al. 1997; Imro et al. 1999) or with surface peptides acid extracted from tumour cells (Nair et al. 1997) and then used as a vaccine in animal models. This technology will be discussed further below in relation to dendritic cells and in chapter 3.

1.7.5 Dendritic cell based vaccines

Current evidence suggests that both a CD4 and CD8 response may be required for tumour rejection (Ali et al. 2000; Chaux et al. 1999; Mancini et al. 1999; Bona et al. 1998; Farzeneh et al. 1998; Girolomoni and Ricciardi-Castagnoli 1997; Hermans et al. 1998; Lasarte et al. 1992; Morse and Lyerly 1998; Herlyn and Birebent 1999). In order to involve both types of effector lymphocytes, efficient antigen presentation and appropriate co-stimulation is essential. Since dendritic cells express MHC class I, class II and co-stimulatory molecules (B7 and CD40) they represent an ideal delivery system for tumour antigen based immunotherapy. Indeed, these co-stimulatory molecules have been shown to be essential to induction of anti-tumour immunity by vaccine-based therapies (Zitvogel et al. 1996; Diehl et al. 1999)

The use of dendritic cells in an adjuvant-like manner pulsed with synthetic peptides (Mayordomo et al. 1995) showed that rejection of established murine lung tumours could be obtained. A further study by the same group showed that pulsing DC with p53 peptides generated CTL capable of lysing a variety of tumours expressing p53, thus suggesting that this therapy can be applied to several tumours with a shared tumour antigen (Mayordomo et al. 1996). The effectiveness of peptide-pulsed DC-based therapy in murine models has been demonstrated by other groups both in therapy and prophylaxis, even against the weakly immunogenic tumour B16 (Zitvogel et al. 1996; Celluzzi et al. 1996). Equivalent studies in human melanoma patients have shown that this type of vaccine is safe, well tolerated and easy to administer (Nestle et al. 1998; Chakraborty et al. 1998).

Clinical responses are variable, but some regression of tumours, including metastases was observed in both studies.

The use of DC in a manner similar to the whole cell vaccine strategy discussed above has also been researched. Perez-Diez et al (1998) showed that DC engineered to express a whole tumour antigen, MelanA/MART1, were able to induce both CD4 and CD8 anti-tumour responses in an in-vivo mouse model. The immunisation with a tumour cell-dendritic cell fusion (Gong 1997) is nearest to the whole cell vaccine strategy discussed above. Following mucin-1 transfection into tumour cells and fusion with dendritic cells, mucin-1 expressing transgenic mice were immunised with the fused cells (Gong et al. 1998). Upon challenge with parental or mucin-1 transfected cells the mice remained tumour free. CTL collected from the mice and assayed for cytotoxicity demonstrated above 35% cytotoxicity at a 100:1 (effector:target) ratio. Serum samples also showed the presence of anti-mucin antibodies, thus demonstrating the requirement and ability to achieve both cell-mediated and humoral immunity.

At present, the clinical use of these vaccines is experimental. However, studies to date demonstrate that the approaches being investigated are effective and feasible. It is possible that augmentation of DC vaccines with other adjuvants (for example, IL-12) could further improve the effectiveness of these vaccines (Gabrilovich et al. 1996).

1.8 Aims of study

Whilst ensuring that the functioning of the immune system, ability of tumours to evade immunsurveillance and the need for adjuvant are considered when designing a cancer vaccine, the primary goal should be to isolate and identify target antigens towards which the immunotherapy is directed. This study was concerned with the isolation and identification of the antigens expressed on tumour cells with a view to their inclusion within a cancer vaccine. The fulfillment of the study aim was dependent on the achievement of the following goals:

- The standardisation of a protocol to isolate tumour cell surface expressed peptides (mild acid elution technique).

- Purification of peptide-containing eluate via removal of contaminant proteins and buffer salts.

- Concentration of peptide-containing eluate using column chromatography (HPLC).

- Sequencing of isolated peptides using electrospray-mass spectrometry.

- Identification of the protein origin of the peptide sequence using protein sequence databases.

- Application of standardised procedure to cell lines and/or clinical patient material expressing tumour antigens of interest.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General laboratory consumables and equipment

a) Glassware (storage)

Soaked and washed in Presept (Johnson and Johnson), rinsed in tap water then soaked in distilled water, left to dry overnight and autoclaved before use. Glassware used to store peptides was then washed in chromic acid (potassium dichromate - Sigma), 1M dissolved in distilled water) and coated with Sigmacote (Sigma) to prevent peptide binding to charged groups on glass.

b) Plastic consumables

'Universal' tubes (30ml)	Bibby Sterilin
Polypropylene tubes (15, 50ml)	Sarstedt
FACS tubes	Elkay
Cryovials (1.5ml)	Helena Biosciences
Centrifuge tubes (50ml)	Sarstedt
Eppendorfs (250μl, 1.5ml)	SLS
25, 75, 150, 500cm^2 tissue culture flasks	Nunc
6-, 24-, 96-well plates	Nunc
96-well flexi-plates	Falcon
Disposable pipette tips (5-1000μl)	Sarstedt
Disposable pipette tips (0.5-10μl)	SLS
Plastic pasteur pipettes	SLS
1, 2, 5, 25ml pipettes	Helena Biosciences
10ml pipettes	SLS
Latex examination gloves	Maxxim Medical

c) Equipment

Centrifuges	Beckman Avanti
Sonic bath	Semat
Microscopes	Weiss
Gamma counter	Canberra Packard

Flow Cytometer (at QMC, Nottingham)	Coulter
HPLC pumps	Milton Roy, ConstaMetric 300
HPLC columns	Phenomenex
Mass spectrometer (Quadrupole ion-trap)	Finnigan MAT

2.1.2 Tissue Culture

a) Table 2.1 Cell lines

Cell line name	Type	Source	Culture Media
CT26	Mouse colon carcinoma	Richard Vile, Hammersmith Hospital	Dulbecco/10% FCS
CT26.cl25	As above, transfected with β-galactosidase	Richard Vile, Hammersmith Hospital	As above, with 500μg/ml G418
FM3	Melanoma	J. Zeuthen, Danish Cancer Institute, Copenhagen	Dulbecco/10% FCS
GERL 3.1	Melanoma	Ludwig Institute, Brussels	Iscoves/10% FCS
HB54	Hybridoma	ATCC	RPMI/10%FCS 2mM glutamine
K562	Lymphoblastoid	J. Zeuthen, Danish Cancer Institute, Copenhagen	RPMI/10%FCS 2mM glutamine
K562-A3	As above, transfected with HLA-A3	A. Dodi, Anthony Nolan Trust, Royal Free Hospital	As above, with 500μg/ml G418
MCF-7	Breast carcinoma	ICRF, London	Dulbecco/10% FCS

SaOs-2	Osteosarcoma	Clinical Biochemistry, University of Sheffield	Dulbecco/10% FCS
SaOs-2 175, 273, vector	As above, transfected with mutant p53	Stephanie McArdle, University of Sheffield	As above, with 500μg/ml G418
T2	Lymphoblastoid	Paterson Institute, Manchester	RPMI/10%FCS /glutamine
T2-A3	As above, transfected with HLA-A3	Pathology, University of Iowa	As above, with 500μg hygromycin
W6/32	Hybridoma	ECACC	RPMI/10%FCS /glutamine

b) Growth media and supplements

Dulbecco's Modified Eagle Medium (DMEM)	Gibco
RPMI medium	Gibco
Iscoves (DMEM, HEPES, glucose, glutamine)	all Gibco
X-Vivo 15 (DC/CTL generation)	Bio-Whittaker
Foetal calf serum (FCS)	Gibco

c) Chemicals/Solutions used for tissue culture

Trypsin	Gibco
Versene	Gibco
Phosphate buffered saline (PBS) (1 tablet dissolved per 100ml water)	Oxoid
Trypan Blue (diluted to 0.1% solution with sterile PBS)	Gibco
White cell count fluid (0.2% acetic acid)	BDH

2.1.3 Flow Cytometry and cellular characterisation

a) Table 2.2 Antibodies

Antibody	Specificity	Amount used	Source
YTH470.5	β2-microglobulin protein	25μl of 1:100 dilution	Serotec
7C6	bcr-abl protein	50μl neat	Abcam
NA1/34	CD1a	10μl neat	Serotec
RPA-T4	CD4	10μl neat	Serotec
LT8	CD8	10μl neat	Serotec
FMC63	CD19 (PE conjugated)	10μl neat	Serotec
BY63	CD86 (B7.2) (PE conjugated)	10μl neat	Serotec
DO7	Mutant and wild-type p53 protein	5μl of 1:10 dilution	Dr. G. Thompson Univ. of Nottingham
HB-54	HLA-A2	25μl neat supernatant	ECACC
MHC I	HLA-A, -B, -C		Serotec
MHC II	HLA-DP, DQ, DR (FITC conjugated)	20μl neat	Pharmingen
W6-32	HLA-A, -B, -C	25μl neat supernatant	ECACC
HC10	MHC class I heavy chain	100μl neat supernatant	H. Ploegh (hybridoma) Purified - A. Dodi
FITC	Anti-mouse secondary	25μl 1:128 dilution	Sigma
FITC	Anti-rat secondary	25μl 1:60 dilution	Sigma
PE	Anti-mouse secondary	25μl 1:20 dilution	Terra Nova

Incomplete (serum/additive free) RPMI used to wash cells between staining steps

All antibodies diluted in incomplete RPMI

b) Intracellular staining solutions (all reagents from Sigma)

PBA: PBS, 0.5% BSA, 0.1% sodium azide

PBA + 10% FCS

Saponin Buffer: PBA, 50mM D-glucose, 0.04% saponin

Fixative: sodium chloride 6.38g

boric acid 1g

EDTA-2K 0.2g

sodium tetraborate 0.2g

dissolve above in 1 litre 0.5% formaldehyde

c) X-gal staining for presence of β-galactosidase

0.1M sodium phosphate buffer pH7:

Na_2HPO_4 1.78g

NaH_2PO_4 1.38g

Each salt dissolved in 100ml and added together to make 100ml pH7 buffer;

Na_2HPO_4 63ml

NaH_2PO_4 37ml

Fixative: 0.1M sodium phosphate buffer pH7

Magnesium chloride 1mM

Glutaraldehyde 0.25%

X-gal: X-gal (Sigma) 20mg/ml stock diluted to 0.2% in dimethylformamide (DMF)

DMF/X-gal solution diluted 1:10 in 100ml X-gal buffer (filtered before use)

X-gal buffer: (reagents from Sigma)

1mM Magnesium chloride (20mg)

150mM Sodium chloride (870mg)

3.3mM $K_4FE(CN)_6.3H_2O$ (139mg)

3.3mM $K_3FE(CN)_6$ (100mg)

60mM Na_2HPO_4 (852mg)

40mM NaH_2PO_4 (552mg)

2.1.4 Acid elution

All reagents used were of high analytical grade. Water was ultrapure (distilled, deionised) dispensed from an Elga filter unit.

Citrate phosphate buffer: 0.131M citric acid (BDH), 2.52g

0.066M sodium phosphate (Sigma) 0.94g

dissolve above in 100ml water and pH to 3.3 with 10M sodium hydroxide (Sigma)

2.1.5 Slot-Blot

Flowgen slot-blot apparatus

Vacuum pump attached to tap

Nitrocellulose membrane (Fisher Scientific)

β_2-microglobulin protein (Sigma)

Primary antibody: Rat antibody to human β_2-microglobulin 1:100

Secondary antibody: Goat anti-rat IgG alkaline phosphate conjugate (Sigma, used at 1:1000)

Detection: nitroblue tetrazolium (NBT) 15mg/ml

(both Bio-Rad) 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) 30mg/ml

Development (alkaline phosphate buffer): 0.1M Tris HCl

(Reagents from Sigma) 0.1M sodium chloride

0.005M magnesium chloride

Solution pH altered to 9.5, 10ml used per blot with 50µl each of NBT and BCIP.

2.1.6 Antibody purification

Protein A sepharose column (Amersham Pharmacia Biotech)

Phosphate buffer pH7 (0.1M) (page 51)

Elution Buffer

Citric Acid pH4 (0.1M)

Neutralising Buffer

Tris pH9 (1M)

2.1.7 Mini-gel for HC10 purity (all reagents Sigma)

12.5% Resolving Gel (for 2 gels)

2120µl Accugel (Acrylamide stock)

2120µl 1.5M Tris Buffer

2540µl ultrapure water

60µl ammonium persulphate (APS – stock concentration 100mg/ml)

6µl TEMED

3.4% Stacking Gel (for 2 gels)

660µl Accugel

1380µl 0.5M tris pH 6.8

3460µl ultrapure water

60µl APS

6µl TEMED

Running Buffer (10x)

30.27g Trizma base (0.25M)

150.14g Glycine (2.0M)

10.0g SDS (1%)

1 litre ultrapure water

Reducing Buffer

2.5ml 3.4% stacking gel buffer

2ml glycerol

400mg SDS

200mg DTT

15.5ml ultrapure water

few grains bromophenol blue

Coomassie Blue stain (All reagents Fisher)

0.5g Coomassie Blue R-250

150ml methanol

50ml acetic acid

300ml ultrapure water

Destain

100ml glacial acetic acid

300ml methanol

600ml ultrapure water

2.1.8 Biochemical clean-up and separation strategies

a) Metal Chelate: 1ml HiTrap sepharose chelating column (Amersham Pharmacia Biotech)

all reagents from Sigma except hydrochloric acid from Fisher

Metal Ion: Copper (II) sulphate 1mg/ml

Wash buffer: 0.1M sodium phosphate buffer pH7

Sample buffer: 0.1M sodium phosphate buffer pH7

Elution buffer: 0.5-0.1mM hydrochloric acid

b) TCA precipitation: 72% trichloroacetic acid (TCA)

72g TCA (Fisher Scientific) added per 100ml water, 100µl of this was added per ml sample prior to centrifugation to precipitate proteins.

c) Cation Exchange: 1ml S (sulphonyl) column (Bio-Rad)

30 ml 0.1M hydrochloric acid (Sigma)

3ml 0.1M sodium hydroxide (Sigma)

pH paper (Whatman)

d) HPLC: acetonitrile (BDH)

Glacial acetic acid (Fisher Scientific)

Trifluoroacetic acid (TFA - Sigma)

e) Standard peptides: H2-K^D-restricted gp70 peptide (SPSYVYHQF)

H2-K^D-restricted β-galactosidase peptide (TPHPARIGL)

HLA-A2-restricted p53 peptide (LLGRNSFEV)

HLA-DR-restricted hepatitis B peptide (TPPAYRPPNAPIL)

HLA-A3 restricted bcr-abl peptide (KQSSKALQR)

HLA-B8-restricted bcr-abl peptide (GFKQSSKAL)

All peptides synthesised by John Keyte at Queens Medical Centre, Nottingham, UK.

f) Tryptic digest of BSA

BSA	1mg/ml (Sigma)
Trypsin (Type IIS)	1μg/μl (Sigma) in 0.1M sodium phosphate buffer pH7

2.1.9 Immunological assays

a) Blood separation: Leucopacks provided by the British Transfusion Service, Sheffield, UK

Ficoll-Hypaque (Amersham Pharmacia Biotech)

b) Cytokines:

	specific activity	supplier
IL-2	$1.8x10^7$ U/ml	Chiron Technologies
IL-4	$1.0x10^8$ U/mg	R+D Systems
IL-7	$2.5x10^6$ U/mg	R+D Systems
GM-CSF	$8.19x10^7$U/mg	Immunex
TNF-α	$5x10^7$ U/ml	Boehringer Ingelheim

c) Proteins and peptides: β_2-microglobulin (Sigma)

HLA-A2 restricted tyrosinase peptide (YMDGTMSQV)

HLA-A2 restricted gp100 peptide (KTWGQYWQV)

HLA-A3 restricted bcr-abl peptide (KQSSKALQR)

HLA-A3 restricted p53 peptide (RMPEAAPPV)

d) CD4 depletion: CD4 dynabeads (Dynal)

e) Cytotoxicity assay Chromium (^{51}Cr – Amersham, 1.85 MBq added to each target cell population).

2.2 Methods

2.2.1 Acid Elution

Confluent cells were washed twice with serum-free RPMI and following aspiration of all remaining liquid, citrate phosphate buffer at pH 3.3 was added to the cells. Adherent cells were washed whilst remaining adherent to the tissue culture flask and suspension cell lines were centrifuged (1200 rpm for three minutes) prior to elution, the cell pellet then being resuspended in the acid. Following the acid wash, the citrate phosphate buffer was removed to a silanised glass bottle and the cells neutralised by the addition of serum-containing medium. The peptide containing-citrate phosphate buffer was stored at –80°C until required.

2.2.1.1 Standardisation of acid elution technique

To investigate the length of acid wash required to remove peptide, whether cells could be re-eluted and viability of cells following elution, the above technique was performed for varying lengths of time and MHC loss investigated by flow cytometry.

2.2.2 Flow cytometry

$2x10^5$ cells in serum free medium were added to a 2 ml centrifuge tube and washed twice more with serum free RPMI media. The cells were centrifuged to remove the medium (1200 rpm for three minutes) and then resuspended in 25µl of the primary antibody and incubated for thirty minutes on ice.The cells were then washed three times in serum free medium and resuspended in 50µl secondary antibody (FITC conjugated) and incubated for thirty minutes on ice. The cells were washed three times and finally resuspended in 500µl fixative fluid and stored at 4°C until analysis. Flow cytometric analyses were kindly performed by Dr. R.A. Robins and Alison Galvin (Department of Immunology) at Queens Medical Centre, Nottingham, UK.

2.2.3 Intracellular staining

10^5 cells resuspended in serum free media were added to a 2 ml centrifuge tube. The cells were centrifuged at 1200rpm for three minutes to remove the media. 1 ml of fixative was then added, the cells resuspended and stored at $4^{O}C$ overnight. The cells were centrifuged the next day to remove the formaldehyde and washed in PBA (1200rpm/3minutes). The washing process was repeated with saponin buffer and saponin/FCS, the cells were then incubated in the primary antibody (anti-p53) for 1 hour in the dark at $4^{O}C$. Following two washes with 1 ml saponin/FCS, the cells were incubated with the secondary antibody (FITC conjugated) for 25 minutes again in the dark and on ice.

The cells were then washed three times each with 1 ml of saponin buffer and finally resuspended in PBA and stored in the fridge until analysis by flow cytometry.

2.2.4 X-gal staining for β-galactosidase

The use of β-galactosidase as a selectable marker in transfection techniques is widespread. The β-galactosidase gene is cloned into a plasmid containing a gene of interest (such as p53 or HLA-A2 which is to be transfected into a cell line). Following transfection, cells which have been successfully transfected can be identified using X-gal staining. X-gal is a yellow substrate, which, in the presence of the β-galactosidase enzyme, is converted into a blue compound. Cells expressing β-galactosidase are thus, following incubation with X-gal are coloured blue thus enabling positive clones to be identified.

CT26.cl25 (transfected with β-galactosidase DNA) were plated in 6 well plates at $1x10^5$ and left overnight in serum containing medium to adhere to the plastic. The cells were then washed gently three times in PBS and 1 ml glutaraldehyde fixative was added. The cells were left at room temperature in the fixative for 15 minutes, following which it was removed and the cells washed three times in PBS as before. X-gal is dissolved in dimethylformamide (20mg/ml) and then filtered prior to further dilution (1:10 in PBS). The diluted X-gal solution (2.5ml) was added to the adherent CT26.cl25 cells and left overnight at $37^{O}C$, removed and the cells washed gently in PBS prior to examination and photography of the plate.

2.2.5 Slot-Blot for β2-microglobulin detection

Following elution, the acid was collected and tested in a slot-blot for the presence of β_2 – microglobulin as would be expected if MHC destabilisation had occurred. The test acted as a confirmation of the flow cytometric data obtained following elution.

The slot-blot (Flowgen) consists of three plastic blocks. The bottom block allows the connection of a vacuum to facilitate concentration of samples onto a nitrocellulose membrane located on top of the middle block. Filter paper (Whatman) cut to the exact size of the middle block was placed underneath the pre-soaked (in distilled water) nitrocellulose membrane to improve the vacuum. The top layer consisted of three rows of sixteen slots allowing 200μl of sample to be introduced at one time. A vacuum was applied to the samples allowing proteins to bind to the nitrocellulose.

For initial experiments, 600μl of sample was applied to the top reservoir but it was later found that 200μl was sufficient for detection. The nitrocellulose membrane was washed briefly three times in PBS and following blocking for 1 hour in 3% Marvel was incubated with primary antibody (rat anti-human β_2M, Serotec) for one hour on a shaker at room temperature. The membrane was then washed three times in PBS and incubated in secondary antibody (goat anti-rat alkaline phosphatase conjugate, Sigma) for fifty minutes on a shaker at room temperature.

The membrane was washed a further three times in PBS and incubated for thirty minutes at room temperature in darkness in 10 ml AP buffer containing 50μl NBT (stock - 60mg/ml) and 50μl BCIP (stock - 30mg/ml). The nitrocellulose was then washed and left to dry overnight between 2 sheets of tissue paper.

In subsequent experiments, to demonstrate specificity of the antibody for β2-microglobulin, the membrane was divided and the samples were applied in duplicate. Following blocking, the membrane was cut in half allowing half to be incubated with primary (anti-β2-microglobulin) antibody as normal. The other half was incubated with primary antibody which had been blocked with β2-microglobulin protein (incubation of antibody with 10 fold excess of protein at room temperature for 1hour).

2.2.6 Antibody purification

A 1ml protein A sepharose column was washed with 5 ml pH7 phosphate buffer to remove the ethanol preservative. A further 2 ml phosphate buffer was then washed through to ensure that the column was equilibrated. Tissue culture medium (100 ml) from the HC10 hybridoma cells containing antibody was pumped onto the column overnight at 200µl per minute. The column was then washed in 5 ml phosphate buffer followed by 3 ml elution buffer. Fractions (1ml) were collected into eppendorfs containing 100 µl neutralising buffer. The fractions were then analysed on a spectrophotometer at 280nm (UV) against a control of elution buffer and neutralising buffer.

2.2.7 Mini-gel for antibody purity

The gel plate was set up after cleaning with ethanol, 3ml resolving gel was added and left to set with 100 µl butanol layered on top. When the resolving gel had set the butanol was rinsed away (using ddH_2O), 2ml stacking gel was poured on top and the well comb inserted while the gel set.

Prior to loading onto the gel, the well comb was removed and 20µl of sample was added to 6.6µl of reducing sample buffer and boiled at 95^0C for 3 minutes. The gel was run at 200V until the samples reached the join between the stacking gel and the resolving gel. The voltage was then decreased to 150V. Once the samples had run the length of the gel, the gel plate was disassembled and the gel was stained in Coomassie blue for 15 minutes. The stain was removed, destain was added and the gel was left overnight to destain following which it was photographed.

2.2.8 Tryptic digest of BSA

BSA (10mg) was dissolved in 10ml ddH_2O and 100µg trypsin dissolved in 1ml 0.1M sodium phosphate buffer pH7 was added. The mixture was incubated at 37^{O}C overnight (16 hours), following which, 1 ml TCA was added and the mixture sonicated and TCA precipitated as described in section 2.2.10 in preparation for mass spectrometric analysis. To ensure that the BSA was digested, previous experiments on a smaller scale (using 1mg/ml BSA and 10µl trypsin) were carried out and 20µl of the mixture was run on a mini gel as above (section 2.2.7)

2.2.9 Metal chelate column

Prior to use, the column must be washed free of preservative using distilled, deionised water (ddH_2O). The column was then loaded with 0.5 ml of the metal ion, copper (II) sulphate, at 1mg/ml. To ensure the copper was bound and that any excess, unbound, was removed, the column was washed in 5ml ddH2O, then 5ml 0.1M sodium phosphate buffer. The sample (mixture of four synthetic peptides, 200nmol each of gp70, p53, β-galactosidase and Hepatitis B) was applied to the column in 0.5ml 0.1M sodium phosphate buffer, pH7 and followed with a further 5ml wash in phosphate buffer. The peptides were then eluted from the column in 3ml of either 0.5 or 1mM HCl, the first millilitre was discarded as it contained the excess salts bound the column, and the following two millilitres were eluted into a silanised glass bottle for further analysis.

2.2.10 TCA precipitation

Trichloroacetic acid (72%, 100µl/ml sample) was added to peptide containing eluate and was sonicated for 10 minutes in an ultrasonic bath at room temperature. The sample was then centrifuged at 15,000g at $4^{O}C$ for 10 minutes and returned to the ultrasonic bath for a further 10 minutes. The supernatant was then removed from the tube taking care not to disturb the pellet and was then applied to the cation exchange cartridge.

2.2.11 Cation Exchange

The column was first washed in 0.1M hydrochloric acid (HCl) and the sample was applied, washed in 30 ml HCl and eluted in 3ml of 0.1M NaOH, the first millilitre being discarded as above (section 2.2.8). Trifluoroacetic acid (70µl) was added to the sample (to improve peak resolution) in preparation for RP-HPLC.

2.2.12 RP-HPLC

The sample (approximately 2 ml) was loaded onto a RP-HPLC pre-column (1x30 mm) via a 3 ml injection loop at 200µl/minute, The pre-column was washed with solvent A (ddH_2O/0.1% TFA) to remove any remaining salt contaminants for 20 minutes. The flow was then redirected using a six port switching valve (Rheodyne 7030) allowing solvent B (acetonitrile/5% glacial acetic acid/0.01%

TFA) to flow through the pre-column and onto the analytical column used for peptide separation (ODS RP-HPLC column, 1x150mm). The elution gradient was then started (0-100% acetonitrile over 45 minutes at 20μl/minute), with 3 fractions being collected from the analytical column per minute into 1.1 ml silanised glass tapered vials.

2.2.13 Mass Spectrometry

The mass spectrometric analysis parameters (for example, voltages, temperature of heated capillary) used were dependent on the peptides used in the majority of experiments carried out and were optimised for the known peptides described in section 2.1.8. For the identification of peptides from the acid eluate mixture, the parameters had been optimised for the generation of multiply charged peptides, which were most appropriate for sequence analysis. Depending on the conditions on the day of analysis, the conditions were altered within a narrow range to ensure optimal ionisation of peptides.

In general, peptide fractions from the RP-HPLC column were injected into a sealed nanospray tip (gold coated borosilicate) and the tip was gently broken against the heated capillary of the mass spectrometer to initiate spraying of the sample into the mass spectrometer. Following the first round of mass spectrometry, generating a parent (precursor) ion spectrum, a second round of mass spectrometry (tandem mass spectrometry) was carried out to determine the sequence of the peptides present within the sample. The fragment (product) ion spectrum was, in the case of known peptides, examined for fragment ion masses corresponding to the predicted amino acid sequence or in the case of unknown peptides, was submitted to a variety of database analyses to determine the amino acid sequence. Database tools used were; SEQUEST (package provided with mass spectrometer), PROWL (www.proteometrics.com) and Protein Prospector (http://prospector.ucsf.edu/).

2.2.14 PBMC isolation from whole blood

A leucopack (containing leucocytes only) was obtained from BTS (Sheffield) and the cells were separated from the serum using Ficoll-Hypaque (Amersham Pharmacia Biotech) as described in the instructions. Briefly, 20 ml PBS was added to the blood and 15 ml diluted blood was layered onto 7.5 ml Ficoll in 20ml universals (Sarstedt). The universals were centrifuged at 400g for 30 minutes at room temperature without brake. The lymphocyte layer (buffy coat) was removed using plastic pasteur pipettes (Fisher) into universals that had been cooled on ice. Ice cold PBS was added to a final volume of 20 ml and the cells centrifuged at 600g for 15 minutes at 4^0C with brake. The cell pellet was resuspended in ice cold PBS and re-centrifuged at 400g for 6 minutes with brake at 4^0C. Following the removal of the buffy coat layer, the serum was collected and centrifuged at 600g for 6 minutes (4^0C). The supernatant was poured off and heat-inactivated at 56^0C for 30 minutes in a water bath. The serum was centrifuged as before and frozen at -80^0C in aliquots until required.

2.2.15 Dendritic cell culture

The freshly isolated PBMC were then plated out at $20x10^6$/well in 6 well plates; each well containing 3ml standard medium (BioWhittaker X-Vivo 15 supplemented with 1% autologous serum). Surplus PBMC were frozen in X-Vivo containing 20% autologous serum and 10% DMSO. The plates were incubated at 37^0C for two hours, after which the non-adherent cells were collected and frozen as above.

The remaining adherent cells were washed gently with standard medium and returned to culture in 2.5 ml standard medium containing 800U/ml GM-CSF and 500U/ml IL-4. (Day –7)

After two days (Day –5), 2.5 ml standard medium was added containing GM-CSF and IL-4 as before. Following a further two days of culture (Day –3), 2.5 ml medium was removed from the wells and replaced with fresh medium of the same amount containing 1600U/ml GM-CSF and 100U/ml IL-4. Two days later (Day –1), 1 ml of medium was removed from the cells and replaced with 1 ml standard medium containing 50ng/ml TNF-α.

2.2.16 Cytotoxic T lymphocyte induction

The dendritic cells were harvested the following day (Day 0). Since they were non-adherent, the medium was collected and the wells washed gently to remove any remaining non-adherent cells. The cells were washed twice and were resuspended in 1ml standard medium containing 50μg peptide and 3μg β_2-microglobulin. (Several peptide stimulations were set up using the same cell population, the cells were divided equally among the peptides with 1ml cell suspension per peptide).

Following 4 hours incubation at 37^0C with resuspension after every hour, the cells were irradiated at 25 Gy (3.40 minutes), washed twice with standard medium and resuspended at $0.3x10^6$/ml. The dendritic cells were then seeded into a 24 well plate (1ml/well) and 1 ml of T-cell suspension (non-adherent cells frozen on Day –7) at $3x10^6$/ml in standard medium containing 1.6μg IL-7 and 1pg/ml IL-12 was added to each well. The plate was then incubated at 37^0C for 7 days (Day +7) during which time the dendritic cells became adherent and the T cells remained non-adherent.

2.2.17 Restimulation of cytotoxic T lymphocytes (CTL)

On Day +7, 1ml of medium was removed from each well and replaced with 1ml standard medium containing 1.6μg IL-7. Five days later, (Day +12), the CTL were harvested and resuspended at $1.5x10^6$/ml. The cells were returned to the incubator until required.

Autologous PBMC (frozen on Day –7) were thawed and resuspended at $4x10^6$/ml in standard medium prior to irradiation at 60 Gy (8.46 minutes). The irradiated PBMC were washed in standard medium and seeded into a 24 well plate (1ml/well) for 2 hours at 37^0C. The non-adherent cells were removed and 0.5ml standard medium containing 20μg/ml peptide and 3μg/ml β_2-microglobulin was added to each well followed by incubation for a further 2 hours at 37^0C.

After 2 hours, the medium was removed, replaced with 1ml/well of the CTL suspension at $1.5x10^6$/ml and returned to the incubator at 37^0C. Two days later, (Day +14), 1ml standard medium containing 20IU/ml IL-2 was added to each well and the plate incubated as before.

The restimulation was repeated on a weekly basis (Days +19 and +26) for two further weeks and IL-2 was added two days after each restimulation as above.

A cytotoxicity assay was then carried out on Day 33 of the culture, 5 days after the final addition of IL-2.

2.2.18 Alternative protocols for the generation of DC and CTL

Alterations to the original protocol were tried to improve the generation of DC and CTL. Altered protocol 1 involved addition of 5% autologous serum during DC generation and at day 0, the use of autologous serum was substituted for 10% FCS. Altered protocol 2 completely substituted the use of autologous serum for 10% FCS and involved the depletion of CD4 cells from the thawed T cell population added to the cultured dendritic cells at day 0.

2.2.19 CD4 T cell depletion

The depletion of CD4 T cells from the thawed cells (non-adherent cells on day –7, thawed for use as T cells on day 0) was carried out as stated in the protocol provided with the magnetic CD4 coated beads (Dynal). The beads were provided at a concentration of $1.4x10^8$/ml and following washing of the beads in PBS/2% BSA they were added at a 3:1 (beads:cells) ratio in approximately 15ml serum free medium. The mixture was gently agitated on a shaker for 30 minutes in a cold room following which the beads were separated from the mixture using the provided magnet, the mixture was removed and centrifuged to allow counting of the CD4 negative cells. The cells were then added to the DC culture at the 10:1 (T cells:DC).

2.2.20 Cytotoxicity assay – Chromium release

The CTL effectors were harvested by collecting the medium and gently washing the wells with standard medium. Target cells were counted and labelled with chromium (1.85MBq) for 1 hour at room temperature and, where appropriate (T2 cells) pulsed with peptide at the same time.

CTL and labelled target cells were then incubated together at effector to target cell ratios of 20:1, 10:1, 5:1 and 2.5:1. The effectors were resuspended in standard medium so that the correct number of cells for the 20:1 ratio were present in 50μl. The remaining ratios were obtained by serially diluting

the cells with standard medium. A 20-fold excess of unlabelled K562 cells were added (in 50 μl) to eliminate any non-specific killing of targets by NK cells. The target cells were then added in 50μl to relevant wells and standard medium was added to bring the well volume to 200μl. All tests were set up in triplicate and incubated for 4 hours at 37^0C.

Wells were set up with target cells alone to determine spontaneous chromium release and 1% Triton X-100 (a detergent used to lyse cells) was added to some of these wells to give a value for maximum chromium release.

Following the incubation, the supernatant was removed and the plate was counted overnight (using a Top Counter, kindly performed by Judith Ramage at City Hospital, Nottingham) and the results analysed for percentage cytotoxicity. Percentage cytotoxicity was estimated using the equation:

$$\frac{\text{Experimental release - spontaneous release}}{\text{Maximum release - spontaneous release}} \times 100$$

For the last four assays, the method by which percentage cytotoxicity was obtained was altered. The acquisition of a gamma counter within our own department enabled the entire assay to be carried out in-situ. However, following the 4 hour incubation (plate A; cells in 200μl supernatant), 100μl of the supernatant were removed from each well into exactly the same order in a clean 96 well plate (plate B). Both plates were then placed in a drying oven overnight, then cut into individual wells and placed in scintillation tubes for counting in the gamma counter. The percentage cytotoxicity was estimated using the calculation above, with the maximum release substituted for maximum incorporation (chromium counts from both Plate A and B). Experimental release counts were obtained by calculation of the amount of chromium released into 200μl (from plate B, multiplied by 2 to obtain counts in 200μl).

2.3 Names and Addresses of suppliers

Abcam Limited

Tennis Court Road, Cambridge, CB2 1QR, UK

Amersham Pharmacia Biotech

Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK

BDH Merck Limited

Fourways, Carlton Road Industrial Estate, Atherstone, Warwickshire CV9 1JH, UK

Bibby-Sterilin

Tilling Drive, Stone, Staffordshire, ST15 0SA, UK

BioRad Laboratories Limited

2000 Alfred Nobel Drive, Hercules, California, 94547, USA

BioWhittaker UK Limited

BioWhittaker House, 1 Ashville Way, Wokingham, Berkshire, RG14 2PL

Boehringer Ingelheim GmbH

55216 Ingelheim am Rhein, Germany

Dynal UK

11 Bassendale Road, Croft Business Park, Bromborough, Wirral, CH62 3QL, UK

European Collection of Animal Cell Cultures (ECACC)

Division of Biologics, Centre for Applied Microbiological Research (CAMR), Porton Down, Salisbury, Wiltshire, SP4 0JG, UK

Elkay Eireann

Unit 6, Costelloe Industrial Estate, Costelloe, County Galway, Eire

Fisher Scientific UK

Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG

Gibco Life Technologies Limited

Washington Road, Abbotsinch Industrial Estate, Paisley, PA3 4EP, UK

Helena Biosciences

Colima Avenue, Sunderland Enterprise Park, Sunderland, Tyne and Wear, SR5 3XB, UK

Immunex Corporation

51 University Street, Seattle, WA 98101, USA

Johnson and Johnson Medical Limited

Coronation Road, Ascot, Berkshire, UK

Nunc A/S

Kamstrupvej 90, Postbox 280, DK4000, Roskilde, Denmark

Phenomenex

Melville House, Queens Avenue, Macclesfield, Cheshire, SK10 2BN, UK

R&D Systems Europe Limited

4-10 The Quadrant, Barton Lane, Abingdon, Oxon, OX14 3YS

Sarstedt Limited

68 Boston Road, Beaumont Leys, Leicester, LE4 1AW, UK

Scientific Laboratory Supplies (SLS)

Wilford Industrial Estate, Nottingham, NG11 7EP, UK

Serotec

22 Bankside, Station Approach, Kidlington, UK

Sigma-Aldrich Limited

Fancy Road, Poole, Dorset, BH12 4QH

Terra Nova Biotechnology

PO Box 13340, St Johns, Newfoundland, A1B4B7, Canada

Whatman International Limited

St Leonards Road, 20/20 Maidstone, Kent, ME16 0LS

Chapter 3: Standardisation of peptide isolation technique

3.1 Introduction

The induction of an immune response is dependent upon the recognition of antigenic molecules by the effector cells of the immune system. As discussed in chapter 1, the method of recognition and type of antigen recognised depends upon the effector cell. Antibodies, produced by B lymphocytes, are involved in the immune response against bacteria and toxins, whereas T lymphocytes are involved in the response to an infected cell. Cytotoxic T lymphocytes recognise infected cells via peptides originating from intracellular proteolytic degradation of protein (antigen) associated with the pathogen, eg viruses (Townsend et al. 1985; Townsend et al. 1986). These peptides are bound to MHC class I molecules and displayed on the surface of the infected cell enabling T cell recognition. The identification of these peptides is crucial to the understanding of cellular immune responses to tumours.

Anti-tumour CTL have been found in cancer patients (studied originally in melanoma) and have been generated in-vitro against various antigens. Research is ongoing into the identification and characterisation of known and "novel" tumour antigens in order to develop vaccine strategies for tumour immunotherapy. Several methods have been employed to identify tumour antigens, including genetic, immunological and biochemical technologies as discussed below.

3.1.1 Genetic Approach

The discovery and identification of the first tumour antigen was enabled using a genetic approach where tumour specific CTLs able to lyse autologous melanoma cells were used to identify the genetic sequence encoding the tumour antigen (Bruggen et al. 1991). The group had previously determined that the lysis of the cell line was mediated by the recognition of six independent antigens. Subclones of the patient-derived cell line (MZ2-MEL) which were not lysed by the CTLs were designated 'antigen loss variants'. To identify that these antigens were indeed responsible for the sensitivity to the CTL, DNA from the CTL-sensitive melanoma cell line clones (E+) was transfected into cosmid libraries and subsequently into an antigen loss variant clone. The CTL-resistant clones transfected with the tumour antigen gene then became sensitive to the CTL.

Comparison of DNA from the original E+ clones and the antigen-loss clones revealed a segment of DNA only expressed in the E+ clones. The DNA segment showed no similarity to gene sequences within known databases and was thus described as the first identified tumour antigen, MAGE-1. Use of this or similar techniques has led to the discovery of (Boel et al. 1995) GAGE (Eynde et al. 1995), tyrosinase (Brichard et al. 1993), MelanA (Kawakami et al. 1994a) and gp-100 (Kawakami et al. 1994) as tumour antigens. This method is, however, labour intensive and dependent on the use of anti-tumour CTL which are not available for all tumours. It also does not provide information on the peptides expressed on the cell surface and thus responsible for induction of CTL. The techniques used to identify tumour antigen peptides are detailed below.

3.1.2 Immunological approach

Peptide binding to MHC is dependent on the presence of certain preferred amino acids (anchor residues) at certain positions within the peptide. Using computer algorithms it has been possible to screen tumour antigen amino acid sequences for, in the case of MHC class I, eight, nine or ten amino acid length peptides containing these specific amino acids. These peptides can then be synthesised and used in MHC stabilisation assays (Gnjatic 1995; Bocchia et al. 1995; Berke et al. 2000).

The stabilisation assay is based upon the evidence that heavy chain, β2-microglobulin and peptide must all be present for a functional MHC class I molecule to be expressed in a stable complex on the surface. This observation has been formed following work with both mouse (Townsend et al. 1989) and human (Cerundolo et al. 1990) cell lines defective in processing and presentation pathways. One of the most used is the human T2 (HLA-A2) cell line, resulting from a fusion between .174 and CEM, two cell lines with different chromosome 6 (MHC coding region) deletions (Steinle and Schendel 1994). The cells are able to produce MHC class I molecules but are deficient in the transporter protein encoding genes TAP1 and TAP2 thus preventing the presentation of endogenously processed proteins (Kovacsovics-Bankowski and Rock 1995). The incubation of the cells with HLA-A2 restricted exogenous peptide (peptide pulsing) stabilises the MHC class I

complexes on the cell surface where they can then be detected with antibody staining (Cerundolo et al. 1990).

Those peptides able to bind and stabilise MHC class I can then be tested for recognition by CTL specific for the peptide. Synthetic analogues of predicted peptides identified by computer algorithms pulsed onto T2 cells have been used in cytotoxicity assays by many researchers to identify the peptides recognised by melanoma reactive CTL (McArdle et al. 2000; Rosenberg 1998; McIntyre et al. 1996; Peoples et al. 1995; Kawakami et al. 1994; Cox et al. 1994).

3.1.3 Biochemical approach

Despite the ability to predict MHC binding -tumour antigen peptides, the above approach does not conclusively determine the exact amino acid sequence of the MHC-bound peptide epitopes. The above method assumes that the amino acid sequence of the protein of interest is not altered during intracellular antigen processing. Indeed, Falk et al (1990) suggested that trimming of the amino terminus of proteasome-generated peptides occurs in the endoplasmic reticulum (ER). Thus, the requirement for the quantitative identification of peptides bound to MHC from tumour cells led to the application of immunoprecipitation to the isolation of these peptides. Indeed it has subsequently been found that N-terminal modified and glycosylated peptides have been shown to be presented to CTL (Elliott et al. 1995; Skipper et al. 1996; Haurum et al. 1999), qualifying the need for this technology.

One of the first approaches to isolate and sequence MHC-bound peptides was pioneered by Falk et al in 1991 using HPLC and Edman degradation. The sequence information provided by this approach was incomplete and led Hunt and co-workers (1992) to develop a method allowing full sequence quantitation. A human B lymphoblastoid cell line (CIR-A2.1) was detergent lysed and the HLA-A2.1 molecules purified from the lysate by immunoprecipitation with the HLA-A2.1 specific antibody BB7.2 attached to a protein-A sepharose column. The peptides were then separated from the MHC molecule by acid extraction. Following centrifugation and concentration, the peptides were fractionated by microcapillary RP-HPLC and analysed on a triple-quadrupole mass spectrometer.

Eight of the 200 peptide ions detected at two times above the background (signal:noise (S/N) =2) were fully sequenced, four of these being identified as cellular proteins. This technique has since been applied to identify MHC-associated tumour antigen peptides recognised by melanoma-reactive CTL cell lines (Cox et al. 1994) and to determine whether predicted tumour-antigen peptides from gp100 and MelanA/MART-1 were naturally presented by tumour cells (Skipper et al. 1999). The immunoprecipitation approach conclusively identifies total cellular MHC-bound peptides, however it has not been confirmed whether all peptide-MHC complexes released from the ER reach the cell surface and thus an approach to isolate only those peptides bound to tumour cell surface-MHC was investigated. Storkus et al (1993) modified a protocol developed by Suguwara et al (1987) which disrupted the MHC class I molecules on the cell surface, and applied the technique to the isolation of surface-bound peptides from tumour cells. An acidic buffer was washed over an (influenza virus infected) adherent cell line monolayer disrupting MHC class I by destabilising the non-covalent bond between the heavy chain and β_2-microglobulin domains. The stability of the MHC class I molecule is dependent on the presence of heavy chain, β_2-microglobulin and peptide (Townsend et al. 1989; Cerundolo et al. 1990), thus destabilisation of β_2-microglobulin causes unfolding of the MHC molecule and subsequent peptide release into the acid buffer which was then collected, concentrated and HPLC-fractionated. The fractions were pulsed onto K4B cells (similar to T2) which were then tested in a cytotoxicity assay with CTL specific for the influenza matrix peptide. Two fractions were identified as containing peptide sensitising K4B cells to lysis by the CTL. Co-elution of the synthetic matrix peptide with the peak suggested that the naturally processed matrix peptide was contained in this peak. Mass spectrometric analysis of the predominant peptide in the peak was found to have a sequence similar to the matrix peptide. Despite the inability to precisely match the peptide sequences, (residues of the same mass cannot be distinguished with mass spectrometry, for example isoleucine/leucine in this case), the evidence suggests that the matrix peptide is presented on the surface of infected cells and was eluted from them thus enabling the K4B peptide pulsed cells to become sensitive to the matrix peptide-specific CTL. This protocol was later applied to the identification of a MelanA/MART-1 peptide from a melanoma cell line (Castelli et al. 1995).

In order to identify novel surface MHC-restricted peptides for the purposes of the present study, it was decided to adopt the above "acid elution" protocol and to apply it to other tumour antigens lacking defined peptide epitopes. The protocol allowed multiple elutions from a cell population thus reducing the number of cells required to generate detectable peptide for mass spectrometry. The results presented in this chapter describes the standardisation of the acid elution technique for several cell lines expressing various tumour antigens.

3.2 Results

3.2.1 Selection of cell lines

Table 3.1 shows details of the cell lines used in these experiments. The three human cell lines, GERL, SaOs and FM3 express high levels of MHC class I as shown by the flow cytometric analysis of W6/32 binding to these cell lines (figure 3.1 a-d), also the majority of the population express the molecules (over 85%). The murine cell line, CT26, does not express a high level of H2-K^D, and only 17% of the cells express any MHC class I. To standardise the acid elution protocol, the GERL 3.1 (MZ2-MEL3.1) cell line was chosen for its ability to grow rapidly. GERL 3.1 expresses MAGE-1, a well characterised tumour antigen which has been extensively documented as being expressed in many melanomas, peptides from this antigen have been determined as having CTL inducing activity (Bruggen et al. 1991; Gaugler et al. 1994; McIntyre et al. 1996; DeSmet et al. 1997; Kirkin et al. 1998). Thus, this cell line provided a model system with which to optimise the protocol.

The other cell lines used to test the protocol following standardisation were chosen for the expression of other well-known tumour antigens and to ensure that the protocol is transferable to other cell and HLA types. The mouse colon carcinoma cell line, CT26, has been transfected with β-galactosidase (CT26.cl25). The use of β-galactosidase as a murine tumour model is also well documented (Wang et al. 1995; Overwijk et al. 1997; Carroll et al. 1998; Tuting et al. 1999) and HLA-binding peptides have been immunologically characterised (Overwijk et al. 1997). The human osteosarcoma cell line SaOs-2, expresses HLA-A2 and has been transfected with mutant p53 (position 175 R to H), the tumour suppressor involved in over 60% of human cancers and thus is the subject of much research in this area. FM-3, a melanoma cell line also transfected with mutant p53 (position 49 R to H) naturally expresses high levels of tyrosinase and gp100, both of which are well-researched tumour antigens. Flow cytometry was performed on each of these cell lines to ensure MHC class I was expressed (figure 3.1a-d) and to confirm tumour antigen expression (figure 3.2a-b), except for the CT26.cl25 cell line where X-gal staining was performed to demonstrate β-galactosidase expression (figure 3.2c). Both MHC class I and tumour antigen expression was as expected indicating that the cell line characteristics had not been lost during cell culture

Table 3.1 Cell line characteristics

Cell line	Tumour type	HLA status	Fluorescence intensity	Percentage stained
GERL 3.1	melanoma	A1 B36 Cw6	579.40	93.18
SaOs-2/175	osteosarcoma	A2 A3 Cw16 Cw47	388.02	99.92
FM3.cl4	melanoma	A2 A3 B7 B44	511.05	85.18
CT26.cl25 (murine)	colon carcinoma	$H2\text{-}K^{D}$ $H\text{-}2I^{A}$	48.47	17.5

Human (GERL 3.1, SaOs-2/175, FM3.cl4) and murine (CT26.cl25) cell lines were stained with anti-MHC class I antibody (W6/32 for human and $H2\text{-}K^{D}$ for murine) to confirm class I expression. Flow cytometry was carried out on a Coulter flow cytometer by Adrian Robins and Alison Galvin at Queens Medical Centre, Nottingham. The raw data was analysed using the WinMDI software from the Scripps Research Institute to generate the fluorescence intensity and percentage cell staining data.

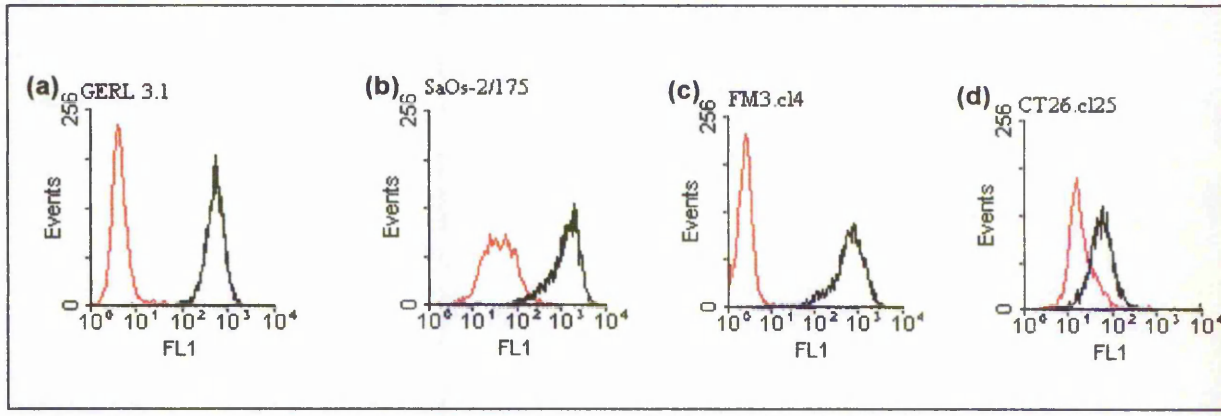


Figure 3.1 MHC class I staining of standard cell lines

Red peaks show negative control (secondary antibody alone to demonstrate non-specific secondary antibody binding) and black peaks MHC class I staining. W6/32, a pan HLA-A, -B, -C antibody, is used to stain a-c and an anti H2-K^D antibody is used to stain d. An FITC conjugated secondary antibody is used to show anti-MHC class I antibody binding as fluorescence.

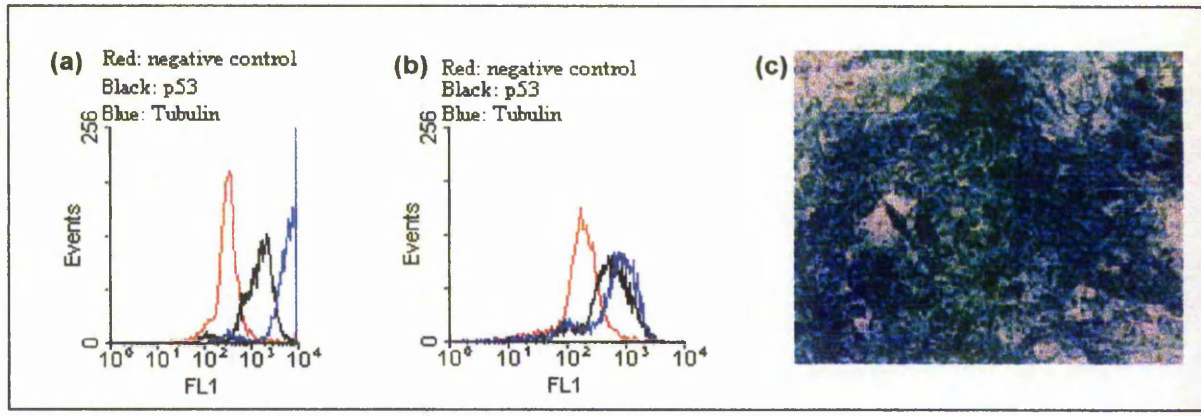


Figure 3.2 Cellular staining to demonstrate expression of tumour antigen

SaOs-2/175 and FM3 cells (a and b) were stained intracellularly (see methods) with anti-p53 antibody (DO7) to ensure these transfected cells continued to express the tumour antigen. They were also stained with an anti-tubulin antibody as a positive control for permeabilisation as tubulin is an intracellular protein. Flow cytometry was carried out and analysed as before (above). CT26.cl25 cells were stained with X-gal causing cells expressing the β-galactosidase gene to turn blue after an overnight incubation.

GERL 3.1 cells cannot be stained for MAGE-1 in this way as an anti-MAGE antibody is not available, however, the presence of this tumour antigen within these cells is well characterised (see text).

3.2.2 Preparation of cell lines for elution

Prior to the addition of acid (citrate phosphate buffer, pH3.3) to the cells, the medium was removed and the cells were washed with serum-free medium to prevent any inhibition of the acid by contaminating proteins contained within the medium, such as foetal calf serum. A comparison between washing with PBS, as is usual in cell culture, and serum free medium was performed using flow cytometry to determine whether MHC class I loss occurs due to the washing of the cells. Figure 3.3a shows dotplots demonstrating that following the PBS wash the cells are less healthy than with the serum free wash. The PBS wash appears to cause cell death, and figure 3.3b shows a loss of MHC from the surface of the PBS washed cells with 73% of cells expressing MHC I compared to 96% of serum free medium washed cells. A T-test performed on this data suggested that the difference was significant ($p=<0.01$).

3.2.3 Length of acid wash

To ensure optimum removal of surface MHC class I molecules whilst protecting cell integrity, the length of acid wash was investigated. T25-sized flasks of GERL 3.1 were taken and acid washed for varying periods of time up to 30 minutes. One flask remained untreated as a control.

Following the acid wash, the cells were washed with serum-containing medium to neutralise the acid and were removed from the flask using trypsin. Flow cytometry was then used to determine loss of MHC class I expression from the cell surface via antibody staining (Figure 3.4). The conformational dependent antibody W6/32 was used to confirm destabilisation of MHC class I as it requires correctly folded MHC molecules to bind (Ferrier et al. 1985; Kahn-Perles et al. 1987; Ladasky et al. 1999). Figure 3.4 a-e shows that the acid treatment of the cells causes a loss in W6/32 reactivity, with very few cells expressing MHC class I after 30 minutes. Figure 3.4f shows that MHC class I expression decreases markedly for the first five minutes, but then begins to level off. The integrity of the cell population can be seen to decrease after prolonged periods in the acid (figure 3.5 a-e), the possibility of cell lysis and thus the leakage of intracellular peptides from these cells is increased, and thus the longer acid treatments were not used in the final protocol.

Figure 3.3a MHC class I expression on GERL 3.1 following PBS or serum free medium wash

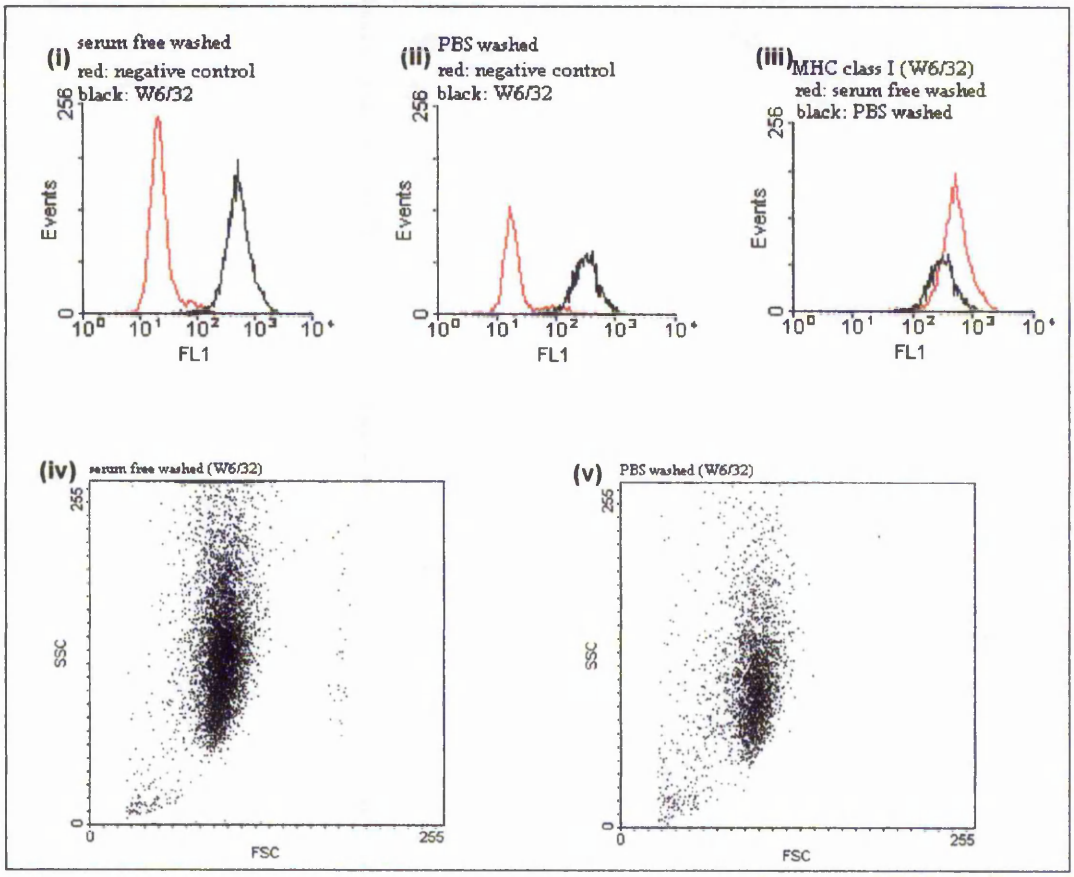


Figure 3.3b Comparison of MHC class I expression following PBS or serum free medium wash

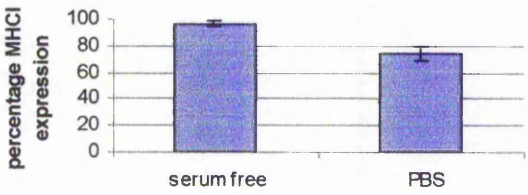


Figure 3.3 Effect of washing cell lines with PBS or serum free medium

GERL 3.1 cells were washed with PBS or serum free medium to remove protein contaminants and then stained with anti MHC class I antibody (W6/32). A sample of cells were stained with secondary antibody alone as a negative control. Raw flow cytometric data was analysed by the WinMDI program to generate histograms (i-iii) depicting fluorescence intensity, dotplots (iv-v) depicting the cell population and percentage staining after each treatment (represented as a graph, figure 3.3b). MHC expression by serum free washed cells was significantly higher ($p=<0.01$) than that by PBS washed cells as tested by a two sample T-test. Error bars were determined by standard deviation between replica samples.

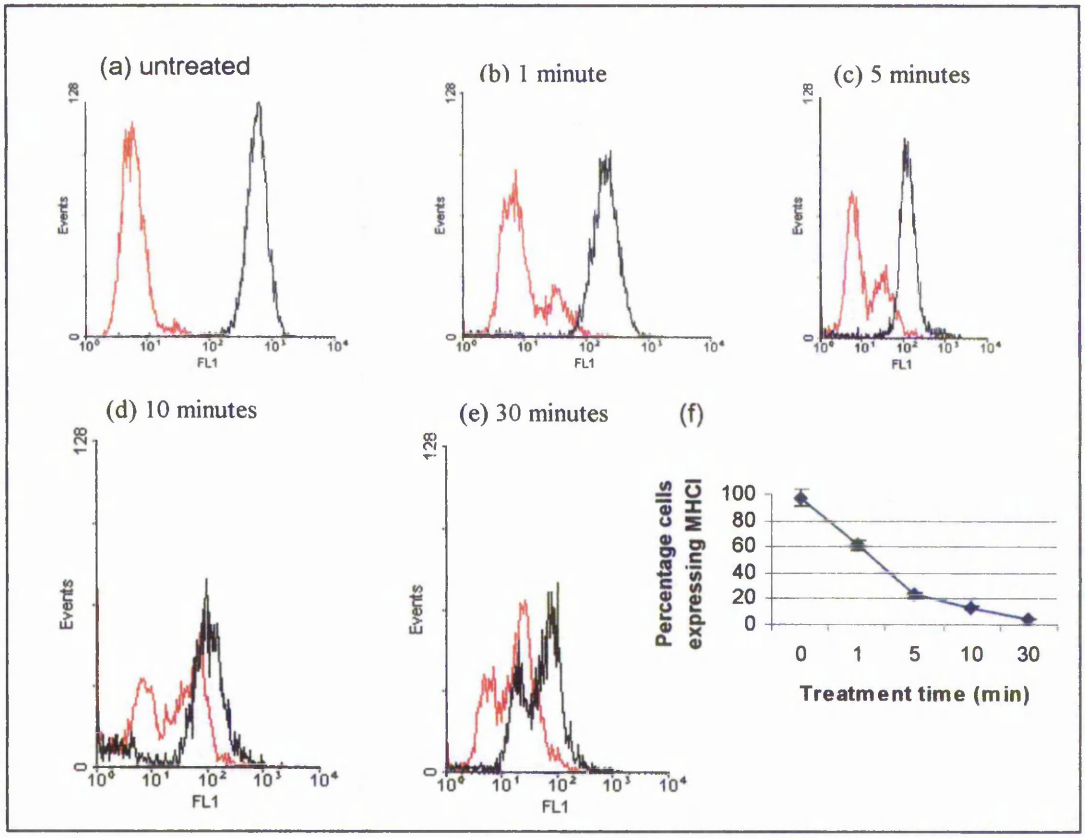
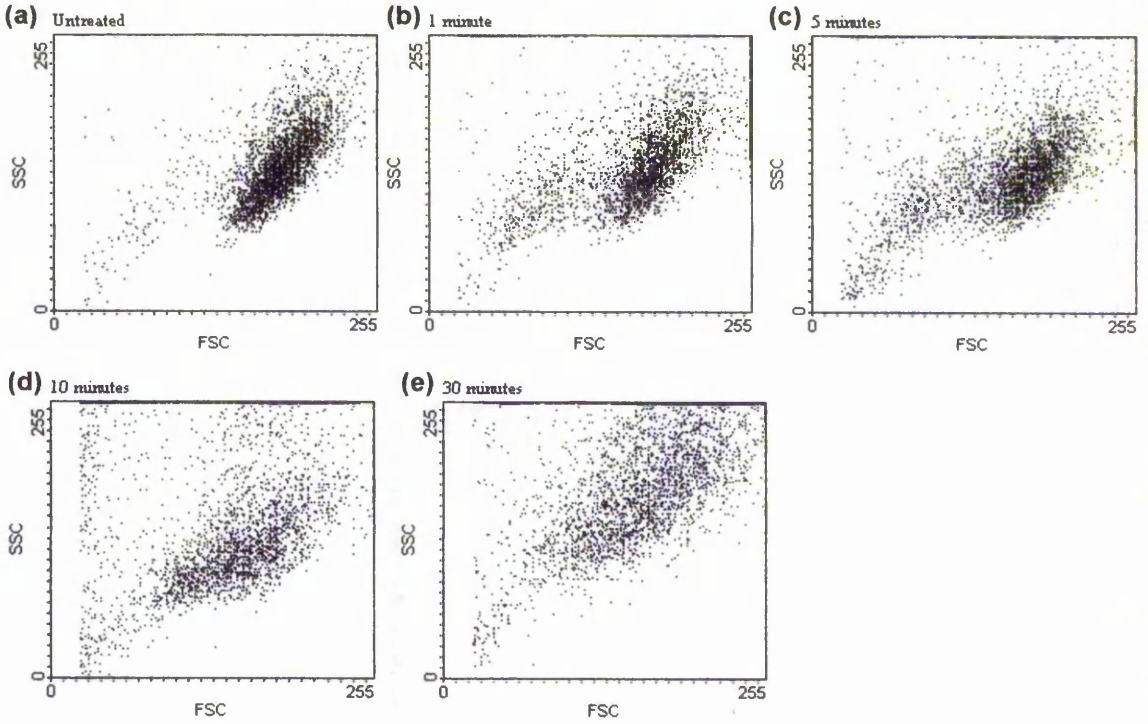


Figure 3.4 Loss of MHC class I expression following acid treatment

GERL 3.1 cells were washed in citrate phosphate buffer 3.3 for varying periods of time in order to determine the optimum length of treatment for the destabilisation of MHC class I. The red peaks represent the background negative staining (non-specific secondary antibody binding) and the black peaks represent MHC class I expression as determined by W6/32 staining. The graph (f) shows the decreasing percentage cellular expression of MHC class I with increasing treatment times. The raw flow cytometric data was analysed using the WinMDI program as before.

Figure 3.5 Dotplots of GERL 3.1 population following acid treatment



Dotplots of the cell antibody stained cell population were obtained using the WinMDI program. Each cell is represented by a single dot in the plots above, and a healthy population of cells is represented by clustered dots as in (a). As the length of the acid treatment increases, the dots become more spaced thus representing differing sizes and granularity and therefore varying viability. Dots clustered near the origin are most likely dead cells, these can be seen to increase as the length of the acid treatment increases.

The dotplots show the population becoming more scattered and an increased amount of cell death in the 10 and 30 minute plots. Taking the results of these three figures together, it was decided that a 5 minute acid treatment would destabilise the majority of MHC class I molecules (only 20% cells retaining MHC class I expression), whilst maintaining cellular integrity.

3.2.4 MHC regeneration

Acid elution was performed as previously described and cells were returned to culture to enable recovery. To ensure that the MHC regenerated and that peptide was re-presented (a requirement in order to perform multiple elutions), four flasks of GERL 3.1 were eluted as described and returned to culture for varying lengths of time (2 hours, 6 hours and 24 hours). Two flasks of cells were set aside as controls, one stained immediately after elution (used to indicate the lowest level of MHC expression) and an untreated flask of cells to show maximum levels. Following the incubation, flow cytometry was again used to quantify MHC expression (Figure 3.6 a-e). The level of MHC class I expression can be seen to increase up to almost the level of untreated cells (figure 3.6f), after 24 hours post-treatment culture 97% of the cells express MHC class I.

3.2.5 Slot Blot for β_2 – microglobulin detection

Following elution, the acid was collected and tested in a slot-blot for the presence of β_2 – microglobulin as would be expected if MHC destabilisation had occurred. The test acted as a confirmation of the flow cytometric data obtained following elution. A rat anti-human-β_2 – microglobulin antibody and an alkaline-phosphatase conjugated anti-rat IgG as secondary antibody were used to indicate presence of β_2 – microglobulin on the nitrocellulose membrane. Antibody binding was visualised using a colourless buffer which was converted into a purple substrate by the presence of bound alkaline phosphatase. The blot is shown in figure 3.7a and demonstrates the presence of β_2 – microglobulin within the acid eluates, the purple bands are indicative of a positive sample. The positive control (β_2 – microglobulin, 1μg/μl) can be seen to generate a positive band similar in intensity to the experimental sample and the negative controls citrate-phosphate buffer and serum free medium were negative. These blots show the β_2 – microglobulin eluted from GERL 3.1,

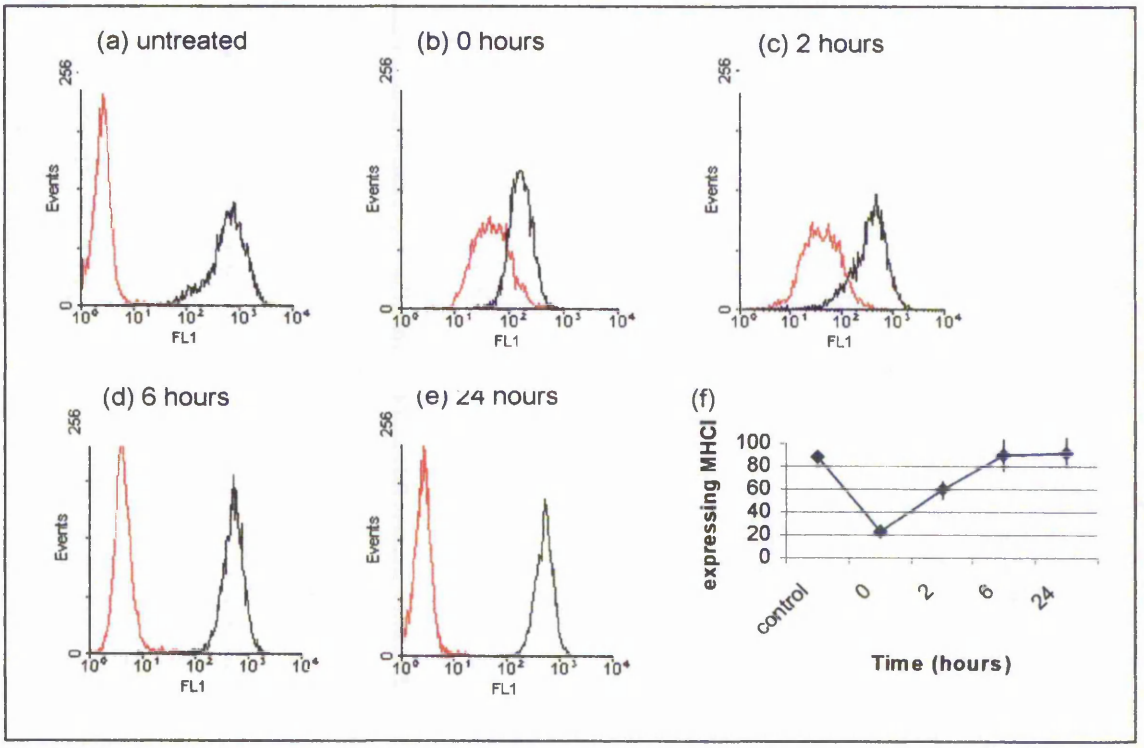


Figure 3.6 Regeneration of MHC class I expression following acid elution

Flasks of GERL 3.1 cells were returned to culture following acid elution and stained for MHC class I expression (using W6/32 antibody) after variable lengths of culture (c-e). An untreated control (a) was included as an indicator of maximum levels of MHC class I expression and one flask of cells was stained immediately following elution (b) to indicate minimum levels of MHC class I expression. Raw data was analysed using WinMDI generating the fluorescence intensity histograms (a-e) and percentage cell staining values which are graphically represented above (f).

Red peaks represent non-specific secondary antibody staining (background) and black peaks represent W6/32 antibody staining (MHC class I expression).

the experiment was repeated with other cell lines (SaOs and CT26.cl25) giving the same result. Incubation of the β_2 – microglobulin antibody with β_2 – microglobulin protein (1 hour, room temperature) was performed to prove that the antibody binding was specific to β_2 – microglobulin. The adsorbed antibody-protein complex and an unadsorbed antibody were used to repeat the above experiment on a fresh sample, the adsorbed antibody was unable to bind to the nitrocellulose membrane whereas the unadsorbed antibody demonstrated that β_2 – microglobulin was bound to the membrane (figure 3.7b)

3.2.6 Acid elution of four cell lines

Following the above optimisation of acid-mediated destabilisation of MHC class I, the protocol was performed on the four cell lines described above in table 3.1. Cells were washed with serum free medium, then acid washed for 5 minutes prior to flow cytometry using W6/32 and anti-β2-microglobulin to detect loss of surface MHC. The results are shown as histograms (fluorescence) and graphs (percentage loss) in figure 3.8 a-d. All four cell lines demonstrate a loss of MHC class I (as a decrease in W6/32 reactivity), the three human cell lines show a similar level of MHC destabilisation, with an average 25% of cells retaining W6/32 reactivity. The murine CT26.cl25 cells despite expressing a low level of MHC class I demonstrated almost total MHC class I destabilisation (decrease in anti-H2-K^D antibody reactivity).

3.2.7 Purification of HC10

To determine whether the release of β_2-microglobulin into the eluate led to a conformational change of the MHC class I molecule following β_2 – microglobulin removal, the HC10 antibody which recognises an epitope on the heavy chain, was used. Unfolding of the heavy chain allows the antibody to bind and thus antibody binding of acid treated cells should increase compared to untreated controls. Since there was no commercial source of this antibody it was purified from hybridoma supernatant using a Protein A sepharose column. This, and an alternative source (Dr. A. Dodi), of the antibody were run on a mini-gel to determine purity (Figure 3.9). It can be seen that the alternative source (lanes 5 and 7) contains less bands compared to that purified as described above

Figure 3.7a Slot blot for presence of β2-microglobulin in acid eluates from three cell lines

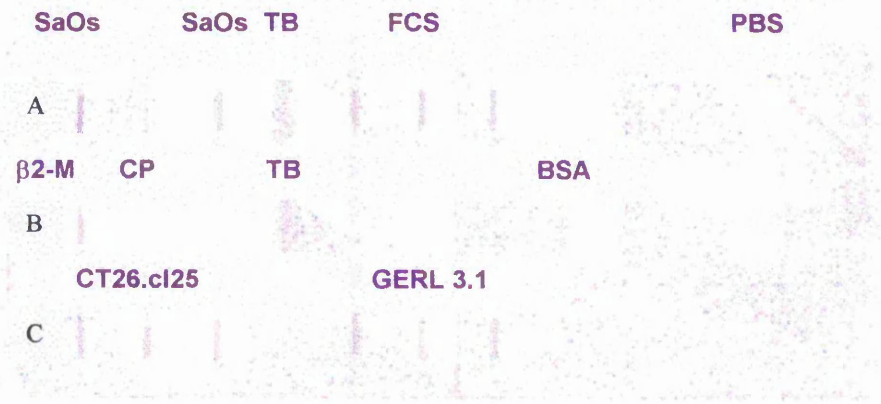
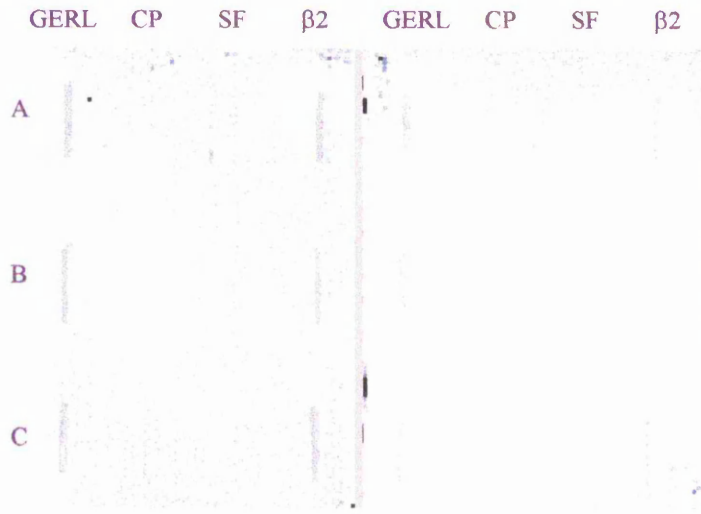


Figure 3.7b Blocking of β2-microglobulin reactivity by adsorption to protein

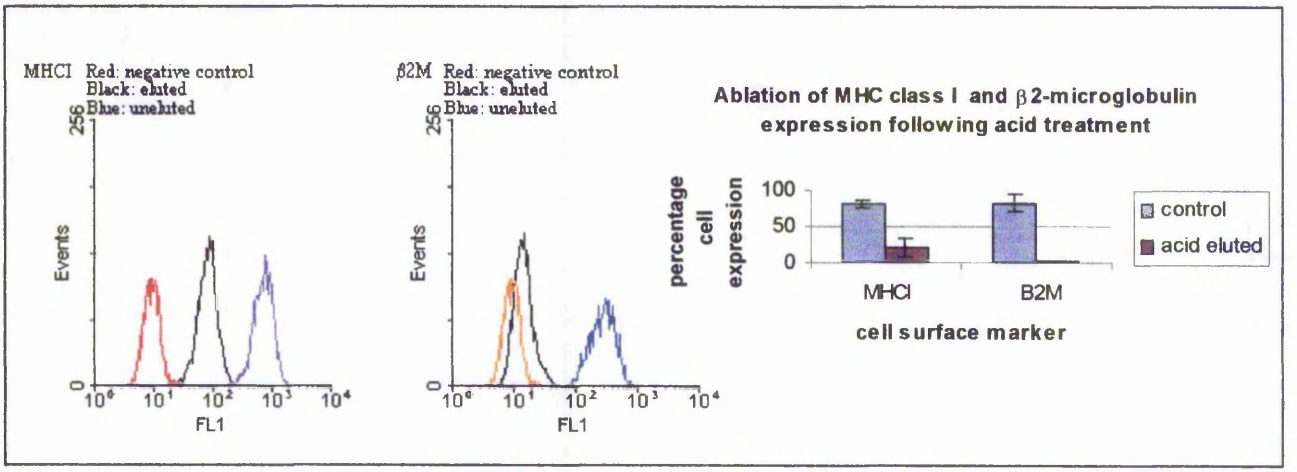


Key:
TB: Trypan Blue
FCS: Foetal calf serum
PBS: Phosphate buffered saline
CP: Citrate-phosphate buffer
BSA: Bovine serum albumin
β2: β_2-microglobulin
SF: Serum free RPMI

Aliquots of citrate-phosphate buffer used to elute cell lines were loaded into the slot-blot apparatus for concentration onto nitrocellulose membrane (3.7a). The membrane was stained with anti-β2-microglobulin antibody and alkaline phosphatase conjugated IgG as the secondary antibody. The staining was visualised with a colourless alkaline phosphatase buffer which was converted to a coloured substrate where antibody had bound.

To ensure that the β2-microglobulin staining was specific to the presence of the protein, an aliquot of antibody was incubated with an identical concentration of -β2-microglobulin protein for 1 hour at room temperature. The samples were concentrated onto the nitrocellulose membrane as before, in duplicate (3.7b). The membrane was cut in half and one half was stained as above (unblocked) and the other half was stained with the antibody-protein complex (blocked). A lack of staining on the nitrocellulose stained with blocked antibody suggests that the antibody binding is specific to the protein.

(A) GERL 3.1



(B) SaOs-2/175

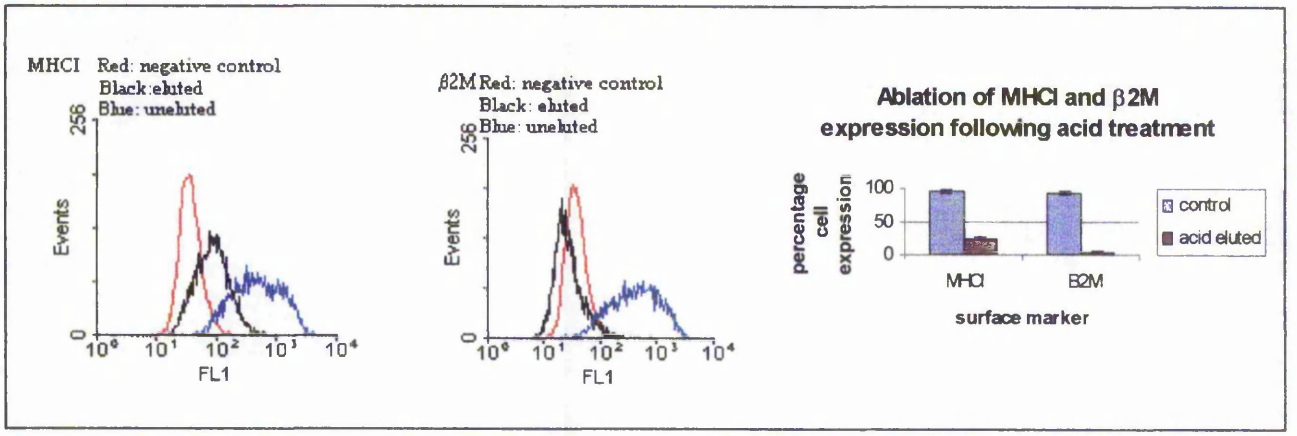


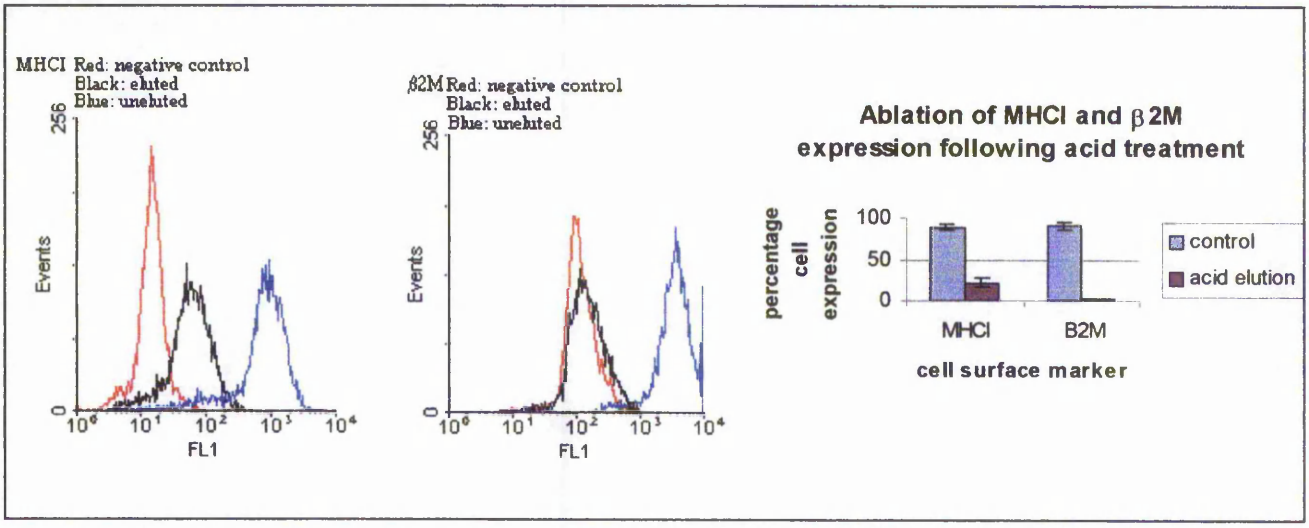
Figure 3.8 Loss of MHC class I and β_2-microglobulin following acid treatment

Cells were acid washed as described in the Methods section. The acid was removed and the cells neutralised by addition of serum free medium and approximately $2x10^5$ cells were aliquoted into tubes for flow cytometry . Following centrifugation the cells were incubated with the primary antibody, either MHC class I or β_2-microglobulin on ice for 20 minutes. The cells were washed and a secondary, FITC conjugated, antibody was incubated with the cells as above, thus enabling bound antibody to be visualised on the cells as fluorescence by the flow cytometer.

An untreated sample was subjected to antibody binding as above to enable comparison and estimation of MHC class I and β_2-microglobulin loss as a result of the acid treatment.

To determine whether any non-specific binding of the secondary antibody had occurred, an aliquot of cells were incubated with secondary antibody alone. These samples were designated negative controls.

(C) FM3.cl4



(D) CT26.cl25

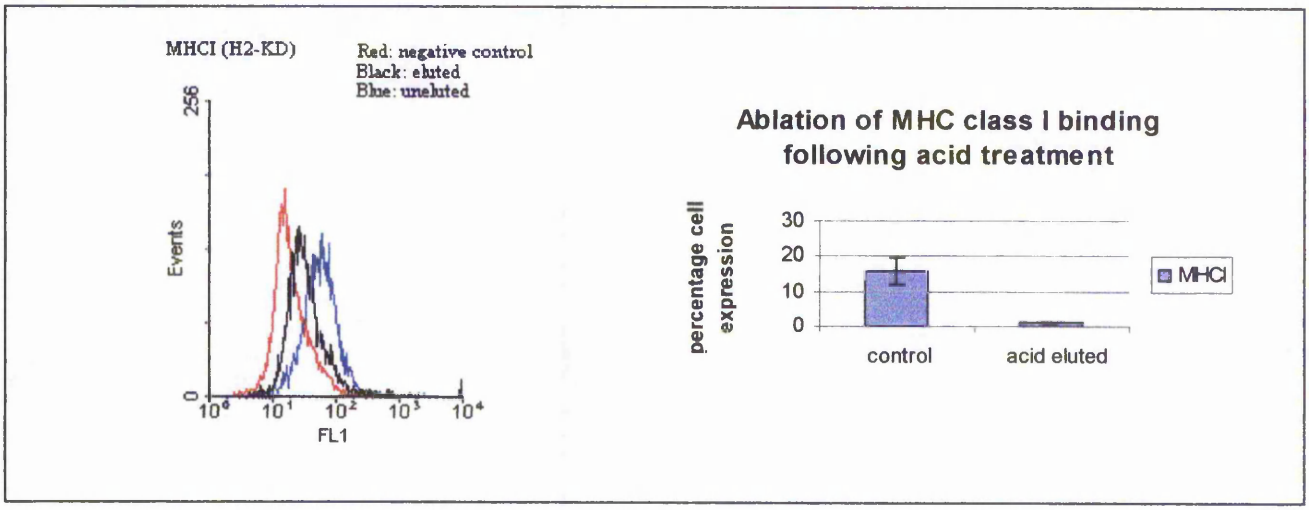


Figure 3.8 Loss of MHC class I and β_2-microglobulin following acid treatment

The cells were treated as explained on the previous page, the CT26.cl25 cells were stained with anti-H2-KD to demonstrate loss of MHC class I from the cell surface following acid elution. These cells were not stained with anti-β_2-microglobulin as a murine β_2-microglobulin antibody was not available.

(lanes 3 and 4). Comparison with the molecular weight markers (lanes 1 and 8) show that both sources contain bands at 50kDa and 15kDa, the masses of the immunoglobulin heavy and light chains respectively. The use of a reducing agent prior to the sample loading and running on the gel causes the dissociation of the chains. The bands within the alternative source of HC10 are much darker than the in-house purified source, suggesting a large amount of the antibody is present and this, together with fewer contaminant bands compared to the in-house purified source, is indicative of the increased purity of the alternative source.

3.2.8 Flow cytometry using HC10

Flow cytometry was performed on acid eluted and untreated control cells to determine how acid elution affected the MHC heavy chain. The use of HC10 should provide additional information on the effects of acid treatment on the MHC molecule (Figure 3.10). Only the alternative source of HC10 was used in this experiment due to its purity. The results obtained at first were confusing, showing an increased HC10 reactivity with untreated GERL 3.1 cells (3.12a) or a complete lack of reactivity (3.12b). Following discussion with Dr. A. Dodi it was suggested that the antibody was better able to bind at very low temperatures. The cells were kept on ice both prior to and following the acid elution, and a large increase in HC10 reactivity was seen on the treated compared to the untreated controls as would be expected (3.12c). This result was not observed with the other human cell lines (not shown).

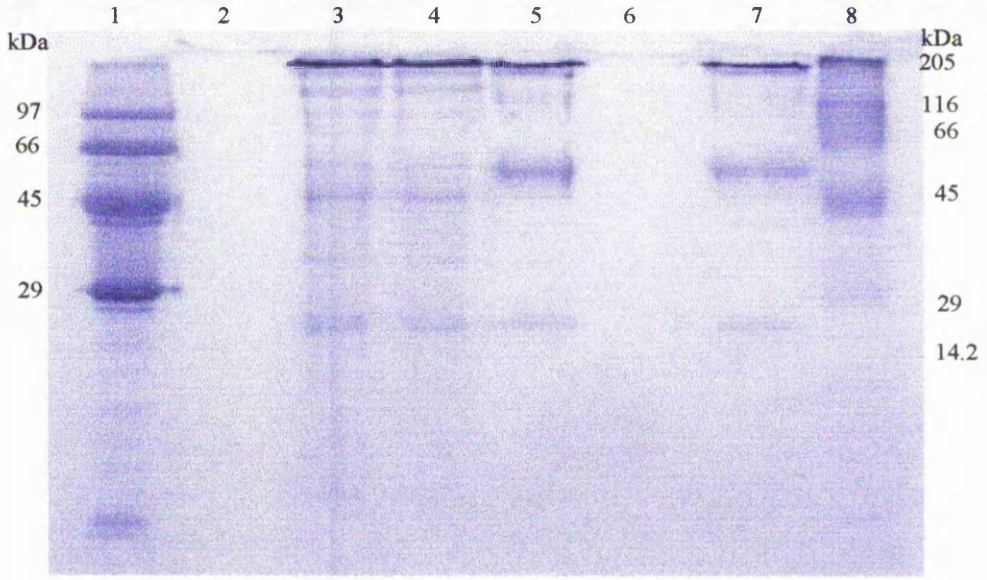


Figure 3.9 Mini-gel of two sources of purified HC10

20μl aliquots of HC10 antibody, purified in-house (lanes 3 and 4) or provided by Dr. A. Dodi (lanes 5 and 7) were run on a minigel as described in the Methods section. The molecular weight markers (lanes 1 and 8) are both from Sigma. Lanes 2 and 6 were left empty.

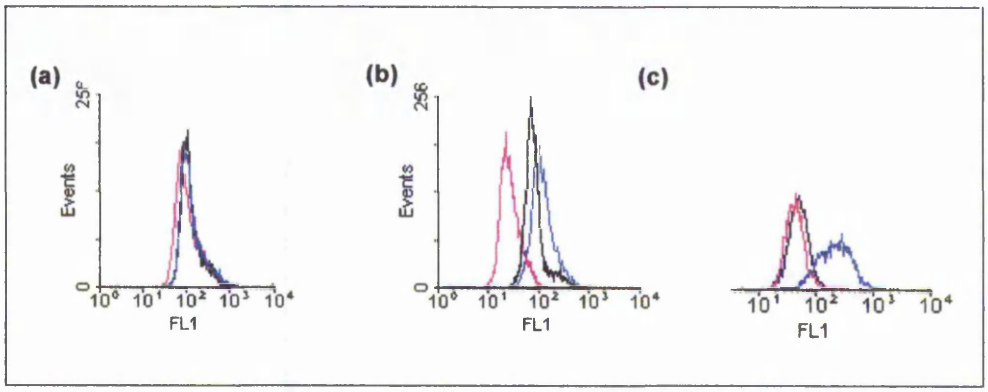


Figure 3.10 HC10 binding to GERL 3.1 cells following acid elution

Following acid elution GERL 3.1 cells were stained HC10 antibody (recognising MHC class I heavy chain only, provided by Dr. A. Dodi) to determine the nature of destabilisation of MHC class I caused by the acid elution. It was expected that the HC10 antibody would bind to eluted cells due to the destabilisation of the MHC, but not untreated cells. The results were confusing, the antibody did not bind to either treated or untreated cells (a), bound to the untreated cells as opposed to the eluted cells (b), or when cells were kept at low temperatures prior to and following the elution, bound to the eluted cells only, with a 50% increase in binding (c).

For (a) and (b) red: negative control, black: eluted, blue: uneluted. For (c) black: uneluted, blue:eluted

3.3 Conclusions

The results shown here provide evidence that the acid elution technique destabilises MHC class I molecules as demonstrated by loss of W6/32 and anti-β2-microglobulin binding on the acid treated cells. The optimum treatment time of five minutes enables the majority of the surface MHC to be removed whilst preventing a large amount of cell death which may contribute intracellular, non-MHC restricted peptides to the eluate. The detection of β2-microglobulin within the eluate confirms the results observed in the flow cytometry. Thus, acid elution can be assumed to remove peptide from the cell surface and is therefore a suitable procedure for use in further studies (Chapter 5).

3.4 Discussion

The flow cytometry results show each of the cell lines express MHC class I thus enabling their use in the study. Figure 3.3 shows that washing with PBS causes some loss of MHC molecules an observation not supported by previous work (Storkus et al. 1993). Washing of the cells with PBS prior to acid elution was of longer duration in the experiments above than used normally to wash cell cultures, in order to ensure that all proteins which might interfere with the destabilising action of the acid are removed. Thus serum free medium was used here to minimise any MHC loss not due to the acid elution.

In accordance with the results by Storkus et al. (1993), the optimum elution time was found to be five minutes, following which the amount of MHC removal varied according to cell line. As shown by flow cytometry, complete regeneration of MHC occurred within twenty-four hours and was almost completed within six hours. Suguwara et al. (1987) showed that regeneration was 80% completed after ten hours while Storkus et al. (1993) demonstrated that CTL recognition of acid eluted targets was restored within eighteen hours. Both studies showed that four hours was not sufficient for regeneration of MHC antigens. The results obtained in the present study and those by Suguwara et al. (1987) and Storkus et al. (1993) show that the cells remain viable after the acid treatment and thus could be re-eluted. This means that fewer cells need to be cultured at one time to generate a high concentration of peptide to be generated, thus making the protocol manageable. Use of the immunoprecipitation technique requires a large starting volume of cells and is therefore fairly

burdensome. However, the acid elution technique, despite not destroying the cells, may induce release of stress proteins which would be presented on the surface following regeneration thus altering the peptide repertoire expressed by MHC class I antigens and further complicating the resulting mass spectrum. To ensure only peptides normally expressed on the surface are contained within the eluate it was decided to perform only one elution. The number of cells used per elution would be determined subsequently when information on the limitations of mass spectrometry was obtained (Chapter 4).

Following the demonstration of the loss of β_2-microglobulin from the cell surface, a slot-blot assay using the eluate from the same cells analysed by flow cytometry was performed to determine whether β_2-microglobulin was released into the acid eluate. This would support the suggestion by Storkus et al. (1993), that upon unfolding of the MHC molecule both β_2-microglobulin and MHC-bound peptide would be released into the eluate. The slot-blots clearly demonstrate the presence of β_2-microglobulin in the eluate and staining is blocked following adsorption of the antibody to the protein prior to staining. This suggests that staining is dependent on the presence β_2-microglobulin which, in these experiments, can only have originated from the cells. β_2-microglobulin positive control was used at a concentration of 1μg/ml and by comparison to the sample eluate it can be seen that the β_2-microglobulin present within the sample is within a similar concentration range.

Finally, the results in figure 3.8 demonstrate that the acid elution causes MHC destabilisation on several cell lines, including the murine carcinoma cell line. It was found that despite binding of anti-β2-microglobulin in the slot-blot using the CT26.cl25 sample, the antibody did not bind to surface β2-microglobulin in the flow cytometric analyses. The reason for this is unknown and since the work by Storkus et al. (1993) only involved the use of human cell lines, the result cannot be corroborated by reference to published work. It can only be assumed that the murine β_2-microglobulin conformation is significantly different from the human conformation or that the epitope determinant is cryptic when complexed with its heavy chain to prevent antibody binding. However, when the β_2-microglobulin is eluted from the cell surface the epitope is avalilable for binding by the (human) antibody as seen demonstrated by the immunoblotting.

The use of the HC10 antibody in these experiments provides confusing information on the fate of the MHC heavy chain following elution. Despite use of a purified source of HC10 (Dr. A. Dodi results shown in figure 3.9), HC10 binding did not always occur either before or after acid treatment (figure 3.10a). In subsequent experiments, the HC10 binding was higher on the untreated cells compared to the treated cells (3.10b), unless the cells were kept at low temperatures prior to and during the antibody binding, where the converse was observed (3.10c). Storkus et al. (1993) showed that binding of HC10 was only observed following acid elution. Suguwara et al. (1987), however, demonstrated loss of antibody binding whether using a conformational dependent antibody (such as W6/32) or an antibody recognising a determinant solely present on the heavy chain (such as HC10). It is possible that during the experiments presented above that either the heavy chain is able to be removed from the surface along with the β2-microglobulin into the eluate, or that the antibody simply is not consistently functional. With the exception of the HC10 results, it can be concluded that MHC class I is destabilised following acid treatment. The loss of β2-microglobulin into the eluate strongly suggests that previously MHC-bound peptide follows the same fate thus making it available for concentration and finally detection via mass spectrometry.

Chapter 4: Purification and Concentration of isolated peptides

4.1 Introduction

The analysis and identification of naturally expressed MHC-bound peptides which have been isolated from biological samples has been performed using relatively few techniques, including HPLC-Edman degradation (Falk 1991) and microcapillary mass spectrometry (Hunt 1992). Improvements in the sensitivity of mass spectrometry over the past ten years has allowed its application as an extremely sensitive method for peptide analysis (deJong 1998). Chemicals used during the isolation of peptides, including those described and used in chapter 3, and biological contaminants such as low molecular weight proteins common in biological samples can cause difficulties in mass spectrometric analysis and must be removed from samples before the peptides can be analysed.

Several researchers utilising mass spectrometric techniques for immunological studies have reported the need to remove contaminants by HPLC and sepharose cartridges (Falk 1991; Hunt 1992; Storkus et al. 1993; Castelli et al. 1995; Skipper et al. 1999).

In addition, the sensitivity of the mass spectrometer can be adversely affected by cationic adducts. Sodium and potassium salts within the sample buffer can bind to peptide ions, thus preventing the identification and sequencing of peptides (Winston and Fitzgerald 1998; Wu et al. 1996; Jackson et al. 2000). This phenomenon is not restricted to salts within the buffer, plastic compounds such as poly-ethylene glycol from storage containers, and several other chemicals can also cause adducts (Wu et al. 1996). Figure 4.1 shows the binding of silica from the silanising agent (see Chapter 2) to a standard peptide thus affecting its mass and thereby its identification. Adduct formation can also cause suppression of the peptide ion signal (Wu et al. 1996). In addition, precipitation of salts on the heated capillary inlet to the mass spectrometer can cause blockage and affect the functioning of the instrumentation. Following removal of contaminants it is important that the sample is concentrated to enable detection of the peptides. Hunt et al (1992) reported that approximately 20 pmol of peptide material can be recovered from 10^8 cells, and several other researchers have noted the low concentration of peptides eluted from MHC on the cell surface together with the need to concentrate the peptides into a small volume for analysis and detection (Tomlinson and Naylor 1995), (Tomlinson et al. 1996; Wu et al. 1996; Winston and Fitzgerald 1998; Brockman et al. 1999).

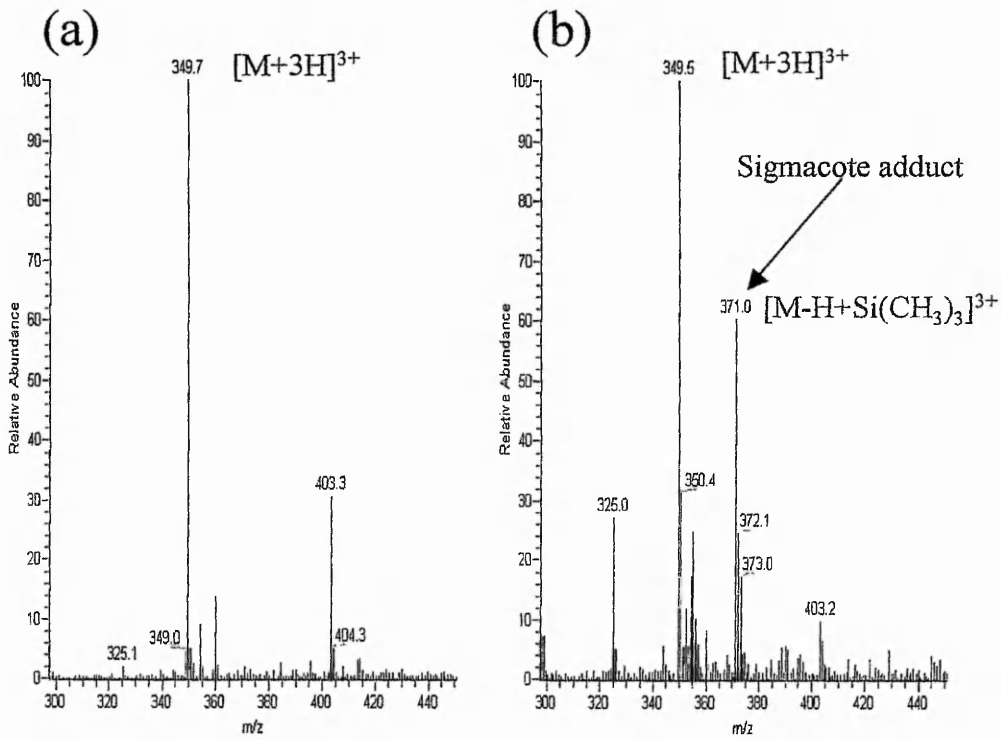


Figure 4.1 Precursor ion spectra showing silica group adduction of triply charged A3 peptide

600 pmol A3 peptide (sequence KQSSKALQR) in acetonitrile/5% acetic acid was added (at room temperature) to glass vials which had been coated with Sigmacote™ just prior to the addition of the peptide solution. The peptide solution was then removed from the bottle and injected into the mass spectrometer. The precursor ion spectrum seen in (a) was obtained and after three minutes the appearance of additional ions, including the m/z 371 ion was seen (b). The m/z 371 ion results from the addition of a silica group, a component of the Sigmacote™, binding to the hydroxyl (OH) group of a serine residue within the A3 peptide. The same result was obtained if the experiment was carried out at 4 or 60°C. Tandem mass spectrometry (see section 4.1.2) was performed on both the m/z 349 and 371 ions generating almost identical fragment (product) ion data. However, the product ion spectrum of the m/z 371 ion contained several additional peaks not belonging to the usual A3 peptide fragmentation pattern (not shown).

Sample clean-up can result in a significant loss of peptide. Since multistep purification processes are common, the efficient concentration of the remaining sample to allow detection is of utmost importance. The following chapter documents the development of a purification and concentration protocol resulting in the production of a sample containing detectable peptides, whilst removing contaminants which affect the stability and resolution of the mass spectrometer.

4.1.1 Mass spectrometry

During these studies we used a Finnigan-MAT LCQ mass spectrometer with an electrospray ionisation (ESI) source. The use of ESI in peptide analysis is well documented (Fisk et al. 1997; Pieper et al. 1999; Heeft et al. 1998; Hogan et al. 1998; Huczko 1993; Hunt 1992; Tomlinson et al. 1996) and thus was used here. Mass spectrometry involves the ionisation of sample molecules and their conversion into a gas phase, the sample ions are then sorted according to mass to charge (m/z) ratio and subsequently analysed. Analysis of peptide presence within samples is performed during the sample introduction to the mass spectrometer and thus a large volume of sample (above 100μl) may be needed if several analyses are to be performed or if detection of peptide is difficult. However, for the majority of applications ESI-MS provides a useful method by which to detect and analyse peptides originating from biological samples.

4.1.2 Mass spectrometric analysis

Peptide analysis using ESI-MS is conducted in two main stages (tandem mass spectrometry – MS/MS). Figure 4.2 is a schematic diagram of the mass spectrometer. The sample is introduced to the mass spectrometer via a narrow metal capillary and becomes charged due to the application of an electrical potential to the capillary. The solution is then sprayed from the capillary as a mist of charged droplets that are evaporated by a warm gas flow; the sample molecules, as ions, are desorped from the solution.(Chapman 1995). The charged ions enter the octopoles where they are channelled towards the ion trap entering via an aperture in the end cap. The ions are sorted by

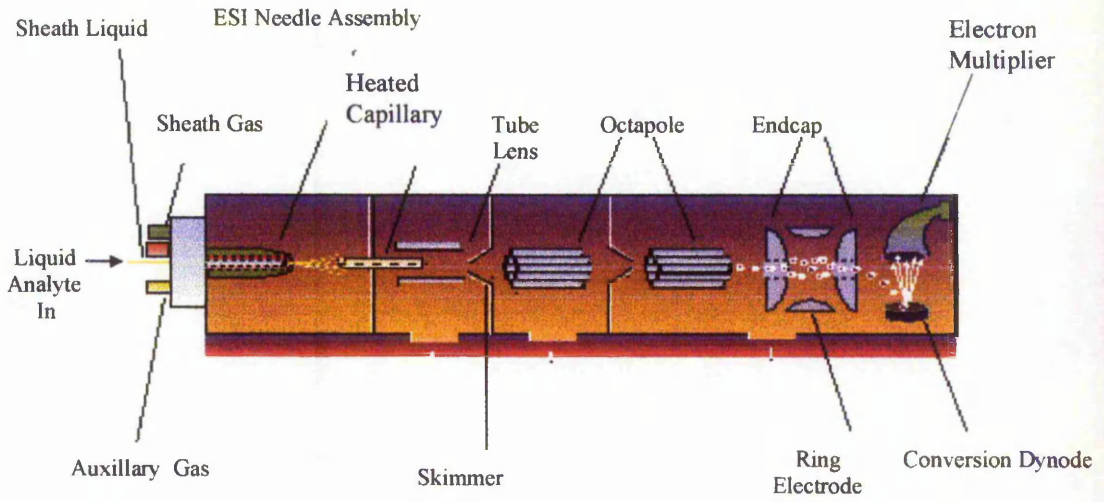


Figure 4.2 Schematic diagram of the Finnigan LCQ quadrupole ion trap mass spectrometer

Samples (liquid analyte) are introduced into the mass spectrometer via the heated capillary inlet (arrowed). The liquid is evaporated due to the temperature of the heated capillary, typically 100^{o}C, leaving the peptide ensconced within droplets which become gaseous ions due to the charge applied to the heated capillary inlet. The ions pass through the tube lens which is also charged and acts to funnel the ions towards the octopoles which, in turn, channel the ions into the ion trap via the endcap. In a single round of mass spectrometry the ions are channelled from the ion trap towards the detector in order of mass generating a precursor ion spectrum. The signals from the ions are multiplied (electron multiplier) to enable them to be seen as peaks on the computer screen attached to the mass spectrometer. In tandem mass spectrometry ions of a single peptide of interest are contained within the ion trap by helium molecules which collide with the peptide ions upon application of RF voltage by the endcaps. This collision (collisionally activated dissociation – CAD) causes the peptide ions to break apart at the bonds between the amino acid residues generating fragment ions representing smaller fragments of the original peptide. This is known as a fragment ion spectrum and the signals are again detected and multiplied by the electron multiplier. Each peptide generates a specific fragment ion spectrum by which it can be sequenced.

mass and exit the ion trap through an endcap aperture on the other side of the ion trap in mass order. The signals from the ions are multiplied by the electron multiplier and generate a precursor ion spectrum of all ion within the sample which is displayed on the computer screen attached to the mass spectrometer. If a particular ion within the sample requires further study, it can be isolated and subjected to fragmentation using tandem mass spectrometry which will allow its identification from a fragment ion spectrum.

4.1.3 Tandem mass spectrometry

Tandem mass spectrometry involves two rounds of mass spectrometry to enable isolation of a particular ion and its subsequent identification. The ions enter the ion trap as above, where an electric frequency is applied to the trap electrodes enabling all but a previously specified ion of interest to be ejected from the trap. Alternating voltages are then applied to the end cap electrodes which increases the energy within the precursor ion. This voltage is known as 'tickle' voltage. The ion begins to vibrate and it then collides with helium molecules causing fragmentation of the ion. RF voltage is applied and the fragment ions leave the trap mass by mass and are recorded by the detector generating a fragment ion spectrum. Peptides fragment into their constitutive amino acids (fragment ions) and are then able to be identified using an appropriate database (Chapman 1996; Hoffman 1996). The fragmentation ion pattern of each peptide is unique, peptides of the same mass but with different amino acid sequences give different fragment ion spectra.

The results displayed in this chapter were obtained in collaboration with Jennie Lill in the Department of Chemistry and Physics, Nottingham Trent University.

4.2 Results

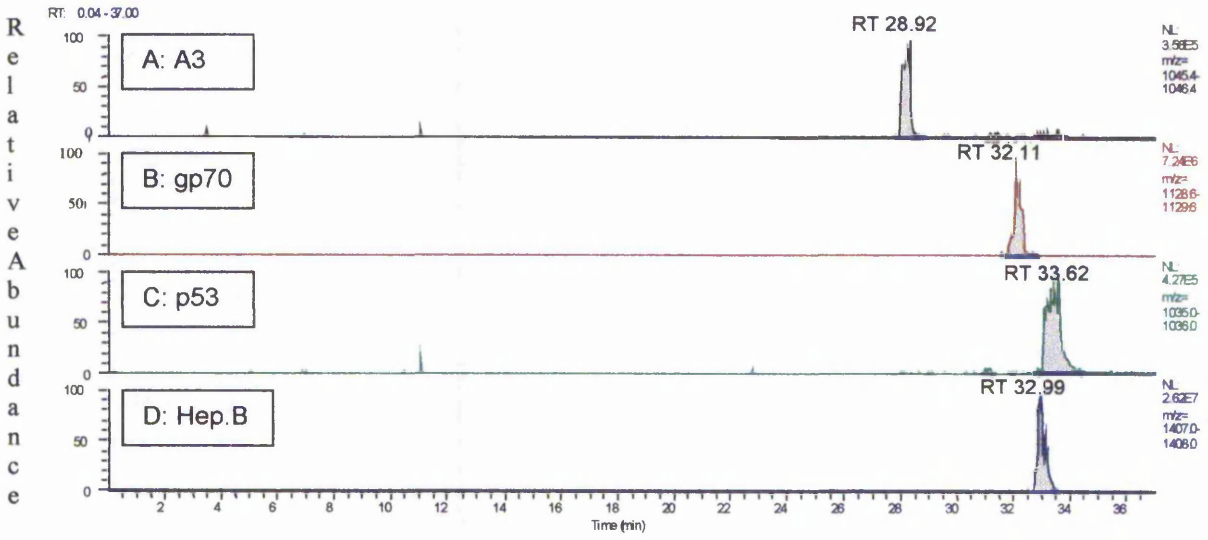
4.2.1 Selection of standard peptides

To ensure that the protocol developed was able to purify and concentrate a range of peptides as would be expected to be present on the cell surface, several standard peptides were selected to determine optimum conditions. Approximately 10,000 distinct peptides are present on the cell surface (Brockman et al. 1999) of differing sequence, hydrophobicity and hydrophilicity, thus a variety of standard peptides able to encompass the range of peptides found was selected. Figure 4.3 shows HPLC-MS traces of four different peptides which were used in the subsequent optimisations. The peptides show differing retention times ranging from 28.92 minutes for the most basic peptide, bcr-abl-A3 (subsequently referred to as A3), to 33.62 minutes for the p53 peptide. The peptide eluting region was thus determined to stretch over approximately five minutes.

4.2.2 Isolation of peptide species using copper complexation

Due to the large number of peptides likely to elute from the cell surface, it was decided that isolation of a subset of the total peptides may prove useful. To simplify the methods by which to identify peptides from the mass spectrum, the isolation of a smaller number of peptides was investigated. Metal chelating columns loaded with copper or nickel are able to separate histidine containing peptides from a peptide mixture. Four peptides, gp70, β-galactosidase, p53 and Hep.B were mixed together to test this method. The gp70 and β-galactosidase peptides both contained a single histidine residue, whereas the other two did not contain histidine and acted as controls for non-specific binding. Figure 4.4a shows the optimisation of the elution buffer. In a comparison between 0.5mM and 1.0mM HCl, the stronger acid was found to elute more efficiently, also removing a small amount of bound Hep. B peptide. Figure 4.4b shows recovery for the four peptides, small amounts of the non-histidine containing peptides (average less than 5% for both) were found along with higher amounts of the two histidine containing peptides. The gp70 peptide recovery was the highest at an average of 69% and the β-galactosidase peptide at 21%.

Figure 4.3 HPLC/MS trace of synthetic peptide mixture



Approximately 200 nmol of each peptide was added to 1ml pH8 water and loaded onto the 4.6mm RP-HPLC column. The gradient was run between 0 and 100% acetonitrile/5% acetic acid over 40 minutes and the peptides were isolated from the into total ion chromatogram (not shown) by inputting the relevant mass into the mass spectrometer software. Panels A-D show the peak relating to each of the synthetic peptides within the mix and their retention times (RT). The figures at the side of the panels relate to the ion concentration and peptide mass as mass to charge ratio (m/z).

A3 peptide origin = bcr-abl fusion region peptide, HLA-A3 restricted, sequence KQSSKALQR

gp70 peptide origin = glycoprotein, H2-K^D restricted, SPSYVYHQF

p53 peptide origin = p53 tumour suppressor, HLA-A2 restricted, LLGRNSFEV

Hep.B peptide origin = hepatitis B virus, HLA-DR restricted, TPPAYRPPNAPIL

Figure 4.4a. Effect of elution buffer molarity on peptide recovery

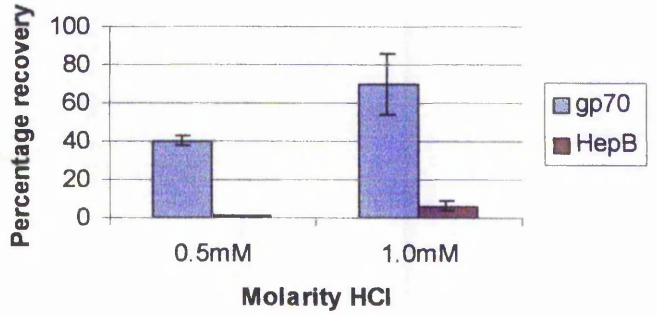


Figure 4.4b. Peptide recovery through metal chelate column

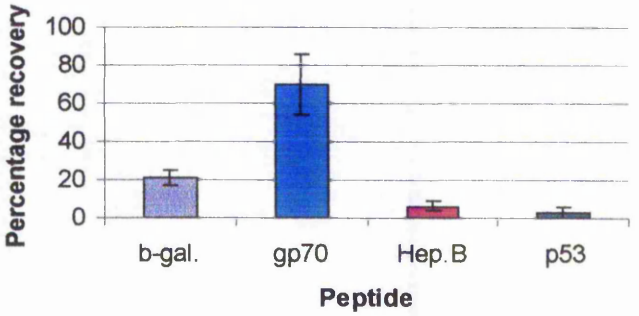


Figure 4.4. Peptide recovery following metal chelate column

Following equilibration, 0.5 ml copper (II) sulphate (1mg/ml) was loaded onto the sepharose chelating column and the column was washed in 5ml 0.1M sodium phosphate, pH7. The synthetic peptide mixture (containing approximately 200nmol of each peptide) was then loaded onto the column in 1ml of the wash buffer. The column was washed as before and the bound peptides eluted in 2ml HCl. The eluate was added to 2ml acetonitrile/5% acetic acid and 20µl was injected into the mass spectrometer. The recovery was calculated by comparison of the column eluate with the same amount of peptide injected directly into the mass spectrometer. In A, different molarities of HCl were compared and the 1mM was found to be most efficient at eluting peptide. This was then used for the subsequent experiment (B) and in future experiments.

4.2.3 Direct mass spectrometric identification of peptides via metal complexation

Following the variable peptide recovery using the metal chelation column, it was decided to attempt direct identification of metal-peptide complexes by mass spectrometry. The technique was first described by Volz et al (1998) for several peptides but had not been applied to MHC class I restricted peptides. Figure 4.5a shows a mass spectrum of a mixture of peptides including those without histidine residues (A3, Hep. B, p53), figure 4.5b shows these same peptides complexed with copper. Although not seen in Figure 4.5b, it was observed that the gp70 peptide complexed most efficiently with copper (results not shown) and figure 4.5c shows the time course over which the copper-gp70 complex was formed in increasing abundance at the expense of the uncomplexed peptide.

4.2.4 TCA precipitation

The development of the protocol to purify and concentrate total cell surface peptides was begun with the removal of any contaminating proteins from the citrate-phosphate peptide containing eluate. The presence of β_2-microglobulin within the eluate had been shown, (Chapter 3), and thus had to be removed from the eluate prior to concentration, together with other contaminating non-MHC associated proteins that may have been attached to the cell surface. Figure 4.6a-d shows the optimisation of the TCA precipitation. Bovine serum albumin was added to act as the protein contaminant. Figure 4.6a shows very low recovery following addition of TCA and centrifugation. It was then determined that the low recovery could be partly attributed to the TCA preventing the ionisation of the recovered peptide (b). Approximately 50% of peptide was unionised in the standard sample resulting in the appearance that it had not been recovered in A. A further improvement in the recovery was facilitated by the addition of a sonication step prior to (c) and also following centrifugation (d). The final protocol shows recovery of peptides at above 55% (compared to less than 25% in a) when corrected for TCA suppression by dilution of the TCA by adding an increased amount of acetonitrile/5% acetic acid:water.

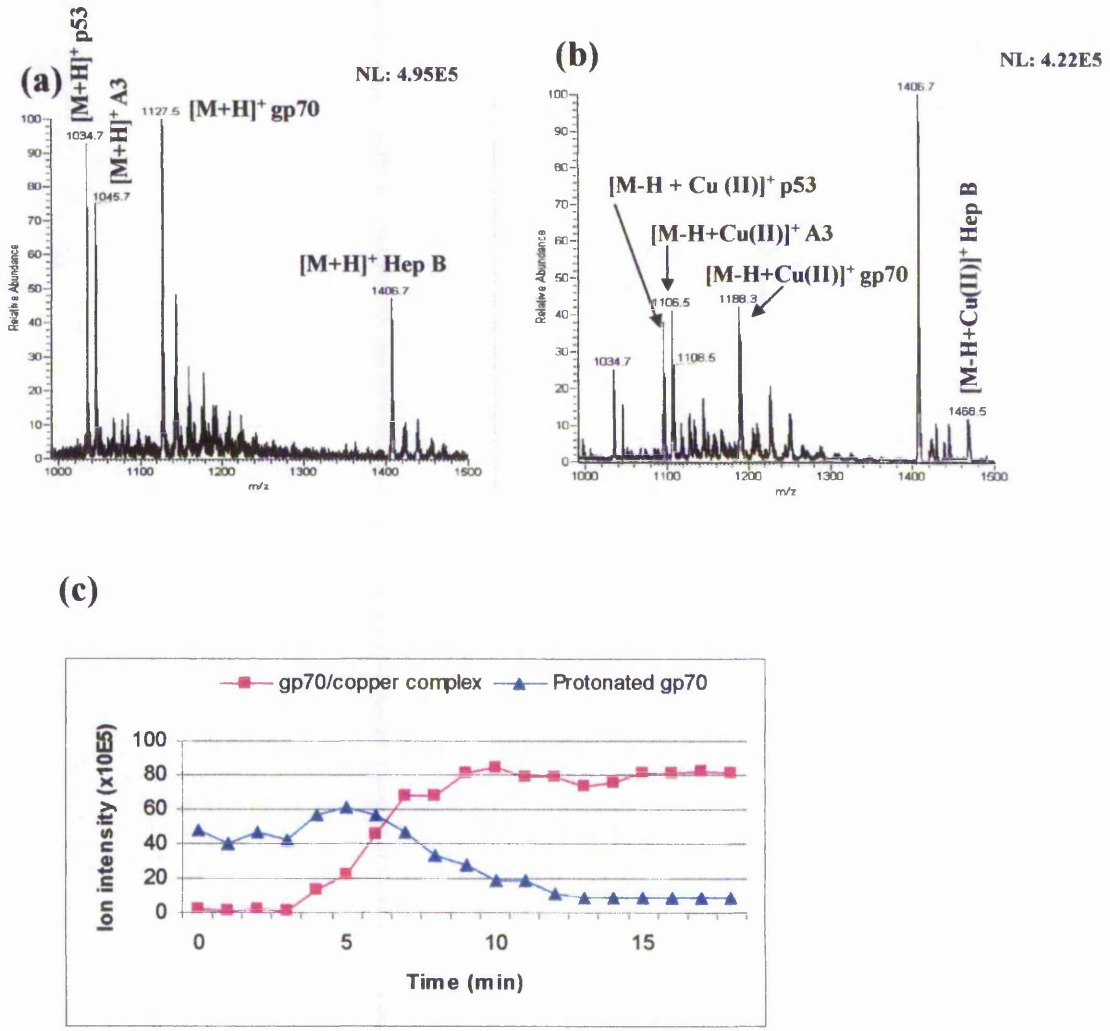


Figure 4.5 Effect of addition of copper (II) acetate to a mixture of peptides during mass spectrometry

A mixture of peptides (300pmol/μl) and 70 molar excess of copper (II) acetate in ddH_2O were injected into the nanospray tip and allowed to mix during introduction to the mass spectrometer. At the beginning of the analysis (a) non-copper complexed peptide peaks were seen with copper complexed peaks (showing a mass increase of 62 mass units due to the addition of copper) becoming more dominant after a few minutes (b), except in the case of gp70 peptide which began almost immediately. The copper complexation to the peptides continued, and in the case of gp70 due to the presence of the histidine residue, was at the expense of the normal protonated variant (m/z1127.5). The progression of the complexation is seen in c, peaking at 10 minutes post addition.

Figure 4.6a Peptide recovery following TCA precipitation

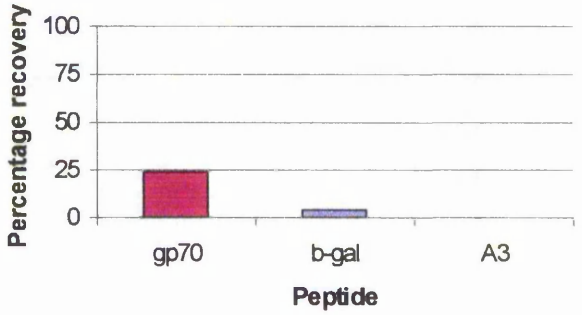


Figure 4.6b Ion suppression effect of TCA

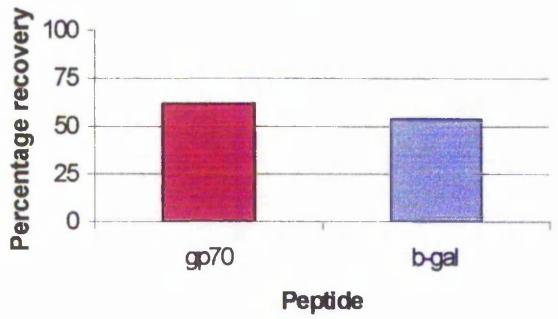


Figure 4.6c Effect of sonication on peptide recovery

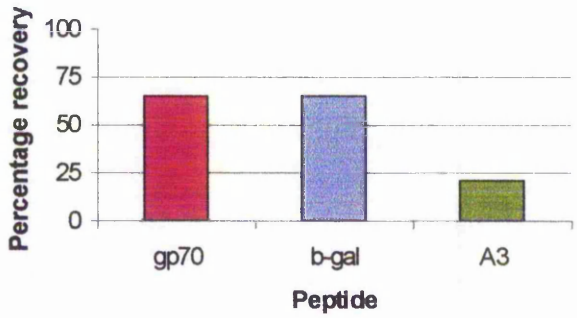


Figure 4.6d Peptide recovery following optimisation

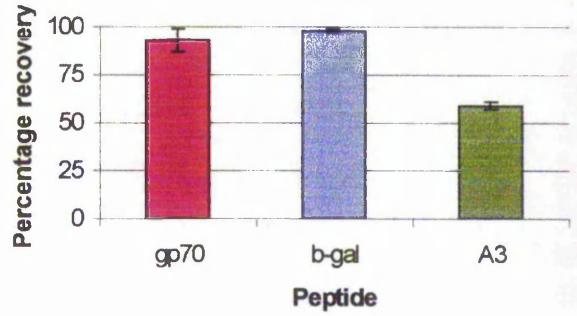


Figure 4.6 Optimisation of TCA precipitation protocol

Approximately 200 nmol of each peptide was added to 200µl bovine serum albumin (BSA at 1mg/ml), 58µl pH8 water and 30µl TCA (final volume 350µl). The mixture was then centrifuged at 15,000rpm for 10 minutes at 4°C, the supernatant removed and 450µl acetonitrile/5% acetic acid was added to bring the total volume to 800µl. Mass spectrometric analysis was performed by introduction of 5µl of the mixture (in triplicate) into the injection port of the mass spectrometer. Percentage peptide recovery was calculated by comparison of the peptide concentration within the mixture to an identical peptide standard consisting of the same peptide concentration in 800µl. For B, the same concentration of TCA used in A was added to an aliquot of the standard peptide mixture and the resulting mixture analysed as before. The suppression effect was quantified by comparison to the standard without addition of TCA. In C, the peptide mixture (as A) was sonicated for 10 minutes prior to centrifugation and otherwise treated as A. A further 10 minute sonication step (following centrifugation) was incorporated into the protocol used in C to produce the results shown in D. This represented the final protocol and was used for all future experiments.

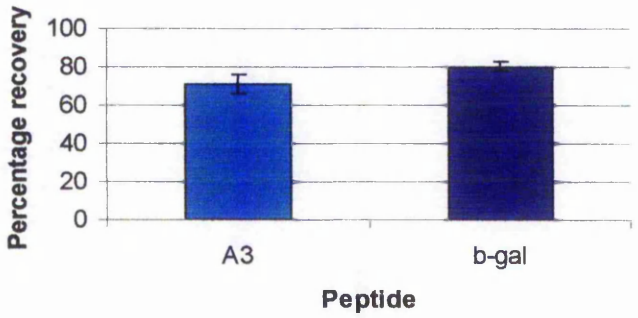
4.2.5 Cation Exchange-HPLC

A number of observations, including that in section 4.3 (ion suppression effect of TCA), resulted in the addition of a further cleanup step prior to HPLC fractionation and concentration. Citrate within the citrate-phosphate buffer appeared to prevent binding of peptides to the HPLC column and the sodium salts also within the buffer caused blockage of the heated capillary inlet preventing efficient functioning of the mass spectrometer (results not shown). A 1ml cation exchange column was used to remove the contaminating chemicals to improve the peptide detection and at the same time to reduce the volume of the eluate from 20ml for ease of loading onto the HPLC cartridge. Peptide recovery was tested for both cation exchange and HPLC together as the function of cation exchange was to facilitate use of the HPLC for peptide fractionation. A high recovery of peptide in excess of 70% was observed which showed the usefulness of this technique (figure 4.7).

4.2.6 Freeze drying

In order to reduce the peptide sample to a volume enabling total peptide to be analysed by the mass spectrometer, a freeze drying technique was investigated. The lyophilisation of peptides would enable total peptide to be reconstituted into a volatile solvent and at a volume convenient for analysis. The peptide sample was reduced to near dryness as reducing to compete dryness caused very low peptide recovery (results not shown). Initial experiments using peptides dissolved in acetonitrile showed that a recovery of 30-60% was obtainable using this technique (Figure 4.8a). However, freeze drying had to be performed after cation exchange – HPLC as the salts within the citrate-phosphate buffer precipitated upon freeze drying (results not shown) preventing mass spectrometric analysis for reasons suggested above (section 4.1). Figure 4.8b shows that peptide recovery following cation exchange-HPLC and lyophilisation was very low, below 20%. Since this may prevent mass spectrometric detection, a more efficient method was investigated.

Figure 4.7 Peptide recovery following cation exchange-HPLC



A peptide mixture (500nmol A3, 65nmol β-galactosidase in 20 ml citrate phosphate buffer pH3.3) was loaded onto the pre-equilibrated cation exchange cartridge and eluted in 2ml with O.1M sodium hydroxide. The pH of the eluate was adjusted to 8 and loaded onto a 4.6mm RP-HPLC column, washed for 20 minutes in pH8 water and a gradient of 0-100% acetonitrile/5% acetic acid/0.01% TFA over 40 minutes (1ml/minute) was used to elute the peptides from the column. 2ml fractions were collected and peptide recovery analysed by injection of 5μl (in triplicate) from each fraction into the mass spectrometer and comparison to an identical peptide standard that had not been fractionated by cation exchange-HPLC.

Figure 4.8a Recovery of peptides following freeze drying

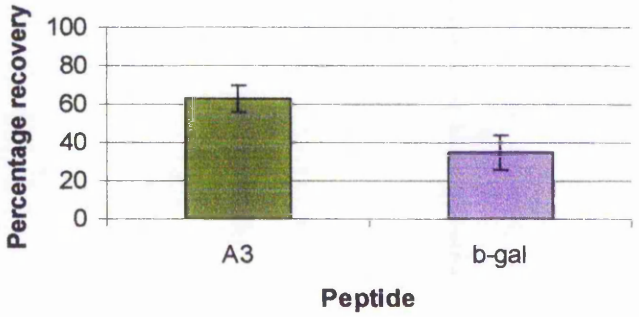
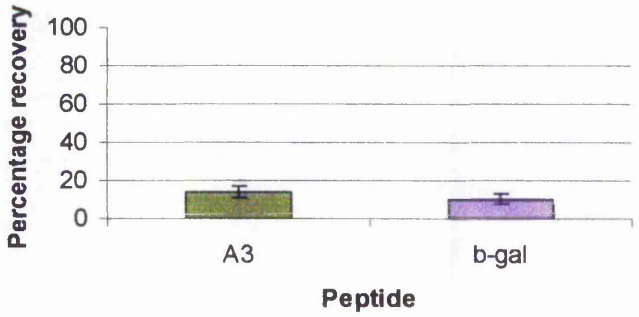


Figure 4.8b Peptide recovery following cation exchange-HPLC and freeze drying



In A, 500nmol A3 and 65nmol β-galactosidase were dissolved in 2ml pH8 water with 5% acetic acid and 1ml was placed into a 1.1ml tapered glass vial with a teflon seal. The liquid was frozen in liquid nitrogen and placed in the freeze dryer for 3 hours or until near dryness. The remaining mixture was then added and treated as previously. The glass vial was then removed from the dryer and acetonitrile/5% acetic acid:water (50:50) was added to reconstitute the mixture to 60μl. The samples were vortexed and sonicated for five minutes. In B, 2ml HPLC fractions were collected following cation exchange and RP-HPLC and treated in the same manner as above. Recovery in both experiments was analysed by 5μl injections (in triplicate) into the mass spectrometer with comparison to identical peptide mixtures which had not been freeze-dried.

4.2.7 Nanospray Ionisation

Figure 4.9 is a schematic diagram of the nanospray source constructed by Jennie Lill and James Stygall in the Department of Chemistry and Physics, Nottingham Trent University. The small gold-coated borosilicate needle (Nano-ES tip, Protana, Denmark) mounted in the PTFE holder was designed to introduce the peptide sample into the heated capillary inlet of the mass spectrometer at low flow rates. Fractions (100μl) were collected from a 1mm (internal diameter) RP-HPLC column (replacing the 4.6mm column used above) and re-fractionated on the same column allowing smaller factions, approximately 20μl, to be collected and loaded into the nanospray tip. The low flow rate enabled each fraction to be sprayed into the mass spectrometer for 1-3 hours allowing detection and identification at low peptide concentrations. Conventional electrospray as used in the sections above enables a short sample run time of a few minutes preventing low peptide concentrations to be detected.

Table 4.1 shows percentage peptide recovery following all stages of recovery and total estimated recovery as analysed by nanospray.

4.2.8 Tryptic digest of bovine serum albumin (BSA)

To test the ability of the protocol to generate peptides for detection and subsequent identification of the original protein, a model system was developed. A tryptic digest of BSA was carried out overnight. Figure 4.10 shows that the digestion reduced the BSA to a mixture of fragment sizes, some of which were expected to be peptides of a similar length to MHC-associated peptides. Larger fragments of BSA were removed by TCA precipitation prior to cation exchange. A prediction, both manually with knowledge of trypsin cleavage sites and with use of a database (Protein Prospector; www.prospector.ucsf.edu/) specifying trypsin as the digestive compound, of peptides produced was performed (table 4.2) and the mass/charge ratio of these peptides was calculated using the same database. A selection of these predicted masses were inputted into the mass spectrometer during the running of the sample and several peptides corresponding with the expected masses of BSA peptides from a tryptic digest were seen. One of these are represented as a full mass spectrometric scan in figure 4.11. Tandem mass spectrometry was subsequently performed to fragment the

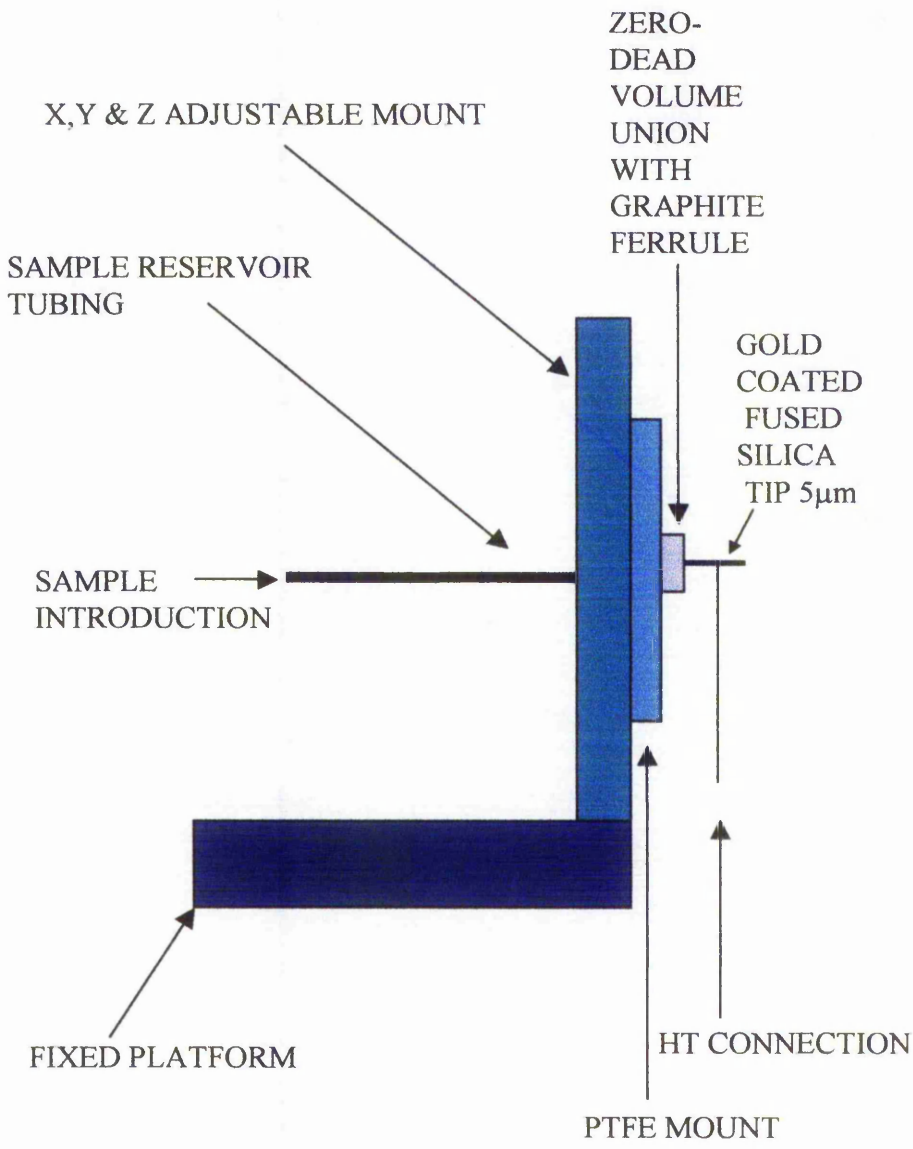


Figure 4.9 Schematic diagram of the nanospray source

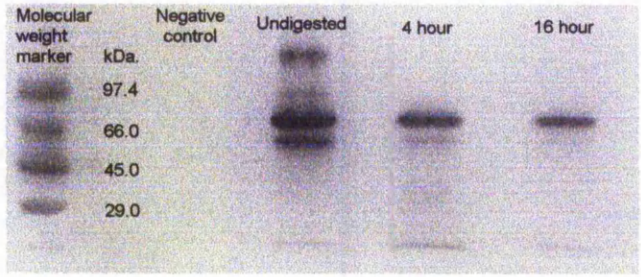
The nanospray source is fitted onto the front of the mass spectrometer in place of the conventional electrospray source. The tip is held in place with a swagelock union and adjusted to line up with the heated capillary inlet using the x,y,z adjustable mount. Tha sample is then introduced into the reservoir and the nanospray tip is gently broken against the heated capillary inlet causing a small aperture to form and spraying of the sample into the heated capillary to be initiated. The HT connection delivers an electrical charge to the nanospray tip causing charging of the molecules within the sample (thus ion formation) as they enter the heated capillary.

Table 4.1 Summary of standard peptide recovery

Separation procedure	Percentage recovery of peptide			Average% recovery
	A3	β-gal	gp70	
TCA precipitation	54	97	88	80
Ion exchange-HPLC	72	82	-	77
Freeze drying	15	10	-	12.5
Average total recovery				10

Samples were analysed for percentage recovery for each separation procedure in individual experiments as detailed above. The samples were analysed by mass spectrometry and the percentage recovery calculated from the original (input) peptide sample. Removal of the freeze drying procedure and replacement with concentration on the 1mm column followed by nanospray ionisation (as opposed to conventional electrospray) lead to an increase in recovery of peptide and sensitivity of detection and thus overall recovery was now approximately 25% (results not shown).

Figure 4.10 Tryptic digestion of bovine serum albumin (BSA).



BSA (1mg/ml) was dissolved in phosphate buffer (0.1M, pH7) and 10μg trypsin (1mg/ml in phosphate buffer) was added. The mixture was left at 37°C for either 4 or 16 hours. After the incubation time, the reaction was stopped with pefabloc (0.4mM) and the samples frozen until the gel was ready to run. The undigested sample consisted of an equal amount of BSA to which trypsin and pefabloc were added at the same time to prevent digestion. The samples were then run on a mini-gel as described in the methods section. Running time 1.5 hours, 100V.

Table 4.2 Predicted peptides resulting from a tryptic digestion of bovine serum albumin

mass	position	#MC	peptide sequence
1249.621	35-44	1	FKDLGEEHFK
1202.679	219-228	2	QRLRCASIQK
1197.557	337-346	1	DVCKNYQEAK
1193.602	25-34	1	DTHKSEIAHR
1177.559	300-309	0	ECCDKPLLEK
1163.631	66-75	0	LVNELTEFAK
1153.694	257-266	1	LVTDLTKVHK
1145.643	236-245	1	AWSVARLSQK
1142.714	548-557	1	KQTALVELLK
1138.567	223-232	1	CASIQKFGER
1115.607	20-28	2	GVFRRDTHK
1108.579	152-160	2	ADEKKFWGK
1083.595	161-168	1	YLYEIARR
1052.450	460-468	0	CCTKPESER
1050.492	588-597	0	EACFAVEGPK
1024.455	499-507	0	CCTESLVNR
1017.580	212-220	1	VLTSSARQR
1015.488	310-318	0	SHCIAEVEK
1014.619	549-557	0	QTALVELLK
1011.420	413-420	0	QNCDQFEK
1002.583	598-607	0	LVVSTQTALA
1001.589	233-241	1	ALKAWSVAR
990.558	210-218	1	EKVLTSSAR
988.567	490-498	1	TPVSEKVTK
987.537	29-36	1	SEIAHRFK
977.451	123-130	0	NECFLSHK
974.458	37-44	0	DLGEEHFK
927.493	161-167	0	YLYEIAR
922.488	249-256	0	AEFVEVTK
918.519	221-228	1	LRCASIQK
906.471	205-211	1	IETMREK
886.415	131-138	0	DDSPDLPK
847.504	242-248	1	LSQKFPK
841.460	483-489	0	LCVLHEK
820.468	229-235	1	FGERALK
818.425	562-568	0	ATEEQLK
817.489	452-459	1	SLGKVGTR
789.472	257-263	0	LVTDLTK
752.357	341-346	0	NYQEAK
733.420	212-218	0	VLTSSAR
725.259	581-587	0	CCAADDK
712.374	29-34	0	SEIAHR
701.401	198-204	0	GACLLPK
689.373	236-241	0	AWSVAR
665.377	156-160	1	KFWGK
660.356	490-495	0	TPVSEK
658.315	118-122	0	QEPER
656.347	24-28	1	RDTHK
649.334	205-209	0	IETMR
649.334	223-228	0	CASIQK
634.378	20-24	1	GVFRR
609.288	524-528	0	AFDEK
590.314	152-156	1	ADEKK
572.363	219-222	1	QRLR
567.325	434-437	1	YTRK
545.341	101-105	0	VASLR
537.282	157-160	0	FWGK
517.298	281-285	0	ADLAK
516.350	545-548	1	QIKK
509.319	558-561	0	HKPK
508.251	229-232	0	FGER
500.246	25-28	0	DTHK

The sequence of BSA was obtained from www.ncbi.nih.gov and was input into the peptide mass tool at www.expasy.com. Trypsin was selected as the enzyme with the maximun number of missed cleavages (#MC) as 2. Peptides larger than 500Da were selected for display. Peptides highlighted in blue were identified from the digest, however for simplicity only the results from the peptide highlighted in red (DLGEEHFK) have been included on the following pages.

peptide into constituent amino acids thus allowing elucidation of the amino acid sequence. The resulting sequence data (as fragment ion spectra) is shown in figure 4.12 and the fragmentation ion pattern was found to correspond to that expected (table 4.3) from the fragmentation of BSA peptides m/z 974.5 (sequence DLGEEHFK). The fragment ion mass was entered into a database (Prowl at www.proteometrics.com) and identified as originating from BSA (table 4.4).

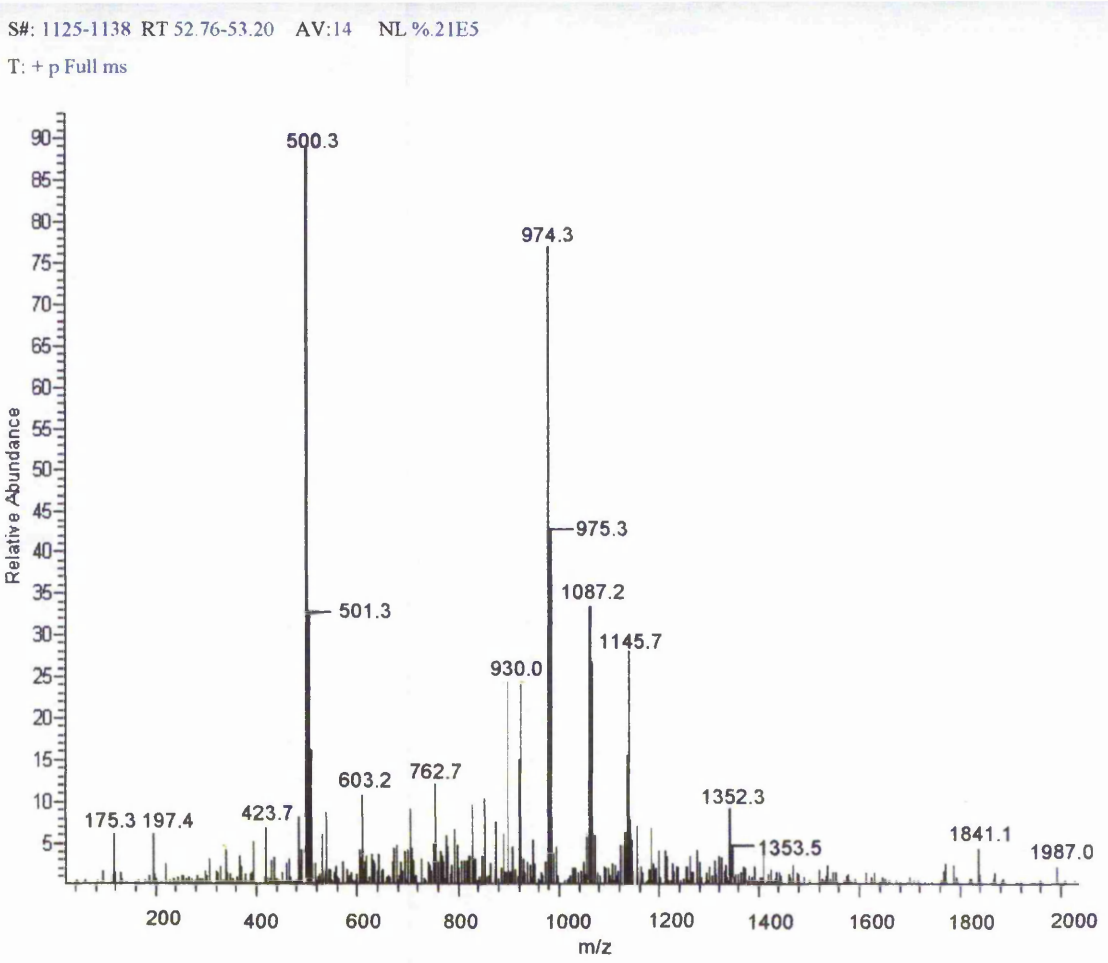


Figure 4.11 Precursor ion spectrum of 974.3

Fractions of the digested BSA sample were analysed by nanospray and predicted peptide masses (table 4.2) were inputted into the mass spectrometer. The 974.3 ion above was thought to originate from BSA (m/z 974.4, sequence DLGEEHFK; highlighted red in table 4.2). Masses identified as occurring within the eluate (present within spectrum for at least 100 scans) were then subjected to tandem mass spectrometry to identify the amino acid sequence.

Table 4.3 Predicted product ions originating from fragmentation of peptide DLGEEHFK (m/z 974.4)

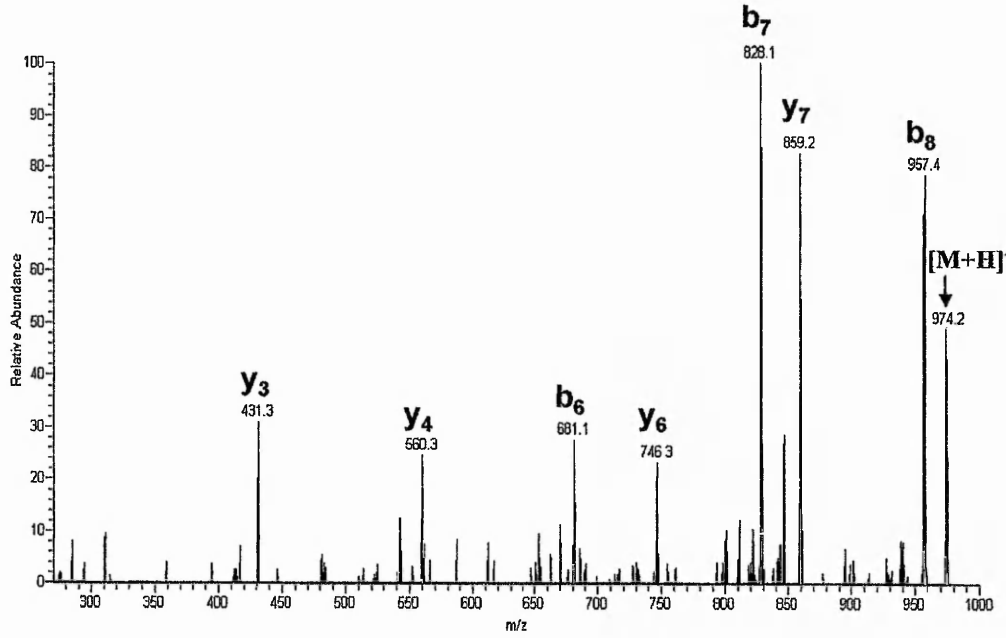
MS-Product Listing

Peptide Sequence	**DLGEEHFK**
Peptide Mass MH^+(average)	**974.4120**
Peptide Mass MH^+(monoisotopic)	**975.0493**
Amino Acid Composition	**D1 E2 F1 G1 H1 K1 L1**

	1	2	3	4	5	6	7
N-terminal ions							
a ions	88.04	201.12	258.15	387.19	516.23	653.29	800.3€
b ions	116.03	229.12	286.14	415.18	544.23	681.28	828.3
b+H_2O ions	---	---	---	---	---	699.29	846.3€
b-H_2O	98.02	211.11	268.13	397.17	526.21	663.27	810.34
C-terminal ions							
y ions	147.11	294.18	431.24	560.28	689.33	746.35	859.43
y-H_2O	---	---	---	542.27	671.32	728.34	841.42
y-NH_3	130.09	277.16	414.21	543.26	672.30	729.32	842.40

The sequence of the BSA peptide, DLGEEHFK was input into the MS Product database at UCSF (www.prospector.ucsf.edu) selecting an ESI-ion-trap as the mass spectrometer. The main fragment ions (monoisotopic masses) are given above, however the immonium ions and internal sequence ions are not displayed. Doubly charged variants of the product ions are achieved by halving the masses given above, ie y7 doubly charged = m/z 430.21. The mass (m) halves as the ion has two protons (charges - z) attached, a phenomenon that readily occurs when ionising peptides for mass spectrometric analysis (de Hoffman 1996).

Figure 4.12 Fragment (product) ion spectrum of peptide m/z 974.2



Tandem mass spectrometry of precursor ion m/z 974.2 generated fragment ions as above. Comparison of predicted fragment ion masses for the peptide and those obtained above confirm the sequence of peptide m/z 974.2 as DLGEEHFK.

Table 4.4 Identification of DLGEEHFK as originating from BSA

Rank	# Unmatched Ions	Sequence	MH^+ Calculated (Da)	MH^+ Error (Da)	Protein MW (Da)/pI	Species	NCBInr.6.24.2000 Accession #	Protein Name
1	0/7	(K)DLGEEHFK(G)	974.4583	-0.2583	69293.9 / 5.82	BOS TAURUS	1351907	(M73993) albumin
1	0/7	(K)DLGEEHFK(G)	974.4583	-0.2583	66141.1 / 5.38	BOS TAURUS	229552	albumin
1	0/7	(K)DLGEEHFK(G)	974.4583	-0.2583	67881.4 / 5.85	MACACA MULATTA	2492797	(M90463) serum albumin
1	0/7	(K)DLGEEHFK(G)	974.4583	-0.2583	69323.9 / 5.82	BOS TAURUS	2190337	(Y17769) bovine serum albumin

Fragment ion masses and the parent ion mass (m/z 974.2) from figure 4.12 were input into the MS-Tag tool at www.proteinprospector.ucsf.edu , using trypsin as the digesting enzyme. The sequence database used was NCBI and searched 30391 sequences (of all species) with the final results using all the inputted fragment ions for the identification. The peptide sequence was confirmed as DLGEEHFK and as originating from bovine serum albumin.

4.3 Conclusion

Use of the purification/concentration protocol described above and summarised in figure 4.13 generates peptides from a model system which can subsequently be sequenced and identified using mass spectrometry (protocol shown in figure 4.14, following final modifications not described here). The identification of BSA from peptide fragment ion spectra demonstrates the feasibility of mass spectrometry in peptide identification from biological samples. The BSA peptides used were present at higher amounts within the sample than would be expected from cell surface MHC-restricted peptides from real cell eluates and thus the next stage was to determine whether the protocol could generate sequence data from a real biological system. Use of standard synthetic peptides demonstrated that the approximate total recovery of peptides was 20% of the starting peptide concentration.

4.4 Discussion

Figure 4.3 shows the single ion chromatograms of the standard synthetic peptides used for the optimisation of the purification/concentration protocol described above. The differing retention times for the peptides suggests that amino acid composition affects the peptide binding to the column. The A3 peptide contains three basic residues (two lysine and arginine) both of which are hydrophilic and thus may contribute to the early elution from the column into the mobile phase during HPLC. This peptide appeared to be the earliest to elute of several tested and as it seemed to be resistant to binding to the column, it was used to optimise the peptide binding conditions for the subsequent stages. The other peptides showed similar binding and elution properties.

The metal chelate studies (section 4.2.1) using the 1ml sepharose column were largely unsuccessful with the β-galactosidase peptide failing to bind to the column. The reason for this could be attributed to the location of the histidine residue within the peptide. A proline residue is located either side of the histidine residue and due to its structure may cause the histidine residue to be less accessible to the column bound copper (Garrett and Grisham 1999). Thus, other histidine containing peptides having a similar structure may not bind to the column. It was this conclusion that led to the use of direct complexation via mass spectrometry (section 4.2.2). Despite more efficient complexation with

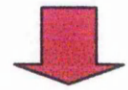
Figure 4.13 Standard protocol for isolation of peptides from surface MHC class I molecules

Acid elution of >2x10^9 cells

* Wash cells in serum free medium, pellet if suspension cell line.

* Add 20ml acid, gently agitate flask/tube ~5 minutes, RT, pH 3.3 citrate phosphate buffer.

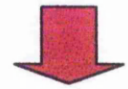
* Remove buffer to silanised glass bottle, add Hep. B peptide spike.



TCA precipitation

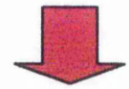
* Add 2ml 72% TCA, sonicate 10 min

* Centrifuge 15000g, 4^{O}C, 10 min, sonicate 10 min, remove supernatant.



Cation Exchange

* Load 20ml supernatant onto preconditioned cation exchange cartridge, wash in 30ml HCl (0.1M).



* Elute in 2ml NaOH (0.1M), load onto HPLC setup (figure 4.15)

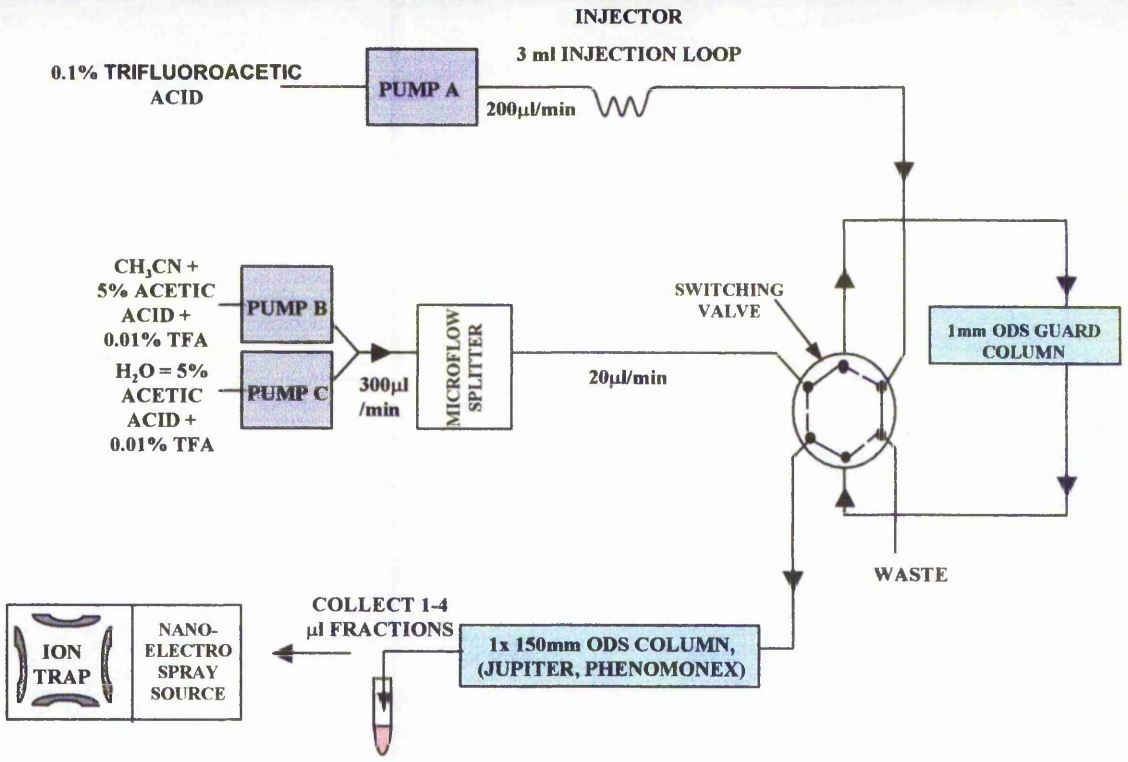


Figure 4.14 RP-HPLC/MS configuration for purification and concentration of peptides eluted from MHC class I molecules

The cation exchange eluate (2ml) is injected via the 3ml injection loop into a flow of water/0.1%TFA from pump A at 200µl/minute. The sample is washed onto the guard column via a switching valve. The peptides within the sample bind to the guard column and the flow from pump A continues through the column to wash salts and contaminants away to waste (20 minutes). The position of the switching valve is then altered and the flow from pumps B (20%) and C (80%) elutes the peptides from the guard column onto the analytical (1x150mm) column with acetonitrile gradient from 0-100% over 45 minutes . Fractions (1-4µl, approximately 1 every 20 seconds) are collected into 1.5ml tapered vials after the concentration acetonitrile from the pumps reaches 19%. The fractions are analysed in sequence by injection into the mass spectrometer via the nanospray source represented in figure 4.9.

the majority of peptides using the direct method, similar results (to the column studies) with the β-galactosidase peptide and the Hep. B peptide (both peptides containing histidine and proline residues) were obtained. A lower amount of copper complexation was seen with these two peptides (results not shown). Since non histidine – containing peptides were found to bind to the original column, albeit to a lesser degree, it seemed likely that copper may bind all peptides. In non-histidine containing peptides, experimentation revealed that this was due to non-specific C-terminal binding (Creaser et al. 2000). The use of copper complexation could therefore be used as a confirmation that given peaks within a spectrum were peptides thus simplifying identification of peptide ions within a biological sample. Indeed, this was performed and is described in chapter 5.

The purification stages of the protocol (sections 4.2.3-4.2.6) were found to be necessary as problems with mass spectrometric analysis of biological samples were identified by ourselves. TCA precipitation was identified as a simple method to remove the β_2-microglobulin known to be present in the citrate-phosphate buffer following elution (chapter 3). Improvements in peptide recovery following sonication of the sample suggested that at least some of the peptides were becoming bound to the larger proteins within the sample and thus being pelleted during the centrifugation step. Sonication may have enabled them to detach from the large proteins, thus preventing their removal from the supernatant and allowing their subsequent detection. The ion suppression effect of the TCA and sodium adducts, together with the phosphate precipitation within the mass spectrometer strongly indicated the need for further purification of the sample prior to HPLC and mass spectrometry. Cation exchange enabled removal of contaminant ions such as sodium and phosphate, and replaced the TCA with a less ion suppressive agent allowing the peptides to be subjected to RP-HPLC in an appropriate buffer. The use of sodium hydroxide in the cation exchange process did not contribute to ion suppression as sodium could be reduced to a baseline level following a twenty minute wash protocol (HPLC), this was not the case with the citrate-phosphate buffer (contained sodium phosphate). It was possible that the presence of phosphate created more problems than sodium

Several researchers (Castelli et al. 1995; Storkus et al. 1993; Hunt 1992) had addressed the need for concentration of the peptides within the sample and found freeze drying a viable method to

achieve this. Whilst freeze drying the peptides dissolved in acetonitrile diminished recovery within acceptable limits (section 4.2.5), our findings were that freeze drying combined with the necessary purification techniques reduced peptide recovery to levels which may not have permitted detection of peptide from real cell eluates. Freeze drying of peptides contained within the citrate-phosphate buffer (thus avoiding cation exchange-HPLC) caused concentration of the salts within the buffer, such high salt concentrations would interfere with the sensitivity of the mass spectrometer (Jackson et al. 2000; Winston and Fitzgerald 1998; Wu et al. 1996; Tomlinson et al. 1996). Development of an HPLC elution gradient allowing bound peptides to be eluted in a relatively small volume over an extended period of time (due to low flow rates), enabled the use of a smaller column (1mm internal diameter) and the elution of peptides into smaller volumes. The peptides were therefore concentrated into a small volume (several 4μl fractions) for analysis by nanospray-mass spectrometry. The small volumes increased the probability of detecting peptides contained within the fraction and thus acted as an efficient concentration technique. The low flow rates used with the nanospray allowed tandem mass spectrometry to be carried out for extended periods of time, thus fractions containing several peptides could be analysed and peptides identified.

With the optimal systems in place, the tryptic digest of BSA was used to provide a model for the isolation and identification of peptides from a biological sample. Despite the BSA not being completely digested (figure 4.10 shows a band of remaining, undigested BSA) several peptides were identified as originating from BSA although for simplicity only one has been included in this section. The peptide was present in high concentrations as suggested by the high ion count (10^6 ions) and upon tandem mass spectrometry, the fragment ion masses matched those predicted for the peptide. The confirmation of the peptide as belonging to BSA was obtained by inputting the fragment ion masses into another database (MS-Seq, www.prospector.ucsf.edu/) which was able to identify the peptide as belonging to BSA. The results from the previous two chapters, including the acid elution technique and the ability to sequence peptides and identify their protein of origin suggests that the protocol described herein is viable as a method by which to isolate and identify tumour-associated MHC-restricted antigens from real cell eluates. The following chapter describes results obtained from the application of this protocol to tumour antigen sequencing and identification.

Chapter 5: Identification of peptide antigens from tumour cell lines and clinical samples.

5.1 Introduction

In order to destroy infected cells, cytotoxic T cells must recognise peptides bound to MHC molecules on the surface of the target cell. Tumour cells express some peptides originating from intracellular proteins that may be tumour-specific and thus could allow the generation of tumour cell specific T cells. As discussed in chapter 1, cytotoxic T cells are an important constituent of any immune response, including that against tumours. The characterisation of MHC bound peptides from tumour cells is therefore important to the development of immunotherapeutic strategies against cancer. Several studies (Skipper et al. 1999; Theobald et al. 1997; Castelli et al. 1995; Storkus et al. 1993; Hunt 1992) and results described in chapter 3 have demonstrated that it is possible to isolate MHC bound peptides from tumour cells. However, a limitation of the technique is that a large number of cells are required to generate sufficient concentrations of peptides for analysis. Studies discussed in chapter 4 suggest that 10^9 cells generate approximately 1 nanomole (nmol) of peptide consisting of 10000 distinct peptides (Brockman 1999) and that peptide detection at this level is extremely difficult (Tomlinson et al. 1996). Following purification, peptide loss is likely to have occurred causing the amount of detectable peptide to have further diminished (to femtomole (fmol) levels), potentially making it undetectable by mass spectrometry. To ensure peptides isolated from tumour cell samples are detectable, in excess of 1 nanomole of peptide would need to be originally present to allow peptide detection and allow for peptide loss during biochemical clean-up protocols. The production of such high numbers of cells was found to be a limiting factor and thus the use of rapidly growing, non-adherent tumour cells was advocated. Leukaemic cells are both rapidly growing and many express a novel and specific tumour antigen, the bcr-abl fusion protein. Thus the characterisation of peptides from these cells may enable development of tumour specific immunotherapy directly against specific CTL epitopes expressed only by CML cells.

5.1.1 Chronic Myeloid Leukaemia

Chronic myeloid leukaemia is a clonal proliferative disorder originating in haematopoeitic stem cells (Fialkow et al. 1980), most common in middle age (Zingde 1998) and has an increased incidence in survivors of atomic disasters (Thijsen et al. 1999). CML is characterised by leucocytosis – the overproduction of white blood cells, which are genetically defective. The leucocytosis can be controlled during the chronic phase of the disease by mild oral chemotherapy (Hehlmann et al. 1993) however, currently curative treatment relies on the use of interferon α (Group 1997) followed by/or allogeneic or autologous bone marrow transplant (Sawyers 1999). Co-administration of GM-CSF and interferon-α has recently been identified as improved treatment for CML (Cortes et al. 1998). The molecular genetics of CML are well characterised and the Philadelphia chromosome (Ph^+) is present in the majority of cases. The Philadelphia chromosome is formed by the 9:22 translocation (figure 5.1) and results in the expression of two novel chimeric gene products (Melo 1996). The bcr and abl genes are normally controlled by checkpoint genes expressed on their original chromosome (Thijsen et al. 1999). Upon translocation this control is lost and the expression of the novel bcr-abl gene results in the continued presence of proteins which have unchecked tyrosine kinase activity which promote uncontrolled cell growth of the CML and ALL cells (Campbell and Arlinghaus 1991), (Daley and Ben-Neriah 1990). These novel proteins represent an ideal tumour specific target for immunotherapy mediated by antigen specific CTLs.

There are two main variants of the bcr-abl fusion protein resulting from splicing of different regions of the two genes together, and almost all Ph^+ patients express one, or occasionally both, of the two variants (Melo 1996). Figure 5.2 is a schematic diagram showing how the variants originate, in the b3a2 variant (pictured, breakpoints arrowed in blue) the first three exons (termed b3) of the bcr gene are spliced to exons 2-11 (termed a2) of the abl gene (Kurzrock et al. 1998). The b2a2 variant is less often observed (Thijsen et al. 1999), and is formed due to the first two exons of the bcr gene (b2 breakpoint arrowed in grey) fusing to exons 2-11 of the abl gene (a2). Both variants transcribe a 210kDa bcr-abl protein with

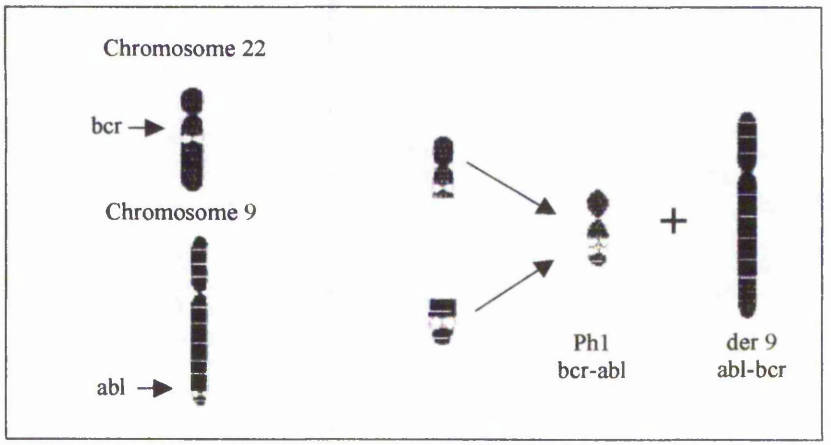


Figure 5.1 Production of Philadelphia Chromosome in CML

The translocation between chromosomes 9 and 22 results in two fusion chromosomes. Chromosome 22 contains the bcr-abl fusion and chromosome 9 the abl-bcr. The novel bcr-abl gene produces a 210kDa (p210) fusion protein known to be involved in the pathogenesis of CML. The function of the reciprocal abl-bcr fusion protein is largely unknown, but peptides derived from the protein have been shown to induce CTL in-vitro (Berke et al 2000).

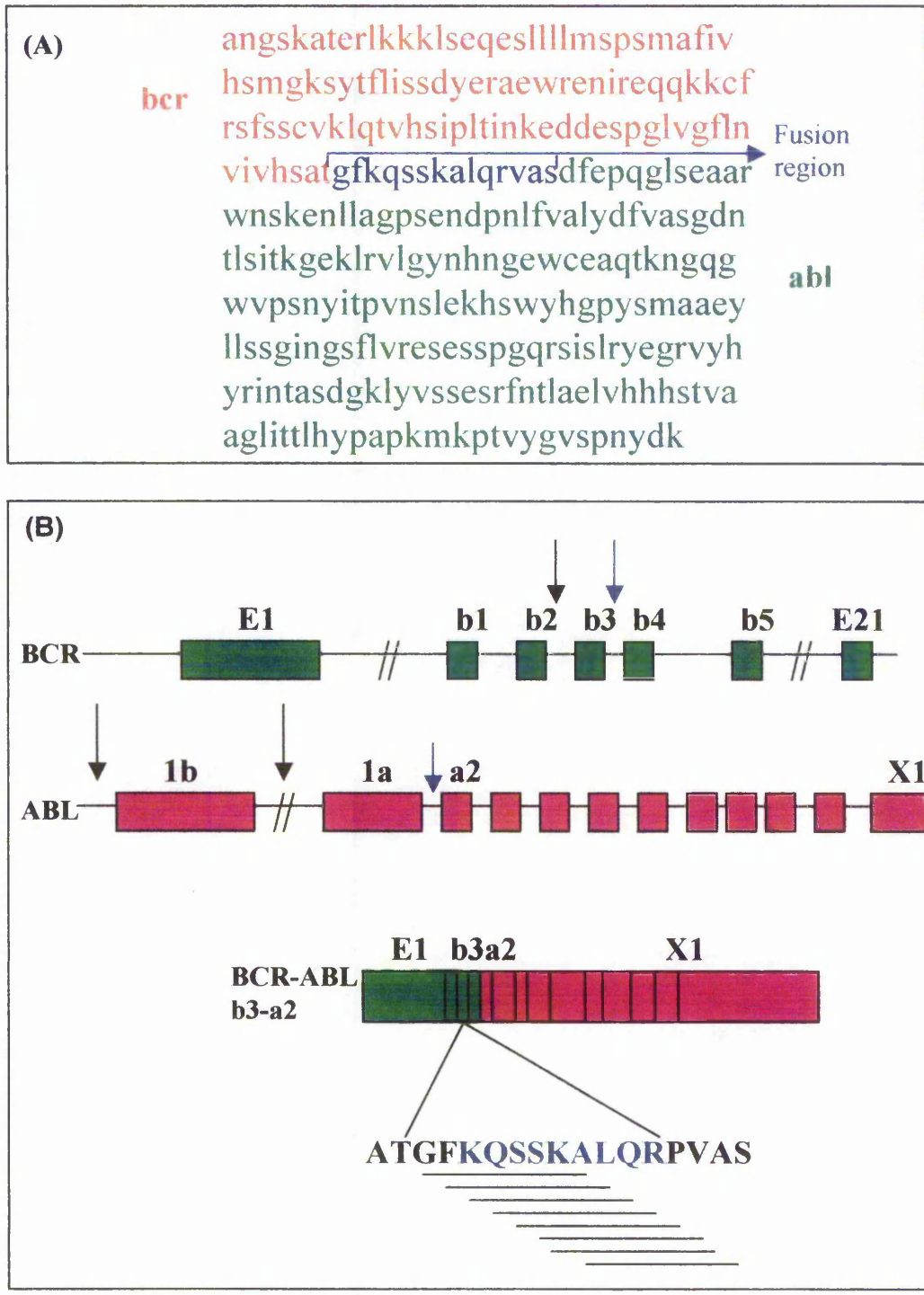


Figure 5.2 Schematic representation of the bcr-abl fusion protein

(A) shows the amino acid sequence of the bcr and abl oncogenes. The central lysine within the fusion region (blue) is specific to the fusion protein, forming as a consequence of the fusion. A schematic representation of abl and bcr genes is seen in (B), showing breakpoint regions (indicated by arrows). The most common breakpoints leading to the b3a2 p210 fusion product are indicated by blue arrows. Exon 3 (b3) of bcr and exon 2 of abl (a2) are spliced together forming the most common fusion, b3a2. The amino acid sequence of the junctional region is displayed and peptides resulting from these are underlined, the HLA-A3 restricted peptide is highlighted in blue.

tyrosine kinase activity (Sawyers 1999; Melo 1996), but the fusion regions have different amino acid sequences thus generating a different series of peptides which could be presented by MHC molecules on the cell surface (Momigliano-Richiardi et al. 1994). The b3a2 variant (fusion region sequence, GFKQSSKALQRPVAS, seen in figure 5.2) contains an amino acid at the centre of the fusion region (lysine), not previously present in either bcr or abl genes, which has been formed entirely as a result of the fusion. Thus b3a2 bcr-abl can be characterised as having a tumour-specific mutation. The b2a2 variant does not contain any novel amino acids and this may account for the reduced immunogenicity of this variant (Bocchia et al. 1996), peptides from the fusion region of this variant are also less able to bind MHC class I molecules (Cullis et al. 1994; Bocchia et al. 1995). The b3a2 fusion therefore receives more interest and appears more relevant immunologically. The A3 peptide (HLA-A3 restricted) used in standardisation processes described in chapter 4 is generated from the b3a2 splicing variant and contains this novel amino acid.

Major histocompatibility antigen (MHC) peptides corresponding to the bcr-abl junction have been shown to contain binding motifs for HLA-A3, HLA-A11 and HLA-B8 antigens, as demonstrated using in-vitro peptide stabilisation assays (Bocchia et al. 1995; Berke et al. 2000), and have also been shown to be capable of inducing both $CD4^+$ and $CD8^+$ lymphocyte responses in-vitro (Berke et al. 2000; Norbury et al. 2000; Osman et al. 1999; Nieda et al. 1998; Bocchia et al. 1996; Bosch et al. 1996). Although vaccination of CML patients with peptides corresponding to the break point region of the bcr-abl fusion protein can induce both DTH and CTL responses, it remains to be demonstrated whether they are expressed by MHC antigens on CML cells.

5.1.2 Selection of tumour cells for acid elution

a) K562 Leukaemic cells

The K562 cell line, is a lymphoblastoid cell line that has lost expression of surface MHC molecules and thus is sensitive to natural killer cell lysis (see sections 1.1.3 and 1.6). It naturally expresses the tumour-specific fusion protein bcr-abl and grows rapidly. The transfection of HLA-A3 into this cell line enables its use as a simplified model system for MHC-restricted presentation of bcr-abl junctional region peptides (see section 5.1.1). The parental K562 and transfected cell line were provided by The Anthony Nolan Bone Marrow Trust at the Royal Free Hospital, London for this purpose.

b) CML patient blast cells

Since large numbers of leukaemic blast cells expressing bcr-abl are removed from patients at initial diagnosis by leucapheresis and at later stages in the chronic phase of the disease (Dr. R.E. Clark, personal communication), this represented an ideal clinical tumour cell sample for acid elution. Patient samples were obtained from the Royal Liverpool University Hospital, and were eluted at Liverpool (using the protocol stipulated in Chapter 3) to ensure the cells were viable and to prevent cell lysis. This allowed the rapid isolation of surface bound peptides only. RT-PCR of mRNA from the cells was performed at Liverpool by Dr. L-H Wang to confirm the splicing variant, and thus to provide an indication of which peptides would be presented by HLA molecules on the cell surface.

5.2 Results

5.2.1 Peptide prediction

The sequence of the bcr-abl fusion protein was input into a database (Syfpeithi at University of Tübingen and BIMAS at NIH) with different HLA restrictions, paying particular attention to those HLA molecules known to bind peptides from the junctional region (HLA-A3, A11 and HLA-B8). The results are displayed in table 5.1. The HLA-A3 associated peptide KQSSKALQR (hereafter referred to as A3) can be seen to completely span the fusion region and also includes a novel amino acid, lysine, thus it is of most interest as a novel tumour antigen derived peptide. It can also be seen that it has a low binding affinity, however, its use in several studies (see section 5.1) to generate CTL responses to this sequence suggests that this may not be a critical issue. The mass of the HLA-A3 binding peptide from bcr-abl (KQSSKALQR) and the product ion masses resulting from tandem mass spectrometry of this peptide was predicted using MS Predict at UCSF (http://prospector.ucsf.edu/) (table 5.2).

5.2.2 Characterisation of K562 transfectants

The parental (untransfected) and HLA-A3 transfected K562 cell lines were analysed by flow cytometry to confirm their HLA status. W6/32 (pan HLA-A, -B, -C) and GAP-A3 antibodies were used to stain the cells to determine HLA-A3 expression. The level of expression in the untransfected cells was close to zero (0.97) and of the transfected cells only 12% expressed the HLA-A3 molecule (figure 5.3a). The GAP-A3 antibody did not bind to the cells well, most likely due to inadequate concentrations of the antibody within the supernatant. The level of bcr-abl expression was also variable as shown by intracellular staining (figure 5.3b), with the untransfected cells expressing the highest amount (22% of cells positive) compared to only 10% of the transfected cell line. The p53 and tubulin antibodies in this experiment acted as a positive internal control to ensure that the cells were permeabilised. The cells show variable staining of both proteins in replicates of the experiment accounting for the large error bars seen in figure 5.3b. Upon subjection to acid elution the transfected cells (10^9 cells) demonstrate a 40% reduction in HLA-A3 expression (7% Figure 5.4) and

Table 5.1 Predicted HLA-A3 binding nonamer peptides

Pos	1	2	3	4	5	6	7	8	9	Score
262	S	**L**	R	Y	E	G	R	V	**Y**	27
229	P	**V**	S	R	N	A	A	E	**Y**	25
113	I	**V**	H	S	A	T	G	F	**K**	23
166	A	**L**	Y	D	F	V	A	S	**G**	23
185	K	**L**	R	V	L	G	Y	N	**H**	23
281	K	**L**	Y	V	S	S	E	S	**R**	23
322	T	**V**	Y	G	V	S	P	N	**Y**	23
177	T	**L**	S	I	T	K	G	E	**K**	22
273	R	**I**	N	T	A	S	D	G	**K**	22
126	A	**L**	Q	R	P	V	A	S	**D**	21
242	G	**I**	N	G	S	F	L	V	**R**	21
55	R	**E**	N	I	R	E	Q	Q	**K**	20
105	G	**L**	Y	G	F	L	N	V	**I**	20
111	N	**V**	I	V	H	S	A	T	**G**	20
151	N	**L**	L	A	G	P	S	E	**N**	20
10	R	**L**	K	K	K	L	S	E	**Q**	19
73	S	**V**	E	L	Q	M	L	T	**N**	19
117	A	**T**	G	F	K	Q	S	S	**K**	19
248	L	**V**	R	E	S	E	S	S	**P**	19
268	R	**V**	Y	H	Y	R	I	N	**T**	19
70	S	**L**	T	S	V	E	L	Q	**M**	18
77	Q	**M**	L	T	N	S	C	V	**K**	18
88	T	**V**	H	S	I	P	L	T	**I**	18
141	S	**E**	A	A	R	W	N	S	**K**	18
187	R	**V**	L	G	Y	N	H	N	**G**	18
224	S	**W**	Y	H	G	P	V	S	**R**	18
302	T	**V**	A	D	G	L	I	T	**T**	18
14	K	**L**	S	E	Q	E	S	L	**L**	17
20	S	**L**	L	L	L	M	S	P	**S**	17
22	L	**L**	L	M	S	P	S	M	**A**	17
32	R	**V**	H	S	R	N	G	K	**S**	17
43	F	**L**	I	S	S	D	Y	E	**R**	17
85	K	**L**	Q	T	V	H	S	I	**P**	17
121	K	**Q**	S	S	K	A	L	Q	**R**	17
127	L	**Q**	R	P	V	A	S	D	**F**	17
170	F	**V**	A	S	G	D	N	T	**L**	17
195	G	**E**	W	C	E	A	Q	T	**K**	17
295	E	**L**	V	H	H	H	S	T	**V**	17
310	T	**L**	H	Y	P	A	P	K	**R**	17
316	P	**K**	R	N	K	P	T	V	**Y**	17

The sequence of the b3a2 bcr-abl protein (see section 5.1.2) from the NCBI protein database was pasted into the 'Syfpeithi' HLA peptide binding prediction database (www.uni-tuebingen.de/kxi) and HLA-A3 selected as the MHC type. The peptides are shown in order of binding strength determined by preferred binding residues at positions 2 and 9 (in bold) with residues at positions 3, 6 and 7 (underlined) having a lesser influence. The peptide with the highest score represents an ideal HLA-A3 binding peptide containing preferred residues. HLA-A3 preferred residues are any of L, M, I, V, S, A, T, F, at position 2 and K, R, Y, at position 9. The HLA-A3 binding peptide KQSSKALQR used in this study (denoted A3) lacks one preferred residue explaining its low ranking, however it spans the fusion region and contains a novel amino acid formed by the fusion and thus explains its interest.

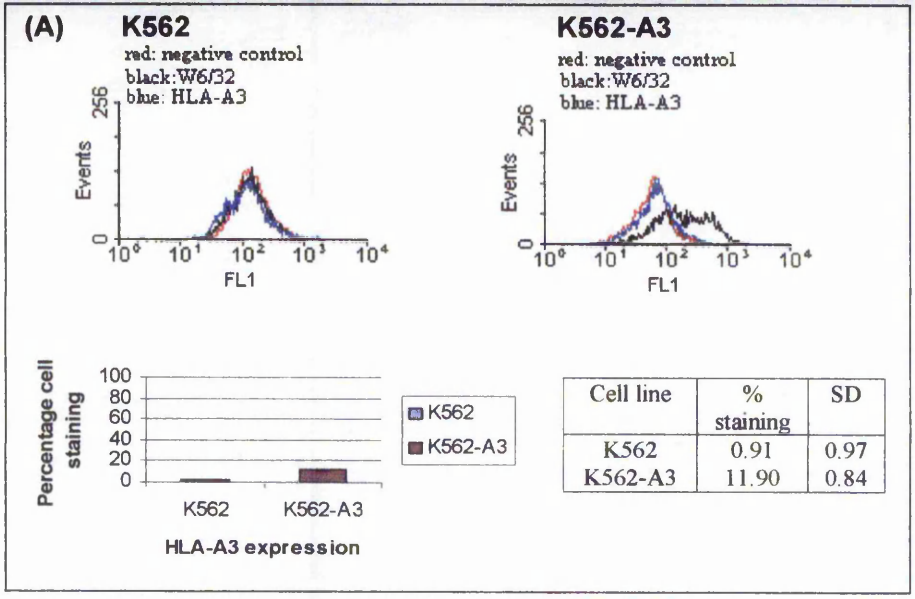
Table 5.2 Predicted product ions originating from fragmentation of peptide KQSSKALQR (m/z 1045.6)

MS-Product Listing

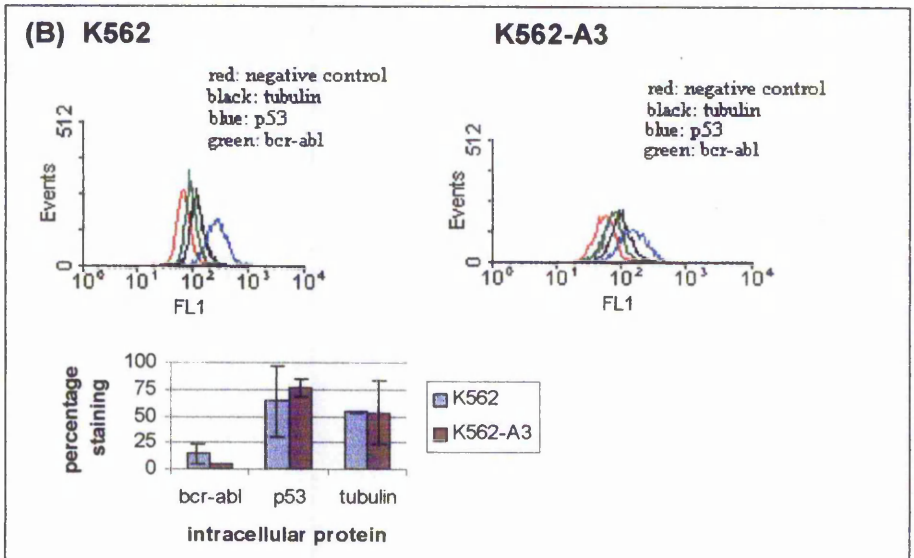
Peptide Sequence	**KQSSKALQR**
Peptide Mass MH^+(average)	**1046.2194**
Peptide Mass MH^+(monoisotopic)	**1045.6118**
Elemental Compositon	C43 H81 N16 O14
Amino Acid Composition	**A1 K2 L1 Q2 R1 S2**

	1	2	3	4	5	6	7	8
N-terminal ions								
a-NH_3 ions	84.08	212.14	299.17	386.20	514.30	585.34	698.42	826.48
a ions	101.11	229.17	316.20	403.23	531.33	602.36	715.45	843.51
b-H_2O ions	---	---	326.18	413.21	541.31	612.35	725.43	853.49
b-NH_3 ions	112.08	240.13	327.17	414.20	542.29	613.33	726.42	854.47
b ions	129.10	257.16	344.19	431.23	559.32	630.36	743.44	871.50
b+H_2O ions	---	---	---	---	---	---	761.45	889.51
C-terminal ions								
y ions	175.12	303.18	416.26	487.30	615.39	702.43	789.46	917.52
y-H_2O	---	---	---	---	---	684.42	771.45	899.5
y-NH_3	158.09	286.15	399.24	470.27	598.37	685.40	772.43	900.49

The sequence of the A3 peptide, KQSSKALQR was inputted into the MS Product database at UCSF (www.prospector.ucsf.edu) selecting an ESI-ion-trap as the mass spectrometer. The main fragment ions (monoisotopic masses) are given above, however the immonium ions and internal sequence ions are not displayed. Doubly charged variants of the product ions are achieved by halving the masses given above, ie y7 doubly charged = m/z 395.23. The mass (m) halves as the ion has two protons (charges - z) attached, a phenomenon that readily occurs when ionising peptides for mass spectrometric analysis (de Hoffman 1998).



Cell line	% staining	SD
K562	0.91	0.97
K562-A3	11.90	0.84

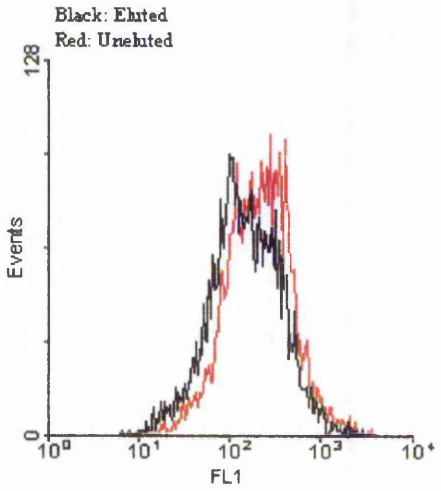


	% staining			SD		
Cell line	bcr	p53	tub	bcr	p53	tub
K562	14.61	63.62	53.41	9.78	33.83	1.15
K562-A3	5.03	73.50	53.18	0.01	8.31	29.70

Figure 5.3 Characterisation of K562 and K562-A3 transfectants

K562 and K562 transfected with HLA-A3 were stained with 25µl GAP-A3 (neat supernatant) or W6/32 and subsequently 25µl FITC secondary antibody (1:128 in IRPMI) at 4°C for 25 minutes each (A). To confirm bcr-abl expression, cells were stained with anti bcr-abl, DO7 (anti-p53) or anti tubulin following permeabilisation with saponin (0.4%) and subsequently with FITC secondary antibody as above. Cells were analysed by flow cytometry and the percentage staining calculated using WinMDI software.

Figure 5.4 Acid elution of K562-A3



Cell line	Fluorescence value	Percentage staining	% removal MHC
K562-A3 uneluted	214.39	11.30	
K562-A3 eluted	148.38	6.90	**39.4**

K562-A3 transfectants were washed in serum free medium then subjected to acid elution for 5 minutes in pH3.3 citrate phosphate buffer at room temperature. HLA-A3 expression on eluted and uneluted K562-A3 cells was analysed by flow cytometry using the anti HLA-A, -B, -C antibody W6/32 and FITC conjugated secondary antibody. The values are corrected for background fluorescence and the percentage cell staining was calculated using WinMDI software.

approximately 50% reduction in β_2-microglobulin (not shown). Cell viability was largely unaffected as tested by trypan blue exclusion and confirmed by the regrowth of an aliquot of eluted cells. Untransfected K562 was also eluted as a control to ensure that any peptides isolated were MHC-restricted, however, flow cytometry was not performed since they did not express detectable levels of MHC class I; these cells remained viable as tested by trypan blue and continued to grow when returned to culture.

5.2.3 Purification and mass spectrometric analysis of acid eluate

The acid eluate from the K562 transfectants was processed as described in chapter 4 and fractions tested by nanospray mass spectrometry. Citrate phosphate buffer, that had not been used to elute cells, was run through the protocol and also analysed by mass spectrometry to ensure that peptides were not present within the system prior to running the sample. The A3 peptide was present at low levels and thus the precursor ion (m/z 1045.0) was not seen in the primary spectrum. The mass of the triply charged variant (m/z 349.0) of the A3 peptide was inputted into the computer attached to the mass spectrometer and tandem mass spectrometry was performed. The fragment ion pattern produced is seen in figure 5.5a. By comparison, the predicted pattern in table 5.2 confirms that the peptide is indeed A3. To confirm the pattern and estimate levels of peptide present, a fraction from the blank run was spiked with 2.3 fmol A3 peptide (figure 5.5b). The fragment ion pattern observed is almost identical and the ion count for both samples is similar suggesting that a comparable amount of peptide is present in the fraction. The majority of the A3 peptide present eluted in a single fraction (spread between four fractions, peak width = approximately 1 minute).

5.2.4 Detection of A3 peptide in CML patient blast eluate

CML blasts were obtained from CML patients (table 5.3) by leucaphoresis at Royal Liverpool University Hospital. Acid elution of the blasts was carried out on site using the protocol described in chapter 3 as transporting the cells might affect their viability and

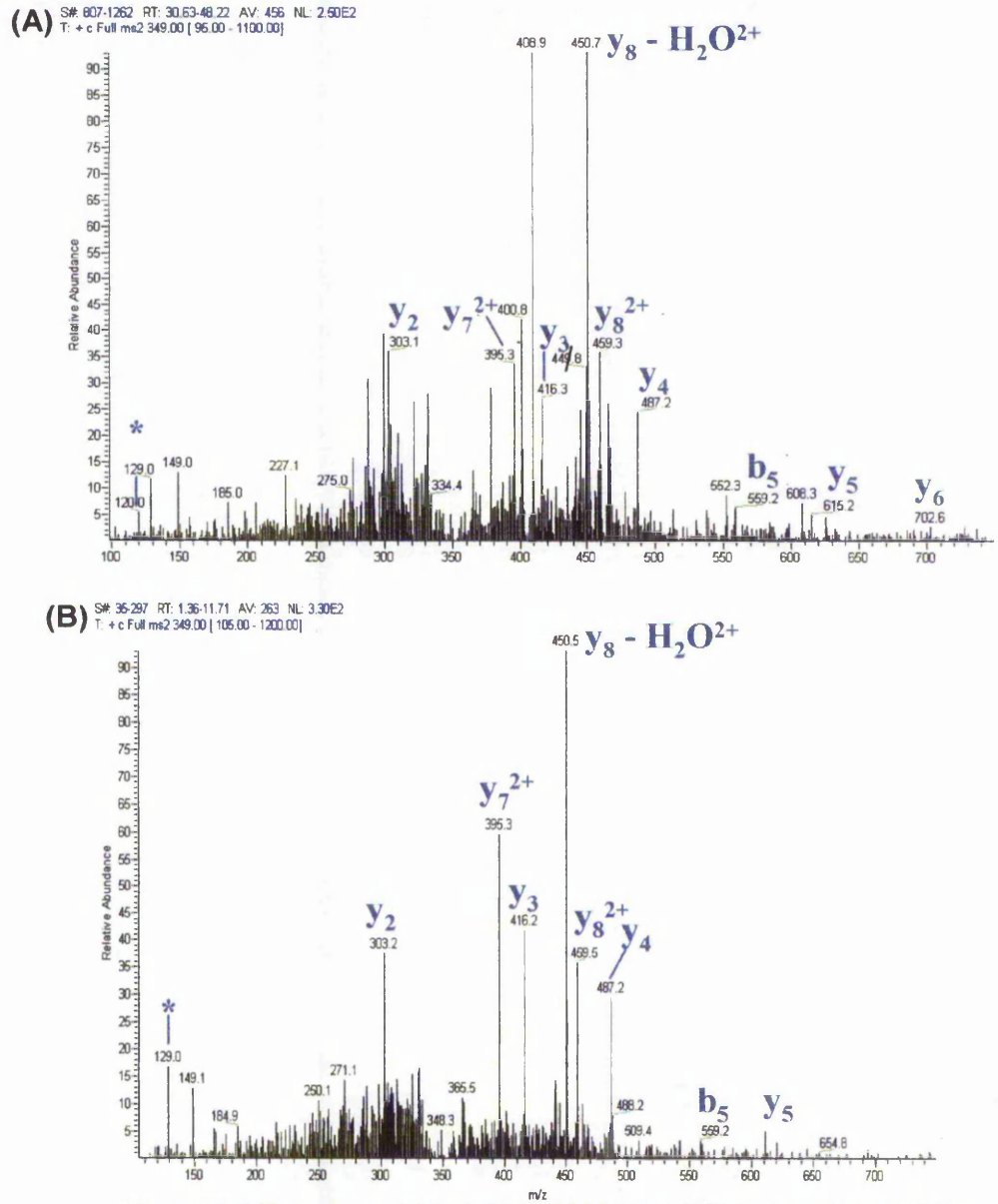


Figure 5.5 Presence of A3 peptide (KQSSKALQR) in transfected K562 cell eluate

Discovery of the triply charged variant of the A3 peptide (m/z 349, not shown) in the K562 eluate led to the subjection of this ion to tandem mass spectrometry. Upon analysis of the product ion spectrum A, several fragments as predicted in table 5.2 were present and have been identified above (in blue). Addition of synthetic A3 peptide (2.3 fmol/μl) to a blank fraction which had not previously contained A3 acted as a final confirmation that the fragment ion pattern seen in A was due to the presence of A3 (B). The star (*) indicates the immonium ion of lysine (K, m/z 129.0) and is also predicted to occur within the fragment ion spectrum (table 5.2).

Table 5.3 Summary table of patient sample data

Sample name	bcr-abl splice type	HLA type	Cell number $x10^9$	Result
JD	b3a2	A3 A29 B7 B44	4	A3 peptide found
CT	b3a2	A1 A3 B7 B8	100	A3, B8 peptides found
JL	b3a2	A2 A3 B51 B60	2.5	A3 peptide found
EM	b3a2	A1 B37 B62	20	A3 peptide negative
JH	b2a2	A2 A3 B8 B44	14	A3 peptide negative

Samples were collected by leucaphoresis at the Royal Liverpool University Hospital, eluted on-site using the given protocol and the eluate was frozen to be transported to Nottingham Trent University. Samples JD, CT and JL were all used as positive controls for the presence of A3 peptide as they were the correct splicing and HLA type. Samples EM and JH were used as negative controls, EM to ensure that the peptide presence was HLA-restricted and JH to ensure it was splicing type (b3a2) restricted.

increase the risk of cell lysis upon addition of the acid. The acid eluate was then frozen and transported to Nottingham where it was thawed and the purification/concentration protocol was carried out as described (Chapter 4). The HPLC fractions were analysed by nano-electrospray mass spectrometry and the A3 peptide was detected in the b3a2 positive patient sample JL as seen in figure 5.6. The fragment ions present were identical to those seen when the synthetic peptide was fragmented and in the K562-A3 eluate (figure 5.5). Inputting the fragment ion masses into a database (MS Tag at UCSF) resulted in identification of the peptide sequence as KQSSKALQR and the protein of origin as the bcr-abl oncogene (table 5.3). Analysis of a HLA-A3 positive, b3a2 negative sample (JH 10^{12} cells eluted) and an HLA-A3 negative, b3a2 positive (EM $2x10^{9}$ cells eluted) was performed to confirm that the peptide seen in the JL sample was obtained from the surface, HLA-restricted and due to the expression of the b3a2 protein. The A3 peptide was not detected in the samples, the splicing variant status of these samples was confirmed by RT-PCR at Liverpool (results not shown).

5.2.5 Detection of HLA-B8 binding bcr-abl derived peptide within CML blast cell eluate

Patient sample CT (b3a2 positive, b2a2 negative, HLA-A2, -A3, B8, B44, $1.4x10^{10}$ cells eluted) also demonstrated presence of HLA-A3 restricted KQSSKALQR (results not shown) in higher amounts than that seen in patient sample JL. The presence of the HLA-B8 binding peptide (GFKQSSKAL) was also seen (figure 5.7), albeit at low levels. Table 5.5 shows the peptide is identified as able to bind HLA-B8 with strong affinity. The product ions predicted to be formed by fragmentation of this peptide are shown in table 5.6 and table 5.7 demonstrates identification of bcr-abl from the B8 peptide product ions found within the eluate.

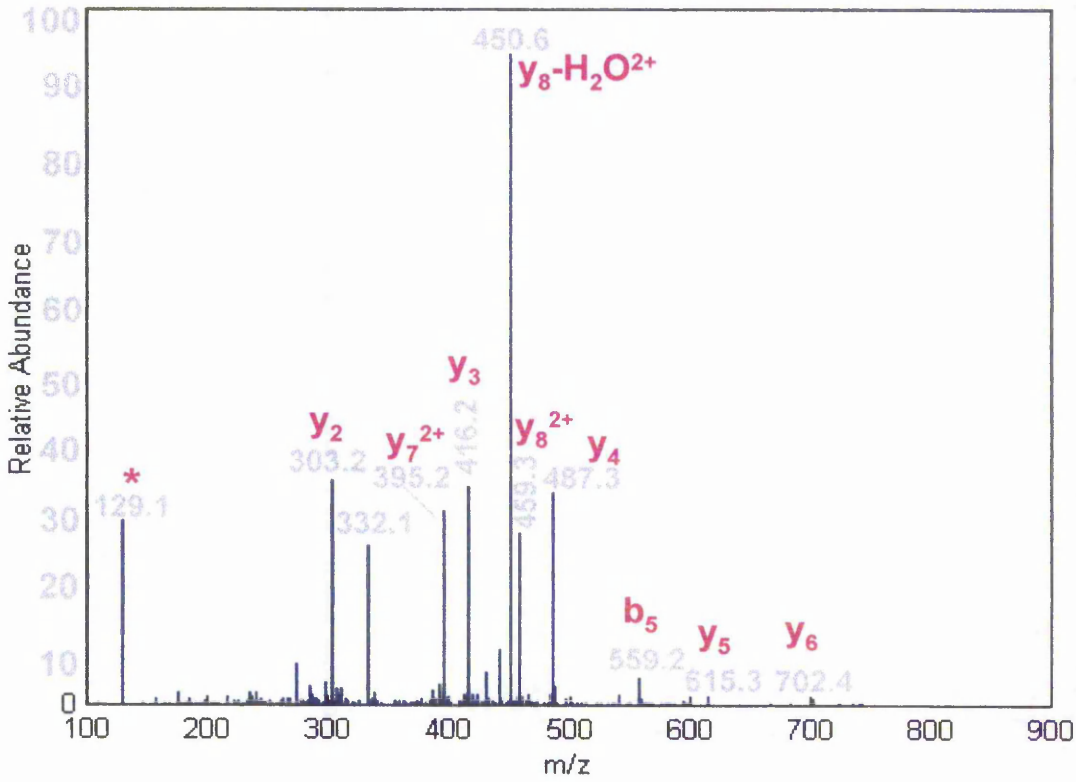


Figure 5.6 Presence of A3 peptide within CML blast cell eluate

CML blast cells collected from an HLA-A3 positive CML patient (JL) at the Royal Liverpool University Hospital were subjected to acid elution as described. The eluate was frozen and transported for analysis at Nottingham Trent University. Sample purification and concentration was performed as described and tandem mass spectrometry revealed fragment ions specific to the A3 peptide.

Table 5.4 Identification of bcr-abl protein from HLA-A3 restricted peptide KQSSKALQR

Result Summary

Rank	# Unmatched Ions	Sequence	MH^+ Calculated (Da)	MH^+ Error (Da)	Protein MW (Da)/pI	Species	NCBInr. 6.24.2000 Accession #	Protein Name
1	0/8	(V)QQSTIGQRAG(P)	1045.539	-0.5547	86099.6 / 6.57	CAENORHABDITIS ELEGANS	1118070	(U41554) Contains similarity to Pfam domain: PF00099 (zn-protease),
1	0/8	(F)KQSSKALQR(P)	1045.611	-0.6275	17118.0 / 5.47	HOMO SAPIENS	180639	(M25946) bcr/c-abl oncogene protein
1	0/8	(F)KQSSKALQR(P)	1045.611	-0.6275	32228.7 / 8.15	HOMO SAPIENS	224526	abl gene
1	0/8	(F)KQSSKALQR(P)	1045.611	-0.6275	15814.5 / 5.50	HOMO SAPIENS	179385	(M25949) bcr/c-abl oncogene protein
1	0/8	(F)KQSSKALQR(P)	1045.611	-0.6275	37242.9 / 8.91	HOMO SAPIENS	4033555	(AJ131466) BCR/ABL (major breakpoint) fusion peptide
2	1/8	(S)QAGSSAGSPAAAA(A)	1045.491	-0.5071	16110.8 / 11.53	CAENORHABDITIS ELEGANS	2911883	(AF047663) W09G12.6 gene product

Product ion masses (m/z 303.10, 416.30, 559.20, 615.20, 702.60, 395.30^{+2}, 450.70^{+2}, 459.30^{+2}) from the spectrum seen in figure 5.6 were input into the MS Tag database at UCSF to enable identification of the protein of origin. The parent ion mass was given as 349.00^{3+} with an error allowance of 1Da either side. The protein matches are given above and bcr-abl was returned by the database as a match for the peptide confirming that the peptide discovered in the JL sample was from bcr-abl.

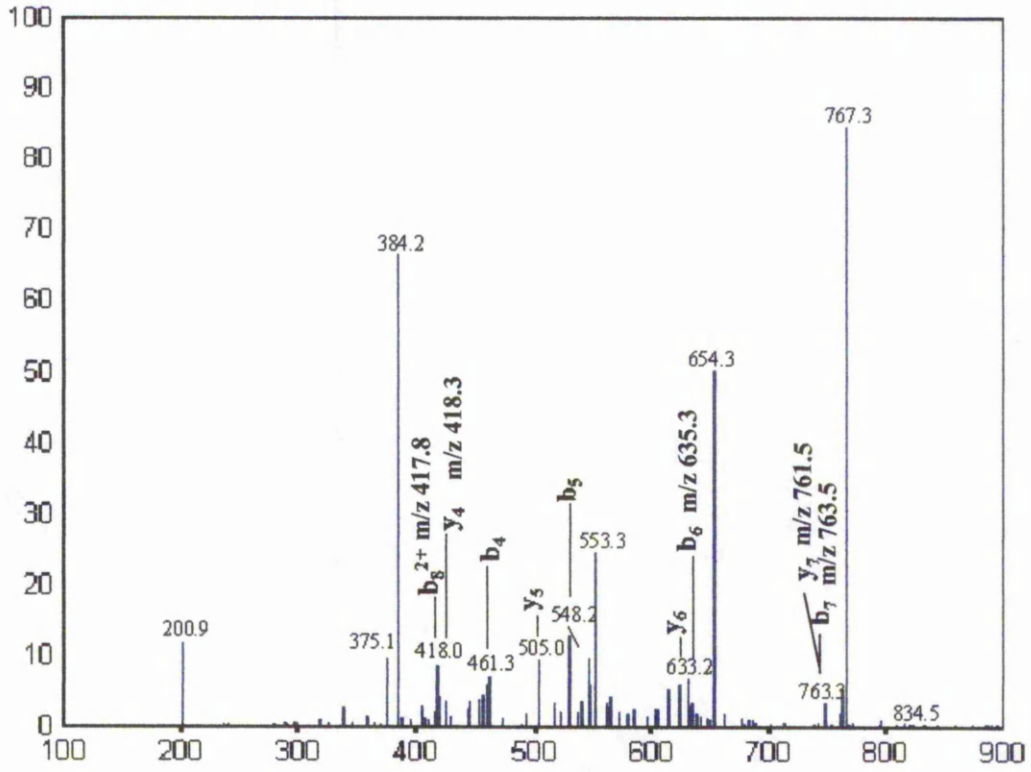


Figure 5.7 Presence of HLA-B8 restricted peptide GFKQSSKAL in CML patient eluate

During analysis of patient sample CT, the HLA-B8 peptide fragmentation ion pattern was found. Due to the low level of ions the blank spectrum (run before the sample eluate, see text) was subtracted from the peptide containing spectrum to remove contaminant ions and enable the peptide ions to be seen more clearly. The larger ions present in the spectrum (m/z 767.3, 654.3, 553.3 and 384.2) are from another peptide (figure 5.8) which is suppressing the B8 peptide ions.

Table 5.5 Predicted HLA-B8 binding nonamer peptides

Pos	1 2 3 4 5 6 7 8 9	Score
10	R L **K** K **K** L S E **Q**	27
255	S P **G** Q **R** S I S **L**	26
119	G F **K** Q **S** S K A **L**	23
181	T K **G** E **K** L R V **L**	22
183	G E **K** L **R** V L G **Y**	21
145	R W **N** S **K** E N L **L**	20
178	L S **I** T **K** G E K **L**	20
285	S S **E** S **R** F N T **L**	20
155	G P **S** E **N** D P N **L**	19
8	T E **R** L **K** K K L **S**	18
14	K L **S** E **Q** E S L **L**	18

The sequence of the b3a2 bcr-abl protein (see section 5.1.2) from the NCBI protein database was pasted into the 'Syfpeithi' HLA peptide binding prediction database (www.uni-tuebingen.de/kxi) and HLA-B8 selected as the MHC type. The peptides are shown in order of binding strength determined by preferred binding residues at positions 3 and 5 and 9 (in bold) . The peptide with the highest score represents an ideal HLA-B8 binding peptide containing preferred residues. The HLA-B8 binding peptide GFKQSSKAL used in this study (denoted B8) binds to HLA-B8 with intermediate to high affinity.

Table 5.6 Predicted product ions originating from fragmentation of peptide GFKQSSKAL (m/z 965.5)

MS-Product Listing

Peptide Sequence	**GFKQSSKAL**
Peptide Mass MH^+(average)	**966.1292**
Peptide Mass MH^+(monoisotopic)	**965.5420**
Elemental Compositon	C43 H73 N12 O13
Amino Acid Composition	**A1 F1 G1 K2 L1 Q1 S2**

	1	2	3	4	5	6	7	8
N-terminal ions								
a-NH_3 ions	---	---	288.17	416.23	503.26	590.29	718.39	789.43
a ions	30.03	177.10	305.20	433.26	520.29	607.32	735.42	806.45
b-H_2O ions	---	---	---	---	530.27	617.30	745.40	816.44
b-NH_3 ions	---	---	316.17	444.22	531.26	618.29	746.38	817.42
b ions	58.03	205.10	333.19	461.25	548.28	635.32	763.41	834.45
b+H_2O ions	---	---	---	---	---	---	781.42	852.46
C-terminal ions								
y ions	132.10	203.14	331.23	418.27	505.30	633.36	761.45	908.52
y-H_2O	---	---	---	400.26	487.29	615.35	743.44	890.51
y-NH_3	---	---	314.21	401.24	488.27	616.33	744.43	891.49

The sequence of the B8 peptide, GFKQSSKAL was inputted into the MS Product database at UCSF (www.prospector.ucsf.edu) selecting an ESI-ion-trap as the mass spectrometer. The main fragment ions (monoisotopic masses) are given above, however the immonium ions and internal sequence ions are not displayed. Doubly charged variants of the product ions are achieved by halving the masses given above, ie b8 doubly charged = m/z 417.72. The mass (m) halves as the ion has two protons (charges - z) attached, a phenomenon that readily occurs when ionising peptides for mass spectrometric analysis (de Hoffman).

MS-Tag Search Results

Result Summary

Rank	# Unmatched Ions	Sequence	MH^+ Calculated (Da)	MH^+ Error (Da)	Protein MW (Da)/pI	Species	NCBInr.56.2000 Accession #	Protein Name
1	0/7	(N)KGFQSSQAL(R)	965.5056	-0.1056	137562.8 / 8.42	HOMO SAPIENS	7657435	(AF181972) NADH/ NADPH thyroid oxidase p138-tox
1	0/7	(Y)LHLPSLTGGA(H)	965.5420	-0.1420	2970.4 / 6.92	HOMO SAPIENS	940086	(U30194) T-cell receptor delta chain
1	0/7	(T)GFKQSSKAL(Q)	965.5420	-0.1420	17118.0 / 5.47	HOMO SAPIENS	180639	(M25946) bcr/c-abl oncogene protein
1	0/7	(T)GFKQSSKAL(Q)	965.5420	-0.1420	37242.9 / 8.91	HOMO SAPIENS	4033555	(AJ131466) BCR/ABL (major breakpoint) fusion peptide
1	0/7	(T)GFKQSSKAL(Q)	965.5420	-0.1420	32228.7 / 8.15	HOMO SAPIENS	224526	abl gene
1	0/7	(T)GFKQSSKAL(Q)	965.5420	-0.1420	15814.5 / 5.50	HOMO SAPIENS	179385	(M25949) bcr/c-abl oncogene protein

Table 5.7 Database identification of bcr-abl from HLA-B8 restricted peptide

Product ion masses (m/z 418.20, 461.20, 504.90, 548.00, 633.20, 763.30, 835.20 selected from the spectrum seen in figure 5.7) resulting from fragmentation of the precursor B8 peptide ion were input into the MS Tag database (at UCSF) and the identification of the original protein sought. The parent (precursor) ion mass was entered as 964.00Da with an error of 1Da allowed. The database scanned 43419 sequences and the table above shows the matches returned by the database. The bcr-abl protein is selected as an origin for the B8 peptide.

5.2.6 Detection of unknown, novel proteins within CML blast eluate

During analysis of patient sample CT unknown peptides not associated with bcr-abl were seen, upon fragmentation their sequence was elucidated by manual analysis of the spectrum. The sequence PTITSGCRM, spectrum seen in figure 5.8, was then input into the MSProduct database and a number of the product ions resulting from fragmentation of this peptide as determined by the database (table 5.8), were identical to those seen in the spectrum. Attempts to identify the protein of origin were largely unsuccessful, but sequences within the peptide seemed to match with a protein involved in myeloid leukaemia (table 5.9). The second peptide identified, sequence TSQYR – Figure 5.9,

(product ions table 5.10), was, due to its size, smaller than conventional MHC class I restricted peptides. The closest sequence matches, table 5.11, were seen to be glycoprotein or virally associated suggesting that the peptide identified could be part of a commonly occurring or conserved protein. Due to the inability of the mass spectrometer to differentiate between glutamine and lysine, it was possible that the peptide sequence was TSKYR, and this sequence was homologous sequences within a haemoglobin protein (table 5.11). The sequence TSKYR was later found within the sample JH as part of a 9 amino acid length peptide (Jennie Lill – personal communication), which was more homologous to the haemoglobin protein shown in table 5.11. Two other small peptides present within the eluate, of 6 and 7 amino acids length, were also sequenced and found to be homologous to viral proteins. One, sequence CASKLCC, showed striking homology to the E core antigen of Hepatitis B virus (results not shown).

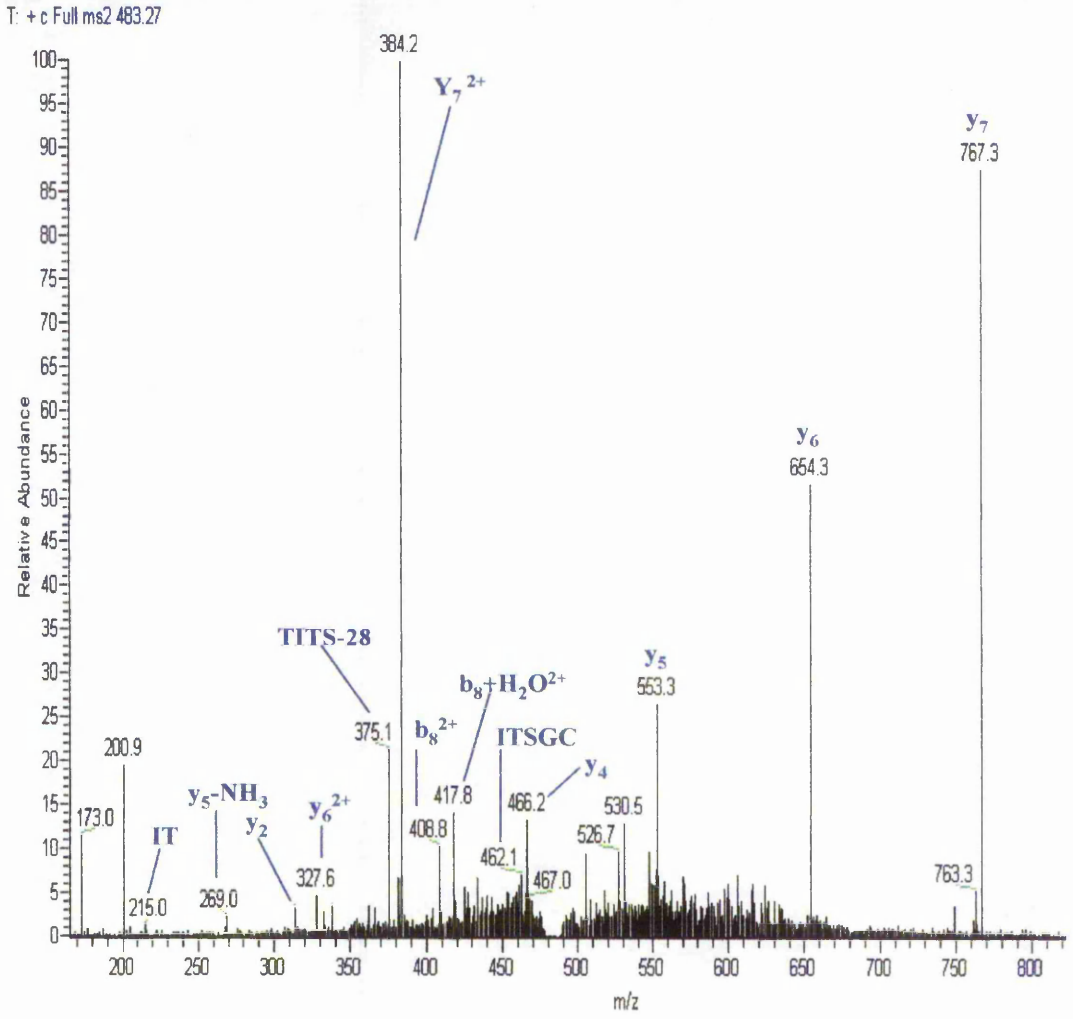


Figure 5.8 Presence of unidentified 9mer peptide (PTITSGCRM) in CML blast cell eluate

CML blast cells from a CML patient (CT) were eluted and analysed by mass spectrometry as described. An unknown peptide, not originating from bcr-abl was found and the sequence elucidated by examination of the fragment ion spectrum above. The postulated sequence was input into a database (MSFrag at UCSF) and the predicted fragment ions were found to be analogous with those within the above spectrum thus confirming the sequence.

Table 5.8 Predicted product ions originating from fragmentation of peptide PTITSGCRM (m/z 965.4)

MS-Product Listing

Peptide Sequence	**PTITSGCRM**
Peptide Mass MH^+(average):	**966.1709**
Peptide Mass MH^+(monoisotopic):	**965.4548**
Elemental Compositon:	C38 H69 N12 O13 S2
Amino Acid Composition:	**C1 G1 I1 M1 P1 R1 S1 T2**

	1	2	3	4	5	6	7	8
N-terminal ions								
a-NH_3 ions	---	---	---	---	---	---	---	771.38
a ions	70.07	171.11	284.20	385.25	472.28	529.30	632.31	788.41
b-H_2O ions	---	181.10	294.18	395.23	482.26	539.28	642.29	798.39
b-NH_3 ions	---	---	---	---	---	---	---	799.38
b ions	98.06	199.11	312.19	413.24	500.27	557.29	660.30	816.40
b+H_2O ions	---	---	---	---	---	---	---	834.41
C-terminal ions								
y ions	150.06	306.16	409.17	466.19	553.22	654.27	767.35	868.40
y-H_2O	---	---	---	---	535.21	636.26	749.34	850.39
y-NH_3	---	289.13	392.14	449.16	536.20	637.24	750.33	851.38

The sequence of the unknown peptide, PTITSGCRM was input into the MS Product database at UCSF selecting an ESI-ion-trap as the mass spectrometer. The main fragment ions (monoisotopic masses) are given above, however the immonium ions and internal sequence ions are not displayed for simplicity. Doubly charged variants of the product ions are achieved as before (table 5.2) and internal sequence ions seen on the spectrum (figure 5.8) were predicted as follows, 'IT' = m/z 215.14; 'TITS-28' = m/z 375.22; 'ITSGC' = m/z 462.20.

Table 5.9 Putative identification of peptide PTITSGCRM

Sequence:TSGCR
gi|5174573|ref|NP_005926.1| MYELOID/LYMPHOID OR MIXED-LINEAGE LEUKEMIA (TRITHORAX (DROSOPHILA) HOMOLOG); TRANSLOCATED TO, 2 Matching: GSGSRTSGCRQAVV (Residues 721 - 725) V gi|347377|gb|AAC37520.1| (L22179) MLL-AF4 DER(11) FUSION PROTEIN [HOMO SAPIENS] Matching: GSSSRTSGCRQAVV (Residues 1821 - 1825) V gi|2136143|pir||I52572 SERINE/PROLINE-RICH FEL PROTEIN, SPLICE FORM 2 - HUMAN Matching: GSGSRTSGCRQAVV (Residues 721 - 725) V
Sequence:TITSG
gi|4758720|ref|NP_004520.1| MYELOID/LYMPHOID OR MIXED-LINEAGE LEUKEMIA (TRITHORAX (DROSOPHILA) HOMOLOG); TRANSLOCATED TO, 3 Matching: DSNLLTITSGQDKKA (Residues 271 - 275) gi|6752305|gb|AB69660.1| (L22179) ALL-AF9 DER(11) FUSION PROTEIN [HOMO SAPIENS] Matching: DSNLLTITSGQDKKA (Residues 270 - 274)
Sequence TITS
gi|341657|gb|AB55978.1| (L12143) ALL-AF4 DER(11) FUSION PROTEIN [HOMO SAPIENS] Matching: TSSYVTITSHVLTAF (Residues 1147 – 1150) gi|2136144|pir||A45268 SERINE/PROLINE-RICH FEL PROTEIN, SPLICE FORM 3 - HUMAN Matching: TSSYVTITSHVLTAF (Residues 721 – 725)

The sequence PTITSGCRM was input into a protein database (www.proteometrics.com) but an exact match for the entire peptide sequence was not found, parts of the sequence were found within the proteins above. There are several variants of the AF-4 protein translocation and of the serine/proline rich protein, not all of which are shown here. These chromosomal translocations occurring in varying leukaemia types concern have been identified in individual or small groups of patients (Nakamura et al 1993, Morrissey et al 1993).

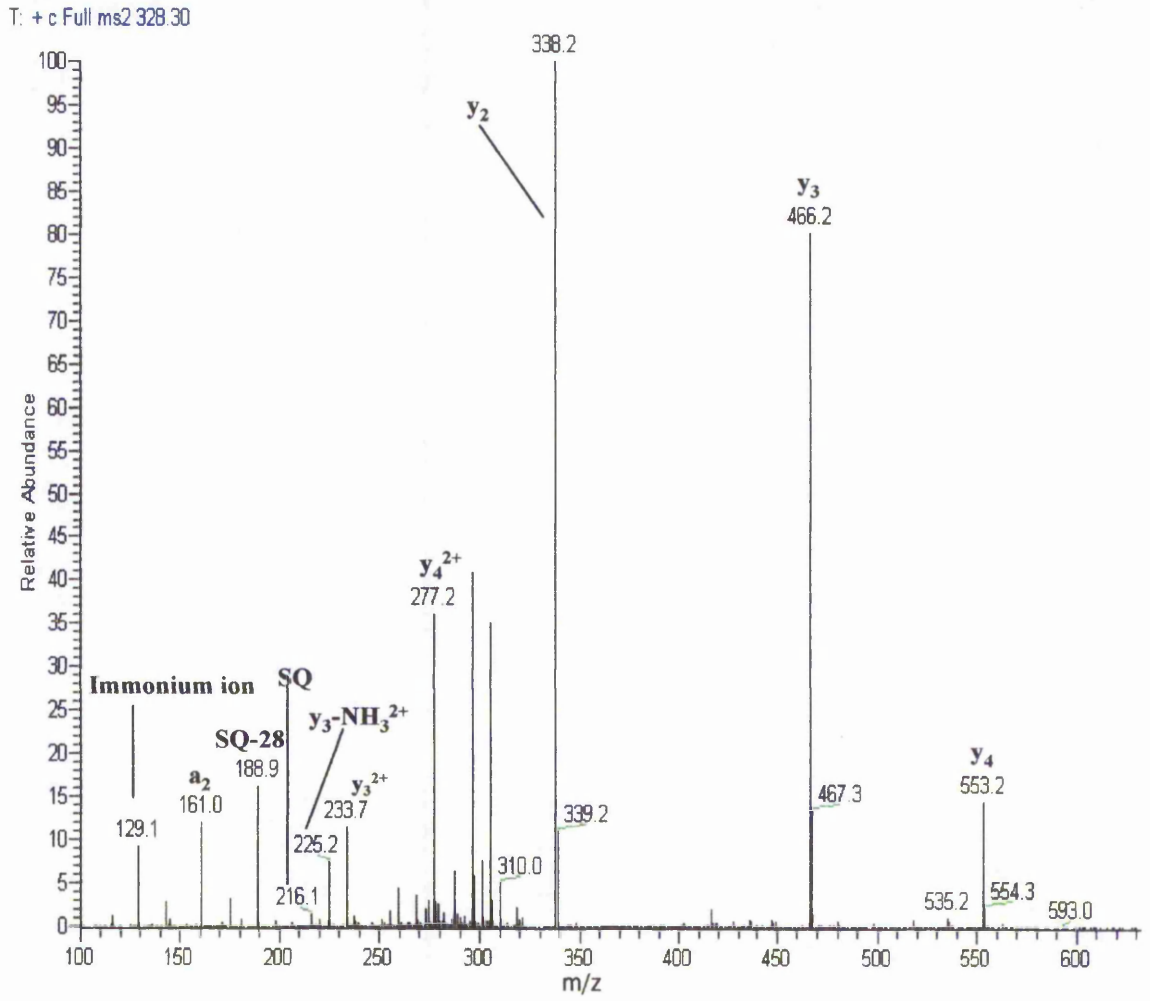


Figure 5.9 Presence of peptide TSQYR within CML blast cell eluate

A second unknown peptide was discovered in the CML blast cell eluate of patient JH. Upon input into the database it was found to correlate strongly with a viral protein from CMV and was also similar to proteins found in HSV and HIV-1.

Table 5.10 Predicted product ions originating from fragmentation of peptide TSQ(K)YR (m/z 654.3)

MS-Product Listing

Peptide Sequence:	**TSQYR**
Peptide Mass MH^+(average):	**654.7467**
Peptide Mass MH^+(monoisotopic):	**654.3575**
Elemental Compositon:	C28 H48 N9 O9
Amino Acid Composition:	**K1 R1 S1 T1 Y1**

	1	2	3	4
N-terminal ions				
a-NH_3 ions	---	---	272.16	435.22
a ions	74.06	161.09	289.19	452.25
b-H_2O ions	84.04	171.08	299.17	462.24
b-NH_3 ions	---	---	300.16	463.22
b ions	102.06	189.09	317.18	480.25
b+H_2O ions	---	---	335.19	498.26
C-terminal ions				
y ions	175.12	338.18	466.28	553.31
y-H_2O ions	---	---	---	535.30
y-NH_3 ions	158.09	321.16	449.25	536.28

The sequence of the unknown peptide, TSQ(K)YR was input into the MS Product database at UCSF selecting an ESI-ion-trap as the mass spectrometer. The main fragment ions (monoisotopic masses) are given above, however the immonium ions (immonium ion of arginine, R, = m/z 129.0) and internal sequence ions are not displayed for simplicity. Doubly charged variants of the product ions are achieved as before (table 5.2) and internal sequence ions seen on the spectrum (figure 5.9) were predicted as follows, 'SQ' = m/z 216.13; 'SQ-28' = m/z 188.14.

Table 5.11 Identification of proteins containing sequence TSQ(K)YR

sequence TSQYR
gi|9625769|ref|NP_040018.1| PP65 (11); UL82 FAMILY Matching: EHPTFTSQYRIQGKL (Residues 370 - 374)
gi|130715|sp|P18139|PP65_HCMVT 64 KD LOWER MATRIX PHOSPHOPROTEIN (PP64) (GP64) Matching: EHPTFTSQYRIQGKL (Residues 360 - 364)
gi|330651|gb|AAA45996.1| (M15120) PHOSPHORYLATED MATRIX PROTEIN (PP65) [HUMAN HERPESVIRUS 5] Matching: EHPTFTSQYRIQGKL (Residues 357 - 361)
gi|476570|pir||WMBETW 65K LOWER MATRIX PHOSPHOPROTEIN - HUMAN CYTOMEGALOVIRUS (STRAIN TOWNE) Matching: EHPTFTSQYRIQGKL (Residues 370 - 374)
gi|852439|gb|AAA68053.1| (U12409) ENVELOPE GLYCOPROTEIN, V1-V3 REGION [HUMAN IMMUNODEFICIENCY VIRUS TYPE 1] Matching: QIDGNTSQYRLINCN (Residues 180 - 184)
gi|9629628|ref|NP_044911.1| CYCLIN D HOMOLOG Matching: VDQHFTSQYRKVLTT (Residues 45 - 49)
sequence TSKYR
gi|4504345|ref|NP_000508.1| HEMOGLOBIN, ALPHA 2 Matching: VSTVLTSKYR (Residues 138 - 142)
gi|2134729|pir||I37141 ALPHA GLOBIN - HUMAN Matching: VSTVLTSKYR (Residues 124 - 128)
gi|183805|gb|AAA52632.1| (J00157) PSEUDO-A-THAL [HOMO SAPIENS] Matching: VSTVLTSKYR (Residues 81 - 85)
gi|8569443|pdb|1DKE|A CHAIN A, NI BETA HEME HUMAN HEMOGLOBIN Matching: VSTVLTSKYR (Residues 137 - 141)
gi|1731407|sp|P52739|Z131_HUMAN ZINC FINGER PROTEIN 131 Matching: LLADITSKYRQGDRK (Residues 70 - 74)

Both sequence TSKYR and TSQYR were input into the database (PROWL) as both have the same mass (K/Q cannot be distinguished by the mass spectrometer). The results for each are given above with the sequence occuring in completely different proteins. However the matches for each of the sequences are from very similar proteins. Matches for TSQYR all having viral/glycoprotein origins and for TSKYR haemoglobin origin.

5.3 Conclusion

The results demonstrate that peptides can be isolated and identified using the protocols described in the previous chapters. The A3 peptide was identified from the K562 transfectants and from clinical samples confirming the processing and MHC class I presentation of the bcr-abl protein. The discovery of other peptides within the eluate was expected due to the large number of MHC bound peptides present, however, the identification of these proved to be elusive. This suggests that the technique can be used to determine the presence of peptides, but is dependent upon the presence of the sequences within databases for identification of their protein origin.

5.4 Discussion

The protocol developed in chapters 3 and 4 has been used to isolate and identify tumour associated cell surface antigens. Use of the protocol led to the discovery of b3a2 bcr-abl peptides within the eluates of patient samples. CML blast eluates from patients JL, CT and JD (all of whom were b3a2 and HLA-A3 positive, and in the case of CT also HLA-B8 positive) contained the expected HLA restricted peptides proven by sequencing and confirmed by database identification of bcr-abl from the sequence data. These findings prove that the bcr-abl protein is intracellularly processed and that resulting peptides are presented on the surface of cells in a MHC-restricted fashion. The prediction of the occurrence of these peptides due to anchor residues in the case of bcr-abl seems inaccurate as the A3 peptide is low in the ranking for MHC binding (table 5.1). Thus this would confirm observations (section 1.7.4) that strong binding peptides are not necessarily the most likely to generate CTL. Indeed, binding studies (Bocchia et al. 1995) showed that the A3 peptide does not bind with strong affinity to MHC antigens, but is still able to induce CTL activity against CML cells (Bocchia et al. 1996; Norbury et al. 2000; Osman et al. 1999; Greco et al. 1996). The finding that these peptides are indeed present on the surface associated with MHC class I molecules confirms that the CTL activity observed against CML cells in these studies is MHC restricted and provides indications that CML immunotherapy

using these antigens may be possible. Studies performed by A. Dodi at the Anthony Nolan Bone Marrow Trust using MHC tetramers (McMichael and O'Callaghan 1998) have identified a population of naturally occurring anti-bcr-abl CTL (HLA-A3 KQSSKALQR restricted) within two of the patients (JL and JD) used within this study (Dodi et al. In Preparation). It is possible that the existing natural immunity towards these antigens could be boosted using vaccine therapy.

It is likely that the bcr-abl peptides identified in this study are MHC-restricted since lysis of the cells did not occur and MHC loss was evident in the model K562 system. However, peptides may be present on the surface that are not bound to MHC antigens. This may be particularly true for the smaller peptides identified (sequences TSKYR, CASKLCC), although it has been shown that MHC class I can present peptides as small as five amino acids, at least, in murine models (Gulden et al. 1996). This research involved purifying total cellular MHC, but CTL recognition was mediated by a pentapeptide. It is possible that the CTL were primed by a larger peptide containing the sequence of the pentapeptide and that the presence of the pentapeptide on the target cells was sufficient for TCR binding. Whether these findings translate to other antigens and humans remains to be determined. It is known from work with T2 lymphoblastoid cells (Cerundolo et al. 1991) that the optimal length of MHC class I binding peptides is 8-9 amino acids giving rise to stable peptide- MHC class I complexes. The presence of smaller virally homologous proteins within the eluted cells is not surprising, as the CML patients from whom the cells were obtained would most likely be immunocompromised, either as a result of oral chemotherapy (Thijsen et al. 1999) or the low numbers of functional white blood cells to fight infections (Zingde 1998). However, a database search revealed that the TSQYR sequence could have originated from a CMV associated protein, pp65 (table 5.11). This protein is known to be expressed in CML cells and is suggested to interfere with integrin functions in the blast cells potentially explaining the lack of blast cells adhering to the bone marrow (R.E. Clark - Personal communication). It is also similar to another 65kDa phosphoprotein known to be present in some cases of CML

and suggested as a potential tumour marker (Hanausek et al. 1996). Another possible match for this sequence is Cyclin D, a protein which is known to be overexpresed in breast carcinomas (Michalides et al. 1996; Steeg and Zhou 1998).

This "novel" peptide was one of many peptides present within the sample, any one cell population may be expected to generate a large population of peptides (possibly 10000 distinct peptides from 10^{10} cells, (Brockman et al. 1999). Due to the time required to isolate and identify a single peptide within the sample (and having a limited amount of sample), it is impossible to sequence all, or even the half of the peptides within the sample, thus only one peptide is presented here. The peptide PTITSGCRM was present as a major peptide component at levels equal to the A3 peptide and thus the levels of protein would have been expected to be present at a comparable level to the bcr-abl. The closest protein match for this peptide was AF-4 which is found in many splice variants mainly in acute leukaemias. Since the patient sample was obtained from during the chronic phase of CML, it is unlikely that this is an exact match. However, such is the complex nature of translocations in leukaemias, that as the disease progresses the number of mutations and translocations increase in a seemingly random fashion (Zingde 1998). It is possible that the particular translocation within this patient is unique or rarely observed. It is also entirely possible that the databases used to identify the protein simply did not contain the sequence of the peptide. Two databases (Swiss-Prot and NCBR) were used on two different database programs thus the number of available sequences searched was extensive and it would be more likely that the protein was unique and thus far unsequenced. The use of this protocol to identify tumour antigens relies on the previous identification of the antigens. However, the discovery of new, previously unidentified antigens using this protocol may provide more targets for immunotherapy.

Chapter 6: Generation of cytotoxic T lymphocytes *in-vitro.*

6.1 .1 Cell mediated immunity

Infected cells are lysed by cytotoxic T cells following recognition of antigen via MHC-peptide complexes on the target cell surface (section 1.2.6). Following binding of the MHC-peptide complex to the T cell receptor and appropriate co-stimulation, (Janeway, 1992), the T cells are activated to undergo proliferation resulting in a population of T cells specific for the antigen contained within the MHC complex and able to kill cells expressing the antigen (see section 1.2.6). Following discovery of tumour specific/associated antigens, it became important to prove whether the antigen could initiate a specific immunological response leading to the destruction of tumour cells by T cells specific for that antigen. As discussed in sections 1.5-6 and 3.1, many approaches have made use of in-vitro CTL generation assays enabling CTL specific for a given antigen of interest to be produced under in-vitro stimulation and tested against tumour targets expressing the original antigen. The immunogenicity of a given antigen can be measured using a chromium release assay and appropriate CTL effectors and tumour targets.

6.1.2 CTL generation assay

The in-vitro generation of CTL is now a readily accepted and widely used technique, however, the development and use of such protocols requires knowledge of the role of cell populations and cytokines in the generation of an immune response. Dendritic cells (DC), T helper cells and the cytokines GM-CSF, IL-4, TNF, IL-7 and IL-2 are all important in the generation of cytotoxic T cells (Pawelec et al. 1997; Hart 1997; Peters et al. 1996; Romani et al. 1994).

6.1.3 Dendritic cells

The generation of cytotoxic CD8+ T cells requires signals delivered by both antigen presenting cells (APC) and T helper cells (T_h). DCs are professional and the most potent APC, in that they alone are able to activate naïve T cells and have high levels of co-stimulatory and antigen presenting molecules (MHC) on their surface (section 1.4 for detailed discussion). Current evidence (Ridge et al. 1998; Bennett et al. 1998; Schoenberger et al. 1998; Bertholet et al. 1997) suggests that CD4 T_h cells can activate DC, which in turn activate CD8+ CTL. Activated T cells express CD40 ligand and

CD4 T_h cells activated by APCs can in turn activate DC via their CD40 ligand interacting with CD40 on the DC surface. The DC is then thought to undergo differentiation (Ridge et al. 1998), upregulating the co-stimulatory molecules CD80 and CD86 (Schoenberger et al. 1998, Bennett et al. 1998), ICAM-1, TNFα, (Bennett et al. 1998) and IL-12 (Schoenberger et al. 1998) also potentially involved. DCs activate CTL mainly by CD28-CD80/86 interactions leading to IL-2 production and T cell proliferation (Ridge et al 1998, Schoenberger et al. 1998, Bennett et al. 1998, Minami et al. 1993, Fraser et al. 1991).

The activation process requires all three cells and since the likelihood of all three being present at the same time and location is low, this sequential model of activation offers an alternative model. Interestingly, virally infected APC are able to directly activate CTL (Ridge et al. 1998).

6.1.4 *In-vitro* generation of dendritic cells

The importance of DCs in the immune response established, the *in-vitro* culture of DCs in order to generate CTL against a given antigen is thus essential. Methods by which this is achieved vary and are numerous. DCs are known to originate from several precursor populations (Peters et al 1996, Hart 1997), but the easiest obtainable source of DC precursors is PBMC in the blood (myeloid DC). PBMC can be separated from other blood cells using Ficoll or similar gradient methods. The PBMC are collected in the buffy coat as a pure population (Young and Steinman 1988). Sallusto et al. (1994) and Romani et al. (1994), amongst others, have identified essential cytokines for the maturation of PBMC into DC. These researchers used GM-CSF as it was found to be essential to differentiate precursor cells into DC, indicated by the appearance of cell surface markers, such as CD34, characteristic of the myeloid origin of DCs. IL-4 was also found to be essential in that it suppresses the development of the PBMC into macrophages (possibly by downregulation of CD14), thus further promoting DC development.

Towards the end of the DC generation, which for human DCs takes 7 days, TNFα is added as it appears to be able to prolong the viability of cultured DC (Koch et al. 1990), induces CD40 (McLellan et al. 1996) and acts to activate the antigen presenting function of the DC by acting as a danger signal (McKenzie et al. 1995 and section 1.4). Mature dendritic cells are characterised by

expression of CD86, MHC II and are negative for the B cell marker CD19 (Gallucci et al. 1999; Santin et al. 1999; Peters et al. 1996, Hart 1997). There is a lack of understanding of whether CD1a expression is essential to generate CTL, however DC generated using the Sallusto et al (1994) and Romani et al. (1994) protocols were potent activators and expressed CD1a, an observation also made by Gallucci et al (1999). Work has suggested that CD1a expression is necessary for generation of CTL (McArdle et al. 2000, Hart 1997), potentially acting as a recognition marker for the T cell receptor (Hart 1997).

6.1.5 *In-vitro* generation of CTL

Following the generation of mature DCs able to present antigen, characterised by cell surface expression of CD80/CD86, CD40 and high concentrations of MHC I and II (Gallucci et al. 1999, Hart 1997), the addition of CTL into the culture for stimulation by the DC is required. PBMC originally isolated from whole blood are incubated for 2 hours allowing adherence of a proportion of cells (usually between 3-6% Sallusto et al. 1994) to the culture vessel. The remainder of the cells are retrieved and stored (frozen in liquid nitrogen) until DC generation is complete. The stored cells are then added to the culture as a source of CTL precursors. The presence of IL-7 and IL-2 during this phase of the culture acts to maintain healthy T cells and induce their proliferation (IL-7) and to supplement endogenous cytokines for the activation of CTL to antigen present on the DC (IL-2). Weekly re-stimulation of the developing T cells is carried out using freshly adhered PBMC pulsed with the relevant peptide of interest, subsequent to the initial activation of the T cells which was carried out by DC. Other APC, such as macrophages may also be used to stimulate the T cells (Sallusto 1994, Romani 1994, Colaco 1998).

6.1.6 Cytotoxicity assay

Target cells, such as tumour cells or T2 cells (section 3.1.2) pulsed or unpulsed with the relevant or irrelevant peptide, can be labelled with chromium-51 and incubated with the generated CTL (usually for 4 hours). The cytotoxic potency of the CTL is indicated by measurement of released chromium caused by lysis of the target cells by the CTL. The use of T2 cells within these assays is

widespread; the addition of exogenous peptide to these cells allows binding of the peptide to the MHC and thus stabilisation of MHC on the cell surface (section 3.1.2). Thus, to determine whether CTL lysis of target cells is peptide specific, pulsed T2 cells can be included in the assay. The inclusion of a control of T2 either unpulsed or pulsed with an irrelevant peptide acts as additional confirmation of peptide specific, CTL mediated, lysis. Lysis of T2 pulsed with relevant peptide, but not unpulsed or irrelevant peptide would prove that the peptide was specifically recognised by the CTL. To ensure that the lysis is specific and CTL mediated, unlabelled (cold) K562 cells are also incubated with the target cells to act as a target for non-specific natural killer cell mediated lysis (section 1.1.3). The killing of the unlabelled K562 does not contribute to the amount of chromium released and thus any chromium released will be as a result of either spontaneous release (controlled for within the assay) or CTL mediated lysis of the target cells.

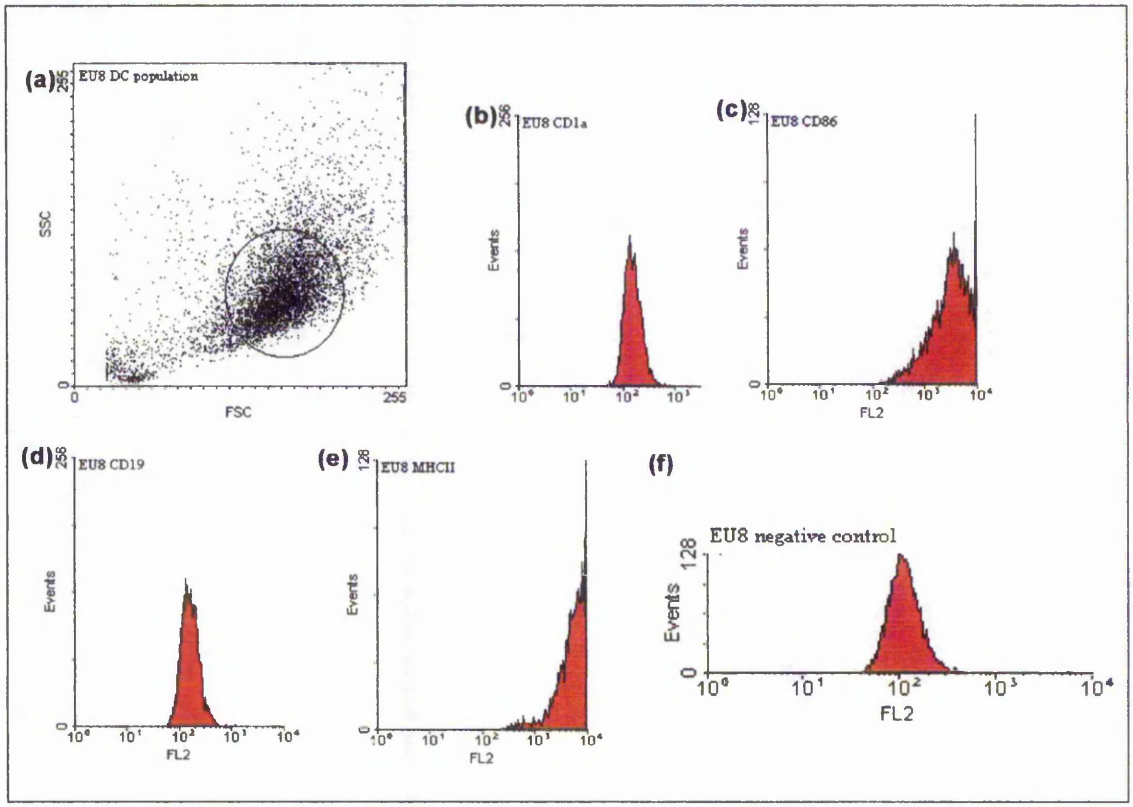
As part of the EUCAPS (European Concerted Action for Peptide Sensitisation), a standardised CTL assay was to be developed within several laboratories based on a consensus of the best available protocol in current use (EU4-8 in the following results), which would subsequently be used to test the immunogenicity of any peptides discovered through the work presented in chapters 4 and 5 (SH1-7). The development and use of such an assay is described in the following pages.

6.2 Results

6.2.1 EUCAPS protocol for generation of DC and CTL

Dendritic cells were generated as detailed in the EUCAPS protocol from HLA-A2 positive leucopacks obtained from BTS (section 2.2.14) and following 7 days of culture (Day 0) were harvested and an aliquot was phenotyped by flow cytometry. The remainder were returned to culture to stimulate naïve T cells to either tyrosinase or gp100 HLA-A2 restricted peptide epitopes. The results of the DC phenotyping are displayed in histograms (tables 6.1, 6.2 and figures 6.1, 6.2), obtained from analysis of the staining with the WinMDI software program (Scripps Research Institute), which are representative of the phenotype obtained from all 6 assays carried out (EU3-8). The histograms show the DC population to be CD1a and CD19 negative, CD86 (B7.2) and MHC class II positive. Table 6.1 and figure 6.2 show percentages of cell surface expression of the markers and fluorescence intensity of the staining. Mature DC are positive for CD86 and MHC II, but negative for CD19, a B cell marker (Hart 1997, Peters et al. 1996). The results obtained confirm that the DC generated are mature and capable of presenting antigen. It is interesting to note that the last assay performed shows the highest percentage of DC obtained (ranging from 30 to 59%) and highest expression of the positive markers CD86 (ranging from 22.15 to 97.53) and MHC class II (34.15 to 99.04). CD1a expression is not induced on the DCs using this protocol, unlike that of Sallusto et al. (1994). The fluorescence intensity shows an extremely high intensity of MHC class II staining and a lesser level of CD86, (the first two (EU4 and 5) assays show a low staining intensity for CD86, the reason for this is unknown), approximately 5000 and 3000 arbitrary units respectively compared to around 150 to 200 for the negative control and CD19. The DC generated express these two markers at high levels as suggested for mature DCs (Hart 1997, Gallucci 1999). However, when tested in the chromium release assay cytotoxicity was not observed with any T cells generated using this protocol with the exception of gp100 specific CTL against FM3 melanoma cells in assay EU4 (20% cytotoxicity). T2 cells pulsed with the gp100 peptide were not killed and the result was not able to be repeated. A further complication was that the number of T cells generated fell with each re-stimulation, once to levels too low to use in a cytotoxicity assay (EU5).

Figure 6.1 Representative cell surface marker expression – dendritic cells generated from EUCAPS protocol



Dendritic cells were stained for surface markers (CD1a, CD19, CD86, MHCII) by flow cytometry after 7 days of culture (day-7). The antibodies used were PE conjugated except MHCII which was FITC conjugated. The dendritic cell population was gated (panel a) and the percentage cells occurring within the gate recorded (shown in table 6.1 and figure 6.2a). Dendritic cells are suggested to be CD86 and MHCII positive, but CD19 negative. CD1a expression is variable and expression may depend on the method used to generate the dendritic cells (EUCAPS meeting discussion). The histograms above are representative of the dendritic cells generated using the protocol detailed in section 2.2.15

Table 6.1 Characteristics of dendritic cells (DC) generated by EUCAPS protocol

	Percentage cells stained				
Assay	DC	CD1a	CD19	CD86	MHCII
EU4	30	NT	0.05	22.15	34.13
EU5	35	NT	0.47	36.99	62.1
EU6	50	1.41	4.97	32.25	58.21
EU7	52	1.74	0.5	94.75	97.85
EU8	59	0.45	1.08	97.53	99.04

Table 6.2Fluorescence intensity of dendritic cell cell surface marker staining

	Fluorescence intensity				
Assay	CD1a	CD19	CD86	MHCII	negative
EU4	NT	142.72	152.9	9390.98	142.52
EU5	NT	171.98	192.53	8411.88	170.47
EU6	243.22	168.99	790.14	2495.62	152.35
EU7	237.1	170.07	2205.81	3240.62	135.45
EU8	169.13	170.33	3380.08	5868.39	125.1

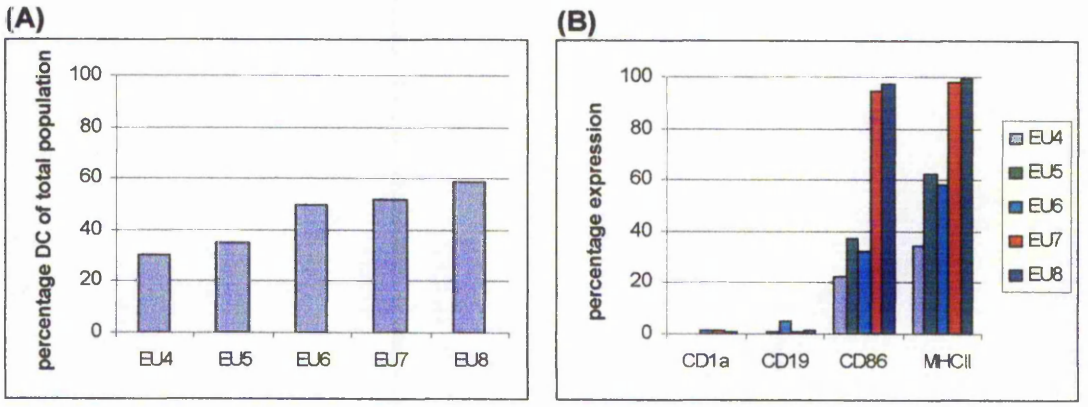


Figure 6.2 Characteristics of dendritic cells generated by EUCAPS protocol

Floating cells collected after 7 days of culture (day 0, see section 2.2.15) were assumed to be dendritic cells (DC) and were stained using antibodies to surface markers CD1a, CD19, CD86 and MHCII. Representative histograms are shown in Figure 6.1, however, expression varied between assays. Table 6.1 gives percentage DC of the total floating cell population (represented in graph form in figure 6.2a) and percentage cell surface marker expression for each assay (represented in figure 6.2b).

6.2.2 Modified protocol 1 – further attempts to generate cytotoxic T cells (SH1-4).

Following the conclusion of the EUCAPS programme, it was decided that modification to the protocol could be made to test a peptide identified as being tumour specific in chapter 5. The HLA-A3 restricted peptide (KQSSKALQR) from the bcr-abl fusion region has been identified by other researchers as able to generate CTL (Berke et al. 2000; Norbury et al. 2000; Osman et al. 1999; Nieda et al. 1998; Bocchia et al. 1996; Bosch et al. 1996), and thus it was decided to use this peptide to confirm those the validity of the CTL generation protocol. The provision of HLA-A3 transfected K562 ensured that a relevant target was available. K562 cells naturally express the bcr-abl fusion protein and the transfection of HLA-A3 into the cells allowed cell surface expression of the HLA-A3 restricted peptide (KQSSKALQR), in the context of MHC class I.

DC generated using the EUCAPS protocol were not CD1a positive, and since this may have been a reason as to the lack of T cells capable of specific cytotoxicity, the protocol was altered (section 2.2.18), This mainly involved using an increased percentage of autologous serum (5% to attempt to induce CD1a expression), reducing the interference of the cells in culture, supplementing with cytokines only once a week and the use of FCS following generation of DC to reduce the risk of contamination and improve the health of the T cells. The results are shown in figure 6.3 and 6.4, and tables 6.3 and 6.4. The first observation is that the percentage of cells expressing DC markers within the cells harvested on day 0 is higher than previous assays, however since the percentage was increasing with the use of the earlier protocol it is likely that this is a result of improvements in technique and handling the cells. CD1a expression does occur in one of the assays (20% in SH2), however the cells from this assay were lost due to contamination. Expression of CD86 and MHCII were observed at similar levels, but CD86 expression was much more variable with this protocol ranging from 39-77%. Interestingly the cells with the lowest amount of CD86 and lack of CD1a are those which generated cytotoxic CTL. Figure 6.5 shows the cytotoxic effect of CTL generated using this protocol (SH1), at a 20:1 effector:target ratio, 8.2% cytotoxicity ($p=< 0.01$) is observed against HLA-A3 transfected K562. It is possible that the higher expression of CD86 and CD1a on the DC may have enabled the generation of more potent CTL. The other two assays which were tested for

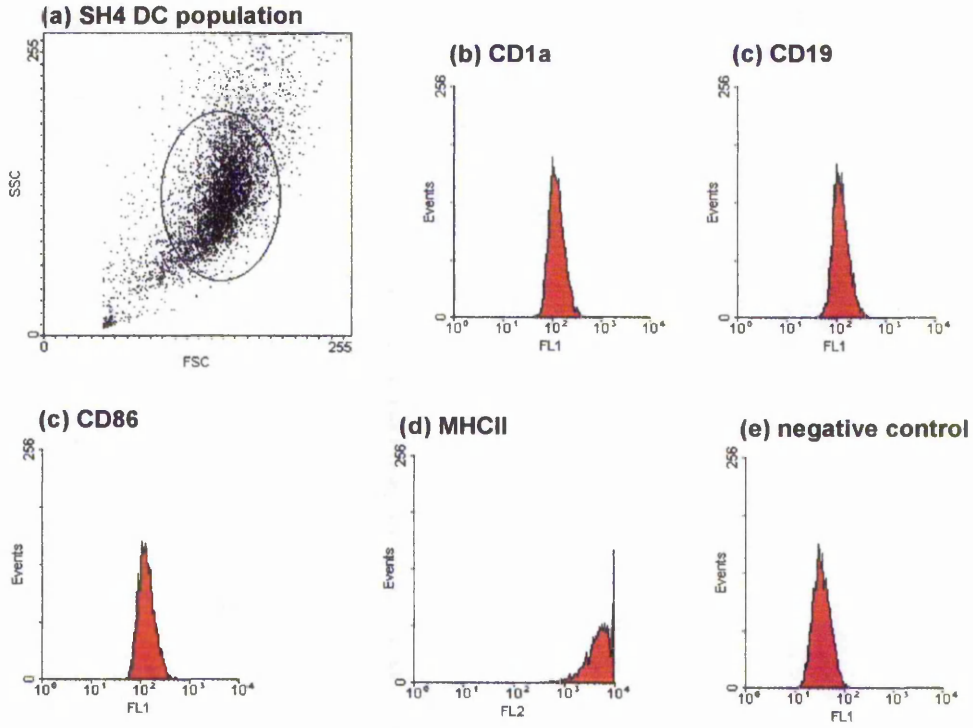


Figure 6.3 Characteristics of dendritic cells generated using modified protocol 1

Dendritic cells were generated as explained in section 2.2.18 with the substitution of FCS for autologous human serum on day 0. The cells were phenotyped for cell surface markers using flow cytometry as explained previously. The histograms above are representative of cell surface marker expression for assays SH1-4

Table 6.3 Characteristics of DC generated using modified protocol 1

	Percentage cell staining					
assay	DC	CD1a	CD19	CD86	MHCII	negative
SH1						
SH2		23.88	0.3	77.18	89.44	1.72
SH3	56.6	2.74	3.5	62.08	72.26	2.35
SH4	75.26	0.68	0.38	44.7	84.22	3.5

Table 6.4 Fluorescence intensity of staining of DC generated using modified protocol 1

Fluor. Int.	CD1a	CD19	CD86	MHCII	negative
SH1					
SH2	232	124.88	1129.29	7233.09	248.45
SH3	215.71	215.22	1096.91	1737.08	185.34
SH4	139.53	119.51	763.56	2801.98	159.34

Dendritic cells were generated using the same protocol as described in section 2.2.15 the protocol was altered following generation with the addition of FCS (protocol 1). The DC generated here were used to generate T cells specific to an HLA-A3 restricted peptide. The cells were phenotyped following 7 days of culture (day 0) using the flow cytometric technique described previously.

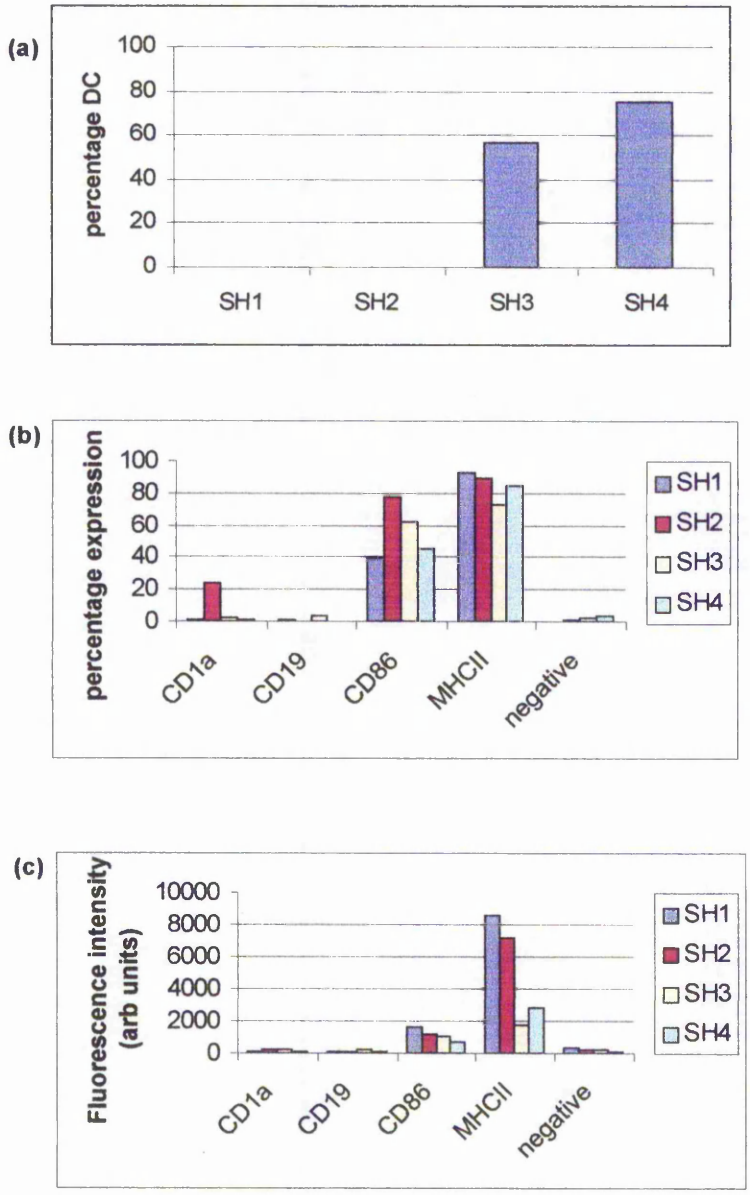


Figure 6.4 Graphical representation of dendritic cell characteristics of assays SH1-4

The above figures are a graphical representation of the data in tables 6.4 and 6.5, showing percentage of DC present within total cellular population (as figure 6.1), percentage expression of the surface markers CD1a, CD19, CD86, MHC II, and fluorescence intensity of the surface marker staining. The background staining level is represented by the 'negative' columns.

Table 6.5 Percentage cytotoxicity - SH1 T cell (generated against an HLA-A3 restricted peptide) – mediated killing of three targets

	E:T ratio					
% cyto	20:1	+/-SD	10:1	+/-SD	5:1	+/-SD	2.5:1	+/-SD
T2 alone	4.967	3.1	4.054	0.7	3.961	4.4	2.414	2.5
T2pep	4.651	4	5.019	1.5	3.591	2.4	3.570	6.5
K563-A3	8.387	3.7	6.111	2.5	4.258	4.9	4.147	5.7

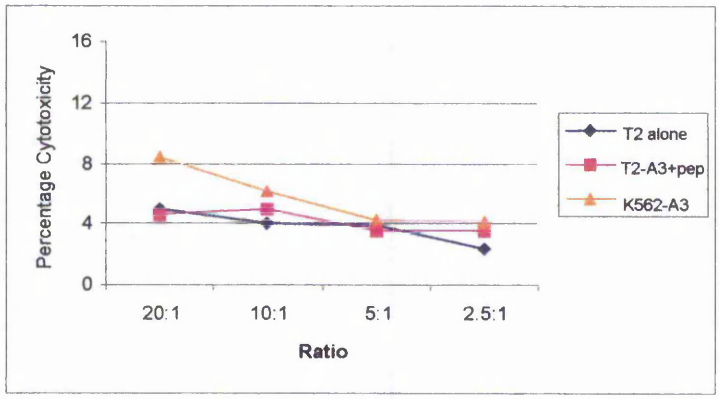


Figure 6.5 Percentage cytotoxicity mediated by SH1 T cells against three targets

SH1 T cells were generated using modified protocol 1 (section 2.218) against the HLA-A3 restricted peptide KQSSKALQR. The cytotoxic ability of the T cells was tested after three restimulations (first with dendritic cells, characterised in figures and tables 6.3, 6.4), then with autologous PBMC. Both types of stimulator cell were pulsed with the relevant peptide. Cytotoxic ability was measured using a standard 4 hour chromium release assay (section 2.2.20) and targets used were K562 (transfected with HLA-A3) which naturally express the peptide, T2 cells pulsed and unpulsed with the peptide. All targets were incubated with a 20 fold excess of cold (unlabelled) untransfected K562 to quench any non-specific NK cell killing. A one tailed, two sample T-Test showed that the K562-A3 killing was significantly different to the T2 unpulsed ($p =< 0.01$).

cytotoxicity against K562-A3 target cells (SH3 and 4) did not demonstrate any killing and thus further attempts to improve the protocol were made.

6.2.3 Phenotyping of T cells from SH3 and 4.

Following the failure of the majority of the assays to generate cytotoxic T cells, the phenotype of the T cells from two of the assays, SH3 and 4, was characterised to investigate the CD4:CD8 ratio. The predominance of CD4 cells would prevent the generation of CD8 CTL populations and cytokines produced by CD4 cells may suppress the generation or activation of CD8 T cells. Figures 6.6, 6.7 and tables 6.6 and 6.7 show the phenotype of the T cells at different stages of the assay. The T cells were collected and frozen (in liquid nitrogen) until the end of the assay when they were thawed and stained with anti CD4, CD8, MHC class I and class II monoclonal antibodies. Unstimulated PBMC were used as a comparison. The results showed that cells collected at day –7 (these cells were frozen and returned to culture at day 0 to be stimulated as T cells by the generated DC) have roughly equal amounts of CD4:CD8 and MHCI:II. As the cells are re-stimulated, (T0 day 0, T1 day 12, T2 day 19) the CD4 expression is increased over the CD8 expression. Cells re-stimulated a third time were unavailable for staining, but it can be suggested that the predominance of CD4 cells throughout the assay may have suppressed the generation of potent CD8 CTL. If CD4 cells were present during the cytotoxicity assay at higher levels than the CD8 cells they would not contribute to cytotoxicity and thus a lower overall level of cytotoxicity would be seen.

In light of these results, further modifications were made to the assay, including a CD4 depletion step at day 0 as used within other protocols (McArdle et al. 2000, Rivoltini et al. 1999, Imro et al. 1999)

6.2.4 Modified protocol 2 – further attempts to generate CTL (SH5-7).

To attempt to improve cell surface expression of CD1a and since a source of contamination was proved to be the human serum, it was decided to use 10% FCS throughout the protocol as the serum supplement. Work carried out within our laboratory showed that only use of FCS generated

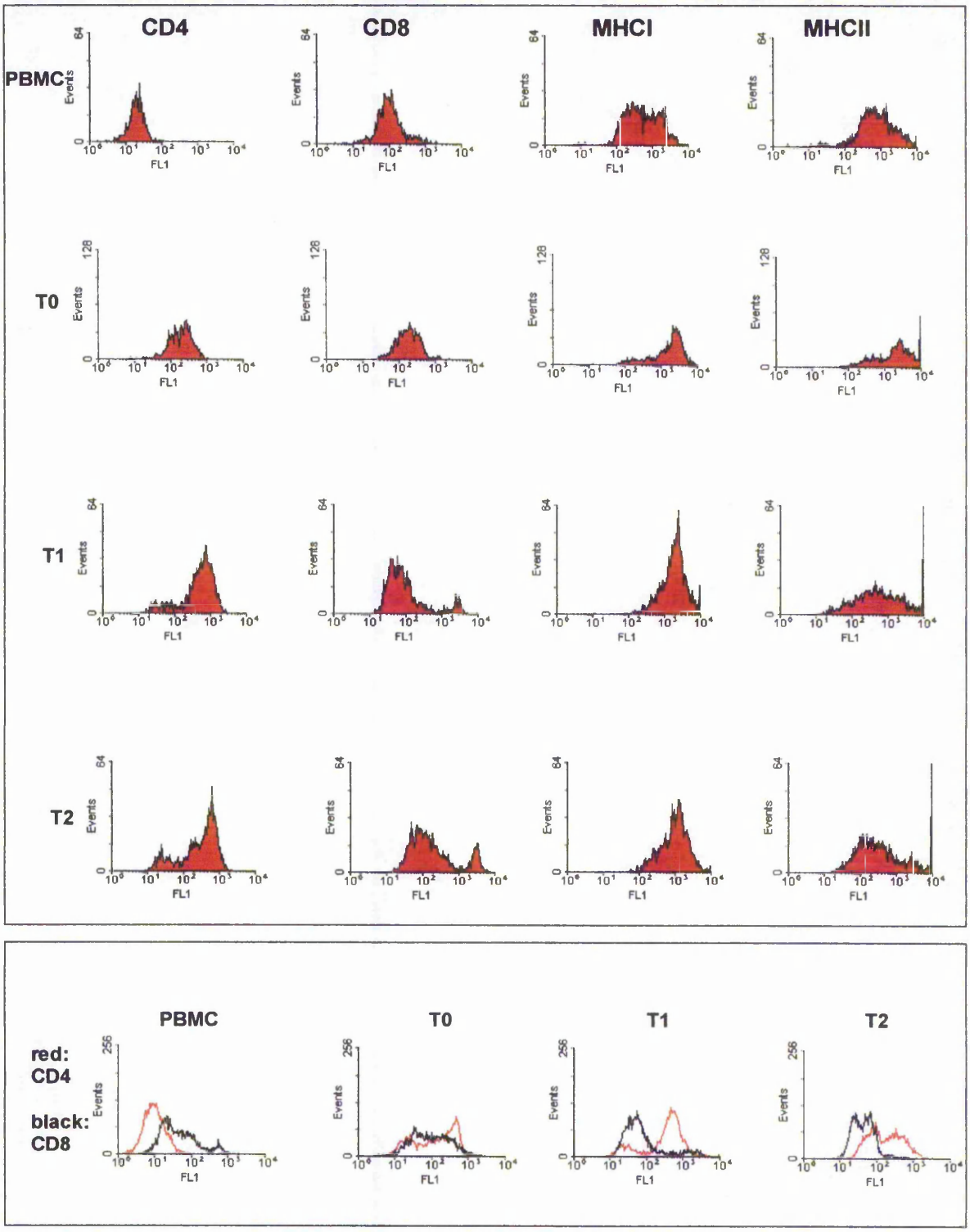


Figure 6.6 Phenotype of T cells generated by modified protocol 1

T cells generated using modified protocol 1 (section 2.2.18) were phenotyped at various stages during the assay period of 33 days. Freshly harvested PBMC were used as a comparison, T0 were floating cells collected after 4h and frozen down to be used as T cells on day 0, T1 were T cells after 1 round of restimulation (day 12) and T2 after after 2 rounds of restimulation (day 19). The above histograms are representative of two assays, SH3 and 4, and were analysed by flow cytometry as described previously.

Table 6.6 Phenotype of T cells from assays SH3 and 4

surface marker	Percentage cells staining							
	PBMC	+/-SD	T0	+/-SD	T1	+/-SD	T2	+/-SD
CD4	0.1	3.76	60.5	3.89	76	13.7	50	5.8
CD8	4	5.87	51.2	5.98	20	20.2	5	14.6
MHCI	40	6.87	67.64	7.92	95	3.4	76	15.2
MHCII	40	3.97	52.62	5.73	53	9.5	80	9.5

Table 6.7 Fluorescence intensity of T cell surface marker staining

surface marker	Fluorescence Intensity			
	PBMC	T0	T1	T2
CD4	21.11	186.51	187.04	293.4
CD8	106.5	180.51	259.77	157.6
MHCI	527.77	1390.63	969.68	1047.36
MHCII	741.6	1541.16	355.97	1114.98

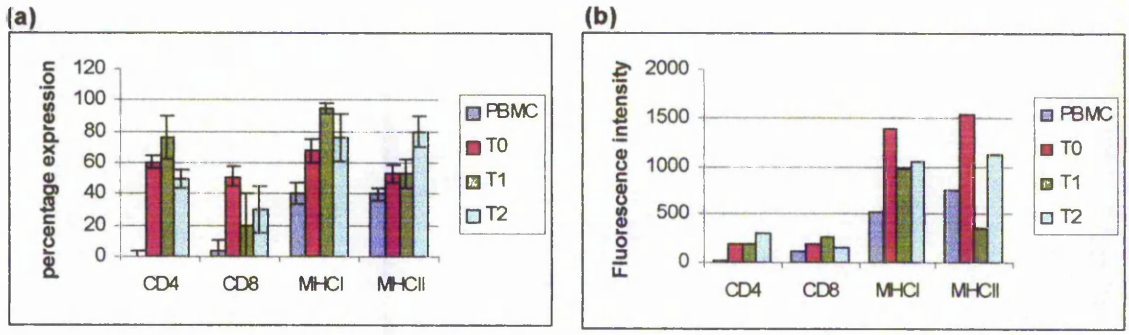


Figure 6.7 T cell phenotyping (assays SH3 and SH4)

T cells used in assays SH3 and 4 were phenotyped at various stages of the assay as described in legend to figure 6.6. The T cells were stored in liquid nitrogen until the final batch of T cells (T2) were harvested from the assay. The expression of CD4 and CD8 on the T cells determines whether the cells are cytotoxic (CD8) or helper (CD4) T cells and this was cited as a potential reason for the failure of the assays so far. The data presented in tables 6.6 and 6.7 are graphically represented in figures 6.7(a) percentage expression of cell surface markers and in (b) the fluorescence intensity of the staining (SH3 only).

CD1a positive DC (S. McArdle – personal communication). These changes led to contamination free assays and all cells survived for longer periods of time, allowing an increased number of cells to be used in the final cytotoxicity assays and thus the effector:target ratios used in the assays for these three experiments (SH5-7) were higher. The results of the T cell phenotyping suggested that enrichment of CD8 T cells may improve the assays and so T cells used for stimulation by DC at Day 0 were first CD4 depleted using magnetic CD4 coated beads (section 2.2.19).

DC generated using this protocol (figures 6.8 and 6.9, tables 6.8 and 6.9) formed a larger part of the total harvested cell population, ranging from 53 to 80%. CD1a expression was increased on these DC, SH6 showing 20% and SH7 a lesser amount at 7%, however this looked to be an improvement compared with previous results. CD86 and MHCII expression was also fairly high, but the fluorescence intensity of the staining was lower than all the previous assays, this does not seem to be an accurate indication of cell surface marker expression on these cells as the percentage cells expressing the markers was comparable with cells showing high staining intensity. Only one of these assays (SH6 – figure 6.10) demonstrated any cytotoxicity, 9% at a 100:1 ratio ($p = <0.01$), which did not decrease linearly with decreasing effector cell concentration as expected and seen in SH1. The low level of cytotoxicity at such a high effector concentration suggests that these CTL are not very potent, despite the supposed improvements made to the assay. The expression of CD1a does not seem to necessarily correlate with the induction of strong CTL activity.

6.2.5 NK mediated killing of target cells.

During these last assays (SH5-7), it became apparent that the condition of the target cells was, on occasion, poor (extremely slow growing) and that this, and not lack of specific CTL, could contribute to the failure of the cytotoxicity assay. The T2-A3 cells used in assay SH5 did not incorporate chromium, and thus it was impossible to see if the CTL were able to kill them, those target cells able to incorporate chromium released high background levels of the radioisotope. The NK source was PBMC isolated from whole blood of a healthy donor which were then incubated with the target cells in the standard 4h chromium release assay (figure 6.11). NK killing of K562-A3 was highest at 36% at a 100:1 effector:target ratio. Lysis of T2-A3 pulsed and unpulsed was comparable at 27 and 21%

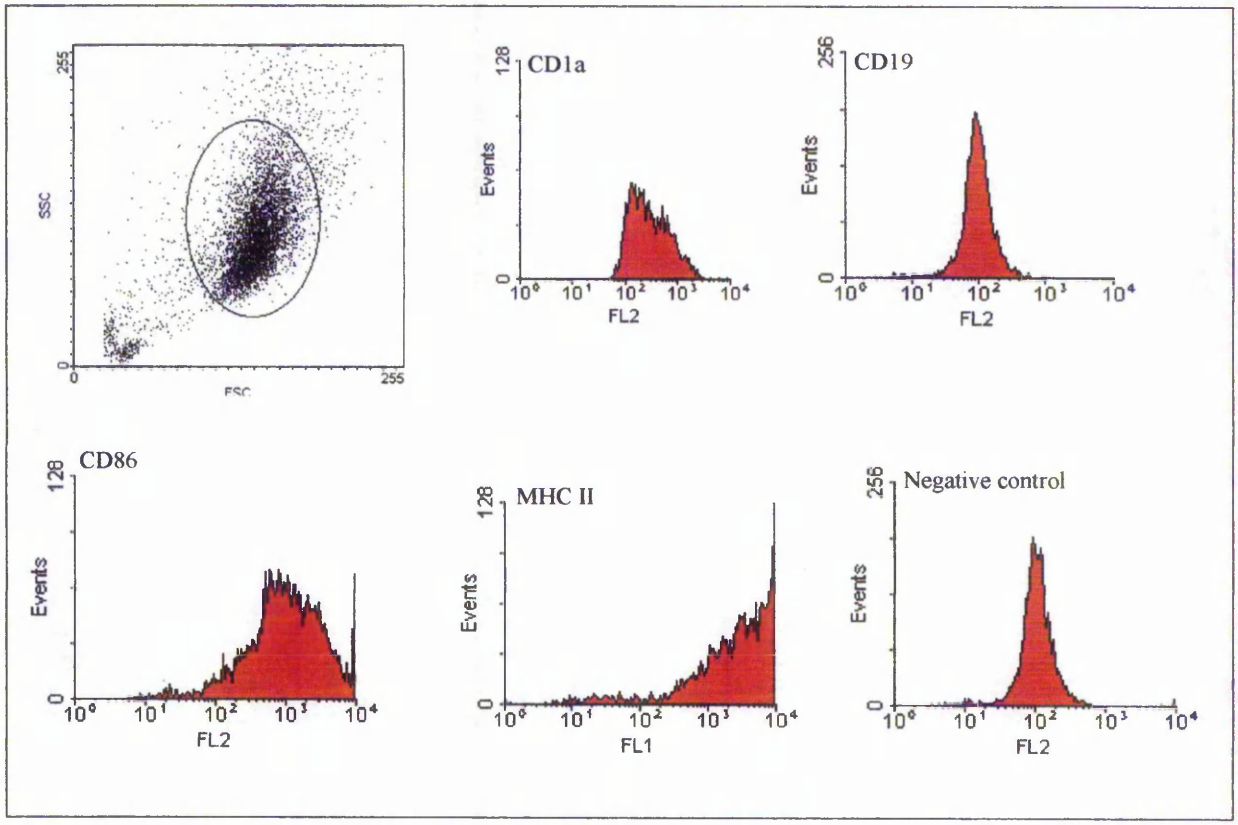


Figure 6.8 Characteristics of dendritic cells generated using modified protocol 2

Dendritic cells were generated using the protocol in section 2.2.15, but FCS was used as the serum supplement for the entire assay as opposed to autologous human serum. The phenotype of the dendritic cells was analysed using flow cytometry on day 0 (after 7 days of culture) as described. The histograms above are representative of assays SH5-7 and in contrast to previous assays, can be seen to have some CD1a expression (SH6 DCs)

Table 6.8 Characteristics of DC generated using modified protocol 2

	Percentage cell staining					
assay	DC	CD1a	CD19	CD86	MHCII	negative
SH5	80.94	0.58	0.45	74.36	90.28	1.56
SH6	53.88	20.28	1.16	77.36	68.9	1.05
SH7	73.76	6.78	1.74	80.04	91.08	2.56

Table 6.9 Fluorescence intensity of staining of DC generated using modified protocol 2

Fluor. Int.	CD1a	CD19	CD86	MHCII	negative
SH5	75.7	77.43	1133.53	5280.23	145.38
SH6	172.78	114.68	1397.64	2111.62	108.3
SH7	104.47	92.99	899.08	2622.95	168.36

Dendritic cells were generated using the same protocol as described in section 2.2.15 the protocol was altered following generation with the substitution of autologous serum for FCS throughout the entire protocol (protocol 2). The DC generated here were used to generate T cells specific to an HLA-A3 restricted peptide. The cells were phenotyped following 7 days of culture (day 0) using the flow cytometric technique described previously.

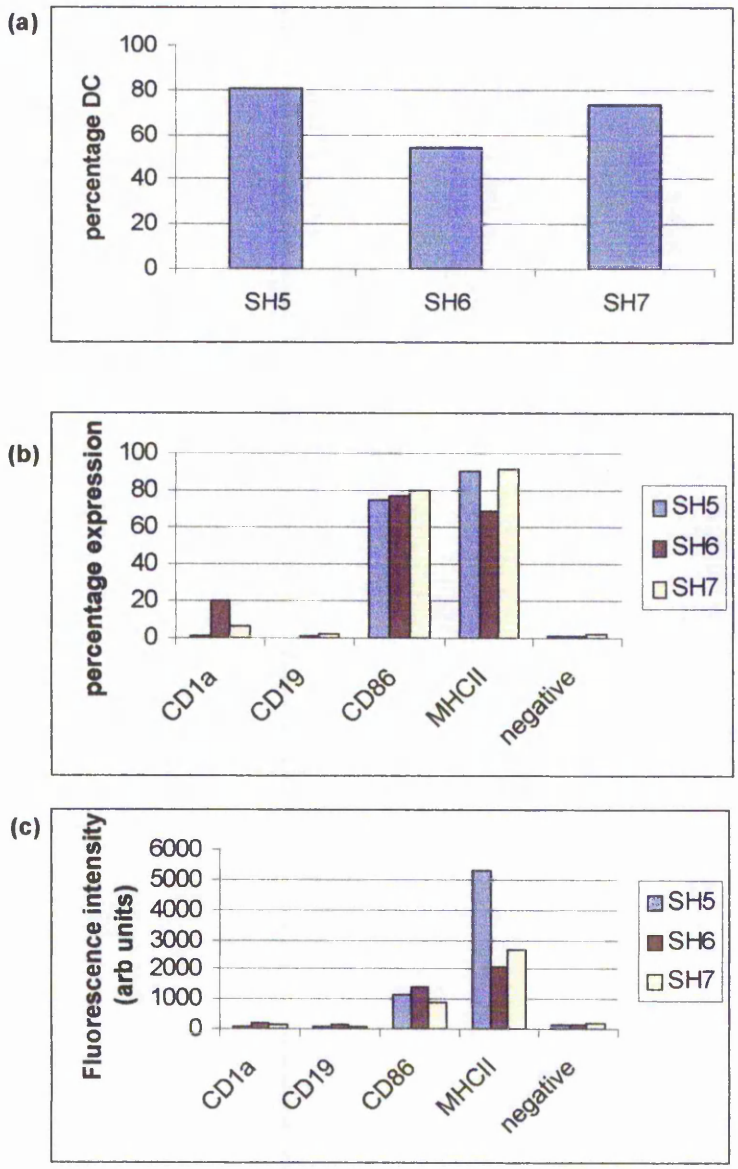


Figure 6.9 Graphical representation of dendritic cell characteristics of assays SH5-7

The above figures are a graphical representation of the data in tables 6.8 and 6.9, showing percentage of DC present within total cellular population (as figure 6.1), percentage expression of the surface markers CD1a, CD19, CD86, MHC II, and fluorescence intensity of the surface marker staining.

Table 6.10 Percentage cytotoxicity - SH6 T cell (generated against an HLA-A3 restricted peptide) – mediated killing of three targets

	E:T ratio											
% cyto	100:1	+/- SD	50:1	+/- SD	25:1	+/- SD	12.5:1	+/- SD	6.75:1	+/- SD	3.75:1	+/- SD
T2-A3 (P)	4.06	2.1	4.74	2.8	0	0.7	0	4.2	0	3.6	0	3.0
T2-A3 alone	0.27	3.6	0	2	0	1.4	0	6.3	0	3	0	2.5
K562-A3	9.03	9.1	0	3.6	2.75	3.6	2.5	5.6	0	2.1	0	4

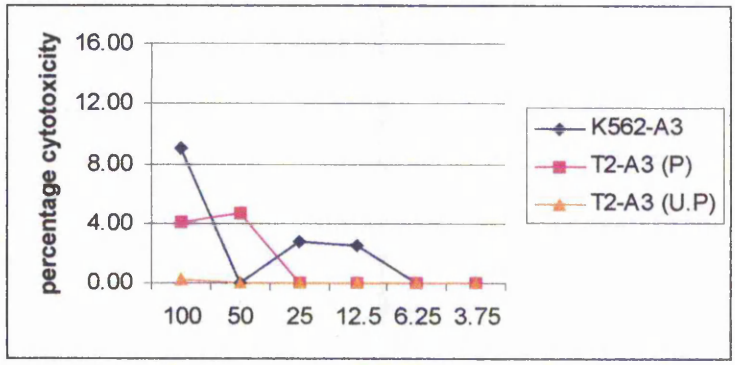


Figure 6.10 Percentage cytotoxicity mediated by SH6 T cells against three targets

SH1 T cells were generated using modified protocol 2 (section 2.2.18) against the HLA-A3 restricted peptide KQSSKALQR. The cytotoxic ability of the T cells was tested after three restimulations (first with dendritic cells, characterised in figures and tables 6.8, 6.9), then with autologous PBMC. Both types of stimulator cell were pulsed with the relevant peptide. Cytotoxic ability was measured using a standard 4 hour chromium release assay (section 2.2.20) and targets used were K562 (transfected with HLA-A3) which naturally express the peptide, T2-A3 cells pulsed and unpulsed with the peptide. All targets were incubated with a 20 fold excess of cold (unlabelled) untransfected K562 to remove any non-specific NK cell killing. A one tailed, two sample T test showed that the K562-A3 killing was significant when compared to the unpulsed T2-A3 ($p = <0.01$).

respectively and lysis of k562 was the lowest at 17%. Due to the sensitivity of K562 to NK (lack of HLA molecules) it would be expected that these cells would demonstrate the highest amount of lysis and not the HLA transfected line as was seen, however all target cells were able to be lysed and the effect decreased in a linear fashion with the lower concentrations of effector cells (NK).

Table 6.11 Percentage cytotoxicity – Unstimulated PBMC harvested from a healthy donor against four targets.

Ratio	Percentage cytotoxicity							
	K562-A3	+/-SD	T2-A3 (P)	+/-SD	T2-A3 (U.P)	+/-SD	K562	+/-SD
100:1	36.16	13	27.42	5.6	21.57	11	17.93	11
50:1	27.21	15	14.65	3.6	12.17	3.7	7.24	1.5
25:1	14.92	3.5	13.41	5.2	7.48	6.3	6.60	4.5
12.5:1	6.54	8	12.03	0.7	2.72	4.6	4.48	4.9
6.35:1	2.59	5.3	1.95	2.1	0	9.5	1.37	7.3
3.75:1	1.93	4.5	14.97	7.8	0	7.5	0	1.7

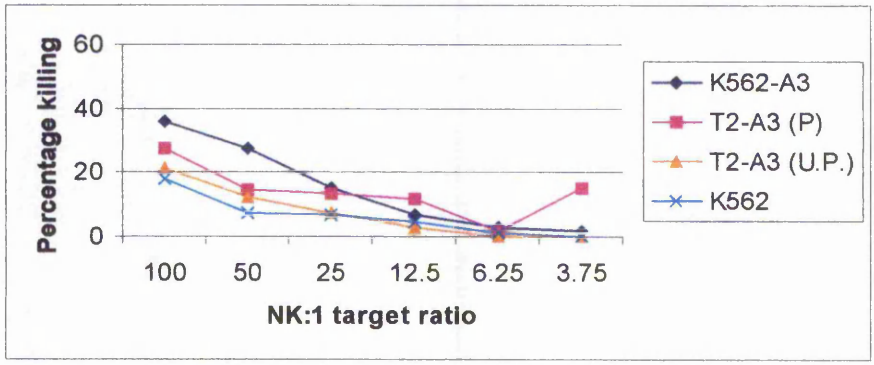


Figure 6.11 NK mediated cytotoxicity against four targets

PBMC were isolated from 20ml fresh blood by Ficoll-Paque (as decribed section 2.2.14) and used as a source of natural killer (NK) cells. To test whether targets used in cytotoxicity assays SH1-7 were able to be killed in a non-specific manner, they were labelled with chromium and the cytotoxic ability of the NK cells against these targets was tested in a standard 4 hour chromium release assay (section 2.2.20). The targets used were HLA-A3 transfected K562 (K563-A3; naturally expressing the HLA-A3 restricted peptide KQSSKALQR), T2-A3 (T2 transfected with HLA-A3) pulsed (P) and unpulsed (U.P) with the peptide and untransfected K562 (K562).

6.3 Conclusion

This study, undertaken to assess the ability of the HLA-A3 restricted bcr-abl peptide to elicit a CTL response using the protocols presented in section 2.2.15-2.2.19, proved to be unsuccessful. Three results were obtained showing a low level of CTL mediated cytotoxicity, but it could not be conclusively determined whether the cytotoxicity was peptide specific as killing of peptide pulsed T2 or T2-A3 cells was not demonstrated. The presence of high numbers of CD4 cells using these protocols was observed and the cultures were depleted of them, however this did not improve cytotoxicity.

6.4 Discussion

Dendritic cells generated using three separate protocols showed a comparable level of expression of the cell surface markers characterising mature DC (Gallucci et al. 1999, Hart 1997, Peters et al 1996). The expression of CD1a, which has been suggested to be important for the generation of cytotoxicity (McArdle et al. 2000, Hart 1997) was variable, and DC expressing CD1a did not seem to induce potent CTL. At a meeting of participants of EUCAPS (London, February 6-8, 2000), similar findings to those reported above (assays EU3-8) were obtained. Although some laboratories were able to generate CTL using the first protocol, the majority found it to be unsuccessful (Pawelec 2000). Modifications to the protocol were advised in the literature, including the use of 5% autologous serum in assays SH1-4 was suggested (from a protocol published by Celis et al. 1994), and used in the generation of bcr-abl peptide specific CTL (Bocchia et al. 1996). The depletion of CD4 T cells is a widespread technique used in many protocols, including that of Bellone et al. (1994). However, neither of these techniques resulted in the improved generation of CTL in *in-vitro* culture for the purposes of this study.

A possible reason for the lack of CTL generation could be the weak binding of the peptides to the MHC on the DC; peptides may not bind at all to the DC/PBMC and this would prevent peptide specific T cells being produced. In the case of the EUCAPS protocol, the peptides had been shown to be strong binders (EUCAPS meeting, 4-7 February 1999) and therefore this is unlikely. The bcr-abl peptide used in assays SH1-7, however, demonstrates a lower binding affinity. Bocchia et al.

(1995) indicated that the peptide had intermediate binding, and Berke et al. (2000) characterised it as binding weakly, however the latter study demonstrated 70% stabilisation of MHC class I (assuming that the control peptide was 100% binding). CTL generated using this peptide appear to be of a low to intermediate potency for CTL induction, Bocchia et al. (1996) demonstrating 22-27% killing at 12.5: effector:target ratio. Vaccination of CML patients with 5 bcr-abl fusion region peptides (Pinilla-Ibarz et al. 2000) resulted in DTH and T cell proliferation responses, however, they were unable to demonstrate CTL mediated killing against peptide pulsed autologous PBMC using a chromium release assay. Other researchers (Buzyn et al. 1997; Yotnda et al. 1998) were able to demonstrate peptide specific CTL activity using a chromium release assay, but again at intermediate levels. A recent report by Norbury et al. (2000), suggested that the HLA-A3 restricted bcr-abl peptide used in the assays above (SH1-7), was able to generate peptide specific CTL capable of lysing peptide pulsed target cells and CML patient blast cells at a comparable level. The protocol used T cell blasts as APC cultured in GM-CSF for 14 days in 96 well plates and fresh PBMC as T cells. Three (one week between each) restimulations of T cells in the presence of peptide, β2-microglobulin, IL-7 and IL-12 were carried out and the cytotoxicity of each individual well was tested. The results showed that 35% of wells contained T cell able to kill peptide pulsed PBMC and 34% of wells contained T cells able to kill HLA matched CML patient blasts. This technique first suggested by Plebanski in 1995 and used by McIntyre et al. (1996) may enable the identification of peptides inducing low frequencies or less potent CTL which is not possible with the bulk culture techniques used above.

Despite the low binding affinity of the bcr-abl peptide, T cells were seen to proliferate after stimulation with peptide pulsed DC on day 0 (not shown). The proliferation of T cells after DC stimulation (which was most evident in the SH1-7 assays) would depend upon the recognition by the T cells of a peptide-MHC complex (as discussed in section 1.2.6 and 6.1). Whether the peptide recognised was the bcr-abl peptide cannot be confirmed by the results presented here. Since the K562-A3 target cells were presenting few other peptides than the bcr-abl (although only 12% of cells expressed MHC, chapter 5), due to their lack of other class I MHC it is likely that the CTL were

recognising the bcr-abl peptide expressed on these cells. The absence of CTL mediated killing of T2-A3 peptide pulsed cells would suggest that the CTL were not killing in a peptide specific manner. However, the T2-A3 cells were not growing optimally, indeed, in assay SH5 these cells did not incorporate chromium, and at the EUCAPS meeting (London, 6-8 February, 2000) it was suggested that the T2 cells not in logarthmic growth and of high viability would resist CTL killing. Thus, it would appear that the status of the target cells can also lead to a negative result in a cytotoxicity assay. It is also entirely possible that the expression of the bcr-abl peptide on the K562-A3 target cells was too low for the CTL to detect and that the problem was related to the target cells used in the cytotoxicity assays and not the CTL generation protocol. These conclusions led to an assessment of NK cell susceptibility in the final assay. The NK cells isolated from a healthy donor were able to lyse the K562-A3 at a higher level than the untransfected K562 (36% compared to 17% at 100:1 effector:target ratio). NK mediated killing of pulsed T2-A3 was not very different to that of unpulsed and thus it would seem that the reported inhibition of NK by the expression of MHC class I (section 1.1.3) was not observed here, or at least is related to other HLA molecules not expressed on these cells. However, the ability of the NK to lyse the cells, albeit at a fairly low level, demonstrated that the target cells were able to be lysed and suggests that the absence of cytotoxicity in these assays was due the inability of the CTL induction protocols to generate peptide specific CTL.

Chapter 7 Final Discussion

Cancer is the second leading cause of death in western civilisation, in Britain 1 in 3 people are affected by cancer, and 1 in 4 people will eventually die as a result of their disease (Office of National Statistics, England and Wales). Despite many years of research, since the discovery of Coley's toxins in 1894 to the present day, a cure for all types of cancer has still not been found.

The failure of conventional treatments to cure cancer has increased interest in the use of immunotherapy to treat cancer. The use of approaches to enhance T cell mediated immune responses against tumours is widespread (section 1.5-1.7) and, since the identification of tumour-specific antigens (Bruggen et al. 1991) enabling the generation of tumour-specific cytotoxic T cells, much research has been concentrated on development of T cell vaccine strategies for cancer therapy.

Peptide-MHC complexes on the surface of infected or tumour cells are responsible for the activation of specific T cells through their binding to the T cell receptor (TCR – section 1.2.6). Thus, the identification of the MHC-binding peptides originating from a given tumour antigen could allow the development of specific T cell immunotherapy to treat tumours. Prediction of MHC-binding peptides from a tumour antigen is easily achieved by the use of algorithms readily available on the Internet, such as Syfpeithi and BIMAS (section 1.7.4). Peptide epitopes identified in this manner have been synthesised and used to vaccinate melanoma patients alone (Jager et al. 1996a) or with adjuvants such as GM-CSF (Jager et al. 1996b), IFA (Salgaller et al. 1996) and IL-2 (Rosenberg et al. 1998). Some clinical responses have been seen (section 1.7.4), and work continues to identify other appropriate and improved adjuvants. The main advantages of peptide-based vaccines are that they are less labour intensive to produce and can easily be adapted to include several peptides, of both class I and class II restriction, to circumvent tumour escape and to provide stimuli able to initiate an effective anti-tumour immune response (section 1.7.5). The use of peptides also enables tailoring of the immune response to specific epitopes which may enable a tumour specific response, minimising the risk of immune cell destruction of non-tumour tissue.

The majority of methods used to identify tumour antigen peptides for use within cancer vaccines (section 3.1) are not able to conclusively demonstrate the expression of the tumour antigen as peptide on the cell surface where it is able to activate T cells to destroy a tumour. The main method used has been immunoprecipitation, in which tumour cells are lysed using detergents and the MHC-peptide complexes are purified from the lysate by affinity column chromatography. This technique does, however, isolate total cellular MHC, including those not expressed on the surface of the cell. It has not been proven whether every MHC-peptide complex trafficks to the cell surface from the endoplasmic reticulum (ER) and thus, peptides not relevant to the anti-tumour immune response may also be identified. The identification of cell surface located MHC-peptide complexes would, in light of their importance in initiating the T cell response, be more relevant in the development of a specific peptide-based cancer vaccine. The use of a surface isolation technique coupled with mass spectrometric analysis has not been extensively demonstrated, and the present study details the optimisation of such a technique together with identification of tumour-specific antigens from cell lines and clinical patient material.

The surface elution technique used in the present study was adapted from Storkus et al (1993) and results contained herein show that the destabilisation of MHC class I following washing with an acid buffer, as shown in section 3.2, enables the isolation of surface MHC-associated peptides (chapter 5). Desalting and concentration procedures required to produce a peptide sample suitable for mass spectrometric analysis are described in chapter 4, the combination of which is entirely novel. Several technologies were evaluated for their ability to purify and concentrate the peptide-containing eluate. Size exclusion chromatography as used in the Storkus (1993) procedure was attempted and rejected due to the inability of this technology to purify large sample volumes in one step. Freeze drying of samples prior to RP-HPLC fractionation was found to profoundly reduce peptide concentration whilst concentrating salts contained within the buffer, causing interference with RP-HPLC and mass spectrometry as described in section 5.1. The procedures finally adopted were able to purify and concentrate peptides even from samples containing many peptides (BSA digest and CML blast eluates). The identification of peptides within the BSA digestion demonstrated the validity of the purification protocol and mass

spectrometry for the identification of peptides within a biological and complex mixture. The application of these procedures to MHC-bound peptide identification from cell lines and subsequently clinical material has enabled the conclusive identification of tumour-specific antigens (bcr-abl) expressed on the surface of tumour cells, which has not been previously reported. Castelli et al. (1995) were able to sequence a peptide, acid eluted from a melanoma cell line using the Storkus (1993) technique, by mass spectrometry. Skipper et al. (1999) were unable to reproduce this result, but were able to identify peptides from a lysate of a melanoma cell line immunoprecipitation and mass spectrometry. To the best of my knowledge, this study is the first to identify surface-bound peptides from clinical patient material, and to conclusively confirm that the intracellular processing of bcr-abl protein results in surface expression of HLA-restricted fusion region peptides. The A3 and B8 peptides identified have been used in a number of studies to generate CTL (Bocchia et al. 1996; Nieda et al. 1998; Yotnda, et al. 1998; Osman et al. 1999; Norbury et al. 2000), which were then used to lyse bcr-abl expressing tumour cells or peptide pulsed cells. The confirmation that the bcr-abl protein is processed intracellularly, resulting in the MHC-restricted expression of peptides known to generate specific CTL, suggests that peptides originating from bcr-abl could be used within a cancer vaccine to treat tumours expressing the protein. Low frequencies of CTL specific for bcr-abl peptides have been identified within patient PBMC, (Greco et al. 1996, Yotnda et al. 1998, Dodi et al. Manuscript in preparation), however, since the tumour is able to progress, this response must be inadequate and augmentation of the natural anti-bcr-abl could be undertaken using a vaccine containing the peptides identified within this study.

The identification of "novel" proteins within the patient blast cell eluates suggests that other peptides may be expressed at similar levels to the known peptides on these cells. The development of a successful cancer vaccine would most likely require the inclusion of several peptide epitopes from different proteins and HLA restrictions to minimise the problems caused by tumour escape (section 1.6). Thus, the identification within this study of two bcr-abl peptides, with different HLA-restrictions (HLA-A3 and –B8), and the presence of an additional peptide (origin unconfirmed, but not bcr-abl associated) suggests that a multi-epitope vaccine would be a

viable option. Since the bcr-abl protein is only expressed within tumours, this provides the opportunity for direct immunotherapy exclusively against tumours. However, care must be taken to exclude bcr or abl region peptides, as these proteins are expressed in many other tissues and immune destruction of healthy tissue must be minimised or avoided.

The results from the present study demonstrated that it was not possible to generate potent CTL against the bcr-abl HLA-A3 restricted peptide using the protocols described in section 2.2. However, the reasons for this are likely to be mainly of a technical nature, relating to the quality of the in-vitro-generated dendritic cells and the targets used within the chromium release assay. The CTL generation technique used was a bulk culture method, which generates a large number of CTL as a single population. The CTL method suggested by Plebanski et al. (1995) and used successfully by Norbury et al. (2000) is a semi-cloning method, performed in 96 well plates, each well containing few cells, thus enabling concentration of peptide specific CTL, increasing the likelihood of positive cytotoxic T cell activity being observed. The CTL induction protocol used within the present study is in need of optimisation if more potent CTL are to be generated.

A limitation of the database identification technique used within the present study to identify the protein origin of peptides is that not all proteins involved in tumours have been sequenced and thus they are not found within databases. The advance of the human genome project, (working draft sequence of entire genome completed, and available on The Genome Database; http://gdbwww.gdb.org), may reduce these problems. However, unless mutated proteins found within tumour patients are sequenced, this information may be of limited use.

Future applications of the technology contained within the present study include the identification of peptide epitopes from other tumours and tumour-antigens. The tumour suppressor, p53, which is involved in 60% of human tumours has been shown to generate CTL and antibodies (section 1.5) and immunotherapy developed utilising this protein could be applied to the treatment of several different tumour types. Tumour cell lines expressing p53 are widespread and could be used together with the technology described to determine naturally processed epitopes. Indeed,

several p53 expressing cell lines were characterised and eluted for use in this study (section 3.2), however, time constraints have prevented the analysis of peptides eluted from these cells. The treatment of other pathogenic conditions which have been shown to be T cell mediated, such as autoimmune disorders, could also benefit from the identification of the T cell epitopes, using the protocol described, mediating the tissue destruction characteristic of these diseases.

Finally, the development of a reproducible and rapid CTL generation assay could be used in partnership with the technology described above to provide an indication of the usefulness of peptides identified prior to the use of an immunotherapy in a clinical setting. The advance of ELISPOT technology may provide such an assay and the ability to identify and characterise antigens for use in patient therapy. The ELISPOT technique identifies antigen-specific CTL by their production of IFN-γ in response to the antigen responsible for their original activation. The technique has been used to assess the CTL response to hepatitis C virus (HCV – Lechner et al. 2000), Epstein-Barr virus (EBV – Yang et al. 2000), the influenza matrix protein (Dunbar et al. 1998) and bcr-abl peptide antigens (Berke et al. 2000). A further application of this technology would be to monitor clinical responses to vaccination as shown by Asai et al. (2000) in melanoma patients responding to a dendritic cell vaccine.

Peptides identified during the course of this study are now available for inclusion in a cancer vaccine. Dendritic cell vaccines, which are able to provide class I, class II and co-stimulation are becoming more widespread (section 1.7) and can be altered to initiate CTL against any peptide antigen. Ali et al. (in preparation) have used DISC-HSV (section 1.7.2) in conjunction with unpulsed dendritic cells to treat murine tumours, achieving 100% rejection. A peptide specific variant of this vaccine would enable the application of antigen specific immunotherapy.

Vaccination strategies for the future have, through work carried out recently, been identified as requiring the inclusion of both class I and class II epitopes (Ali et al. 2000, Bona et al. 1998, Chaux et al. 1999, Mancini et al. 1999, Farzeneh et al. 1998, Lasarte et al.1992), co-stimulation

i.e. B7-CD28, (Bellone et al. 1994, Nair et al. 1997) and, more recently, OX-40 (Walker et al. 2000, Weinberg et al. 2000) and 'danger signals' (i.e. heat shock proteins) to facilitate the recruitment of macrophages and phagocytic cells (Fuchs and Matzinger 1996, Todryk et al. 2000). In essence, anti-tumour responses may be dependent on the orchestration and involvement of the entire repertoire of immune system mediators as occurs in response to other pathogens and this should be taken into consideration when designing a vaccine to treat cancer.

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Abstracts:

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24th Annual Meeting of British Mass Spectrometry Society, 1999

36th Annual Congress of Royal Society of Chemistry, 1999

23rd Annual Meeting of the British Mass Spectrometry Society, 1998

35th Annual Congress of the Royal Society of Chemistry, 1998

Presentations:

4th Meeting of European Concerted Action in Peptide Sensitisation (EUCAPS), London, 2000

Tumour Immunology Affinity Group (British Society for Immunology) Meeting, Cardiff, 1999

Scandinavian Immunology Society Meeting, Copenhagen, 1998