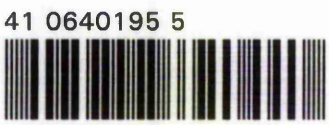

The development of novel immunotherapy strategies for prostate cancer

Richard John Parkinson

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School of Biomedical and Natural Sciences
Nottingham Trent University
Clifton Lane
Nottingham
NG11 8NS

NOTTINGHAM TRENT UNIVERSITY

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The development of novel immunotherapy strategies for prostate cancer

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Abbreviations

Abbreviation	Meaning
2-ME	2-MercaptoEthanol
Ab	Antibody
Ag	Antigen
APC	Antigen Presenting Cell
BSA	Bovine Serum Albumin
CD4 / CD8	Cluster of Differentiation
cFLIP	cellular FLICE Inhibitory Protein
CPE	CytoPathic Effect
CTL	Cytotoxic T-Lymphocyte
DC	Dendritic Cell
DISC-HSV	Disabled Infectious Single Cycle-Herpes Simplex Virus
DMSO	DiMethyl SulphOxide
ER	Endoplasmic Reticulum
FCS	Foetal Calf Serum
FITC	Fluorescein-IsoThioCyanate
GMCSF	Granulocyte-Macrophage Colony Stimulating Factor
HLA	Human Leukocyte Antigen
IFA	Incomplete Freund's Adjuvant
IFN	Interferon
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Media
KLH	Keyhole Limpet Haemocyanin
LAMP	Lysosomal-Associated Membrane Protein
LB	Luria-Bertani medium
LCMV	Lymphocyte ChorioMeningitis Virus
LPS	LipoPolySaccharide
MAGE	Melanoma AntiGen E
MFR	Mean Fluorescence ratio
MHC	Major Histocompatability Complex
NPCP	National Prostate Cancer Project
PAP	Prostate Acid Phosphatase
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PIN	Prostatic Intraepithelial Neoplasia
PSA	Prostate Specific Antigen
PSCA	Prostate Stem Cell Antigen
PSMA	Prostate Specific Membrane Antigen
RDA	Representational Difference Analysis
RPA	Ribonuclease Protection Analysis
RPMI-1640	(media developed at the) Roswell Park Memorial Institute
SAGE	Serological Analysis of Gene Expression
SDS	Sodium Dodecyl (lauryl) Sulphate
SEREX	SErological analysis by Recombinant cDNA EXpression
TAP	Transport Associated Protein
TCC	Transitional Cell Carcinoma
TCR	T-Cell Receptor
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TRAIL	TNF-Related Apoptosis Inducing Ligand

Abstract

This thesis examines two separate approaches to prostate cancer immunotherapy. The first aims to employ a viral vector (DISC-HSV) for the systemic delivery of a multi-epitope vaccine in order to induce immune responses against several prostate cancer antigens simultaneously. Initially, it is intended to establish the feasibility of delivering a multi-epitope vaccine using DISC-HSV. The design and construction of a multi-epitope vaccine employing well-known cancer epitopes is described.

The succeeding chapters will describe the identification of novel prostate cancer epitopes, and the testing of their immunogenicity in both human and transgenic animal models. The discovery of an entirely novel, immunogenic, class-I epitope derived from prostate acid phosphatase, designated PAP.135, is described. This peptide was predicted to be immunogenic by analysis of the PAP amino-acid sequence, and subsequently exhibited strong HLA-A2 binding in a T2 binding assay. Its ability to excite an immune response has been demonstrated in a transgenic mouse model, and this has been shown to be mediated through the induction of cytotoxic T-lymphocytes (CTL). Early data in a human model system, employing peripheral blood monocytes to establish CTL cultures have supported these findings.

The second approach employs the same viral vector to deliver a cytokine gene (GMCSF) to tumour cells, with the aim of inducing anti-tumour immunity. The feasibility of this approach is established by the successful testing the ability of the virus to infect prostate cancer cells *in vivo* and *in vitro*, with the expression of virally encoded gene products. The physiological effects of infection by this agent on the prostate cancer cells are examined in relation to gene expression.

Although several strands of related work are presented here in sequence, it should be remarked that the experiments are not presented in chronological order; indeed many of these experiments were undertaken concurrently. Work on the multi-epitope vaccine constructs began at the start of the project, whereas the confirmation of the immunogenicity of PAP.135 was very late in the study period. Therefore, PAP.135 was not (and indeed could not) be included in the multi-epitope construct described in chapter three.

Introduction

Contents

The prostate gland

The prostate is a glandular organ that forms part of the male reproductive system (McMinn 1994). It lies on the urogenital diaphragm, posterior to the retropubic space and anterior to the rectum (see figure 1.1a). It is fused with the urinary bladder superiorly and is perforated by the urethra, which transverses its entire length. The gland consists of multiple acini of various sizes embedded in a fibromuscular stroma. Numerous small ducts open into the urethra.

The gland contributes approximately 30% of the volume of seminal fluid (Campbell 2002). Its secretions, along with those of the other male accessory sex organs, add high concentrations of a variety of substances to the seminal fluid, including fructose, citric acid, spermine, prostaglandins, zinc, proteins, immunoglobulins, proteases, esterases and phosphatases. With the exception of certain specific enzymatic activities, the physiological functions of many of these compounds remain obscure. They are thought to provide nutritional support for spermatozoa, optimise the environment for fertilization and possibly also have a microbicidal function (Campbell 2002).

Prostate cancer

The impact of prostate cancer on men's health is considerable. It is one of the most common cancers worldwide, causing illness and early death in hundreds of thousands of sufferers each year (Quinn and Babb 2002; Quinn and Babb 2002). As a cause of cancer death in men, adenocarcinoma of the prostate is second only to lung cancer, with a lifetime risk of dying from prostate cancer of approximately 3% in most western countries (Quinn and Babb 2002).

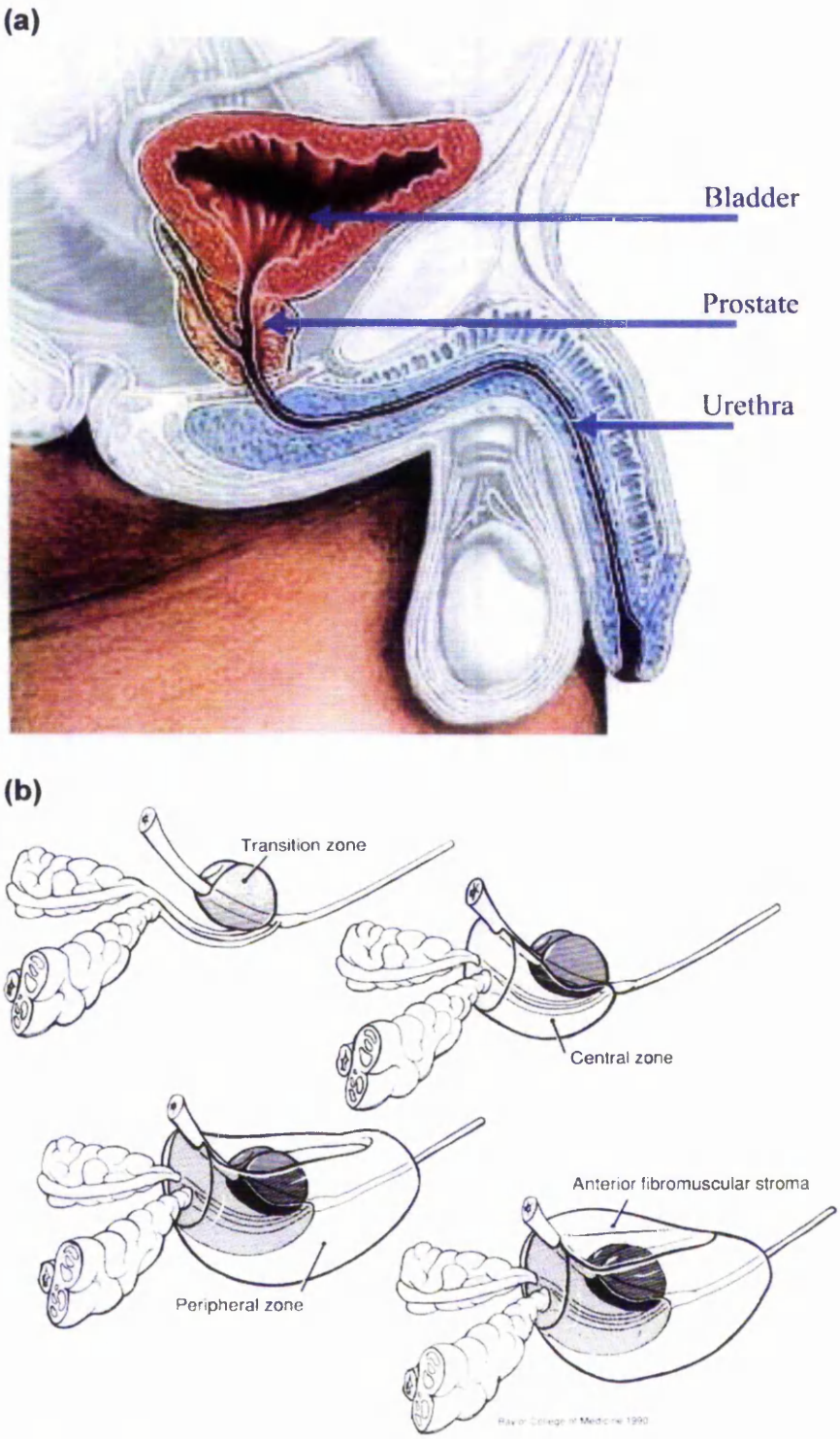


Figure 1.1: Anatomy of the prostate gland.

The prostate gland lies on the urogenital diaphragm, posterior to the retropubic space and anterior to the rectum **(a)**. It is fused with the bladder above and is perforated by the urethra, which traverses its entire length. The gland may be divided into zones **(b)**. 75% of cancers arise in the peripheral zone, 20% in the transitional zone and 5% in the central zone.

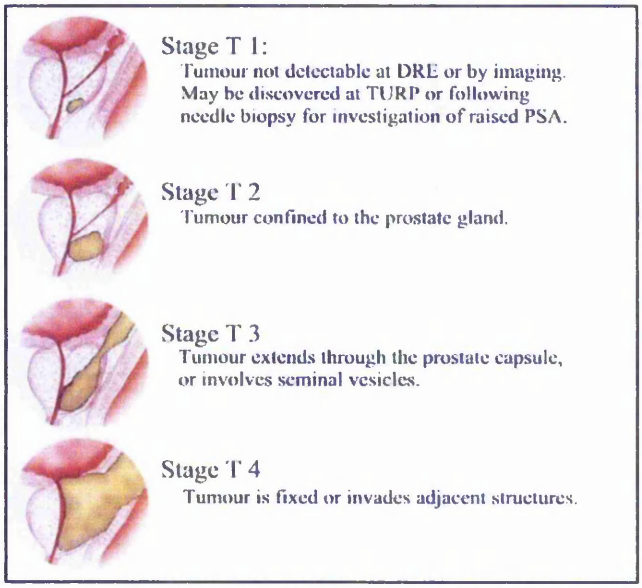
Tumours usually arise in the peripheral zone of the gland, the second commonest site being the transitional zone (McNeal 1988) (see figure1.1b). Prostate cancer is primarily a disease that affects men over the age of 50 years. Other risk factors include race (e.g. african Americans, northern Europeans) and family history.

The growing popularity of Prostate Specific Antigen (PSA) testing has increasingly led to the presentation of subclinical prostate cancer (i.e. in asymptomatic patients). Progression to locally advanced and metastatic stages is associated with the development of symptoms and complications, including bladder outflow obstruction, perineal pain, ureteric obstruction, bleeding, bone pain, pathological fractures and spinal cord compression. Prostate cancer treatments aim to reduce morbidity and mortality through eradication of localised tumours or palliation of locally advanced and metastatic disease.

Current treatments

Curative treatment of organ-confined prostate cancer can only be reliably achieved by early intervention with radical surgery, radiotherapy or brachytherapy (D'Amico *et al.* 1998) with prostatectomy offering a disease free survival at 10 years of between 68% and 80% (Amling *et al.* 2000; Gretzer *et al.* 2002). The presence of extraprostatic extension significantly reduces the chances of long-term cure (Partin *et al.* 1993), with more advanced stages being associated with worse prognoses. The TNM staging system for prostate cancer (Sobin and Wittekind 2002) classifies organ-confined disease to stages pT1 and pT2 (see figure 1.2).

(a)



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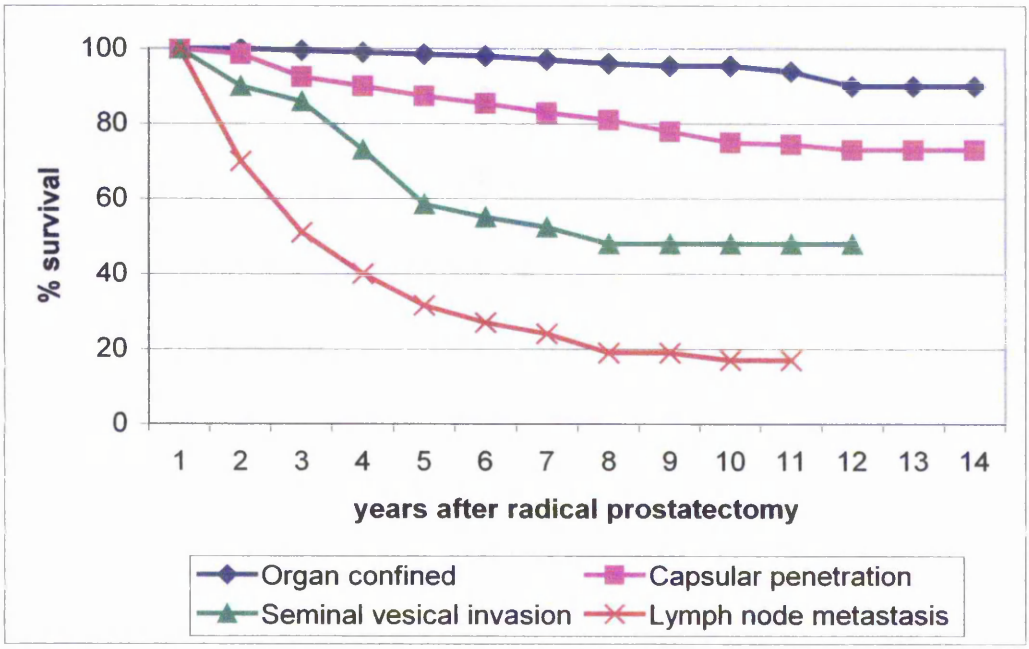


Figure 1.2: Prostate cancer staging.

The TNM system provides a means of describing cancers according to the extent of the primary tumour (T), the involvement of lymph nodes (N), and the presence of distal metastases (M). The following diagram illustrates the "T" stages of prostate cancer **(a)**. The long-term survival of patients with prostate cancer is dependent on the pathological stage, with more advanced stages being associated with a poor prognosis **(b)**.

Most prostate tumours are initially androgen-dependent, and may be effectively treated by hormone ablation (Huggins and Hodges 1941). This forms the mainstay of treatment for advanced tumours. Such treatment is only palliative as most tumours become refractory within 18 months (Robinson *et al.* 1995). The role of chemotherapy is limited, principally due to the slow growing nature of most prostate tumours. For most patients, the benefits of chemotherapy are outweighed by the side effects of treatment. Other forms of treatment are not widely used, but some examples are summarised in Table 1.1.

Therapy	**Mode of action**	**Clinical effects**
Estracyt	Estramustine Phosphate (microtubule inhibition) + oestrodiol	30-60% response, but toxicity is limiting
Pamidronate	Bisphosphonate	Symptomatic relief of bone pain (Diener 1996)
^{89}Strontium	Radioactive isotope localising to bone metastases	Improves bone pain, but probably inferior to external beam radiotherapy (Oosterhof *et al.* 2003)
EGFR inhibitors	Signalling pathway implicated in cell proliferation, apoptosis, angiogenesis and metastatic spread	Early phase I data show some response (Blackledge *et al.* 2000)
Suramin	Growth factor and angiogenesis inhibition	Benefits in bone metastases, but toxic (Braddock *et al.* 1994)
Atrasentan	Endothelin antagonist (effects on growth factors and osteoclast/blast activity)	May reduce progression & morbidity, with minimal morbidity (Nelson 2003)
Anti-sense RNA	Inhibition of e.g. bcl-2	Possible benefits in combination with taxanes / vinca alkaloids (Gleave *et al.* 2003)

Table 1.1: Alternative systemic approaches to prostate cancer therapy.

Unfortunately, many of these treatments are associated with side effects and confer limited benefit. The need for new treatments in all stages of prostate cancer, but most particularly for advanced disease, is not disputed. Prostate cancer may be an ideal target for immunotherapy: the organ is not essential for life, it is relatively easily accessible, and there are a number of well-known putative target antigens, which are largely prostate specific.

Principles of immunotherapy

Immunotherapy for cancer entails the exploitation of the various effector arms of the immune system to destroy tumour cells. These immune system components include antibodies, T-lymphocytes and natural killer cells.

The concept that the immune system is capable of attacking cancer cells is supported by sporadic cases of spontaneous tumour regression in some tumours, including melanoma and renal adenocarcinoma, and strategies of enhancing this anti-tumour immunity in order to provide new cancer treatments have been investigated for over a century. In the 1890s, William Coley began treating cancer patients with inoculations of bacterial extracts to activate general systemic immunity, with the idea that some might be directed against the tumour (Coley 1893). Regression of inoperable tumours was observed in some cases, although the technique was limited by toxic side effects.

More recently, there has been an increase of interest in cancer immunotherapy, largely attributable to improved understanding of the molecular basis of immune recognition and immune rejection of cancer cells (Houghton *et al.* 2001). This section will give a background to the scientific basis of immune surveillance and the concepts surrounding the development of immunotherapies for cancer treatment.

In the past, it was thought that the prostate might be an immunologically privileged site due to the scarcity of lymphatic vessels within the gland, and that it would therefore be insensitive to immunotherapy (Gittes and McCullough 1974). However, evidence has emerged that suggests the importance of the immune response in prostate cancer, including the finding that deficiency or absence of tumour-infiltrating lymphocytes (TIL) are independent risk factors for progression of prostate tumours (Vesalainen *et al.* 1994). Furthermore, early studies of non-specific immune therapies such as BCG in patients with prostate cancer showed a small advantage in terms of survival and symptoms (Guinan *et al.* 1982). These findings have triggered a resurgence in interest in immunotherapy for prostate cancer and numerous studies exploring different approaches are underway (Tjoa and Murphy 2000).

Cell-mediated immunity

Antibody therapies, either used alone or in combination with chemotherapy, have been shown to be highly effective in mediating tumour regression in some haematological malignancies (Colombat *et al.* 2001). However, their effects on solid tumours have been less promising, and the majority of responsive solid tumours are those that overexpress certain growth factor receptors (Cobleigh *et al.* 1999). Anti-tumour immune responses are mediated more effectively by T-lymphocytes (adaptive immunity) than antibodies (innate immunity) (Greenberg 1991); hence, the production of cell-mediated immune responses has become paramount in immunotherapy research (Rosenberg 1999). Preclinical and clinical research is focused on inducing a cellular antitumour immune responses, with one of the main goals being the generation highly specific CTLs.

Requirements for lymphocyte activation

The initiation of an immune response occurs when an antigenic peptide physically interacts with the T-cell receptor (TCR) on the surface of a T-lymphocyte. TCRs interact with peptides "presented" by cell-surface Major Histocompatability Complex (MHC) molecules. Foreign peptides presented on a cell, for example following viral infection, are recognised by T-cells and the cell is destroyed. Neo-antigens (abnormal self proteins arising during neoplastic transformation) may be recognised by the immune system as foreign and are therefore potential targets for T-cell mediated cellular killing.

Central to the successful induction of adaptive immune responses is the interaction of T-cells with antigen presenting cells (APC). Through phagocytosis, such cells constantly "sample" their environment, processing internalised proteins and presenting fragments of these proteins (peptides) on the cells surface. These peptides can be detected by T-cells, which may become activated and proliferate under favourable conditions. Dendritic cells are the most important and widely studied members of the antigen presenting cell "family", and appear to have a pivotal role in the cellular immune response. An understanding of the functions and interactions of these cells is essential to cancer immunology.

MHC molecules

The MHC describes a region of genes, located on chromosome 6 in humans, that encode proteins that are responsible for the rejection of tissue between different species or members of the same species (Trowsdale *et al.* 1991). These cell surface proteins allow the immune system to differentiate "self" from foreign cells, and also present peptides for inspection by T-lymphocytes.

The cell surface MHC molecules were first identified on white blood cells, and are synonymously known as human leukocyte antigens (HLAs). There are two major types of MHC antigens termed *class I* and *class II.* Virtually all nucleated cells express MHC class I antigens, whereas class II antigens are primarily found on certain cells of the immune system, including B cells, monocytes, macrophages and dendritic cells (DC). Each individual has a total of six HLA molecules, inheriting two class I and one class II antigen from each parent. The HLA molecules are polymorphic, based on multiple alleles (alternative genes at each locus). Over 150 types have now been serologically defined making it very unusual for two unrelated individuals to share the same six HLA antigens.

T-cells recognise antigens through presentation of antigen fragments (epitopes) by MHC molecules on the surface of cells (see figure 1.3). MHC class-I molecules consist of a large 44 kDa alpha chain, which is associated non-covalently with the smaller 12 kDa $ß_2$-microglobulin. The alpha chain is anchored to the cell membrane by its transmembrane segment and includes the peptide-binding cleft, yet both α and β components are required for presentation of peptides. MHC class I molecules present peptides of 8-10 amino acids in length, derived principally from intracellular cytoplasmic proteins, digested by the proteosome and ultimately presented to CD8+ T-cells (e.g. cytotoxic T-cells), which may destroy the presenting cell if foreign or bacterial peptides are detected. In this way, the presentation of viral proteins enables the immune system to identify infected cells. The mechanisms involved in MHC class-I presentation of intracellular peptides are illustrated in figure 1.4.

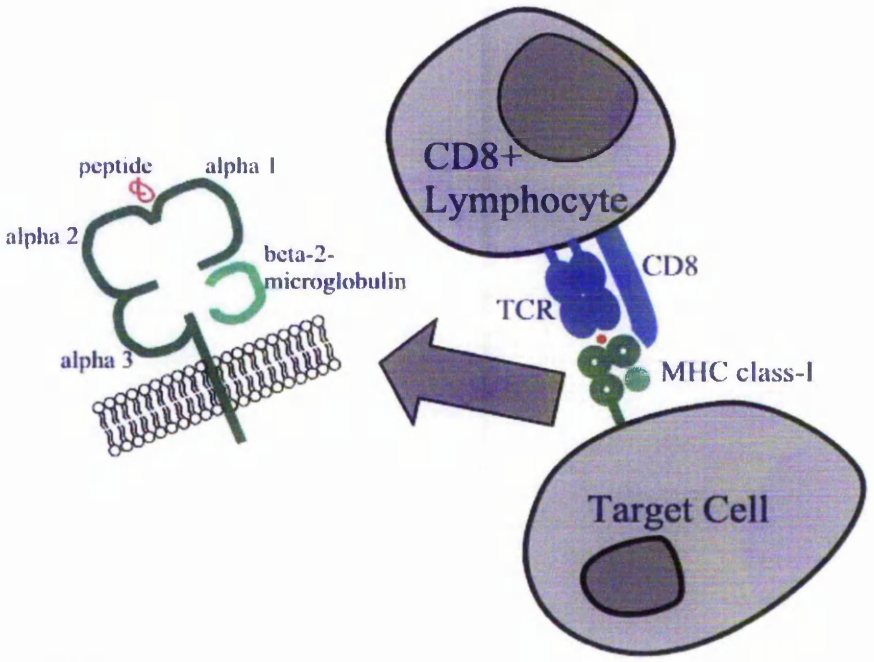


Figure 1.3: Presentation of peptides to CD8+ lymphocytes by MHC-class I

MHC class-I molecules are present on all nucleated cells, and consist of a large alpha chain, which is associated non-covalently with the smaller $ß_2$-microglobulin. The alpha chain is anchored to the cell membrane by its transmembrane segment and includes the peptide-binding cleft, yet both components are required for presentation of peptides. CD8+ lymphocytes are able to interact with peptide presented by MHC class-I molecules, and may destroy the presenting cell if foreign or bacterial peptides are detected.

MHC class-II molecules are present only on cells of the immune system such as antigen presenting cells, and consist of two alpha (34kDa) and two beta (28kDa) chains with a peptide binding cleft between (see figure 1.5). Extracellular proteins engulfed by immune cells such as APCs, and digested in intracellular endosomes producing shorter peptides, which are then presented by MHC class II molecules on the cell surface (see figure 1.6). Peptide presented by MHC class-II may be

recognized by T-cell receptors (TCR) on T-helper lymphocytes expressing CD4, which may then activate other immune cells producing and augmenting an immune response against the original protein. MHC class-II mediated antigen recognition is pivotal in the orchestration of immune responses directed towards either extracellular or an intracellular pathogens.

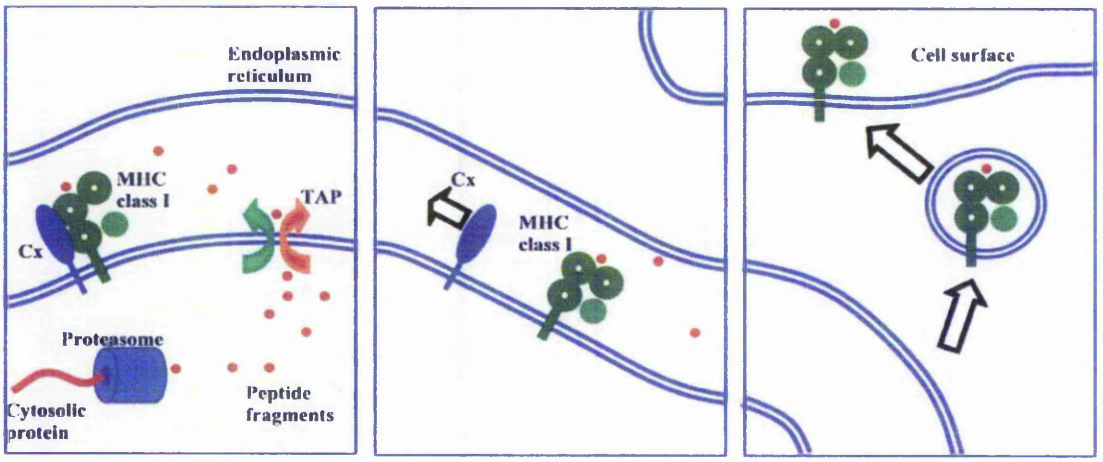


Figure 1.4: Class I intracellular processing pathways

The source of peptides for MHC class-I binding is the degradation of cytosolic proteins by the proteosome, a large protease complex. Peptide fragments generated by the proteosome are conveyed into the endoplasmic reticulum (ER) by the Transporter for Antigen Processing (TAP). MHC class-I molecules are assembled in the ER, complexed to a membrane bound protein, Calnexin (Cx). In this complex, the MHC molecule is retained in the ER and is prevented from being transported to the cell surface. Release of MHC molecules from calnexin is dependent on the binding of peptides, which induce folding of the MHC molecule. The peptide/MHC complex is then transported through the Golgi apparatus to the cell surface.

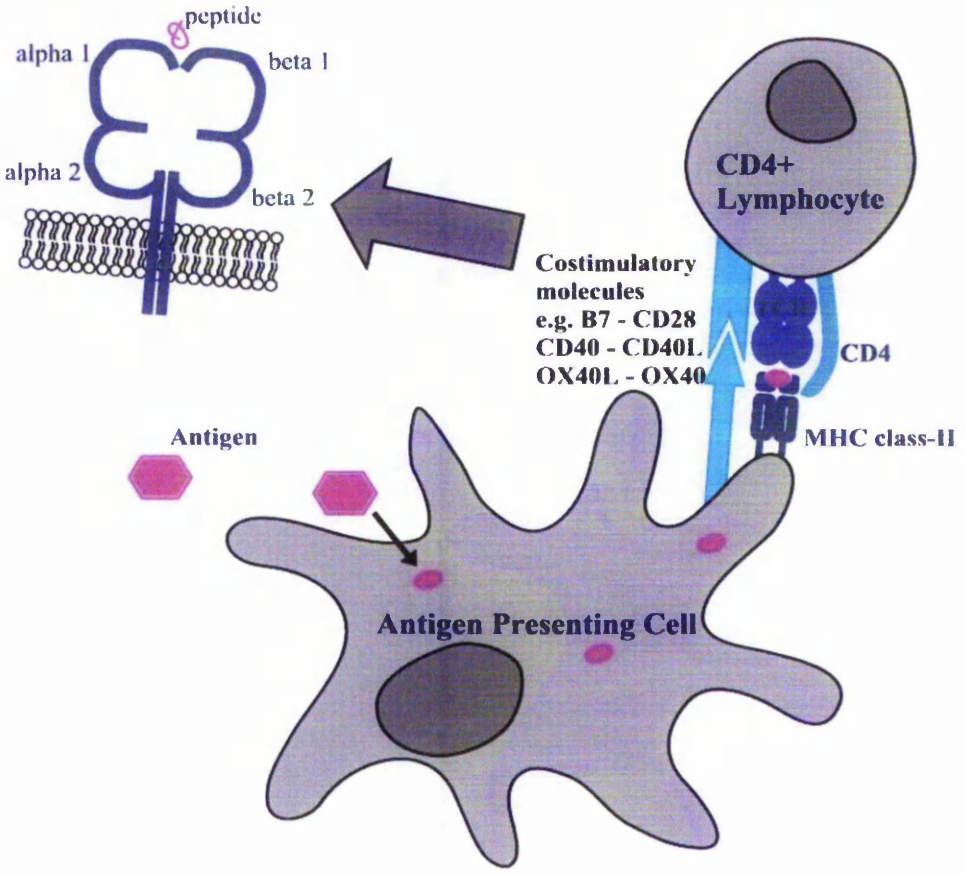


Figure 1.5: Presentation of peptides to CD4+ lymphocytes by MHC-class II

The MHC class-II molecule is present only on cells of the immune system, such as antigen presenting cells. It consists of two alpha and two beta chains with a peptide-binding cleft between. Peptide presented by MHC class-II may be recognized by T-cell receptors (TCR) on T-helper lymphocytes expressing CD4, which may then activate other immune cells against the original protein. Co-stimulatory molecules expressed by both cells are necessary for effective stimulation of an immune response.

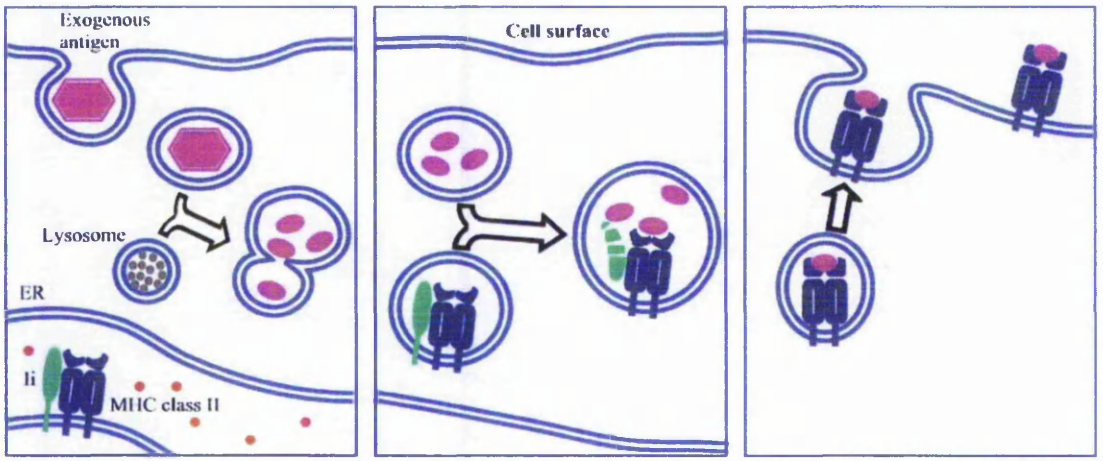


Figure 1.6: Class II intracellular processing pathways

The source of class-II peptides may be bacteria or parasites that replicate within intracellular vesicles. Alternatively, proteins may be engulfed by phagocytic cells and digested by proteases within acidified endosomes. MHC class-II molecules are synthesized within the ER, where the invariant chain (Ii), or class-II γ-chain, prevents binding of MHC class-II molecules with intracellular peptides present within the ER lumen. The invariant chain also directs its transport through the Golgi apparatus to the acidified endosomes containing engulfed extracellular proteins. The activated endosomal proteases cleave Ii, generating a smaller fragment that cannot prevent peptide binding, but remains bound to MHC class-II preventing its transport to the cell surface. With further degradation of the Ii fragment, the peptide/MHC class-II complex is transported to the cell-surface.

For both MHC class-I and class-II, certain sequences from the antigen protein are favourable for effective binding to the MHC molecule and hence presentation; these sequences are dependent on the MHC haplotype (MHC restriction). To facilitate the interaction of lymphocytes with other cells, the expression of other cell-surface molecules, such as cell-adhesion molecules (e.g. CD3) is required. In addition, the expression of co-stimulatory molecules (e.g.. CD28) is necessary for the effective stimulation of an immune response. This is described in more detail below.

Activation versus tolerance

The failure of the immune system to eradicate emerging tumours does not appear to be attributable to an inability to recognize newly transformed cells, but rather that these cells do not activate the immune system sufficiently to provoke a response capable of destroying them.

The interaction of an MHC/peptide complex with a T-cell receptor may lead to the activation of the T-cell and ultimately cell destruction. In certain circumstances, the T-cells do not become activated even in the presence of a foreign antigen, and the immune system becomes tolerant to the antigen encountered. The outcome of antigen recognition by T-cells is determined by the context in which the antigen is encountered. The recognition of foreign antigen in association with tissue damage or inflammation is more likely to result in immune activation, whereas immune tolerance is induced in the absence of such an appropriate "danger signal" (Matzinger 1994).

This effect is mediated through the release of cytokines and the expression of co-stimulatory molecules such as B7.1 (CD80) or B7.2 (CD86) by APC when "danger" is perceived (see figure 1.7a) (Kearney *et al.* 1995). The resting T-cell then progresses through the cell cycle from G_0 to clonal proliferation. The importance of such interactions is illustrated by the regression of experimental prostate tumours following the inhibition of CTLA-4, a competitive antagonist of B7/CD28 interaction (Kwon *et al.* 1997; Kwon *et al.* 1999).

The activation of CTL is also dependent on $CD4^+$ T-cell responses both directly (e.g. via IL-2 release) and through the induction of the expression of costimulatory molecules by APC (e.g. via activation of CD40 on APC by CD40-ligand on activated $CD4^+$ T-cells) (Ford *et al.* 1999).

(a)

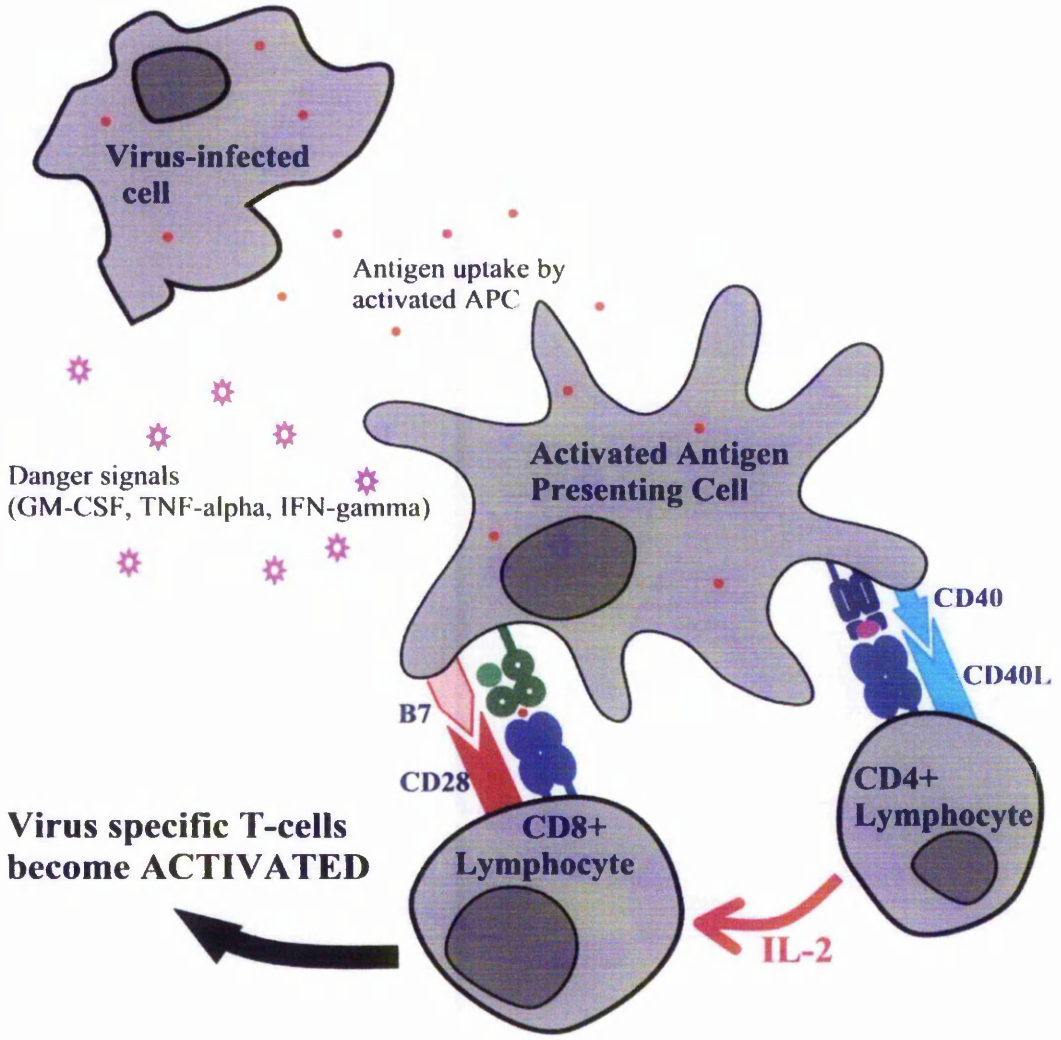
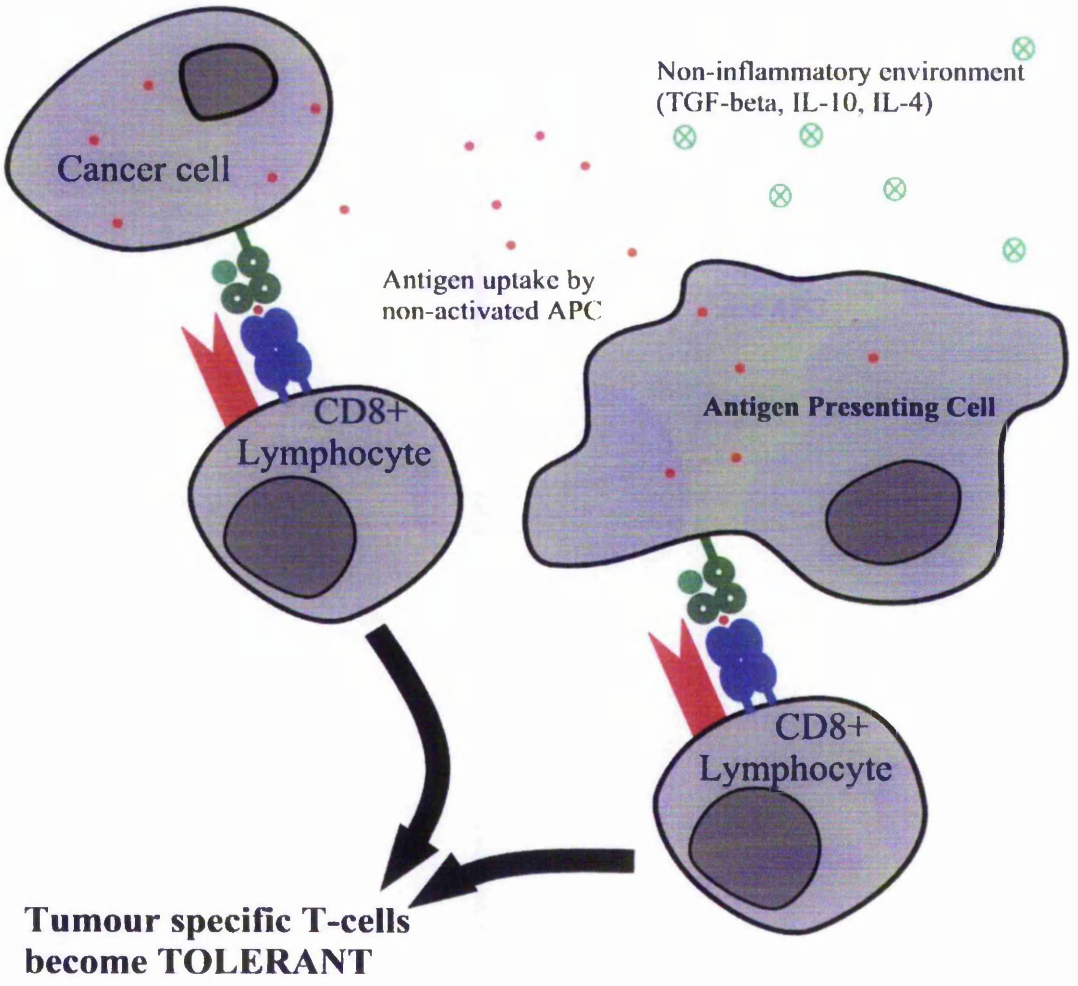


Figure 1.7: T-cell activation and tolerance

The possible responses of T-cells encountering foreign antigen are exemplified by the typical reactions to tumour cells and virally infected normal cells. The inflammation and tissue destruction that accompanies viral infection (or injection of antigen mixed with adjuvant) causes activation of local APCs and the expression of costimulatory molecules (such as B7), which can interact with complementary molecules on the T-cell (such as CD28) resulting in activation **(a)**. $CD4^+$ lymphocytes are also instrumental in these processes. In contrast, endogenous expression of tumour neo-antigens in the absence of "danger signals" results in the presentation of antigenic peptides either by non-activated APC (lacking expression of costimulatory molecules such as B7) or by the tumour itself, and the usual outcome is immunological tolerance **(b)**.

(b)



Promotion of $CD8^+$ and $CD4^+$ T-cell responses may be elicited through the transient expression of inflammatory cytokines such as IL-2, IL-12, IFN-γ and most notably GM-CSF (Watanabe *et al.* 1989; Fearon *et al.* 1990; Dranoff *et al.* 1993; Toda *et al.* 1998; Ali *et al.* 2000; Ali *et al.* 2002) via the activation of professional APCs such as dendritic cells (DC). The ability to promote T-cell activation in response to specific antigens is also contingent upon the simultaneous expression of co-stimulatory molecules such as B7.1/B7.2 (CD80/CD86), OX-40L and CD40 by APC through

direct interaction with cognate receptors (e.g. CD28, OX-40 and CD40L) upon the T-cell surface (Weinberg 2002). Engagement of OX40 enhances proliferation and cytokine production by CD4+ T-cells in vitro (Gramaglia *et al.* 1998) as well as survival of Ag-specific CD4+ T-cells in vivo (Gramaglia *et al.* 2000).

A number of other accessory molecules may support or enhance costimulation such as CD2 engaging leukocyte functional antigen-3 (LFA-3, CD58) and LFA-1 engaging ICAM. The interaction of B7 with the cognate CD28 molecule on the T-cell results in activation of protein kinase C and other signal transduction pathways (Lenschow *et al.* 1996), and evidence supports the contention that expression of co-stimulatory molecules by circulating APC can be stimulated by inflammatory cytokines such as GM-CSF (Markowicz and Engleman 1990; Caux *et al.* 1992).

When a foreign antigen is detected in the absence of tissue destruction or other "danger signals", the immune system may become tolerant to the antigen encountered. This probably represents a protective mechanism through which the possibility of overstimulation of the immune system against a harmless entity is minimized, and also protects against immune reactivity against self-peptides. Tolerance may occur as a result of various mechanisms (see below). Antigens that are present in the serum, such as PSA and PAP, are particularly tolerogenic. Creating the conditions under which immune tolerance can be overcome is pivotal to the success of cancer immunotherapy.

Mechanisms of tumour evasion of the immune response

Evidence of successful treatment of some tumours with immunotherapies, and evidence of early tumour suppression in animal models supports the hypothesis that immunosurveillance has the potential to detect and destroy malignant neoplasms

(Dunn *et al.* 2002). In the early stages of tumour growth, failure to excite immune attack may be attributable to lack of danger signals (see above). Instead of immune activation, reactive T-lymphocyte clones are rendered impotent through various mechanisms. For example, without the necessary danger signal, B7 expression by APC will be insufficient for activation of CD28 on T-cells (Schwartz 1990), and so the detection of presented antigen by naïve T-cells will induce anergy. Later re-exposure to antigen, even in the presence of B7, will not be sufficient to overcome this effect. Another mechanism of T-cell control implicated in tumour evasion involves suppressor T-cells ($CD4^+$, $CD25^+$), which regulate immune activation and prevent autoimmunity (Sakaguchi *et al.* 2001). Such cells secrete immunosuppressive cytokines such as IL-4, IL-10 and TGF-β (Asseman *et al.* 1999; Seddon and Mason 1999) (Powrie *et al.* 1996), and downregulate not only IL-2 but also the B7 expression and IL-12 secretion by APC necessary for CTL stimulation (McHugh and Shevach 2002).

Later tumours may become inherently more immune activating: unregulated growth may damage surrounding tissues or lead to tumour ischaemia and necrosis, and genetic dysregulation may produce the emergence of neo-antigens. Thus, the growth of tumours implies their evasion of immunosurveillance, and may result from the selection of clones of tumour cells by Darwinian mechanisms.

Immunosuppression may be systemic in advanced cancer, but the local tumour environment is often immunosuppressive for a number of reasons. The secretion of various soluble factors may abrogate the immune response, including VEGF, $PG\text{-}E_2$, TGF-β and IL-10, which negatively regulate T-helper and macrophage responses, and also reduce natural killer cell (NK) activity (al-Sarireh and Eremin 2000). These

factors have frequently been shown to be associated with poor prognosis or disease progression, although it is likely that this can be related, in part, to their functions as angiogenesis and growth factors.

The loss of MHC class-I molecules or deficiencies in class-I processing is associated with tumour invasion, metastasis and recurrence (Garrido *et al.* 1997). This may occur through a variety of mechanisms, including mutations of the β_2-microglobulin gene, defects in antigen processing (e.g. LMP-2 and LMP-7 down-regulation), defects in transport pathways (e.g. TAP-1 and TAP-2 deficiencies), and selective loss (partial or complete) of MHC molecules. MHC loss has been described in many tumours, including melanoma, colorectal adenocarcinoma, prostate carcinoma and breast carcinoma (in which over 50% of tumours exhibit total loss of HLA class-I).

Even in the presence of normal MHC expression, the expression of tumour antigens may be lost, and this appears to be associated with increased tumour aggressiveness. For example, the loss of gp100, MART-1 and tyrosinase expression by melanoma has been associated with disease progression (de Vries *et al.* 1997). Furthermore, gp-100 peptide immunisation has been shown to produce a reduction of expression of gp100 (but not other antigens), possibly mediated through selection pressure (Riker *et al.* 1999).

Fas ligand (Fas-L) and TNF related apoptosis inducing ligand (TRAIL) are "death receptor" systems that play a role in immune surveillance. The interaction of the Fas receptor (Fas-R) on the surface of target cells and Fas-L on CTL initiates an intracellular chain reaction (the "caspase cascade") resulting in apoptosis of the target (Berke 1995). Tumour cells have been shown to reduce their expression of the Fas receptor (Strand *et al.* 1996), or increase their expression of caspase inhibitors such as

cFLIP (Irmler *et al.* 1997) rendering them insensitive to Fas-L mediated attack (see figure 1.8). Evolution of tumour cell phenotypes that express Fas-L may even allow such cells to reverse the Fas attack and elicit destruction of tumour infiltrating lymphocytes, thus evading the immune response (Strand *et al.* 1996). Experimental evidence for this is not yet conclusive.

TNF-Related Apoptosis Inducing Ligand (TRAIL) is a cell-surface protein that, like Fas-L, may induce cell-death by interaction with a cell-surface receptor. Mutations at multiple sites along the signalling pathway, including receptor loss and deficiencies in the caspase system, may prevent TRAIL mediated apoptosis.

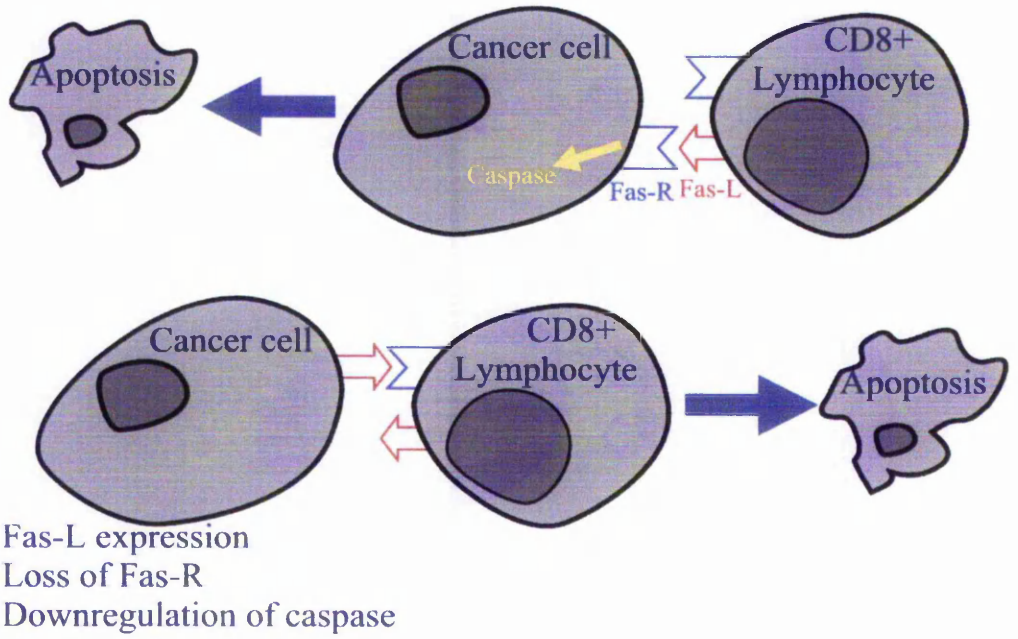


Figure 1.8: Evasion of Fas-L mediated immune attack

Destruction of tumour cells by Fas-L mediated attack may be abrogated by loss of Fas-R expression or defects in the apoptosis-inducing caspase cascade. Tumour cell clones that express Fas-L may also produce a counter-attack on lymphocytes.

Importance of MHC class-II responses

Most investigations into tumour antigens recognised by T-cells have focussed on MHC class-I restricted antigen recognition by $CD8^+$ cytotoxic T-lymphocytes (CTL). However, $CD4^+$ T-cell (T-helper or T_H) responses, which are MHC class-II restricted, are also essential for the generation of antitumour immune responses (Ostrand-Rosenberg 1994; Topalian *et al.* 1994), and have a role in priming both CTL and non-specific effector immune mechanisms, including eosinophil activation and the generation of reactive oxygen species by macrophages (Ostrand-Rosenberg 1994; Tepper *et al.* 1998). There is accumulating evidence of the synergistic effects of targeting both class-I and class-II immune pathways for the production of effective cellular immune responses (Ahlers *et al.* 1997; Wilson *et al.* 1999; Ahlers *et al.* 2001).

In antitumour immune responses, $CD4^+$ lymphocytes contribute effectively (Toes *et al.* 1999), and the effectiveness of vaccination strategies has been shown to be abrogated in the absence of $CD4^+$ T-cells (Ali *et al.* 2000; Cohen *et al.* 2000). Furthermore, adoptive transfer of activated $CD4^+$ T-cell clones specific for murine leukaemia can confer systemic antitumour responses in tumour-bearing hosts (Pardoll and Topalian 1998), supporting the importance of $CD4^+$ T-cells in cancer immunotherapy.

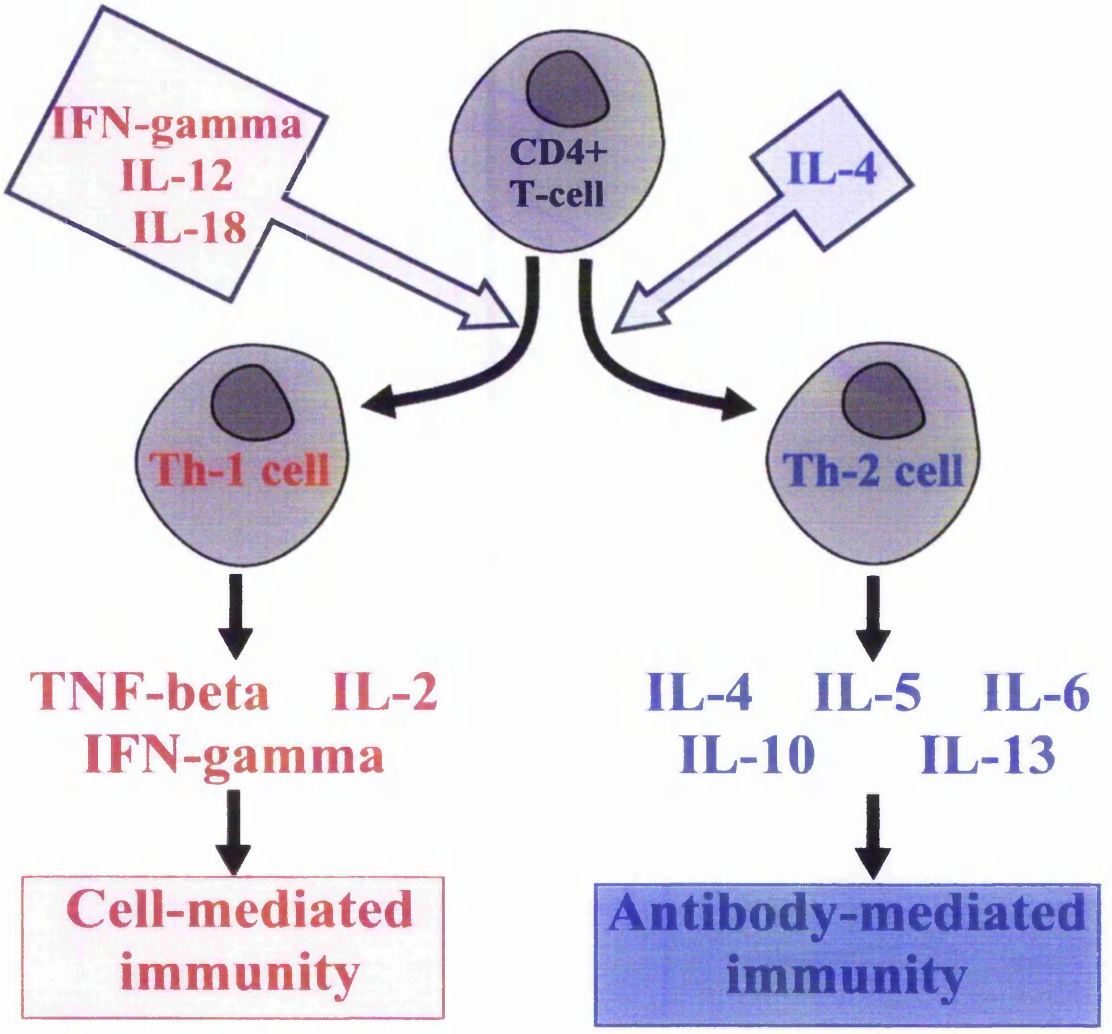


Figure 1.9: T-helper cell responses

T_H-1 cells are produced by the activation of CD4+ T-cells in the presence of IL-12, IL-18 and IFN-γ. The production of IL-2, TNF-β and IFN-γ by these cells promotes a cell-mediated immune response. Also, IFN-γ and IL-12 inhibit the formation of Th2 cells. T_H-2 cells are produced in the presence of IL-4 and secrete IL-4, IL-5, IL-6, IL-10 and IL-13. IL-4 stimulates class switching in B cells and promotes the synthesis of IgE. It also acts as a positive-feedback device promoting more CD4+ T-cells to enter the T_H-2 pathway and blocks expression of the IL-12 receptor thus inhibiting CD4+ T-cells from entering the T_H-1 path. IL-5 attracts and activates eosinophils. IL-10 inhibits IL-12 production by DCs, thus inhibiting CD4+ T-cells from entering the T_H-1 pathway. IL-13 also promotes the synthesis of IgE antibodies. Thus, the two pathways of T-helper cell activation produce different and mutually inhibiting effects on the immune system.

Two distinct varieties of $CD4^+$ T_H cells are recognised: T_H-1 cells participate in cell-mediated immunity and facilitate the expansion of CTL, whereas T_H-2 cells provide help for B-cells and hence promote antibody-mediated immunity. These effects are mediated through the release of regulatory cytokines such as TNF-β, IL-2 and IFN-γ (T_H-1), and IL-4, IL-5, IL-6, IL-10 and IL-13 (T_H-2). The type of T_H response is dependent on cytokine production and each response is inhibitory to the other (see figure 1.9). The production of an effective T_H-1 response is highly desirable (if not essential) for the activation of CTL-mediated cytotoxic responses and is therefore an essential component of vaccine design.

Innate immunity and immunotherapy

Although this thesis explores cell-mediated immune responses to cancer, the importance of innate immunity should not be overlooked. Innate immunity refers to cellular components that serve as the first line of immunological defence but without resulting immunological memory. The innate system comprises monocytes and macrophages, neutrophils, eosinophils, natural killer (NK) cells and the complement cascade.

Innate responses are more rapid than adaptive responses, but lack specificity for target molecules. This broad spectrum attack diminishes the ability of innate mechanisms to single out cancer cells, but innate immune components can produce anti-cancer effects without undue toxicity to normal cells. A number of animal models have demonstrated that innate effectors, such as monocytes, macrophages and eosinophils, can induce tumour cell killing (Kleinerman *et al.* 1983; Giavazzi *et al.* 1984; Hung *et al.* 1998). NK cells also are known to play important roles in tumour killing and tumour cell immune surveillance (Berke 1995; Rees and Mian 1999).

The innate and adaptive parts of the immune system cannot be regarded as wholly separate, as there is considerable interaction and cooperation between the two, from the initiation of the immune response to the final outcome. Upregulation of innate immunity has been employed as a method of augmenting T-cell responses toward tumours. For instance, the release of inflammatory mediators such as GMCSF by dendritic cells plays a central role in controlling specific T-cell responses. Natural killer cells have also been shown to be synergistic with adaptive immune responses, and can improve T-cell activation. The interaction of NK cells with dendritic cells increases the production of cytokines, and the rapidity of the NK response enables the activation of T-cells to be accelerated (Chang *et al.* 2005).

Cooperation between adaptive and innate immune responses also occurs at later stages of the immune response. Activated cytotoxic T-cells exert some of their antitumour effects through recruitment of innate effectors, such as eosinophils and monocytes (Hung *et al.* 1998). Thus, innate immunity functions as a critical component in the induction of immune-mediated antitumour activity, both through direct anti-tumour effects and through synergy with the adaptive arm of the immune system.

Identification of targets for cancer immunotherapy

Immunotherapy strategies aim to produce cellular killing in a tissue specific manner, targeting tumour without damaging the surrounding normal healthy tissues. It is therefore necessary to identify immune targets that are present on tumour cells but not on normal cell types. As well as such cancer-specific antigens, antigenic targets can be selected that are strongly upregulated in cancer cells in comparison with other tissues (cancer-associated antigens), or which are expressed exclusively on cancer cells and non-essential tissues (such as cancer-testis antigens).

Cancer-associated antigens

These antigens are expressed by normal cells during differentiation and are reactivated during neoplastic transformation, or are expressed by normal cells but overexpressed in tumour cells. For example, cancer-testis antigens are developmental antigens expressed exclusively by malignant cells and normal testis: this group includes NY-ESO-1 (Jager *et al.* 1998) and the MAGE, BAGE and GAGE families of antigens (van der Bruggen *et al.* 1991; Van den Eynde *et al.* 1995). Another cancer associated antigen, her2/neu, is overexpressed in breast cancer and others due to gene amplification (Hudziak *et al.* 1987), while MART-1/melanA and gp100 are both overexpressed in melanoma (Coulie *et al.* 1994; Cox *et al.* 1994). The use of such antigens as immune targets may be associated damage to any normal tissues that express them. If the affected normal tissues can be regarded as non-essential (for example, in the case of normal prostate cells or melanocytes), then such antigens may be considered for inclusion in cancer vaccine design.

Cancer-specific antigens

These include antigens encoded by mutated genes, which are expressed only on transformed cells. The absence of expression on normal cells makes these antigens attractive targets as toxicity to normal tissues will be minimised. Examples of cancer-specific antigens include the ras oncoprotein (resulting from point gene mutations) (Bos 1989), bcr-abl (an aberrant fusion protein resulting from chromosomal translocation) (Heisterkamp *et al.* 1985; McLaughlin *et al.* 1987), E6 and E7 (viral proteins expressed by cancer cells due to HPV infection) (Stoler and Broker 1986), and mutant p53 (multiple point mutations) (Levine *et al.* 1983). Their specificity for cancer cells makes them attractive immune targets for immunotherapy.

The identification of suitable antigenic targets requires careful analysis of the expression patterns of proteins by neoplastic and non-neoplastic cells. Western blotting and immunohistochemistry have been used to determine the specificity of a variety of cancer antigens. Other techniques, including RT-PCR and SAGE will be described later.

Identification of epitopes derived from cancer antigens

A number of methods may be used to identify T-cell epitopes for cancer-associated antigens. Peptides eluted from MHC molecules purified from cancer cells can be pulsed onto antigen presenting cells (APCs) and tested for reactivity with specific anti-tumour lymphocytes (Hunt *et al.* 1992; Cox *et al.* 1994). Tandem mass spectrometry can be used to identify the peptide sequence (Skipper *et al.* 1999), which can then be compared with known proteins by searching databases such as Genbank® (http://www.ncbi.nih.gov/Genbank).

Alternatively, cDNA libraries derived from tumour cells can be screened for genes encoding antigenic peptides capable of producing an immune response. The lymphocyte proliferation assay technique was pioneered by Boon (Boon 1993). Tumour-specific CTLs were tested for their ability to recognise target cells transfected with tumour-derived DNA. The amino-acid sequences of epitopes presented by reactive targets can then be inferred from the nucleotide sequence of the DNA used to transfect them. This technique requires the ability to culture CTL clones and appropriate tumour cell-lines, which limits its application in certain tumour types (e.g. breast carcinoma).

Another approach that aims to identify tumour antigens through the screening of tumour cDNA libraries is SEREX (serological analysis by recombinant cDNA expression). In this technique, cDNA derived from fresh tumour specimens is transfected into *E.Coli.* bacteria. The recombinant proteins produced are blotted onto nitrocellulose membranes and tested for their reactivity with IgG derived from patients' sera (Sahin *et al.* 1995). Subcloning of reactive colonies identifies immunogenic peptides, which can then be sequenced and checked for homology with known proteins.

An indirect method of epitope identification, often referred to as "reverse immunology" has been used successfully to identify the effectiveness of candidate peptides, selected because of the predicted suitability of their amino-acid structure using computer algorithms (Drijfhout *et al.* 1995; Kawashima *et al.* 1998). The abilities of predicted epitopes to bind MHC molecules with high affinity can be tested *in vitro* (e.g. using a T2 binding assay – see Chapter 4) (Salter and Cresswell 1986; Hosken and Bevan 1990). The affinity and avidity of MHC binding have been shown

to be accurate predictors of effective CTL epitopes (Nijman *et al.* 1993; van der Burg 1996). The ability of epitopes to stimulate peptide specific T-lymphocytes can then be confirmed *in vitro*, for example using transgenic mice (Pascolo *et al.* 1997; Firat *et al.* 1999).

Candidate prostate tumour-associated antigens as immune targets

High levels of expression in prostate cancer coupled with low levels of expression in normal tissues other than prostate are essential characteristics for immune targets in this disease. A number of peptides have been identified as good potential targets for prostate cancer immune therapy (see Table 1.2) (Saffran *et al.* 1999). The apoptosis associated peptides p53 and survivin may also be suitable immune targets for prostate as well as other cancer immunotherapies. Further details of candidate target-antigens may be found in chapter 3.

Target Antigen	Function	Immune Therapies
PSA	Serine protease	mAb conjugate vaccine
PSMA	Homology to NAALADase*	mAb therapy Dendritic cell vaccine
PSCA	Unknown	mAb therapy vaccine
PAP	Acid phosphatase	mAb conjugate vaccine

* N-acetylated α-linked acidic dipeptidase mAb = monoclonal antibody

Table 1.2: Candidate prostate cancer antigen targets

Prostate Specific Antigen (PSA) is a 34 kDa serine protease and a member of the human kallikrein gene family, produced exclusively by the ductal and acinar epithelial cells of the prostate gland, and found at high concentrations in the seminal fluid (Li and Beling 1973). Serum levels of PSA are raised in men with prostate cancer (Oesterling 1991), and PSA levels correlate with cancer stage (Catalona *et al.* 1993) and prognosis (Stamey and Kabalin 1989), making it a clinically useful marker for the detection and evaluation of prostate cancer.

PSA expression is maintained in the majority of both androgen dependent and independent prostate cancer, and is therefore a good potential immunotherapy target. Its ability to induce specific T-cell responses has been confirmed in murine and human systems, both *in vitro* and *in vivo* (Correale *et al.* 1997; Xue *et al.* 1997; Correale *et al.* 1998; Kim *et al.* 2001; Heiser *et al.* 2002), and numerous CTL epitopes have been defined.

Prostate Specific Membrane Antigen (PSMA) is a 100 kDa type II integral membrane glycoprotein comprised of 750 amino acids, first described in the LNCaP prostate cancer cell-line (Horoszewicz *et al.* 1987). The ability of PSMA to act as a target for CTL has been demonstrated. CD4+ and CD8+ lymphocytes derived from normal subjects showed strong proliferative responses following exposure to PSMA derived peptides (Corman *et al.* 1998). Murphy and colleagues demonstrated that intact PSMA derived from LNCaP membranes or baculovirus recombination can stimulate proliferation of T-cells from both healthy individuals and prostate cancer patients (Lodge *et al.* 1999). MHC class-II epitopes derived from PSMA capable of inducing CD4+ T-cell responses have also been described (Kobayashi *et al.* 2003; Schroers *et al.* 2003).

Prostate Stem Cell Antigen (PSCA) is a 123 amino acid cell-surface glycoprotein, isolated by representational difference analysis from the LAPC-4 xenograft model of prostate cancer (Reiter *et al.* 1998). It has 30% homology to stem-cell antigen 2 (SCA-2). PSCA mRNA expression is highly upregulated in both androgen-dependent and androgen-independent prostate cancer tumours, as well as high-grade prostate intraepithelial neoplasia (PIN). Dannull and colleagues have identified three peptides, derived from PSCA, which are capable of stabilizing the HLA-A2*0201 molecule on the cell surface (Dannull *et al.* 2000). One of these (amino-acid residues 14-22) was capable of eliciting a PSCA-specific T-lymphocyte response in a lymphocyte culture from a patient with advanced prostate cancer, and CTLs lysed both peptide-pulsed targets and three prostate cancer cell-lines.

Prostatic Acid Phosphatase (PAP) is a 354 amino acid protein with a molecular weight of 41kDa secreted by the prostate. Its gene is located at chromosome 3q21. It is thought to have a role in the regulation of prostate growth, and also may affect sperm motility and fertility.

It is highly prostate specific (Lam *et al.* 1989; Solin *et al.* 1990; Sinha *et al.* 1998), and upregulated in prostate cancers. Increased circulating PAP levels are associated with advanced stage and poor prognosis (Jacobs and Haskell 1991). PAP derived peptides have been used to induce antigen-specific CTL responses in human studies (Peshwa *et al.* 1998; Inoue *et al.* 2001), and MHC class II epitopes that are able to stimulate human T_H responses *in vitro* have also been identified (McNeel *et al.* 2001).

Other peptides that are largely prostate-specific and could act as potential immune targets include hk2 (KLK2) (Magklara *et al.* 2000), hk4 (KLK4) (Obiezu *et al.* 2002), NKX3.1 (Gelmann *et al.* 2003) and DD3 (Bussemakers *et al.* 1999). The cancer-associated antigen trp-p8, which has been shown to be upregulated in most prostate cancers, has also been proposed as a potential immune target, and an HLA-A*0201-restricted CTL epitope has been shown to activate CTL responses capable of lysing prostate cancer cells (Kiessling *et al.* 2003). CTL-epitopes derived from the Prostein antigen have also been published (Friedman *et al.* 2004; Kiessling *et al.* 2004). Expression in non-prostatic tissues and variable expression in prostate cancer has limited the usefulness of most of these as immune targets.

Strategies for immunotherapy

This section will discuss the various approaches to cancer immunotherapy under evaluation for prostate cancer therapy. Novel cancer treatments are often evaluated in patients with advanced disease. This heterogeneous group is characterised by wide spectra of tumour loads and systemic problems, and their tumour biology is often markedly unpredictable, making the assessment of treatment response problematic. Thus the interpretation of the data from clinical studies of immunotherapy may be hampered by the variety of methods used to assess the response to treatment. The use of survival as an end-point is precluded by the long natural history of prostate cancer, which would render the necessary study period prohibitively long.

Serum PSA levels have been adopted as a surrogate marker for clinical response. In hormone-refractory prostate cancer, reductions in PSA of greater than 50% lasting for four weeks or more have been associated with improvements in pain and prolonged survival (Small *et al.* 2001). Some investigators have assessed changes to the rate of

increase of PSA following initiation of treatments, which is likely to be more sensitive to subtle changes. These methods do not correlate with improvements in clinical parameters, and can only be interpreted as a sign of some biological effect of the agent used.

Immune responses can be used as endpoints in studies of immune therapies. Methods used to assess CD8+ T-cell responses include cytotoxicity assays and cytokine production assays (including ELISA and ELISPOT). Delayed-type hypersensitivity (DTH) testing can be used to determine whether a patient can mount an immune response to the immunogen, consistent with the induction of antigen-specific T-cells.

Passive immunotherapy

Passive immunotherapy describes the administration of an activated immune effector component to cancer patients; for example, monoclonal antibodies (mAb) specific for various tumour-associated antigens. Anti-PSMA antibodies, particularly those directed against the extracellular epitopes of PSMA, have been used therapeutically (Holmes 2001), but with limited success. Deb *et al* reported no objective clinical responses and only transient subjective improvements in 25% of subjects receiving radio-labelled anti-PSMA antibody, and marrow toxicity was universal (Deb *et al.* 1996). The results of studies of newer monoclonal anti-PSMA antibodies are awaited (Bander *et al.* 2003; Bander *et al.* 2003; Nanus *et al.* 2003).

Another example of passive immunotherapy is the adoptive transfer of tumour infiltrating lymphocytes (TILs). TILs can be isolated from a tumour and activated *in vitro* by incubation with IL-2, before being administered to the patient. Such TILs are capable of lysing autologous tumour targets (Rosenberg *et al.* 1986; Topalian *et al.* 1988), but their application in human trials has been limited by toxicity associated

with the high doses of IL-2 required (Higuchi *et al.* 1991). An alternative is the infusion of lymphocyte activated killer (LAK) cells, which consist mostly of natural killer (NK) cells. Prolonged survival and the prevention and regression of pulmonary metastases have been shown in rat models of prostate cancer following LAK therapy combined with IL-2 (Tjota *et al.* 1991). However, there have been no reports of human trials.

Whole-cell vaccines

Whole-cell vaccines attempt to stimulate tumour-specific T-cells using injections of irradiated tumour cells harvested from the patient (autologous) or from other individuals (allogeneic). The practical difficulties in establishing and maintaining human prostate cancer cells *in vitro* (Peehl and Stamey 1986) has lead to difficulties in obtaining sufficient quantities of autologous cells for multiple vaccinations. There has therefore been an increasing interest in allogeneic vaccines, which weigh the advantage of cost and ease of use against the disadvantage of having a narrower shared antigenic spectrum with the host's tumour. The validity of whole-cell vaccination is dependent on the capacity of the vaccine to induce a stronger immune response against tumour-specific antigens than against ubiquitously expressed self-antigens within the tumour. An additional immune stimulus is also required in order to amplify the immune response generated against the cells.

Transduction of tumour cells with cytokine genes is one method of enhancing the immunogenicity of such vaccines. Granulocyte-macrophage colony stimulating factor (GMCSF) is one of the most potent immune stimulating cytokines (Dranoff *et al.* 1993) (see below), and whole-cell vaccines transduced with the GMCSF gene have shown tumouricidal immune responses in human clinical trials of melanoma (Soiffer

et al. 1998) and renal cell carcinoma (Simons *et al.* 1997). Autologous cancer cells transduced *ex vivo* with GMCSF genes have been used in phase I/II trials in eight patients with advanced prostate cancer (Simons *et al.* 1999). Evidence of an immune response at the injection site was reported, including recruitment of T-cells, dendritic cells and eosinophils. DTH reactions against non-transduced tumour cells were noted in two out of eight patients prior to vaccination and in seven out of eight patients following vaccination, indicating the successful generation of a tumour specific cellular immune response, although there were no accounts of clinical outcomes.

In the Dunning rat prostate cancer model, IL-2 transduced tumour cells were capable of curing animals with subcutaneously generated tumours, and induced immune memory that protected against future tumour challenge (Moody *et al.* 1994; Vieweg *et al.* 1994). However, this method was less effective in orthotopically induced tumours.

Cytokine transduced tumour cells are not necessarily superior to tumour cells administered with an immune adjuvant (Hock *et al.* 1993). One such approach uses a protein derived from *Mycobacterium vaccae*, SRL172. Delayed tumour growth and a reduction in metastases were demonstrated in both the Copenhagen and Lobund-Wistar rat models for prostate cancer using SRL-172 co-injected with autologous cells (Hrouda *et al.* 1998), or allogeneic cells (Hrouda *et al.* 2000). A phase I/II trial of the allogeneic vaccine was completed at the end of 2000, and examined sixty patients with hormone-refractory disease. No significant decrease in PSA was demonstrated, although evidence of increased cytokine production, antibody proliferation and T_H-cell proliferation were reported (Eaton *et al.* 2002).

Up-regulation of heat-shock protein expression (e.g. hsp70) by whole-cell vaccines has been shown increase immunogenicity. In a rat model of prostate cancer, heating of tumour cells to 42°C for one hour increased hsp70 expression. Subsequent vaccination conferred protection against tumour challenge, which was associated with a T_H-1 response (Todryk *et al.* 2004). This method allows enhancement of the immune response without the need for genetic modification or the addition of potentially toxic adjuvants, although this is almost certainly at the expense of potency.

The production of whole-cell vaccines is still a complex and expensive multistep procedure and, although effective against subcutaneous tumours, they are far less effective in orthotopically implanted tumours in rats (Vieweg *et al.* 1994). *In vivo* and *in situ* gene transfection systems may be preferable (Thompson 1999; Ali *et al.* 2000). Such systems would allow tumours to be transfected with cytokine genes rendering them immunogenic without the need to remove cancer cells from the patient (see below).

Peptide vaccines

Activation of immune responses against selected immunodominant tumour antigens may provide greater control over antitumour immunity. Vaccines based on peptides, viruses, bacteria and DNA can all be used to activate immune responses against a specific antigen. This requires the identification of the most potent tumour rejection antigens and an efficient mechanism by which the peptide can be delivered to the immune system. Peptide vaccination depends on the loading of empty MHC molecules on APCs, and it is necessary to target the peptide to APCs to prevent

loading of MHC molecules on other cells, which could induce tolerance rather than immune activation (Aichele *et al.* 1995). This can be achieved by carefully selecting the route of administration of the vaccine and the use of immune stimulating adjuvants within the vaccine formulation.

Adjuvant approaches to enhance the response to peptides include the covalent association with lipopeptidic immunostimulants, or the encapsidation of the peptides into liposomes. Strong adjuvant effects have also been observed with the block copolymer adjuvant P1005 (Yang *et al.* 1996). Conjugation of peptides to larger molecules results in enhanced immunogenicity, particularly if the carrier proteins contain strong T-helper epitopes. Direct covalent fusion, cross-linking with glutaraldehyde and other chemical processes have been used. Diphtheria toxoid, Tetanus toxoid, Pseudomonas aeruginosa toxin A, β-galactosidase, Brucella abortus (killed bacteria), keyhole limpet haemocyanin, Influenza virus haemagglutinin and nucleoprotein, and Hepatitis B core and surface antigens are examples of carrier proteins that have successfully improved the immunogenicity of peptides (O'Hagan and Valiante 2003). Some of them (e.g.. Brucella) appear to induce T-cell independent responses; most of them, however, act by providing T-cell help or facilitating their presentation, functions that require their covalent fusion with the peptides.

JBT1001, also known as *Onco Vax-P*, is a peptide vaccine consisting of recombinant PSA with lipid A formulated in liposomes (McElrath 1995). Lipid A is an endotoxin derived from the outer monolayer of the outer membranes of most Gram-negative bacteria, and is used in this context as an immune stimulant (adjuvant). T-cells reactive to PSA were detected following vaccination in 8/10 patients with advanced

prostate cancer, compared with 2/10 prior to treatment (Meidenbauer *et al.* 2000). The absolute numbers of reactive cells were low, however, and consisted predominantly of $CD4^+$ cells. The size of the study group prevented correlation of responses to clinical outcomes. A further study of 18 patients showed similar results, with an increase in PSA-reactive T-cells in a small number of patients (Meidenbauer *et al.* 2002).

Globo-H-KLH is a hexasaccharide (globo-H) conjugated to the carrier protein keyhole limpet Haemocyanin (KLH). Globo-H is expressed on numerous normal tissues, but is upregulated in some cancers, including prostate (Slovin and Scher 1999; Wang *et al.* 2000). A study of 20 prostate cancer patients with recurrent disease examined the effects of five subcutaneous inoculations of Globo-H-KLH given with QS-21 immune adjuvant over 26 weeks (Slovin *et al.* 1999). Globo-H specific IgM and/or IgG responses were demonstrated in all subjects, and these were sustained for around 19 weeks. A small decline in the rate of PSA increase was seen in some subjects. However, these changes were too slight and short lived to be clinically significant, and concerns remains that an immune response sufficient to produce clinically beneficial anti-cancer effects will also cause significant toxicity in normal tissues that also express Globo-H.

Dendritic cell vaccines

The central role of dendritic cells (DCs) in the initiation of immune responses has been alluded to (Steinman 1991; Fong and Engleman 2000). Many groups have used either directly isolated or GMCSF-induced DCs as antigen carriers for tumour vaccination. DCs can be loaded with antigen in the form of tumour extracts or lysates, or short peptides covering the known CTL epitopes of antigens; other approaches involve ex *vivo* transduction of DCs with genes encoding antigens.

In vitro studies have determined the ability of DCs pulsed with prostate tumour lysates to induce CTL capable of lysing PSMA specific targets (Tjoa *et al.* 1996); CTL proliferation was also detected following incubation with DCs pulsed with CTL epitopes derived from PSMA. In a small phase I clinical trial using a similar approach, evidence of immune responses were detected, with some improvements in clinical outcomes (Pandha *et al.* 2004).

A phase I clinical trial of the administration of autologous DCs pulsed with PSMA peptides demonstrated their ability to induce an antigen specific cellular immune response (Murphy *et al.* 1996). 51 patients with advanced prostate cancer were treated with PSMA peptides alone, DCs alone or DCs pulsed with PSMA peptides. 7 out of 51 subjects had a partial clinical response continuing for at least 100 days after treatment (Tjoa *et al.* 1997). Of the 19 patients given PSMA-pulsed DC, 5 had a partial clinical response; there were no toxic effects. Following on from this work, 25 patients received autologous DCs pulsed with PSMA peptides in a phase II study, producing a partial clinical response in six (24%) and a complete response in two (8%), as defined by National Prostate Cancer Project (NPCP) criteria and a 50% reduction in PSA (Murphy *et al.* 1999). Clinical responses were maintained for a mean of 150 days (Murphy *et al.* 1999). In this, and further trials of PSMA-pulsed DC, a total of 95 patients with locally recurrent (37) or hormone-resistant metastatic disease (58) produced 3 complete and 16 partial responses (Simons *et al.* 1999; Tjoa *et al.* 1999), although some patients also received other treatments concurrently.

Cellular immune responses in these studies were slight, however, and immune responses were not associated with clinical effects. Although these studies are among the few to show clinical improvements following immune therapy, the treatment

groups have been too small to draw conclusions regarding efficacy. Possible immunosuppression due to advanced cancer and age and the mixture of HLA phenotypes of the subjects have also hampered the interpretation of the data, probably contributing to type-2 error.

A similar approach (Provenge) has used DC pulsed with peptides derived from PAP. Selected peptides from PAP have been shown to bind to HLA-A2, and induce peptide specific CTL in leukocytes derived from healthy blood donors. Antigen specific cell lysis was demonstrated in peptide-pulsed targets and tumour cells (Peshwa *et al.* 1998). A vaccine formulation consisting of autologous DC pulsed with a fusion protein PAP/GMCSF was administered in two infusions to 13 patients with hormone-refractory prostate cancer (Burch *et al.* 2000). These were followed by three subcutaneous injections of the fusion protein without DC. A significant (>50%) reduction in PSA was seen in three patients, and all evaluable subjects showed a significant proliferation of T-lymphocytes reactive to the PAP/GMCSF fusion protein. In a further study of 31 patients with hormone-refractory prostate cancer, 38% of subjects developed immune responses to PAP (Small *et al.* 2000). Three patients exhibited a reduction in PSA of at least 50%, and 3 exhibited a 25-49% decline in PSA. More recently, Provenge has been used in a randomised, placebo-controlled trial of 127 patients with hormone refractory prostate cancer (Small *et al.* 2003). There was a trend towards increased time to disease progression, but this did not reach statistical significance. Subset analysis revealed a possible benefit for patients with Gleason grade ≤ 7, although further study will be required to ascertain the validity of these results.

Another phase I/II DC vaccine trial has used mouse PAP as an immunogen (Fong *et al.* 2001). Such xenogeneic homologues of tumour antigens are sufficiently different to the self antigen to render them immunogenic, and have sufficient similarities such that the immune effectors resulting from treatment can cross-react with it. All patients developed T-mediated immunity against mouse PAP following vaccination, and 11 out of 21 developed immunity to human PAP. Six out of twenty-one patients had evidence of stable disease following treatment, which lasted for over a year in three.

Vieweg's group have also investigated the use of autologous dendritic cells transfected with RNA encoding PSA. DCs were generated from normal subjects and prostate cancer patients and transfected with PSA mRNA *in vitro.* These DCs were able to elicit CTL mediated lysis of target cells expressing PSA (Heiser *et al.* 2000). Because the entire PSA RNA was transfected, rather than short epitopes, the vaccine was not HLA restricted. In a phase I trial of 13 prostate cancer patients, the vaccine was well tolerated and PSA-specific CTL were detected in all patients (Heiser *et al.* 2002). A separate study employed dendritic cells pulsed with recombinant PSA, administering the vaccine to patients with biochemical recurrence following radical prostatectomy. The treatment was well tolerated, and a modest decrease in PSA was seen in eleven out of twelve patients, although this was transient (Barrou *et al.* 2004).

Macrophages transduced with a murine IL-12 adenoviral vector have been evaluated in an orthotopic mouse prostate cancer model (178-2 BMA). Such macrophages had been shown to secrete IL-12 and demonstrated increased expression of MHC class-I and class-II molecules (Satoh *et al.* 2003). Intra-tumoural injections of these macrophages suppressed the growth of both primary tumours and lung metastases and also prolonged survival; this was associated with infiltration by CD4+ and CD8+ T-lymphocytes.

DNA vaccines

Injection of naked nucleic acids can induce local inflammation and activation of APCs (Pardoll and Beckerleg 1995). It has been demonstrated that these cells present antigens encoded by the DNA administered (Fu *et al.* 1997). A vaccine comprised of influenza nucleoprotein DNA protected animals from influenza challenge (Montgomery *et al.* 1993), and there has been some success at providing systemic tumour protection using DNA vaccines encoding tumour antigens (Irvine *et al.* 1997).

A vaccine consisting of PSMA and CD86 DNA delivered using a plasmid vector has been used in a phase I/II trial of prostate cancer patients (Mincheff *et al.* 2000). There were delayed hypersensitivity reactions in all subjects, and evidence of a clinical and/or PSA response in a small number. Weiner *et al* used a mammalian expression vector to deliver human cDNA encoding PSA in mice (Kim *et al.* 1998) and primates (Kim *et al.* 2001). An antibody response against PSA was observed, and both T-helper cell and cytotoxic T lymphocyte responses against PSA were detected.

Multi-epitope vaccines

Vaccine strategies that deliver entire antigen proteins may induce an immune response against a single immunodominant epitope only, with subdominant epitopes being ignored. Failure to engage multiple targets could allow cancer cells to evade immune destruction through antigenic variation and loss. Multi-epitope vaccines overcome these problems by delivering several epitopes derived from one or more tumour antigens simultaneously: short segments of DNA encoding different epitope peptides can be joined in a string-of-beads fashion. Using epitopes derived from proteins rather than the entire proteins has the additional advantage of avoiding the potential hazards of delivering genes and proteins that are involved in neoplastic transformation, such as p53, ras and HER2/neu.

Several workers have used multi-epitope vaccines in mice to induce CTL, T_H and B-cell responses against infectious organisms (Whitton *et al.* 1993; Thomson *et al.* 1996; An and Whitton 1997; Hanke *et al.* 1998; Thomson *et al.* 1998). Protective anti-tumour immunity has also been documented using a multi-epitope vaccine comprising tumour-associated CTL epitopes (Toes *et al.* 1997). Human multiepitope vaccines have produced CTL responses in transgenic mice (Ishioka *et al.* 1999), and a vaccine containing human T-cell epitopes from PSA has been used to produce CTL responses both *in vitro* and *in vivo* using transgenic mice (Correale *et al.* 1998). CTL-mediated cytotoxicity against targets bearing either of two PSA epitopes was observed following stimulation of T-cells with a multi-epitope oligopeptide that comprised both epitopes in tandem. Administration of the multi-epitope DNA has been by direct DNA vaccination, or via adenovirus or vaccinia vectors.

Viral vectors

The potency of viral vaccines is generally much greater than that of naked DNA vaccines. Viruses provide a stronger "danger signal", and their replicative cycle enables a greater amount of antigen to be presented to the immune system. Vaccinia has been used to deliver DNA encoding the PSA protein to patients with recurrent prostate cancer following prostatectomy (Sanda *et al.* 1999; Eder *et al.* 2000). In one study, stable PSA levels were observed for at least 6 months in 14 out of 33 patients, and for 11-25 months in 9 patients (Eder *et al.* 2000). However, the definition of stable disease in this case allowed an increase in PSA of up to 50% for three consecutive monthly measurements. Neither the actual PSA measurements nor the rate of increase in PSA levels prior to treatment were disclosed. A PSA-specific T-lymphocyte response was elicited, however. In a separate trial by the same group involving prostate cancer patients, immune responses were observed in some subjects, as evidenced by an increase in the proportion of PSA-specific T-cells following vaccination (Gulley *et al.* 2002). Furthermore T-cells derived from responding subjects were able to lyse PSA-expressing tumor cells *in vitro*, although no clinical responses were reported.

A vaccinia-based vaccine encoding both the MUC-1 antigen and IL-2 genes has been evaluated in a phase-I clinical trial of patients with advanced prostate cancer. Although only one patient responded, he showed evidence of a T_H-1 immune response with production of MUC-1-specific CTL (Pantuck *et al.* 2004).

Viruses also provide an attractive means of delivering non-antigen DNA into cells. Adenovirus, vaccinia and HSV have all been used extensively for gene delivery in cancer immunotherapy, including cytokine therapy.

Cytokine immunotherapy

Cytokines are peptides that influence the biological functions of cells, and have a central role in the regulation of the immune system. Over 150 different cytokines have been described, and together they help to orchestrate and control immune responses through their effects on cell migration, proliferation, differentiation and activation. Understanding and ultimately modifying the interaction between cancer cells and the immune system is in part dependent upon the study of cytokine dynamics.

Systemic cytokine therapy in the form of subcutaneous administration of IL-2 and/or IFN-α has been successful in a number of cancers, including renal cell carcinoma (deKernion *et al.* 1983; Rosenberg *et al.* 1994) and melanoma (de Gast *et al.* 2000). A pilot study of systemic IL-2 and IFN-α therapy administered to thirteen prostate cancer patients following hormone relapse resulted in partial responses in a small number of patients, and reductions in PSA were also observed (Maffezzini *et al.* 1996). In a phase II study, the subcutaneous administration of GMCSF in patients with hormone refractory prostate cancer resulted in a reduction in PSA levels in 10 out of 22 patients (Small *et al.* 1999). The effects were sustained for a median of 3.5 months. Similar results were obtained in a separate study using subcutaneous GMCSF in sixteen patients with advanced prostate cancer (Dreicer *et al.* 2001). Six patients exhibited a 10-15% reduction in PSA that was sustained for six months but relapsed following completion of the treatment. Unfortunately, systemic administration of cytokines is often associated with significant toxicity, which limits the efficacy of the treatment. Local administration of cytokines has therefore been studied.

Intratumoural injections of cytokines have shown some promising effects. A study of intratumoural TNF-α combined with subcutaneous IFN-α2B in patients with hormone refractory prostate cancer resulted in a reduced prostate volume and PSA in nine out of ten of patients (Kramer *et al.* 2001). The therapeutic use of both IL-2 and murine GM-CSF as a intratumoural viral vaccine has been reported in a variety of tumour cell types, including sarcoma (Ali *et al.* 2000), melanoma (Ali *et al.* 2002; Rees *et al.* 2002) and prostate cancer (Trudel *et al.* 2003). Adenoviral delivery of the IL-12 gene via intratumoural injection significantly inhibited local tumours and metastatic deposits in a murine prostate cancer model (Nasu *et al.* 1999). Survival was only minimally prolonged compared to controls, however.

Cytokines have also been used in combination with vaccine therapy. In the Dunning rat model of prostate cancer, inoculation of irradiated cancer cells transduced with the GMCSF gene prolonged survival compared with cells mixed with soluble GMCSF (Sanda *et al.* 1994), and autologous cancer cells transduced *ex vivo* with GMCSF genes have been used in phase I/II trials in patients with advanced prostate cancer (Simons *et al.* 1999).

These data support the contention that cytokine-based therapies are potentially useful agents in cancer treatment. GMCSF is a potent immune stimulatory cytokine, particularly for recruitment and activation of DCs. The transfection of tumour cells with GMCSF, either *in vivo* (Ali *et al.* 2000) or *in vitro* (Sanda *et al.* 1994), render these cells capable of provoking anti-tumour immune responses.

Whole-cell vaccines *versus* in situ transfection of cytokine genes

As discussed above, the practical application of whole-cell vaccines has proved problematic, not least because of the technical difficulties in producing sufficient quantities of autologous cells necessary for vaccine formulations. Also, such vaccines are less effective in orthotopically implanted tumours than subcutaneous tumour models (Vieweg *et al.* 1994). However, autologous whole-cell vaccines are capable of acting as a source of antigen, and enhanced cellular immune responses have been observed when whole-cells are transfected with cytokine genes.

Some success has been reported using *in situ* transfection of cytokine genes. Using an orthotopic RM-9 mouse model of prostate cancer, significant antitumour cytotoxicity was achieved following transfection with the IL-12 gene (Thompson 1999), and a modified herpes simplex virus, DISC-HSV, has been successfully employed as a vector for cytokine genes in an intra-tumoural vaccination system in mice (Ali *et al.* 2000). These techniques are still highly investigational, but the above data support the validity of the scientific principle and indicate the significant potential of such approaches.

HSV vectors

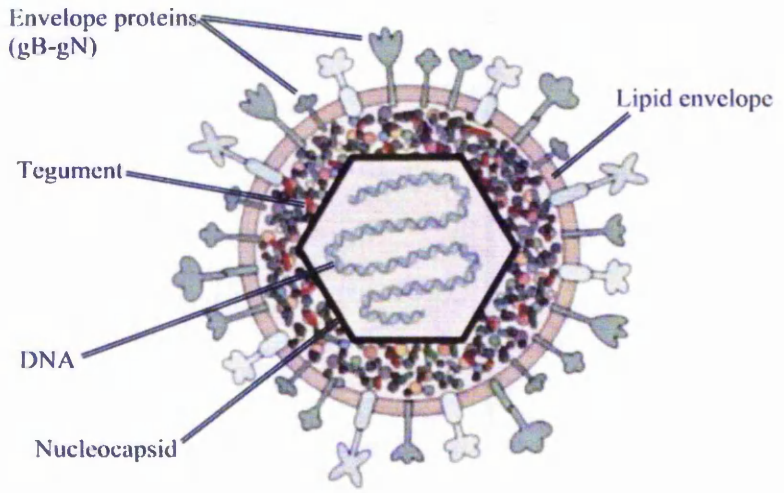
Herpes simplex virus (HSV) is a large, nuclear replicating, icosahedral, enveloped DNA virus, with a genome of approximately 152 kb (Kieff *et al.* 1971). The four major structural components of the virus illustrated in figure 1.10. In total there are 81 genes, and extensive research into the HSV genome has provided detailed information regarding the role of certain genes in infection and virulence (Roizman and Jenkins 1985; Ward and Roizman 1994). Regions of non-essential viral DNA have been identified that may be deleted and replaced with foreign DNA.

HSV virions contain at least 30 proteins (virion polypeptides, VPs) of which 8 are glycoproteins (denoted gB, gC, gD, gE, gG, gH, gI and gJ). These include glyoprotein components of the envelope spikes. Although most of these glycoproteins have a role in viral replication, only three are indispensable: gB, gD and gH.

The viral genome consists of two components: unique long (UL) and unique short (US) regions, each flanked by inverted repeats (Roizman 1996). Its genes are categorized according to the order of their expression following infection of the cell (Roizman and Sears 1993). Immediate Early (IE) genes, also known as alpha genes, include genes responsible for regulatory functions and for the initiation of expression of the next group of genes. Early (E) genes, or beta genes, encode enzymes necessary for DNA replication, for example thymidine kinase. Late (L) genes, or gamma genes, encode structural proteins, surface glycoproteins and others responsible for viral entry, release, cell-to-cell spread, immune modulation and virus-host interactions (Roizman 1996).

Around half the viral genes are non-essential for viral growth. Deletion of non-essential viral DNA provides up to 40-50kb (kilo-bases), which can accommodate foreign DNA without compromising the virus's ability to replicate (Roizman and Jenkins 1985; Ward and Roizman 1994). Extensive research into the HSV genome has provided detailed information regarding the role of certain genes in infection and virulence. Thus, genetic modification of the HSV genome can produce attenuated, replication deficient or replication restricted strains.

(a)



(b)

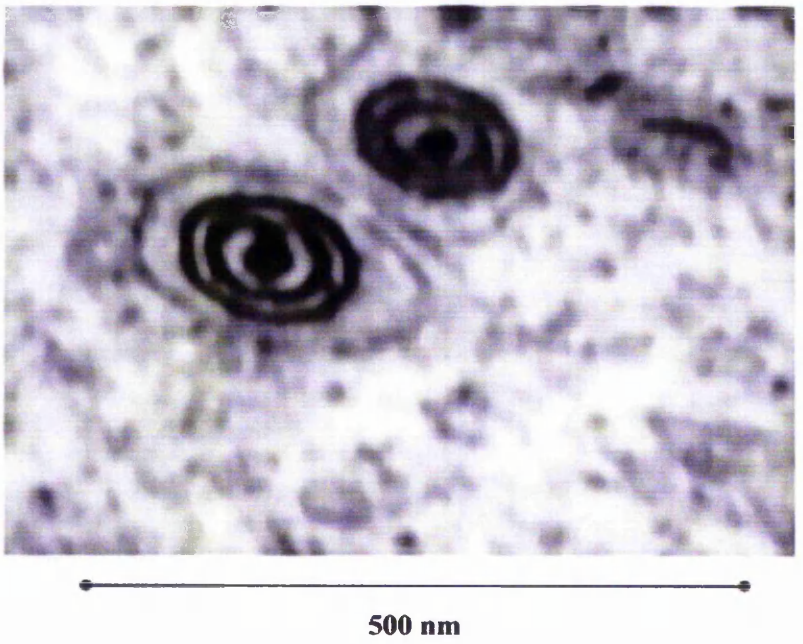


Figure 1.10: Structure of HSV.

All herpes viruses consist of an icosahedral capsid surrounded by a membrane envelope. The capsid contains the viral dsDNA. Between the capsid and the membrane is a layer of protein called the tegument (a). The membrane, tegument and capsid can be seen in the electron micrograph shown (b). The overall virion diameter is approximately 1-200 nm.

Replication of HSV

Fusion of the viral envelope and the cell membrane results from the interaction of envelope glycoproteins with cellular receptors. Endocytosis may occur, but is not essential for viral penetration. Heparan sulphate proteoglycans on the cell surface act as receptors for either gB or gC, which facilitate the initial attachment of the virus. gB and gD are essential for cellular penetration. Capsids are transported by the cellular cytoskeleton to nuclear pores, and viral DNA is released into the nucleoplasm. The virus DNA is replicated in the nucleus where progeny capsids are also formed. Progeny DNA molecules are then packaged into complete but DNA-free capsids. Filled capsids then bud through the nuclear membrane, acquire tegument and membrane in the cytoplasm, and exit the host cell.

Disabled Infectious Single Cycle Herpes Simples Virus (DISC-HSV)

DISC-HSV has been genetically modified by deletion of the gH glycoprotein gene (Forrester *et al.* 1992). The virus is infective but is unable to produce infectious progeny, and is therefore unable to produce a clinical disease state in the host organism. It has safely been used in humans in clinical trials as an anti-HSV vaccine (McLean *et al.* 1994; Hickling *et al.* 1998) and in ongoing immunotherapy trials against melanoma and haemopoietic malignancies (Dilloo *et al.* 1997).

DISC-HSV may provide an immune adjuvant effect and thus enhance immune responses *in vivo*. This relates to the use of DISC-HSV as a vaccine to induce anti-herpes responses. Rather than damping the anti-tumour immunity by producing an immunodominant anti-herpes response, DISC-HSV appears to increase recruitment of immune cells and the production of immunostimulatory molecules, creating a favourable environment for an anti-tumour immune response (Dilloo *et al.* 1997; Ali *et al.* 2002).

Comparison with other viral vector systems

Retroviruses are widely used viral vectors for gene delivery owing to their ability to stably integrate into the host cell genome and thus persist in the progeny of transduced cells. However, they are unable to integrate or be expressed in non-dividing cells. Adenoviruses have therefore been proposed as an alternative gene delivery system, although the efficiency of integration of such viruses is relatively low (Muzyczka 1992). The infection rate of cells in culture with DISC-HSV approaches 100%, which compares favourably with other viral vectors(Rees *et al.* 2002): the infection efficiency of retro-viruses is in the order of 0-3%, and adenoviruses may provide 0-35% infectivity.

Deletion of non-essential viral DNA provides up to 40-50kb which can accommodate foreign DNA without compromising the virus's ability to replicate (Roizman and Jenkins 1985; Ward and Roizman 1994). This compares favourably with other widely used viral vectors such as retrovirus, adenovirus and vaccinia, which are able to carry 5 kb, 7kb and 25kb of DNA respectively. The potential complications of vaccinia use have already been discussed and may limit its applications as a vaccine vector.

DISC-HSV therefore provides efficient infection of host cells and is able to induce expression of large DNA inserts. The ability of this virus to excite a non-specific adjuvant immune reaction may further enhance its use as a vaccine delivery system. The development of clinical applications using DISC-HSV will be facilitated by the capacity to produce high viral titres and the proven safety in both animal and human systems.

Materials and methods

Contents

Reagents

The following reagents and other consumables have been used in these studies. Standard assays and kits were used according to the manufacturers' protocols.

Reagent	Company	Address	Catalogue number
Antibodies			
Anti HLA-A2 antibody (blocking)	Serotec	Oxford, UK	MCA2090XZ
Control for HLA-A2 blocking Ab (IgG2b)	Serotec	Oxford, UK	MCA691XZ
Goat anti-mouse FITC antibody	Sigma	St. Louis, USA	F5262
murine anti-HLA-A2 antibody	ATCC	UK	MA2.1
Rat anti-mouse CD3 FITC	Serotec	Oxford, UK	MCA 500F
Rat anti-mouse CD4 FITC	Serotec	Oxford, UK	MCA 1107F
Rat anti-mouse CD8 FITC	Serotec	Oxford, UK	MCA 1108G
Rat anti-mouse IgG2a FITC (control)	Serotec	Oxford, UK	MCA 1212F
Cytokines			
GMCSF	R&D Systems	Minneapolis	215-GM-025
IL-12	R&D Systems	Minneapolis	219-IL-025
IL-2	R&D Systems	Minneapolis	202-IL-050
IL-4	R&D Systems	Minneapolis	204-IL-025
IL-7	R&D Systems	Minneapolis	207-IL-025
Interferon-γ (IFN-γ)	R&D Systems	Minneapolis	285-IF-100
Lipopolysaccharide (LPS)	Sigma	St. Louis, USA	L-2654
TNF-α	R&D Systems	Minneapolis	210-TA-050
Kits			
Bio-Rad Protein assay	Bio-Rad Laboratories	Hercules, USA	500-0113-5
CellTitre 96 AQueous One Solution Cell Proliferation Assay	Promega	Madison, USA	G3582
DuoSet murine IFN-γ ELISA kit	R&D systems	UK	DY485
DuoSet murine IL-5 ELISA kit	R&D systems	UK	DY405
Dynabeads®	Dynal ASA	Oslo, Norway	
Endofree plasmid maxi kit	Qiagen	Germany	12362
Lipofectamine 2000	Gibco / Invitrogen	Paisley, Scotland	11668-027
Microcon-10 filter kit	Millipore	Bedford, USA	AMI-42 406
Prep-a-gene DNA purification kit	Bio-Rad Laboratories	Hercules, USA	732-6010-1
QIAamp DNA blood mini kit	Qiagen	Germany	51104
QIAfilter plasmid maxi kit	Qiagen	Germany	12263
Wizard DNA clean-up kit	Promega	Madison, USA	A7280
Wizard Plus SV Miniprep kit	Promega	Madison, USA	A1470
Zero blunt-II TOPO PCR cloning kit	Invitrogen	UK	K2800-20
Molecular biology reagents			
1 kilobase DNA ladder	GiboBRL	Paisley, Scotland	10787-018
Big Dye DNA sequencing reagent	QMC	Nottingham, UK	
Deoxyribonuclease I	Sigma	St. Louis, USA	DN-25
dNTP	Bioline	London, UK	BIO-39028
Gel loading solution	Sigma	St. Louis, USA	G2526
Pfu DNA Polymerase	Promega	Madison, USA	M7741

Reagent	Company	Address	Catalogue number
Molecular grade chemicals			
Acetic acid	Fisher Chemicals	England	A/0360/25
Chloroform	Sigma	St. Louis, USA	C-2432
Dimethylsulphoxide	Acros Organics	New Jersey, USA	326882500
Ethanol	BDH Lab supplies	Poole, England	437433T
Ethidium Bromide	Sigma	St. Louis, USA	E1510
Ispopropanol	Sigma	St. Louis, USA	I-9516
Magnesium Chloride ($MGCl_2$)	GibcoBRL	Paisley, Scotland	Y02016
Phenol-chloroform	Sigma	St. Louis, USA	P-3803
Propidium iodide	Sigma	St. Louis, USA	P-4170
Sodium chloride	Sigma	St. Louis, USA	S3014
Collagenase	Sigma	St. Louis, USA	C-0130
Heparin	Sigma	St. Louis, USA	H-3149
Plasticware and disposables			
25G hypodermic needle	Becton-Dickinson	Ireland	
Amicon YC Ultrafiltration Discs	Millipore	Billerica, USA	
Disposable plastic pipette (1,2,5,10,25 ml)	Nunc	EU	
Ependorf tubes (500, 1500 microlitre)	Sarstedt	Germany	
Hypodermic vaccination syringes	Becton-Dickinson	Ireland	
Lumaplate™ 96-well plates	Packard Bioscience	Netherlands	6006633
Pasteur pipettes	Sarstedt	Germany	
Pipette filter tips	Sarstedt	Germany	
Polystyrene test tubes for flow cytometry	Elkay Laboratories	Basingstoke, UK	
Sterile disposable scalpels	Swann-Morton	Sheffield, England	
Sterile screw-cap tubes (15, 50ml)	Sarstedt	Numbrecht, Ger.	
Sterile screw-cap tubes (7, 25ml)	Bibby-Sterilin	Stone, England	
Syringe barrels (2, 5 10, 20, 50ml)	Beckton-Dickinson	Cowley, England	
Tissue culture flask (25,75,150 cm sqd)	Sarstedt	Newton, USA	
Tissue culture plate (6,12,24,48,96 well)	Sarstedt	Newton, USA	
Tissue culture media			
Dulbecco's Modified Eagle Media	BioWhittaker Europe	Verviers, Belgium	BE12-604F
EMEM media	GibcoBRL	Paisley, Scotland	21430-012
Hams F-12 media	Sigma	St. Louis, USA	N-8641
Optimem media	Gibco / Invitrogen	Paisley, Scotland	
RPMI 1640	GibcoBRL	Paisley, Scotland	31870-025
X-VIVO 15	BioWhittaker Europe	Verviers, Belgium	04-418Q
Tissue culture supplements			
2-Mercaptoethanol	Gibco / Invitrogen	Paisley, Scotland	31350-010
Albumin, bovine	Sigma	St. Louis, USA	A-3059
Cyclosporin-A	Sigma	St. Louis, USA	C-3662
Foetal calf serum (FCS) - serum supreme	BioWhittaker Europe	Verviers, Belgium	
Foetal calf serum for lymphocyte culture	PAA labs	Parker Ford, USA	
Fungizone (Amphortericin B)	Gibco / Invitrogen	Paisley, Scotland	15290-026
Hepes Buffer	BioWhittaker Europe	Verviers, Belgium	BE-17-737E
Hydrocortisone	Sigma	St. Louis, USA	H0135
Kanamycin	Sigma	St. Louis, USA	K0129
Non-essential amino acids	Gibco / Invitrogen	Paisley, Scotland	11140-035
Penicillin-Streptomycin	Gibco / Invitrogen	Paisley, Scotland	15140-122
Sodium pyruvate	Gibco / Invitrogen	Paisley, Scotland	11360-039
Testosterone	Sigma	St. Louis, USA	T5035

Reagent	Company	Address	Catalogue number
Miscellaneous reagents			
Agar	Oxoid Ltd	Basingstoke, UK	
Agarose	Bioline	London, UK	
Beta-2-microglobulin	Sigma	St. Louis, USA	
Bovine Serum Albumin (BSA)	Sigma	St. Louis, USA	
Igepal	Sigma	St. Louis, USA	
Incomplete Freund's Adjuvant (IFA)	Sigma	St. Louis, USA	
Isoton-II	Beckman-Coulter	Miami, USA	
LymphoPrep™	Nycomed Labs	Oslo, Norway	
Mitomycin-C	Sigma	St. Louis, USA	
Phosphate buffered saline (PBS)	Oxoid	Hampshire, England	
Protease	Sigma	St. Louis, USA	
SDS (sodium dodecyl sulphate)	Sigma	St. Louis, USA	
Trypan blue	Sigma	St. Louis, USA	
Trypsin	Gibco / Invitrogen	Paisley, Scotland	
Trypsin with versene	BioWhittaker Europe	Verviers, Belgium	
Typtone	Oxoid Ltd	Basingstoke, UK	
Versene	Gibco / Invitrogen	Paisley, Scotland	
Yeast extract	Oxoid Ltd	Basingstoke, UK	

Equiptment

The following items of laboratory equiptment were used.

Equiptment	Model	Company	Address
Centrifuge	Mistral 2000R	MSE	Crawley, UK
Confocal microscope		Lyca	
DynaBead™ separation unit	MPC-1	Dynal ASA	Oslo, Norway
Electron microscope	JEOL 1010	JEOL	USA
Electroparation chamber		Biorad	Hercules, USA
Electrophoresis gel tanks		BioRad	Hercules, USA
Flow Cytometer	Epics XL-MCL	Beckman-Coulter	Miami, USA
Flow hood (tissue culture)	Microflow	Walker Safety Cabinets	Glossop, England
Fluroescence reader	Spectra-Fluor	Tecan	Salzburg, Austria
Gamma counter	TopCount™	Canberra-Packard	Reading, UK
Haemocytometer		Neubauer	
Incubator (tissue culture)		Forma Scientific	Ohio, USA
Microcentrifuge	Mikro-22R	Hettich	Germany
Microscopes		World Precison Implements	USA
Powerpack	Powerpack-300	BioRad	Hercules, USA
Spectrophotometer	DU7	Beckman	Miami, USA
Thermocycler	T-1 thermocycler	Biometra	Germany
Ultrasonicator		Ultrawave	Cardiff, UK

Cell-lines and media

T2 and RMAS/HLA-A2 are lymphoblastoid cell-lines that exhibit a deficiency in MHC class-I expression on the cell surface despite synthesizing normal HLA-A2 heavy chains and $ß_2$-microglobulin (Salter *et al.* 1985; Salter and Cresswell 1986). These cells lack the TAP1 and TAP2 genes located within the MHC class-II region of chromosome 6, which encode the Transport Associated Proteins (TAP) necessary for the transport of oligopeptides from the cytosol into the endoplasmic reticulum. T2 cells express human MHC class-I molecules. The RMAS/HLA-A2 cells used had been previously transfected with HLA-A2.1/K^b transgenic class-I (Pascolo *et al.* 1997). This modification allows the murine CD8 molecule on murine $CD8^+$ T-lymphocytes to interact with the syngeneic α-3 domain of hybrid MHC class-I molecule (Vitiello *et al.* 1991). Cells were grown in suspension in tissue culture flasks containing RPMI 1640 with 10% FCS and 1% L-glutamine supplement. Regular passage of cells was undertaken to prevent over-confluency, typically every two days.

FM3 is a human metastatic melanoma cell-line, which is known to express HLA-A2 and the gp100 cancer antigen (Kirkin *et al.* 1995; Kirkin *et al.* 1999). Cells were propagated in RPMI 1640 media supplemented with 10% FCS, and 1% L-glutamine, with passage every three to four days.

LCL-BM is a HLA-A2 positive human lymphoblastoid cell-line that does not express either PAP or gp100. Cells were grown in suspension in tissue culture flasks containing RPMI 1640 with 10% FCS and 1% L-glutamine supplement.

K562 is a human erythroleukaemic cell-line (Andersson *et al.* 1979) established from pleural effusion of 53 year female with chronic myelogenous leukaemia in terminal blast crisis. The cells are highly undifferentiated and of the granulocytic series. Cells were grown in suspension in tissue culture flasks containing RPMI 1640 with 10% FCS and 1% L-glutamine supplement. K562 were obtained from the European Collection of Cell Cultures (ECACC)

Three human prostate cancer cell-lines were used. PC3 is derived from a bone metastasis of a prostate adenocarcinoma from a 62 year old male Caucasian. The cells exhibit low acid phosphatase and testosterone-5-α-reductase activities, and express HLA A1 and A9 antigens. PC3 cells were propagated in Ham's F12K medium (Sigma 88H2327) with 2mM L-glutamine, supplemented with 1% non-essential amino-acids (GibcoBRL 11140-035) and 10% foetal calf serum (BioWhittaker Europe). Subculture of cells was undertaken every four days.

DU145 is derived from a brain metastasis of a prostate adenocarcinoma. The cell-line is only weakly positive for prostate acid phosphatase activity and does not express prostate specific antigen. DU145 cells were propagated in Dulbecco's Modified Eagle Media (DMEM – GibcoBRL 41966-029) supplemented with 1% non-essential amino-acids, 1% pyruvate (GibcoBRL 11360-039) and 10% foetal calf serum. Passage of cells was undertaken every three to four days.

LNCaP is derived from a metastatic deposit within a left supraclavicular lymph node from a 50 year old caucasian male. The cells are positive for prostatic acid phosphatase, prostate specific membrane antigen and prostate specific antigen, and express androgen and oestrogen receptors (Horoszewicz *et al.* 1983). They also express HLA-A2. LNCaP cells were propagated in RPMI 1640 media (GibcoBRL

31870-025) supplemented with 10% foetal calf serum, 100 U/ml penicillin with 100 μg/ml streptomycin (GibcoBRL 15140-122), 5ng/ml hydrocortisone, and 5ng/ml testosterone. LNCaP is a slow growing cell-line and meticulous care is required to prevent cell-loss. Gentle exchange of culture medium is undertaken every two to three days, but harvesting or replating of cells more often than once per week is not recommended.

CR1 cells are green monkey kidney cells that have been transfected with the herpes simplex virus gH glycoprotein gene. This transfection allows the gH-deficient DISC-HSV to proliferate in CR1 cells. These cells were developed and provided by Xenova Pharmaceuticals Ltd. CR1 were grown in cell-culture at 37°C using RPMI 1640 medium containing 10% FCS and 1% L-glutamine, and require passaging every two to three days.

Construction of multi-epitope vaccine

In order to test the ability of DISC-HSV to act as a vector for a multi-epitope vaccine, two test vaccines were designed comprising class-I epitopes from p53 and class-II epitopes from hen egg lysozyme (HEL). p53 is a tumour suppressor gene present in all cells, and mutations within this gene represent the most common genetic alterations observed in human malignancies (Hollstein *et al.* 1991).

Extensive research has been undertaken into the immunological responses to these proteins, and numerous class-I and class-II epitopes have been described and validated by several laboratories. The design of constructs and primers was undertaken in collaboration with Dr. S. Mian, Nottingham Trent University.

Class-I Multiepitope vaccine construction

Figure 2.1 illustrates the sections of p53 DNA that encode selected class-I epitopes derived from mutant p53. A multi-epitope DNA construct was designed by joining these "minigenes" together sequentially, with additions and modifications to the DNA sequence made in order to improve the efficiency of epitope expression. A well-established murine class-I epitope derived from the human adenovirus E1B $H2D^6$ protein was also included in order to provide a positive control for subsequent testing in a murine MHC-transgenic model system. The final DNA sequence for the p53 class-I multi-epitope vaccine is given in figure 2.2. Its protein translation is also given, along with an explanation of the constituent elements.

p53.65-73 RMPEAAPPV → CGT ATG CCT GAA GCT GCT CCT CCT GTT

p53.149-157 STPPPGTRV → AGT ACG CCT CCA CCG GGT ACT AGA GTG

p53.217-225 VVPYEPPEV → GTG GTG CCT TAC GAG CCT CCA GAA GTA

p53.264-272 LLGRNSFEV → CTG CTA GGA CGA AAC TCG TTC GAG GTC

Figure 2.1: Selected class-I epitopes derived from mutant p53.

Four known class-I p53 epitopes are shown along with the nucleotide sequences of the DNA that encodes them.

(a) Nucleotide sequence

AAGCTTCGCCCTTT.....ACC ATG GCG GCC CCC GGC GCC CGG CGA
CCG CTG CTC CTG CTG CTG CTG GCA GGC CTT GCA CAT GGC GCC
TCA GCA CTC TTT GAG GAT CTA **GCT GCT GCT** CGT ATG CCT GAA
GCT GCT CCT CCT GTT **GCT GCT GCT** AGT ACG CCT CCA CCG GGT
ACT AGA GTG **GCT GCT GCT** GTG GTG CCT TAC GAG CCT CCA GAA
GTA **GCT GCT GCT** CTG CTA GGA CGA AAC TCG TTC GAG GTC **GCT**
GCT GCT GTC AAC ATT CGA AAC TGC TGT TAC ATA **GCT GCT GCT**
TAA TCT AGA GGATA

(b) Amino acid translation

AAPGARRPLLLLLLAGLAHGASALFEDL **AAA** RMPEAAPPV **AAA** STPPPGTRV
AAA VVPYEPPEV **AAA** LLGRNSFEV **AAA** VNIRNCCYI **AAA**

(c) Vaccine components

HindIII restriction site . . . non-coding sequence . . . KOZAK/Start . . .
ER targeting . . . **AAA** . . . p53 A2.1 epitopes (65-73; 149-157; 217-225; 264-272) . . . **AAA** . . . E1B H2D^{6} . . . **AAA** . . . **stop** . . . Xba1 restriction site

Figure 2.2: p53 multi-epitope class-I vaccine construct.

This figure illustrates the constituent parts of the DNA construct that is to be created using PCR. **(a)** illustrated the nucleotide sequence of the DNA vaccine. The amino acid translation of this genetic code is shown in **(b)**. Finally, **(c)** shows an explanation of the functions of each of these components.

Primer design

The p53 class-I multiepitope vaccine DNA sequence was assembled by serial addition of DNA using polymerase chain reaction (PCR). This technique uses multiple oligopeptides, each encoding a section of the vaccine DNA and each overlapping the preceding oligopeptide. Such oligopeptides were used as primers in PCR reactions and thus stepwise fused and amplified yielding the desired long-chain vaccine DNA (307 bases). In this case, oligonucleotide primers were synthesized (Alta Bioscience, UK) as 60mers with 15 bases overlapping (see figure 2.3).

Forward primer #1

5′ TTG AGAT ACC ATG GCG GCC CCC

Reverse primer #1

3′ AGT CGT GAG AAA CTC CTA GAT...CGA CGA CGA GCA TAC GGA CTT CGA CGA GGA
5′ TCA GCA CTC TTT GAG GAT CTA...GCT GCT GCT CGT ATG CCT GAA GCT GCT CCT

Reverse primer #2

3′ GGA CTT CGA CGA GGA...GGA CAA CGA CGA CGA TCA TGC GGA GGT GGC CCA TGA TCT CAC CGA
5′ CCT GAA GCT GCT CCT...CCT GTT GCT GCT GCT AGT ACG CCT CCA CCG GGT ACT AGA GTG GCT

Reverse primer #3

3′ CCA TGA TCT CAC CGA...CGA CGA CAC CAC GGA ATG CTC GGA GGT CTT CAT CGA CGA CGA
5′ GGT ACT AGA GTG GCT...GCT GCT GTG GTG CCT TAC GAG CCT CCA GAA GTA GCT GCT GCT

Reverse primer #4

3′ CTT CAT CGA CGA CGA...GAC GAT CCT GCT TTG AGC AAG CTC CAG CGA CGA CGA CAG TTG TAA
5′ GAA GTA GCT GCT GCT...CTG CTA GGA CGA AAC TCG TTC GAG GTC GCT GCT GCT GTC AAC ATT

Reverse primer #5

3′ CTC CAG CGA CGA CGA CAG TTG TAA...GCT TTG ACG ACA ATG TAT CGA CGA CGA ATT CTT AAG CCTAT
5′ GAG GTC GCT GCT GCT GTC AAC ATT...CGA AAC TGC TGT TAC ATA GCT GCT GCT TAA TCT AGA GGATA

Forward primer #2

5'-AAGCTTCGCCCTTT…ACC ATG GCG GCC CCC

Figure 2.3: PCR primers used for the production of class-I DNA construct.

Oligonucleotide PCR primers are shown. The forward primer is constructed in the 5'→3' orientation. The reverse primers are orientated in the 3'→5' orientation, but the complementary 5'→3' sequence is also illustrated for clarity. The three dots indicate the initial overlapping section of each reverse primer. See text for full explanation of PCR procedures.

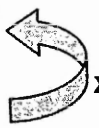
Sequential addition of DNA using PCR

The first PCR reaction used the forward primer #1 and the reverse primer #1. These oligonucleotides were combined in the PCR reaction mixture shown below. This reaction mixture was combined on ice, with the reaction enzyme (pfu) being added last to minimise the risk of enzyme degradation. The template DNA used was an ER-targeting sequence to which the first set pair of primers could anneal during this initial reaction. The denaturation, annealing and extension temperatures were controlled using a Biometra T-1 thermocycler, and are given below.

Reagent	Volume	Final concentration
sterile, deionised water	68µl	
10x pfu reaction buffer	10µl	1x
Dimethylsulphoxide	10µl	10%
50mM dNTP	4µl	0.5mM each of dATP, dCTP, dGTP, dTTP
template DNA	5µl	1ng/ml
forward primer	1µl	0.25µM
1st reverse primer	1µl	0.25µM
pfu DNA polymerase	1µl	25 u/ml
total	**100µl**	

10x pfu reaction buffer consists of 200mM Tris-HCl (pH 8.8 at 25°C), 100mM KCl, 100mM $(NH_4)_2SO_4$, 20mM $MgSO_4$, 1.0% Triton® X-100 and 1mg/ml nuclease-free BSA.

Initial denaturation:	95°C	2 minutes
Denaturation:	95°C	1 minute
Annealing:	40°C	1 minute
Extension:	72°C	1 minute
	4°C	(hold)



x25 cycles

Subsequent PCR reactions were conducted as described above, but with the substitution of the reverse PCR primer with the next reverse primer in the sequence. The template DNA used was the resultant DNA produced from the previous PCR reaction. For each PCR reaction, a negative control reaction was also prepared in which water was added in place of template DNA, thus identifying any DNA contamination of the reagents.

The forward primer utilised in the first PCR was designed so that the initial bases of the sequence would correspond to bases in the template DNA, allowing effective annealing to take place. In order to add a Hind III restriction enzyme binding site to the start of the vaccine sequence, a final PCR was performed, using the second forward primer shown in figure 2.3.

Successful addition of DNA was confirmed after each PCR by diagnostic gel. 1µl aliquots of PCR products, negative controls and a DNA ladder were diluted into 17µl sterile, deionised water and 2µl gel loading solution (Sigma, USA). This was then loaded onto a 1.2% agarose gel containing an ethidium bromide stain. Electrophoresis was performed using a BioRad power pack and gel tank containing 1 x TAE solution, and the DNA was photographed under UV transillumination.

Class-II Multiepitope vaccine construction

Epitopes derived from hen egg-white lysozyme (HEL) and bovine pancreatic RNAse-A were selected for inclusion in the class-II multi-epitope vaccine. The structures of these proteins have been accurately determined and described in some detail. Class-II epitopes have been identified and have been extensively investigated in immunological systems.

Appropriate class-II epitopes were selected for inclusion in the multi-epitope construct (see figure 2.4), and additional DNA added to facilitate epitope presentation. The final DNA sequence for the class-II multi-epitope vaccine is illustrated in figure 2.5. Its protein translation is also given, along with an explanation of the constituent elements.

Primer design

The total length of the HEL class-II multiepitope vaccine DNA sequence is 362 bases. In this case, oligonucleotide primers were synthesized (Alta Bioscience, UK) as 60mers with 18 bases overlapping. These primers are shown in figure 2.6.

Sequential addition of DNA using PCR

The reagent concentrations and PCR conditions used for the construction of the class-II HEL vaccine were similar to those employed for the class-I p53 vaccine, described above. In this case, the DNA template employed for the initial PCR was the class-I p53 vaccine constructed previously. As this vaccine already contained a Hind III restriction site, this precluded the necessity to employ a final PCR step to add such a site, as described for the p53 vaccine.

HEL.46-61		AAC ACC GAT GGG AGT ACC GAC TAC
NTDGSTDYGILQINSR	→	GGA ATC CTA CAG ATC AAC AGC CGC
HEL.116-129		AAG GGC ACC GAC GTC CAG GCG TGG
KGTDVQAWIRGCRL	→	ATC AGA GGC TGC CGG CTG
RNAse-A.43-56		GTG AAC ACC TTT GTG CAC GAG TCC
VNTFVHESLADVQA	→	CTG GCT GAT GTC CAG GCG

Figure 2.4: Selected class-II epitopes derived from HEL and bovine pancreatic RNAse-A.

Three known class-II epitopes are shown along with the nucleotide sequences of the DNA that encodes them.

(a) Nucleotide sequence

AAGCTTCGCCCTTT.....ACC ATG GCG GCC CCC GGC GCC CGG CGA
CCG CTG CTC CTG CTG CTG CTG GCA GGC CTT GCA CAT GGC GCC
TCA GCA CTC TTT GAG GAT CTA AAC ACC GAT GGG AGT ACC GAC
TAC GGA ATC CTA CAG ATC AAC AGC CGC.AAG GGC ACC GAC GTC
CAG GCG TGG ATC AGA GGC TGC CGG CTG.GTG AAC ACC TTT GTG
CAC GAG TCC CTG GCT GAT GTC CAG GCG.CTT AAC AAC ATG TTG
ATC CCC ATT GCT GTG GGC GGT GCC CTG GCA GGG CTG GTC CTC
ATC GTC CTC ATT GCC TAC CTC ATT GGC AGG AAG AGG AGT CAC
GCC GGC **TAT CAG ACC ATC** TAG

(b) Amino acid translation

AAPGARRPLLLLLLAGLAHGASALFEDL NTDGSTDYGILQINSR
KGTDVQAWIRGCRL VNTFVHESLADVQA
LNNMLIPIIVGAALAGLVLIVLIAYLIGRKRSHAG**YQTI**

(c) Vaccine components

HindIII restriction site . . . non-coding sequence . . . KOZAK/Start . . . ER-targeting . . . HEL class II epitopes (46-61; 116-129) . . . RNAase-A class II epitope (43-56) . . . LAMP-1 . . . **stop** . . . XbaI restriction site

Figure 2.5: Class-II multi-epitope vaccine construct.

This figure illustrates the constituent parts of the DNA construct that is to be created using PCR. **(a)** illustrated the nucleotide sequence of the DNA vaccine. The amino acid translation of this genetic code is shown in **(b)**. Finally, **(c)** shows an explanation of the functions of each of these components.

Forward primer

5'-AAGCTTCGCCCTTT ACC ATG GCG GCC CCC

Reverse primer #1

3'-CCG CGG AGT CGT GAG AAA CTC CTA GAT
5'-GGC GCC TCA GCA CTC TTT GAG GAT CTA

Reverse primer #2

3'-CGT GAG AAA CTC CTA GAT.....TTG TGG CTA CCC TCA TGG CTG ATG CCT TAG GAT GTC TAG TTG TCG GCG-5'
5'-GCA CTC TTT GAG GAT CTA.....AAC ACC GAT GGG AGT ACC GAC TAC GGA ATC CTA CAG ATC AAC AGC CGC-3'

Reverse primer #3

3'-GAT GTC TAG TTG TCG GCG.....TTC CCG TGG CTG CAG GTC CGC ACC TAG TCT CCG ACG GCC GAC-5'
5'-CTA CAG ATC AAC AGC CGC.....AAG GGC ACC GAC GTC CAG GCG TGG ATC AGA GGC TGC CGG CTG-3'

Reverse primer #4

3'-TAG TCT CCG ACG GCC GAC.....CAC TTG TGG AAA CAC GTG CTC AGG GAC CGA CTA CAG GTC CGC-5'
5'-ATC AGA GGC TGC CGG CTG.....GTG AAC ACC TTT GTG CAC GAG TCC CTG GCT GAT GTC CAG GCG-3'

Reverse primer #5

3'-GAC CGA CTA CAG GTC CGC.....GAA TTG TTG TAC AAC TAG GGG TAA CGA CAC CCG CCA CGG GAC CGT CCC-5'
5'-CTG GCT GAT GTC CAG GCG.....CTT AAC AAC ATG TTG ATC CCC ATT GCT GTG GGC GGT GCC CTG GCA GGG

Reverse primer #6

3'-CCG CCA CGG GAC CGT CCC.....GAC CAG GAG TAG CAG GAG TAA CGG ATG GAG TAA CCG TCC TTC TCC TCA-5'
5'-GGC GGT GCC CTG GCA GGG.....CTG GTC CTC ATC GTC CTC ATT GCC TAC CTC ATT GGC AGG AAG AGG AGT-3'

Reverse primer #7

3'-TAA CCG TCC TTC TCC TCA.....GTG CGG CCG ATA-5'
5'-ATT GGC AGG AAG AGG AGT.....CAC GCC GGC TAT-3'

Reverse primer #8

3'-CC TCA GTG CGG CCG ATA.....GTC TGG TAG-5'
5'-GG AGT CAC GCC GGC TAT.....CAG ACC ATC-3'

Reverse primer #9

3'-CCG ATA GTC TGG TAG...ATC AGA TCT-5'
5'-GGC TAT CAG ACC ATC...TAG TCT AGA-3'

Figure 2.6: PCR primers used for the production of class-II DNA construct.

Oligonucleotide PCR primers are shown. The forward primer is constructed in the 5'→3' orientation. The reverse primers are orientated in the 3'→5' orientation, but the complementary 5'→3' sequence is also illustrated for clarity. The three dots indicate the initial overlapping section of each reverse primer.

Insertion of construct into DISC-HSV backbone

The multi-epitope DNA sequence includes restriction sites to facilitate the insertion of the construct into the DISC-HSV genome by plasmid reconstruction. Firstly, rapid reproduction of the DNA constructs was achieved by insertion into a prokaryote vector (TOPO), followed by propagation in bacterial cultures. The vaccine DNA was then isolated and transferred into a shuttle vector, PIMJ28, in order to facilitate its insertion into the DISC-HSV genome

Insertion of PCR-product into the Zero blunt TOPO cloning vector

PCR products resulting from pfu DNA polymerase reactions are "blunt" ended, in contradistinction to products produced by taq polymerase reactions, which characteristically add a single deoxyadenosine (A) to the 3' ends of the resulting PCR products (Campbell 1996). The zero blunt TOPO PCR cloning kit was therefore used to insert the vaccine constructs into the TOPO plasmid. An excess of PCR product (5µl) was mixed with 1µl of pCR®4Blunt-TOPO vector and 1µl of salt solution (1.2M NaCl, 0.06M $MgCl_2$), and incubated at room temperature for 10 minutes and then at 4°C for 5 minutes.

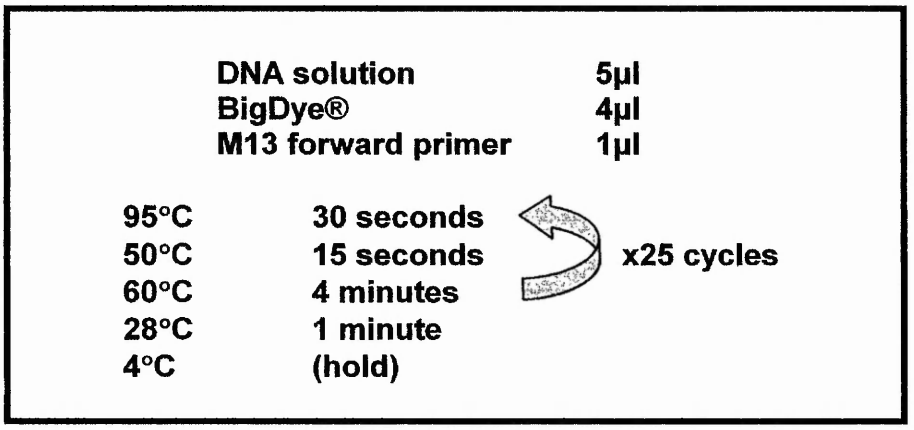
Transformation into TOP10 chemically competent E.Coli bacteria proceeded immediately. 2µl of the cloning reaction was added to one vial of "One Shot" chemically competent E.Coli, and incubated on ice for 30 minutes. The cells were then heat-shocked at 42°C for 30 seconds and once again placed on ice. 250µl room temperature SOC media (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was then added, and the resultant mixture shaken at 37°C for 1 hour at 200rpm. 50µl of transformed cells was spread

evenly over a pre-warmed, kanamycin-selective agar plate and incubated at 37°C overnight. 20 colonies were selected for analysis and transferred into separate tubes containing 5ml LB culture media with kanamycin. Bacteria were grown in culture overnight at 37°C in a horizontal shaker at 80 rpm.

To establish whether the PCR product had been successfully inserted into the TOPO plasmid, bacterial DNA was extracted from these cultures so that a sequencing reaction could be carried out. DNA isolation was carried out using the Wizard Plus SV Miniprep kit and Wizard Plus SV Spin Columns, according to the manufacturers protocol. Briefly, the samples were harvested by centrifugation and resuspended in 250µl Cell Resuspension Solution (50mM TRIS-HCl pH 7.5, 10mM EDTA, 100µg/ml RNAase A). 250µl Lysis Solution (0.2M NaOH, 1% SDS) was then added and mixed by inversion until the suspension cleared. 10µl Alkaline Protease solution was added, mixed by inversion and incubated at room temperature for 5 minutes. 350µl Neutralisation Solution (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid, final pH 4.2) was then added and the suspension mixed by inversion, before being centrifuged at 14000rpm for 10 minutes.

The cleared lysate was transferred to a Wizard Plus SV Spin Column and a vacuum applied to pull all the liquid through the column. 750µl and then 250µl Column Wash Solution (16.8mM potassium acetate, 22.6M TRIS-HCl pH 7.5, 0.109mM EDTA pH 8.0) was added to the column and the vacuum reapplied, and the column was then centrifuged at 14000rpm to remove all traces of liquid. The DNA was eluted from the columns by adding 100µl nuclease-free water and the solution collected in a microcentrifuge tube by centrifugation at maximum speed for 1 minute.

A DNA sequencing reaction was performed following extraction of the plasmid DNA, in order to confirm the presence of the correct DNA insert. EcoR1 restriction digestion was performed to remove the insert from the plasmid. 10µl of miniprep DNA was mixed with 1µl EcoR1 enzyme, 2µl Buffer H and 7µl water, and incubated at 37°C for 1 hour. The digestion products were run on a 0.8% agarose gel in TAE buffer to detect the presence of inserts of the correct size, and a sequencing reaction was performed on those samples, as below. The M13 primer site is present within the TOPO genome.



124µl precipitation mixture was added to the reaction and incubated on ice for 10 minutes before centrifugation at 14000rpm for 15 minutes. After removing the supernatant, the pellet was washed using 250µl 70% ethanol, centrifuged once more at 14000rpm for 5 minutes, and dried. A sequencing gel was performed by Dr. J. Keyte (Queens Medical Centre, Nottingham), and the results compared to the sequence of the original multi-epitope construct.

Bacterial clones confirmed to contain plasmid with a correct DNA insert were single colony purified by streaking-out of bacteria on kanamycin-selective agar plates and incubation at 37°C. A single colony was selected and streaked onto a fresh kanamycin

selective agar plate. This process was repeated three times. A single colony was then transferred to 100ml LB media with kanamycin and propagated in a horizontal shaker at 37°C in order to produce a large-scale preparation of DNA.

DNA extraction was performed using a QIAfilter Plasmid Maxi kit, following the manufacturers protocol, as follows. Following centrifugation of the bacterial culture, the pellet was resuspended in buffer P1 (50mM Tris-Cl pH 8.0, 10mM EDTA, 100μg/ml RNase A). 10 ml buffer P2 (200mM NaOH, 1% SDS) was added and mixed by inversion. After 5 minutes incubation at room temperature, 10ml buffer P3 (3.0M potassium acetate pH 5.5) was added, mixed by inversion and incubated on ice for 20 minutes. This mixture was cleared by running through a QIAfilter cartridge, and applied to a QIAfilter tip. The QIAfilter tip was washed using 60 ml QC wash buffer (1M NaCl, 50mM MOPS pH7.0, 15% isopropanol) and the DNA eluted in 15ml QF solution (12.5M NaCl, 50mM Tris-Cl pH 8.5, 15% isopropanol). The eluted DNA solution was precipitated using 10.5ml isopropanol and then centrifuged at 15000g for 30 minutes at 4°C. The precipitated DNA was washed using 70% ethanol and allowed to air-dry before being resuspended in 50μl water. The resultant DNA solution was then digested using the EcoR1 restriction enzyme to confirm the presence of an insert of appropriate size A sequencing reaction was performed, following the method described above, to confirm the correct sequence of the plasmid insert.

Insertion of multi-epitope construct DNA into shuttle vector PIMJ28

Having produced a large-scale preparation of plasmid DNA and confirmed the correct DNA sequence, the multi-epitope construct was removed from the TOPO cloning vector and transferred into the PIMJ28 mammalian vector to facilitate insertion into

the DISC-HSV genome. This was achieved by restriction digestion using Hind-III and Xba-I enzymes followed by ligation of the excised construct DNA with the decircularised PIMJ28 plasmid. Two restriction digestion mixtures were prepared as below and allowed to react at 37°C for 1 hour.

Reagent	Volume	Reagent	Volume
TOPO/construct DNA	5µl	PIMJ28 plasmid DNA	5µl
Buffer "B"	3µl	Buffer "B"	3µl
Xba-I	1µl	Xba-I	1µl
Hind-III	1µl	Hind-III	1µl
water	20µl	water	20µl
total	30µl	**total**	30µl

The resultant digested PIMJ28 plasmid was purified using the Promega Wizard DNA clean-up kit, following the manufacturer's protocol. The multi-epitope construct DNA was purified using a phenol-chloroform method. The DNA was first diluted into 450µl water, to which 100µl phenol was added and mixed. The DNA solution was centrifuged at 14000rpm for 5 minutes and the aqueous layer recovered. 100µl chloroform was added and mixed, then centrifuged at 14000rpm for 5 minutes, preserving the aqueous layer once again. This DNA solution was concentrated using the Microcon-10 filter kit. Both purified DNA samples were run on a 0.8% agarose diagnostic gel.

Following restriction digestion and purification of the DNA samples, ligation of the multi-epitope construct and the PIMJ28 plasmid DNA was performed. 2.5µg (15µl) insert DNA was added to 2µl (excess) PIMJ28, 2µl ligase buffer and 1µl T4 DNA

ligase, and the reaction mixture placed in ice-water, being allowed to gradually increase to room-temperature overnight. The resultant DNA solution was de-salted by osmotic filtration. Filters were placed on a bath of distilled water and 30µl DNA solution laid on top. The DNA solution was allowed to desalt for 15 minutes before being removed with a sterile pipette.

The PIMJ28/multiepitope construct DNA was subsequently transformed into E.Coli by electroparation. Bacteria in exponential growth phase were cooled on ice for 20 minutes, pelleted, and resuspended in 200µl 100mM dextrose. 20µl DNA was added to the bacteria, electroparated at 2keV, 25F capacitance, and 200Ω resistance, and then transferred to 2ml LB media + 20mM dextrose and incubated at 37°C for 30 minutes in a horizontal shaker. Control transformations were also performed: bacteria plus no DNA (negative control) and bacteria plus empty PIMJ28 plasmid DNA (positive control).

20µl bacteria were incubated on an amoxycillin-selective agar plate at 37°C overnight. The resultant cultures were single colony purified and processed to confirm the presence of a correct DNA insert within the PIMJ28 vector. This involved small and large-scale bacterial preparations, DNA extraction, gel electrophoresis and sequencing as described above. For sequencing, CMV primers were used. The concentration of PIMJ28/multiepitope construct DNA was ascertained by spectrophotometric absorption at 260 and 280nm UV light, using a Beckman Coulter spectrophotometer.

Insertion into DISC-HSV viral backbone

All procedures involving DISC-HSV DNA and/or live virus were performed under sterile conditions in a level-2 isolated laboratory. The insertion of the multi-epitope construct into the DISC-HSV genome required restriction digestion of both the PIMJ28 plasmid and viral DNA, followed by ligation of the resulting DNA strands. DISC-HSV DNA was obtained from Xenova Pharmaceuticals. Both DISC-HSV and PIMJ28 were digested using the Pac1 enzyme at 37°C for 2 hours. Following digestion, purification of DISC-HSV DNA was performed using the phenol-chloroform method described above, and the DNA concentration was quantitated by UV spectophotometry. PIMJ28 DNA was cleaned by gel-purification using the Prep-a-gene DNA purification kit according to the manufacturer's protocol.

The digested DNA solutions were ligated using T4 DNA ligase enzyme as follows. 3μg multi-epitope DNA was added to 1.5μg DISC-HSV DNA, 1μl T4 DNA ligase enzyme and 6μl ligase buffer, and made up to 60μl with water. The resultant mixture was incubated in ice water and allowed to return to room-temperature overnight. A control ligation, using DISC-HSV DNA alone, was also performed to ensure that a functional virus could be produced in the absence of insert DNA.

Transfection of the virus DNA into CR1 cells was performed using Lipofectamine-2000. CR1 cells were grown at $4x10^5$ cells/well in 6-well plates overnight, and then washed twice with serum-free Optimem medium. 60μl DNA was mixed with 12μl lipofectamine-2000 and 328μl water and incubated at room-temperature for 30 minutes. 1600μl Optimem media was then added to the mixture, mixed gently, and overlayed onto two wells (1ml per well). The plates were incubated for 5 hours at 37°C, after which the tranfection mixture was removed and replaced with growth

medium containing full supplements (10% FCS and 1% L-glutamine). The cells were observed for cytopathic effects (CPE), indicating successful viral infection, over the subsequent 7 days. When evidence of CPE was observed, the media from the infected wells was used to infect further wells in order to propagate the virus further.

Following the establishment of viral infection, single-colony purification of viral clones was performed. CR1 cells were seeded at $3x10^5$ per well in 6 well plates, and subsequently infected with DISC-HSV. After 1 hour incubation at 37°C, the media was removed and replaced with 2ml of 1:1 2xEMEM plus low-melting-point agarose at 50°C. Further incubation at 37°C allowed the agarose to solidify, thus permitting viral propagation to continue whilst restricting the spread of individual viral clones between cells. Infected plates were allowed to grow for 2-3 days, until plaques of virally infected cells were visible below the agarose. These plaques were then extracted using a fine-tipped glass pipette and transferred to fresh wells of 6-well plates containing CR1 cells. By repeating this procedure, clones of DISC-HSV were produced. After each round of subcloning, virus was propagated and then viral DNA isolated so that the presence of insert DNA with the correct nucleotide sequence could be confirmed as follows.

Extraction of viral DNA from DISC-HSV infected CR1 cells was performed using the QIAamp DNA blood mini kit, following the manufacturer's protocol. DNA was extracted from 200µl viral supernatant, and was eluted in 200µl water + 0.04% azide. The insert DNA was then amplified by PCR as below, using primers specific for the residual part of the PIMJ28 plasmid.

Reagent	Volume
DISC-HSV DNA	10µl
10 x buffer	10µl
$MgCl_2$ (50mM)	3µl
Taq DNA polymerase	1µl
dNTP (12.5mM)	2µl
Forward primer (10µM) (5' TGACTCACGGGGATTTC)	2.5µl
Reverse primer (10µM) (5' CACAGGCGTGACACGTT)	2.5µl
water	69µl
total	100µl

94°C 1 minute
50°C 1 minute
72°C 1 minute
x 35 cycles

The PCR products were run on a 0.8% agarose gel to identify clone-containing inserts of the correct size. The PCR-amplified DNA from clones with correct-sized inserts was then purified using the phenol-chloroform method and a sequencing reaction was performed on the resultant DNA, as described above. PIMJ28 primers were used for sequencing.

Large-scale preparations of DISC-HSV containing the correct sequence multi-epitope insert were then produced using one of the sub-clones (denominated 13.B.a). Bulk cultures of CR1 cells were infected with virus. Virus was then harvested by detaching cells into the culture media and lysing the suspension by ultrasonication.

Propagation of DISC

CR1 cells were seeded overnight at 37°C in T75 tissue culture flasks with $4x10^6$ cells in 12.5ml culture medium. Allowing for accrual of cells overnight a population of $8x10^6$ was assumed. After discarding the media, the cells were inoculated with virus at 0.1 pfu/cell in serum-free media and incubated at 34°C for 1 hour. Gentle rocking of the flasks every 20 minutes prevented drying of any cells. 14ml serum-free media was added and cultures returned to 34°C. Cells were observed over 2-3 days for evidence of cytopathic effects indicative of successful viral infection.

Cells were released into the culture media using a cell-scraper and extracts from all flasks were pooled. Following centrifugation at 400g for 3 minutes at room temperature, the pellets were resuspended in pre-chilled serum-free media, using 0.5ml for each T75 culture flask harvested. 0.5ml aliquots were placed in 1.5ml sterile plastic tubes and sonicated for one minute in an ice-water bath. Cell-lysis by sonication was confirmed by microscopic evaluation. All samples were combined and mixed before being aliquoted into cyrovials for storage at -80°C. 150µl was preserved in order to titrate the viral concentration.

Titration of viral cultures

CR1 cells were grown overnight in 96-well plates at a concentration of $4.5x10^5$ cells in 100µl. Two plates are required for each virus to be titrated. Serial 1/10 dilutions of virus samples (kept on ice) were made, producing 10^{-4} to 10^{-10} samples. 50µl of virus at various concentrations were added to the wells of 96-well plates containing CR1 cells as shown in figure 2.7.

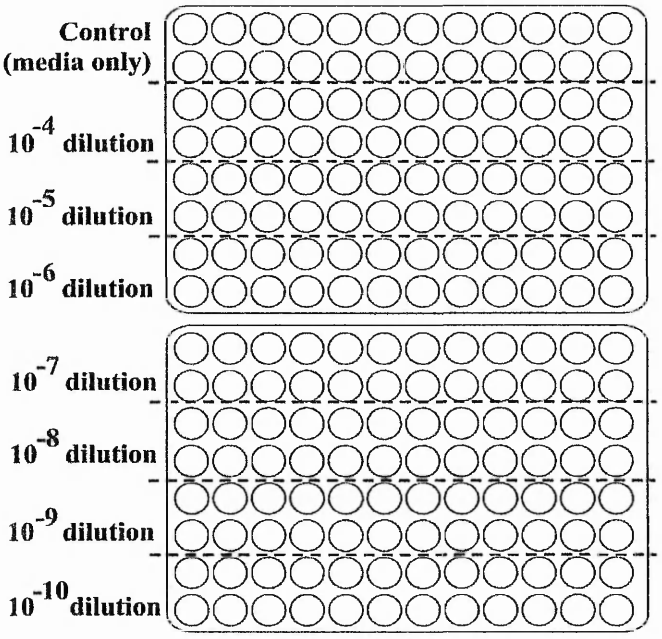


Figure 2.7: Arrangement of 96-well plates for titration of virus.

The plates were incubated at 34°C for 3 days. The number of infected wells (as determined by observation of the development of cytopathic effects) for each dilution of virus was recorded. The titre of the original virus stock was then derived from these data by using a software package (TCID50), developed by Wilson, Coney and Boursnell.

Identification of prostate CTL epitopes

Identification of candidate prostate cancer antigens and class-I epitopes

Candidate prostate cancer antigens were identified by examination of published journal articles identified using the Medline database. The following criteria were used to select antigens for further study: prostate specificity; consistent expression in prostate cancer; expression in all stages of prostate cancer; expression in metastatic tumours. The over-expression of antigen by prostate cancer was deemed a preferred but not essential characteristic. On the basis of the above studies a number of prostate antigens were identified for further examination and their amino acid sequences were obtained from the GenBank® database.

Journal articles describing the identification of MHC class-I prostate cancer antigen epitopes were identified using the Medline database. The oligopeptides described by these articles were noted for comparison with the candidate epitopes identified in this study.

Identification of novel epitopes: MHC binding prediction algorithms

In order to identify candidate HLA-A2 binding epitopes derived from the above prostate antigens, two on-line epitope prediction software resources were used; the URLs are given below.

http://syfpeithi.bmi-heidelberg.com/

http://bimas.dcrt.nih.gov/molbio/hla_bind/

All predicted epitopes were cross-checked for homology with other known human proteins by searching the Blast database (National Institute of Health, USA) to ensure that the peptides derived from prostate cancer antigens did not coexist within other proteins, potentially impairing the prostate-specificity of the epitopes chosen.

Identification of novel epitopes: MHC binding assay

Peptides were supplied as 5 micromoles in lyophilised powder form (Alta Biosciences, UK), and resuspended in 50µl DMSO and 200µl water, producing stock solutions at a concentration of approximately 20mM. These peptide solutions were stored at -80°C until ready for use. For oligopeptides containing 9 amino-acids, the molecular weight is approximately 1000 Da (g/mole), and hence 10mM is approximately equivalent to 10mg/ml. Table 2.1 shows the structure and nomenclature of the test peptides used in these experiments. Test peptides were selected on the basis of tissue specificity for prostate cancer and predicted HLA-A2*0201 binding affinity, as outlined above.

Amino-acid sequence	Name	Source
ALDVYNGLL	PAP.299	(Peshwa *et al.* 1998)
VLAKELKFV	PAP.30	(Peshwa *et al.* 1998)
IMYSAHDTTV	PAP.284	prediction algorithm
ILLWQPIPV	PAP.135	prediction algorithm
ALASCFCFFC	PAP.15	prediction algorithm
GMPEGDLVYV	PSMA.168	prediction algorithm
LLGFLFGWFI	PSMA.34	prediction algorithm

Table 2.1: amino acid sequences of peptides under investigation

In addition, the following well-known HLA-A2*0201-binding epitopes were used as positive controls (Table 2.2). The dominant Influenza A epitope recognized by HLA-A2.1-restricted CTL is derived from the M1 matrix protein (Man *et al.* 1994), and is commonly used as a positive control in HLA-A2 binding assays. gp100 is a melanoma associated antigen, and gp100.154-162 has been shown to act as a CTL target (Kirkin *et al.* 1999). As gp100 acts as positive controls in the immunisation studies that follow, a comparison of HLA-A2 binding affinities with the test peptides was desirable. A solution of 20% DMSO without peptide was used as a negative control.

Amino-acid sequence	Name	Description
GILGFVFTL	flu.58	influenza virus M1 protein
KTWGQYWQV	gp100.154	melanoma antigen

Table 2.2: amino acid sequences of control peptides

T2 cells in exponential growth phase were washed twice in serum-free RPMI by pelleting and resuspension. After counting, the cells were suspended at a concentration of $4x10^6$ cells/ml, and 40µl were added to wells of a round-bottomed 96 well plate. Test peptides were prepared at concentrations of 500µM, 50µM and 5µM by diluting in serum-free RPMI and 10µl were added to triplicate wells, giving final concentrations of 100, 10 and 1µM. A negative control was provided by adding dilute DMSO without peptide to 2 wells. The cells were incubated with peptide overnight at 37°C.

Following incubation, each well was mixed by gentle pipetting, and the cell suspension transferred to a flow-cytometer test-tube. The cells were washed with PBS + 0.1% BSA, and pelleted by centrifugation. The supernatant was discarded and the

cells resuspended in the residual liquid. 20µl of primary antibody (mouse anti-HLA-A2) was added to all tubes, with the exception of one of the two control samples, to which 20µl PBS + 0.1% BSA was added. The tubes were incubated for 30 minutes at 4°C. The antibody was then removed by addition of PBS + 0.1% BSA and centrifugation, and the cells were resuspended in the residual liquid once again. 100µl of secondary antibody (a 1/100 dilution of FITC labelled goat anti-mouse IgG) was added to each tube, including both controls, and the tubes were incubated for 30 minutes at 4°C. The cells were washed twice by the addition of PBS + 0.1% BSA and centrifugation, and then resuspended in 500µl Isoton fixative. Cells were allowed to normalise to room temperature for 20 minutes prior to analysis by flow cytometry with excitation at 488nm using an Epics XL-MCL flow cytometer (Beckman-Coulter, USA). For a summary, see appendix 2.1.

The intensity of immunostaining reflects the avidity of HLA-A2 binding, and this can be quantitated indirectly by comparing the mean fluorescence of cells incubated with a test peptide with the mean fluorescence of cells incubated without peptide (negative control). The relative binding affinity of peptides compared to control is reflected by the mean fluorescence ratio, which can be calculated as follows:

$$\text{Mean Fluorescence Ratio} = \frac{\text{Mean fluorescence of peptide-pulsed cells}}{\text{Mean fluorescence of control cells}}$$

Evaluation of CTL epitopes in a transgenic model system

Mice

HLA-A2.1/K^b transgenic C57 black mice express the product of the HLA-A2.1/K^b chimeric gene in which the α-3 domain of the heavy chain is replaced by the H-2/K^b domain, but the HLA-A2.1 α-1 and α-2 domains are unaltered (Vitiello *et al.* 1991). These mice were a kind gift from Henrik Bo Hansen (Denmark) and were bred under license at Nottingham Trent University.

Reagents

Transport Media:	1 x PBS 100U/ml penicillin + 1000U/ml streptomycin 1% fungizone
CTL Media:	RPMI 1640 1% L-glutamine 10% FCS (PAA labs) 20mM Hepes 50μM 2-mercaptoethanol (2-ME) 50U/ml penicillin + 500U/ml streptomycin 0.25μg/ml fungizone
Digestion Media:	IMDM 50mM 2-ME 100U/ml penicillin + 1000U/ml streptomycin 8mg/ml collagenase 1% DNAse

Peptides were supplied as 5 micromoles in lyophilised powder form (Alta Biosciences, UK), and resuspended in 50μl DMSO and 200μl water, producing stock solutions at a concentration of 20mM. These peptide solutions were stored at -80°C until ready for use. Table 2.3 below gives the structure and nomenclature of the test peptides used in these experiments. Hep-B.128 is a known murine MHC class-II, IA^b-restricted epitope derived from hepatitis B pre-core protein (AAK57285), and acts as a non-specific immune stimulant in the vaccine formulation.

The use of gp100.154 as a control was essential for the testing of cytotoxicity against endogenously processed and presented targets. Flu.58 was also used as a positive control peptide for the MHC-binding assay (see above), and is a highly immunogenic class-I epitope.

Amino-acid sequence	Name	Description
TPPAYRPPNAPIL	hep-B.128	vaccine component (class-II epitope)
GILGFVFTL	flu.58	positive control
KTWGQYWQV	gp100.154	positive control - melanoma antigen
VLAKELKFV	PAP.30	test peptide - (Peshwa *et al.* 1998)
ILLWQPIPV	PAP.135	test peptide - novel

Table 2.3: amino acid sequences of peptides used in the study

Vaccine formulation

Peptides solutions were diluted to a concentration of 10mg/ml (approximately equivalent to10mM) using distilled water. The formulation below was produced under sterile conditions. An excess volume of vaccine was produced to allow for losses during mixing and pipetting.

	4 mice	6 mice
100µg class-I test peptide (per mouse)	50 µl	70µl
100µg class II Hep B peptide (per mouse)	50 µl	70µl
PBS	150µl	210µl
Incomplete Freund's adjuvant	250µl	350µl
	500µl	700µl

Protocol

HLA-A2.1/K^b mice were immunised with an emulsion consisting of a putative class-I peptide epitope mixed with a non-specific class-II peptide derived from hepatitis B virus and incomplete Freund's adjuvant (IFA). The vaccine was delivered as a 100µl intradermal bolus injection at the base of the tail of the test animals, in accordance with Home Office guidelines. After a period of at least 7 and not more than 10 days following immunisation, immunised animals were killed and the spleens recovered into "transport media".

In vitro stimulation of CTL

Under sterile conditions, spleens from immunised mice were gently macerated to break down the connective tissue and facilitate the isolation of splenocytes, and then flushed by injecting 10ml "CTL culture media" under pressure though a fine bore hypodermic needle introduced through the spleen capsule. The effluent was collected and the remaining spleen tissue was diced and digested for 1 hour at 37°C using "digestion media", to release the remaining cells. Following digestion, the tissue was broken down by pipetting, and washed with CTL media producing a cell suspension. The "digested" and "flushed" splenocyte populations from immunised mice were combined and pelleted by centrifugation at 1500rpm (400g) for 3 minutes at 4°C, before being resuspended in 5ml CTL media (see figure 2.8).

The cell concentration was ascertained by counting of under light microscopy using a Neubauer haemocytometer following treatment with 0.6% acetic acid and trypan blue staining to exclude erythrocytes and non-viable cells from the count. The splenocyte suspension from each test mouse was adjusted to a concentration of $5x10^6$ cells/ml, and seeded in 15 wells of a 24 well plate at $5x10^6$ cells/well. One ml of CTL media

containing 20µg peptide was added to each well. The test peptide used for immunisation was added to 12 wells, while 3 control wells received an irrelevant class-I peptide. Splenocyte cultures were incubated for 5 days at 37°C. For summary diagram, see appendix 2.2.

Splenocyte cultures were also established from non-immunised (naïve) mice in order to provide negative control effector cells. *In vitro* processing, including exposure to test and control peptides, was identical to splenocytes from immunised mice.

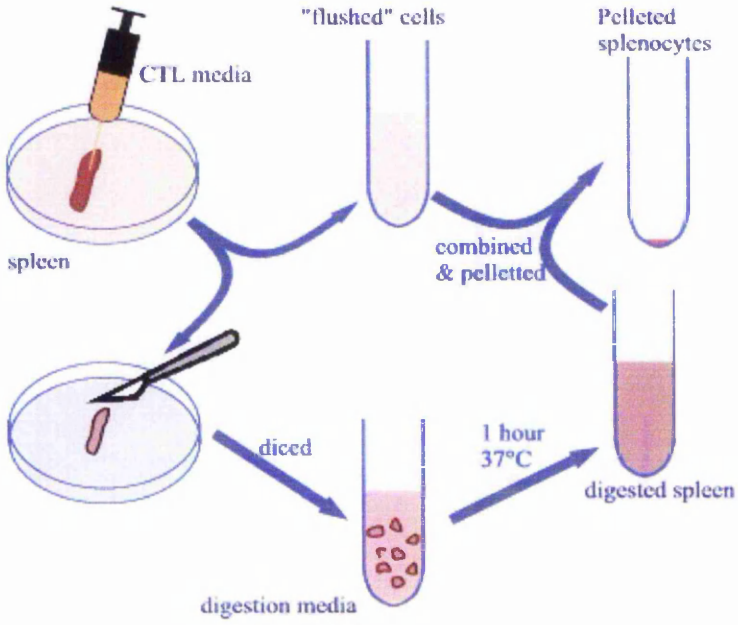


Figure 2.8: Processing of spleens derived from peptide-immunised mice.
Adherent perisplenic fat was removed, and the spleens gently macerated before being flushed with CTL media to remove splenocytes. The remaining splenic tissue was diced and digested in an enzyme mixture to release further cells. These two cell suspensions were combined and washed by centrifugation.

Characterisation of effector cells in culture

During the incubation period, 100µl aliquots of media were removed from 3 test wells and the 3 control wells on days 2 and 5. These were used subsequently for the measurement of IFN-gamma and IL-5 cytokine production using ELISA. Duoset ELISA kits (R&D systems, UK) were used, following the manufacturer's instructions.

Excess splenocytes were analysed by immunostaining for CD4 and CD8 expression followed by flow cytometry in order to characterize the cell cultures further. Cells were washed twice using PBS + 0.1% BSA and divided into 3 aliquots. One of three FITC-labelled antibodies was added to each aliquot: rat anti-mouse CD4, rat anti-mouse CD8, or rat anti-mouse IgG2A (control). After incubation on ice for 20 minutes, cells were washed in PBS + 0.1% PBA and resuspended in 500µl PBS + 0.1% PBA. Cells were allowed to normalise to room temperature before being analysed by flow cytometry with excitation at 488nm using an Epics XL-MCL flow cytometer (Beckman-Coulter, USA). The background level of fluorescence (ie. following staining with the control antibody) was measured. The percentages of cells exhibiting fluorescence above this level following staining with either the CD4 or CD8 antibodies were calculated.

Preparation of target cells for chromium-release assay

On day 4 of CTL maturation, target cells were prepared by incubation with the test peptide used for immunisation, or an irrelevant class-I peptide to be used as a negative control. RMAS cells growing in exponential phase were suspended in 2ml serum-free CTL media containing 40µg peptide and incubated at 37°C overnight. Two aliquots were prepared: one pulsed with the test peptide, and the other pulsed with an irrelevant class-I peptide. Human cell-lines FM3 and LNCaP, which endogenously

express the gp100 (Kirkin *et al.* 1999) and PAP (Horoszewicz *et al.* 1983) tumour antigens respectively, were also used as targets. These were incubated in media containing 100U/ml IFN-gamma for 24 hours prior to the cytotoxicity assay in order to upregulate MHC expression and thus target presentation.

Chromium-release assay

On day 5, target cells were pelleted by centrifugation at 1500rpm (400g) for 3 minutes, and resuspended in the residual media. An amount of ^{51}Cr with a radioactive dose of 1.85MBq was added to each tube of target cells and incubated at 37°C for 1 hour. Target cells were then washed with 5ml RPMI media, pelleted, resuspended in 1ml RPMI media and incubated once again at 37°C for 1 hour. This second period of incubation allowed time for the equilibration of intracellular ^{51}Cr with the culture media, ultimately reducing background levels of ^{51}Cr release during the assay. After a further wash in 5ml RPMI media, the target cells were resuspended and counted, before being diluted to a concentration of $5x10^4$ cells/ml. During this time, splenocytes were recovered from their culture plates by moderate pipetting, pelleted by centrifugation at 1500rpm (400g) for 3 minutes and resuspended CTL media. Following counting, cells were diluted to a concentration of $5x10^6$ cells/ml with CTL media.

Splenocytes at $5x10^6$/ml were seeded in round-bottomed 96-well tissue culture plates. Serial 50% dilutions were performed along 5 adjacent wells, as shown in figure 2.9, producing a reducing concentration of cells in 100µl per well. To each well, 100µl ^{51}Cr-labelled target cells at $5x10^4$/ml were added, producing a range of effector to target ratios ranging from of 100:1 in the first well to 3:1 in the last. For each splenocyte population, two sets of effector cells were seeded. Targets pulsed with the

test peptide were added to one set, whilst the other set received targets pulsed with the irrelevant class-I (negative control) peptide. In addition, triplicate control wells consisted of 100µl target cells mixed with either 100µl media alone (for "spontaneous" or background chromium release), or 50µl media mixed with 50µl 1% SDS (to produce "maximum" chromium release). The plates were incubated in lead-shielded boxes at 37°C for four hours. See appendix 2.3 for summary.

The amount of ^{51}Cr released into the media samples was measured indirectly as follows. After four hours, 50µl media was removed from each well, without disturbing or removing cells or debris, and transferred to the corresponding well of a 96 well Lumaplate™. The Lumaplates were placed in a drying cabinet overnight, allowing the media to evaporate. The activity of each Lumaplate well was measured using a TopCount™ scintillation counter (Canberra-Packard, USA). If the amount of chromium released during the assay is assumed to increase directly with the number of target cells lysed, then the gamma-activity of the media increases proportionally with the cytotoxicity of the effector splenocyte population against target bearing cells. Therefore, the activity of each experimental well was compared to that of the spontaneous and maximum release control wells, and the relative target cell lysis in each well, as determined by chromium release, was quantitated by the following formula:

$$\% \text{ cell lysis} = 100 \times \frac{[\text{experimental release}] - [\text{spontaneous release}]}{[\text{maximum release}] - [\text{spontaneous release}]}$$

Confirmation of CD8 dependence

In order to establish that any observed cytotoxicity against target cells could be attributed only to the action of CD8+ CTL, HLA-A2 blocking antibody (Harig *et al.* 2001) was added to some wells during the chromium-release assay. Other wells received a non-inhibitory control antibody.

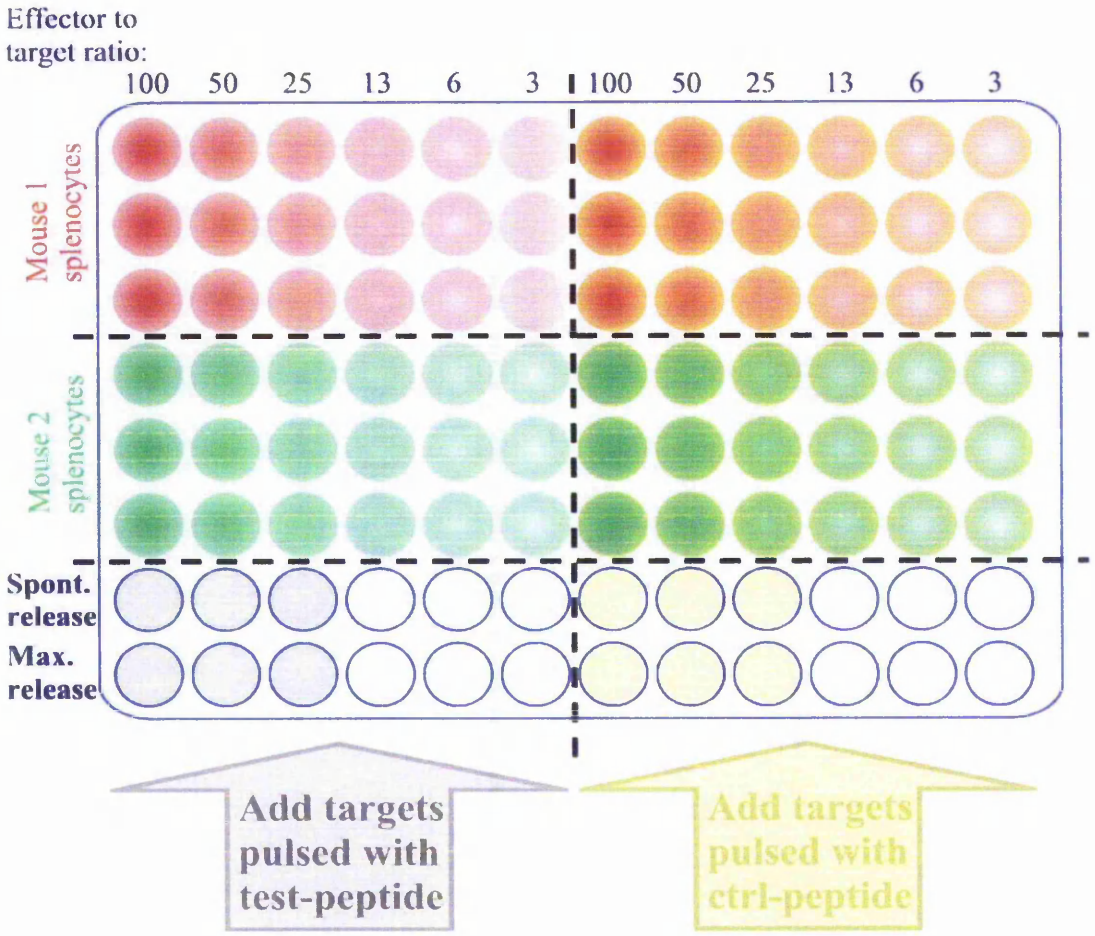


Figure 2.9: Layout of 96-well plates used in ^{51}Chromium-release cytotoxicity assay. Splenocytes were seeded in triplicate wells in columns 1 and 7 of a 96 well plate. Serial 50% dilutions were performed from left to right. Target cells, pulsed either with the test peptide or an irrelevant control peptide, were added to these wells, producing a range of effector-to-target ratios, as shown below. In addition, target cells were added to triplicate wells containing either media alone (spontaneous release) or 0.5% SDS (maximum release).

Activation of naïve human PBMC by MHC class-I peptides

Blood was provided by consenting adult donors. HLA class-I phenotyping was carried out on all donors using an indirect immunofluorescence technique. Donors expressing the HLA-A2 class-I haplotype were selected for use in these experiments.

Positive control peptides employed in the preceding MHC binding assay were also used in these experiments Table 2.4 below gives the structure and nomenclature of the control and test peptides used.

Amino-acid sequence	Name	Description
GILGFVFTL	flu.58	positive control - influenza antigen
KTWGQYWQV	gp100.154	positive control - melanoma antigen
VLAKELKFV	PAP.30	test peptide - (Peshwa *et al.* 1998)
ILLWQPIPV	PAP.135	test peptide - novel

Table 2.4: amino acid sequences of peptides used

Isolation of human PBMC from donated blood

120ml blood was provided by known HLA-A2 donors and 1200 units of heparin added to inhibit coagulation. Heparinised blood was diluted in an equal volume of PBS, and 15ml aliquots of this mixture were gently layered onto 7ml LymphoPrep™ in 25ml universal tubes.

These 16 tubes were centrifuged at 400g for 35 minutes at room temperature with no brake, separating the mixture into layers containing different blood components (see figure 2.10). The "interface" layer was collected into pre-chilled universal containers

by careful pipetting, with particular care taken to avoid the inclusion of cells contained in the lowest "blood" layer. The "interface" cells were washed twice by centrifugation (at 600g with brakes at 4°C for 15 minutes), and resuspension in chilled PBS, and were finally resuspended in 4ml chilled RPMI containing 10% FCS.

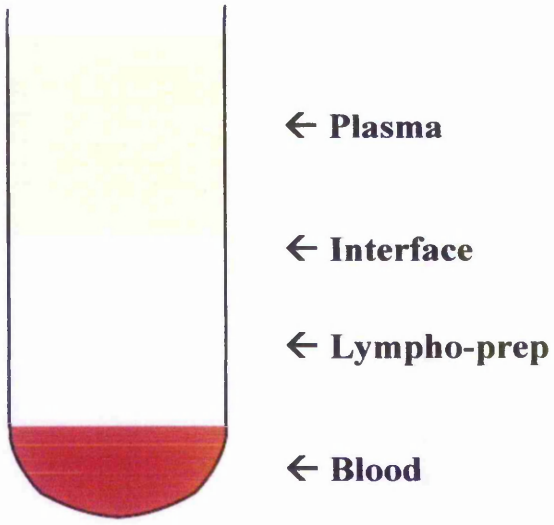


Figure 2.10: Separation of donated blood by centrifugation.

Heparinised blood was layered onto LymphoPrep™ solution and separated into its constituent components by centrifugation. PBMC contained within the "interface" were collected by careful pipetting. The lowest "blood" layer contains erythrocytes and care should be taken to avoid aspirating this layer during this process.

Monocytes were separated from other PBMC by allowing cells to adhere to tissue culture plates, as follows. The PBMC cell concentration was ascertained by counting under light microscopy using a Neubauer haemocytometer following treatment with 0.6% acetic acid and trypan blue staining to exclude erythrocytes and non-viable cells from the count. PBMC were seeded in 6-well plates at a concentration of $10x10^6$ / well in 5 ml RPMI containing 10% FCS. The plates were incubated at 37°C for 2 hours after which time the media and non-adherent cells were removed by pipetting.

These non-adherent cells, consisting primarily of lymphocytes, were pelleted by centrifugation, resuspended in RPMI containing 50% FCS and 10% DMSO at 10^7 cells/ml and frozen at –180°C for later use. The adherent cells were covered with 5ml RPMI containing 10% FCS with 500 U/ml IL-4 and 1000 U/ml GMCSF and incubated again at 37°C. The addition of IL-4 and GMCSF in combination have been shown to stimulate the differentiation of dendritic cells from monocytes (Roth *et al.* 2000), evidenced by reduced CD14 expression (a marker of monocytes, but downregulated on dendritic cells), increased MHC expression and enhanced stimulation of lymphocytes.

After 6 days, the DC were replated in order to stimulate maturation. Adherent cells were washed into the overlying media by pipetting, and then centrifuged at 400g for 4 minutes at 4°C. After counting, the cells were seeded into 24-well plates at $0.5x10^6$ / well in 1ml RPMI containing 10% FCS with 500 U/ml IL-4, 1000 U/ml GMCSF and 10ng/ml TNF-α. TNF-α enhances the maturation of DC, characterised by a reduction in phagocytic activity and up-regulation of costimulatory and adhesion molecule expression, antigen presentation and cytokine production (Schnurr *et al.* 2000). At this point, test peptide was added to the DC culture media at a final concentration of 50μg/ml in order to induce peptide presentation. Approximately $1x10^6$ DC are required for each test peptide to be tested. DC cultures were incubated again at 37°C. Any excess DC were frozen at –180°C for later use. See appendix 2.4.

Generation of CTL

After 2 days, DC were recovered into the culture media and centrifuged at 400g for 4 minutes at 4°C. For each peptide, the pellet was resuspended in 1ml X-VIVO-15 medium containing 10μg of test peptide, and the suspension placed at 37°C until required. X-VIVO-15 has been optimised for the proliferation of lymphocytes. Addition of serum increases CTL proliferation, while serum-free conditions favour NK cells.

Non-adherent cells (lymphocytes) from the same donor were thawed and washed three times. A minimum of $4x10^6$ cells are required for each test peptide. Cells were counted and seeded in 24-well plates at $2x10^6$/well in 500μl X-VIVO-15 media with 10ng/ml IL-7 and 100pg/ml IL-12. The peptide pulsed DC were added to each well at a concentration of $2x10^5$ DC in 500μl (producing a final ratio of lymphocytes:DC of 10:1), and the plates incubated at 37°C.

IL-7 has been shown to provide a potent costimulatory signal to T-cells, promoting proliferation and the generation of CTL (Bhardwaj *et al.* 1996). In similar *in vitro* studies, the addition of IL-7 resulted in significant enhancement of cytotoxicity (Hickman *et al.* 1990; Plebanski *et al.* 1995). IL-12 is important for the generation of CTL responses against synthetic peptides pulsed on dendritic cells: the addition of exogenous IL-12 has been shown to enhance $CD8^+$ T-cell proliferative and cytotoxic effects whereas the absence of IL-12 resulted in very weak responses (Grufman and Karre 2000).

After 3 days, IL-2 was added to each well at a final concentration of 20U/ml. IL-2 is a potent lymphocyte growth factor and its central role in the activation of CTL has been alluded to in the Introduction chapter. On the same day, a second culture of DC was established in order to perform a second round of stimulation. Previously frozen DC were thawed and washed three times by centrifugation and resuspension in PBS. Approximately $5x10^5$ DC are required for each test peptide. These cells were incubated with IL-4 and GMCSF and restimulated by replating after 5 days following the method above. See appendix 2.5.

Restimulation of CTL using 2nd DC culture

Ten days after the initial stimulation of lymphocytes using DC, the non-adherent cells were gently harvested by pipetting, and pelleted by centrifugation. $CD4^+$ lymphocytes were removed using antibody coated magnetic beads as follows. Dynabeads™ were washed twice in PBS + 2% FCS, counted and then mixed with cells in 1ml chilled PBS + 2% FCS at a ratio of 3:1 (beads:cells). The mixture was shaken gently by inversion for 30 minutes at 4°C to allow adhesion. The beads, along with adherent $CD4^+$ cells, were separated from the mixture by placing the cell-suspension in a magnetic field. Cells remaining in suspension (primarily $CD8^+$ CTLs) were removed, washed and resuspended in a volume of at least 10ml at a concentration of either 1 or $2x10^5$ cells/ml (depending on the number of cells available). 100µl of this CTL cell suspension was added to each well of a round-bottomed 96-well plate. Thus, 96 test wells for each peptide under investigation were established.

The second DC culture was harvested as before, suspended in 1ml X-VIVO-15 containing 10μg test peptide and incubated at 37°C [†]. DCs were resuspended at 1 or $2x10^4$ cells/ml (depending on the concentration used for CTL culture) in 10ml X-VIVO-15 containing 10ng/ml IL-7 and 100pg/ml IL-12, and 100μl was added to each well (giving a CTL:DC ratio of 10:1). After 3 days, IL-2 was added to each well at a final concentration of 20U/ml. See appendix 2.6.

Cytotoxicity Assay

After 3 days (day 16 after the first CTL stimulation with DC), target cells were prepared for use in a chromium-release cytotoxicity assay. T2 cells growing in exponential phase were labelled with test peptide(s) by incubating overnight at 37°C in 2ml RPMI containing 12.5μg β_2-microglobulin and 40μg test peptide. A minimum of $0.2x10^6$ cells were prepared for each 96-well plate.

24 hours later (day 17), target cells were labelled with ^{51}chromium, as described previously and suspended at $20x10^3$ cells/ml. K562 cells growing in exponential phase were counted and suspended at $1.2x10^6$/ml. The 96-well plates containing CTL/DC were inspected, and any evaporation of media was replaced to ensure approximately 200μl media per well. Each well was mixed by gentle pipetting, and 50μl transferred to wells on each of 2 fresh 96-well plates (one plate to act as the test plate, and the other a control plate to which target cells stimulated with an irrelevant peptide were added). This volume was replaced with 100μl media containing 200U/ml IL-2 (100U/ml final concentration in wells) and the original plates returned to the incubator at 37°C for use in later experiments.

[†] This procedure can be performed during the 30 minute mixing of lymphocytes and beads above, allowing time for DC to incubate with test peptide whilst the CTL are washed, counted and plated.

50µl K562 and 100µl peptide-pulsed T2 cells were added to each of the wells of the test plates. K562 is used as a bystander cell: any non-specific immune cells within the culture (eg. natural killer (NK) cells) will have activity against the K562, thus diluting their effect on the T2 targets. Specific immune activity (ie. CTL effector cells) will be directly only against T2 cells bearing the target peptide and their effects will not be diluted. To maximise the diluting effect of K562, these cells were in significant excess (K562:T2 ratio of 30:1).

As described previously, the maximum and spontaneous release of ^{51}chromium from target cells was ascertained by mixing T2 target cells with K562 and either 10% SDS (maximum) or media (spontaneous). The plates were incubated at 37°C in a lead-shielded box for 4 hours. 50µl aliquots from each well were transferred to corresponding wells of a Lumaplate™. These were then dried overnight before being read using a TopCount™ scintillation counter (Canberra-Packard, USA) to give a measure of ^{51}chromium release. See appendix 2.7.

The activity of each experimental well was compared to that of the spontaneous and maximum release control wells, and the relative target cell lysis in each well, as determined by chromium release, was quantitated by the following formula:

$$\% \text{ cell lysis} = 100 \times \frac{[\text{experimental release}] - [\text{spontaneous release}]}{[\text{maximum release}] - [\text{spontaneous release}]}$$

The immunogenicity of the test peptides in this model were determined by calculating the difference between the levels of cytotoxicity produced against targets bearing test peptides and that against targets bearing control peptides.

Further restimulations of CTL cultures

The CTL cultures were incubated for 7 days following the first cytotoxicity assay, above. PBMC from the original donor (or DC if available) and PBMC from an allogeneic (HLA mismatched) donor were thawed from storage and washed twice in PBS. These cells were each treated with mitomycin-C (8μg in 1ml RPMI containing 10% FCS) for 2 hours and then washed twice in X-VIVO-15. Autologous cells were incubated with peptide at 10μg/ml for 1 hour at 37°C. Following counting, $120x10^3$/ml autologous cells and $80x10^3$/ml allogeneic cells were mixed in X-VIVO-15 with 50ng/ml IL-7 and 500pg/ml IL-12. 50μl was added to each well of the CTL culture.

After 2 days at 37°C, IL-2 was added to each well at a final concentration of 20U/ml. After a further 5 days (ie. day 14 after first cytotoxicity assay), the chromium-release assay was repeated, following the same procedure as described above. See appendix 2.8.

DISC-HSV AS AN INTRATUMOURAL DELIVERY SYSTEM FOR PROSTATE CANCER IMMUNOTHERAPY

Viruses

The DISC-HSV viruses used in these experiments were supplied by Cantab Pharmaceuticals (subsequently Xenova Group, PLC). Construction of DISC-hGM-CSF and DISC-ß-gal by plasmid recombination, and routine determination of viral titres for infectivity studies are described in detail by Boursnell *et al* (Boursnell *et al.* 1996). A two-stage recombination strategy was employed. Firstly, sodium iodide purified wild-type DISC-HSV DNA and plasmid DNA (pIMMB56) were transfected into complimenting (gH expressing) CR1 cells. DISC-HSV is unable to replicate in normal cell systems, due to the deletion of an essential glycoprotein gene (gH) from the viral genome. CR1 cells, which have been transfected with the gH gene, are permissive to DISC-HSV replication, and may be used to propagate the virus.

The plasmid pIMMB56 contains the ß-galactosidase gene under the control of the SV40 promoter; the expression cassette is flanked by HSV sequences to enable recombination into the viral genome (Boursnell *et al.* 1996). In the second stage, sodium iodide purified viral DNA from stage one and plasmid DNA (pIMR3) were transfected into CR1 cells as before. Plasmid pIMR3 was constructed by ligation of the hGMCSF gene into the shuttle vector pIMMB46. Plasmid pIMMB46 has been adapted to contain the CMV promoter and bovine growth hormone poly(A) addition signal from the plasmid PPRC/CMV (R&D systems, UK). The resulting viruses, DISC-β-gal from stage one and DISC-hGMCSF from stage two, were each passaged three times on BHK gH+/TK- cells in the presence of methotrexate to select for TK+ virus.

Infectivity of cell-lines with DISC-HSVcontaining β-galactosidase reporter gene

PC3, DU145 and LNCaP human prostate cancer cell-lines were cultured in 6-well plates at $2x10^5$ cells per well overnight in medium containing full supplements and 10% serum. The following day the media was removed and the cells washed three times in serum-free media. Cells were infected with DISC-HSV containing the β-galactosidase reporter gene at concentrations of 0, 1, 5, 10, 20, and 50 plaque-forming units (PFU) per cell in a final volume of 300μl serum-free media. After 1 hour at 37°C, the virus was removed and the cells were washed three times with 300ml culture media. 300ml culture media containing 10% FCS was added, and the cells were incubated for 24 hours at 37°C before staining for β-galactosidase activity.

Staining of cell lines for β-galactosidase expression

Following incubation, cells were harvested by washing with PBS and then trypsinisation. Cells were washed by centrifugation and resuspension, and then fixed by resuspension in 0.25% glutaraldehyde and incubation for 20 minutes at 37°C. The fixative was removed from the cells by centrifugation and washing, and cells were resuspended in 1ml "X-gal" staining solution (2mg 5-bromo-4-chloro-3-indolyl-beta-D-galactoside in 1mM $MgCl_2$, 150mM NaCl, 3.3 mM $K_4Fe(CN)_6.3H_2O$ and 3.3 mM $K_3Fe(CN)_6$, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4). Colour was allowed to develop for 2 hours at 37°C at which time the proportion of positively stained cells was ascertained by manual counting under light microscopy using a Neubauer haemocytometer. The optimum concentration of virus for efficient infection of each cell-line was thus determined, and this was used for all subsequent experiments.

Electron microscopy of cell-lines following DISC-HSV/βGal infection

Tissue culture cells were infected with DISC-HSV/βGal and harvested as described above. Cells were washed, centrifuged to a pellet and fixed in 2.5% glutaraldehyde. Further processing was undertaken by Trevor Grey in the department of Pathology, Queen's Medical Centre, Nottingham, and is described here for completeness. After washing in buffer and centrifuging to a pellet, a solid block of cells was formed using the commercial 'Cytoblock kit' (Shandon Inc, Pittsburgh, USA). These fixed, solid blocks of tissues were then washed in cacodylate buffer and secondary fixed in 1% osmium tetroxide. The blocks were subsequently dehydrated in ascending grades of ethanol followed by acetone before being infiltrated and finally embedded in an epoxy based resin (TAAB Resin mix, TAAB, England). After Polymerisation each block was trimmed and 0.5μm sections cut and stained with 1% toluidine blue, for examination under a light microscope. A suitable area, containing potentially infected cells was selected in each case for electron microscopy study. Ultrathin (80nm) sections were cut, mounted on grids and stained with uranyl acetate followed by lead citrate for subsequent examination. Viral samples were prepared for negative stain examination on coated grids using 3% phosphtungstic acid at pH 4.7. Subsequent examination of all samples was performed on a JEOL 1010 electron microscope with an attached slow-scan high-resolution (1600x1200) digital camera attached to a computer with image analysis capabilities (Analysis v3, Germany).

Quantitation of ß-galactosidase expression by cell-lines following DISC-HSV infection

$0.5x10^6$ DU145 or PC3 cells were seeded in $25cm^2$ tissue culture flasks (Sterilin, UK) and incubated overnight at 37°C. Cells were infected with DISC-β-gal at a ratio of 10 pfu per cell for PC3 and 20 pfu per cell for DU145. After 1 hour at 37°C, the media was removed and the cells washed three times with culture media containing 10% FCS, then cultured at 37°C. Cells were harvested from one flask immediately following infection by washing with PBS and then trypsinisation. Further flasks were harvested every 24 hours for five days. Cells were washed and pelleted, and then lysed in a solution of 150mM NaCl, 50mM Tris, 5mM EDTA, 1mM benzamidine, 0.1mM PMSF, 1mM Na_3VO_4, 1mM NaF, 1% IGEPAL CA-630, 0.1% lauryl sulphate, and 0.5% deoxycholic acid. A total protein assay was performed on the cell lysates using Bio-Rad Protein Assay (Bio-Rad laboratories, California USA), and ß-galactosidase activity measured using Promega ß-galactosidase Assay Kit (Promega Corporation, Wisconsin USA), following the manufacturers' protocols. The ratio of β-galactosidase to total protein (arbitrary units) was used as a measure of the expression of the virally encoded gene product.

Infectivity of cell-lines with DISC-HSV containing the hGM-CSF gene

Each cell line was seeded at $2x10^4$ cells per well in 48-well plates and grown overnight at 37°C. Cells were infected with DISC-hGM-CSF at a ratio of 10 pfu per cell for PC3, and 20 pfu per cell for both DU145 and LNCaP, in a total volume of 300µl serum-free media. Negative control samples were mock infected by incubation in a similar volume of media containing no virus. In addition, further control samples were infected with DISC-HSV containing an irrelevant gene: DISC-ß-gal. After 1

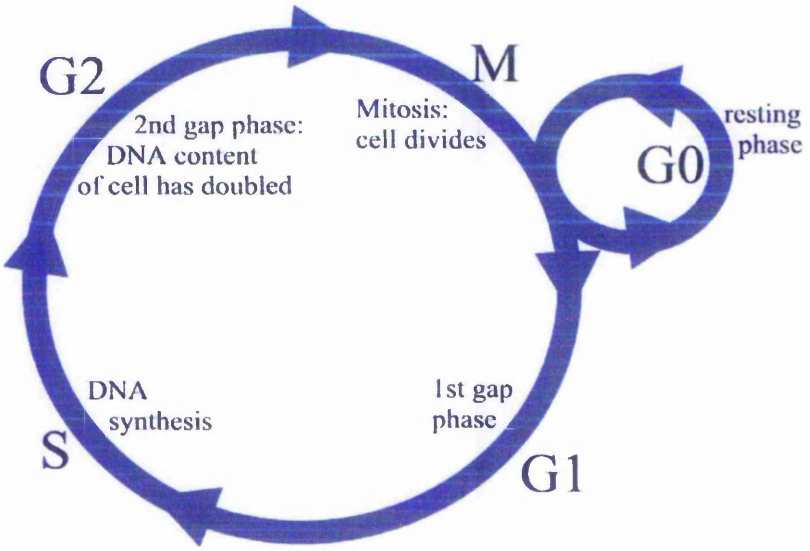
hour at 37°C, the media was removed and the cells washed three times with culture media containing 10% FCS. After the final wash, the media was retained to provide a background level of GM-CSF. The cells were incubated at 37°C for five days with media being collected and replaced at 24-hour time intervals. Supernatants were centrifuged to minimise the risk of cell carryover prior to storage at –80°C. ELISA assays for human GM-CSF were performed using a DuoSet human GM-CSF ELISA kit (R+D Systems, Abingdon UK). The data represent mean values from 2-5 independent experiments (each time point being read in triplicate). Significance testing was conducted at the $p<0.05$ confidence level using a Student's unpaired t-test.

Assessment of proliferation of infected cell-lines

Proliferation was ascertained using a commercial assay kit (CellTitre 96® AQueous One Solution Cell Proliferation Assay). This kit determines the number of viable cells using a colorimetric method that is dependent on NADPH production by dehydrogenase enzymes present in metabolically active cells, and therefore reflects the total metabolic activity of the cell population.

Cells were cultured at $4x10^4$ cells per well in 48 well plates. Cells were infected with either DISC-hGM-CSF or DISC-β-gal as described previously, and negative controls were incubated in media alone. 24 hours following infection, the culture media was removed and replaced with 150ul of media and 30ul of CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, UK). Plates were incubated at 37°C until sufficient levels of colouration had developed in the control wells. Three aliquots of 100ul of proliferation assay solution were placed into triplicate wells of a Nunclon flat bottom 96 well plate and absorbance measured at 492nm using a spectrophotometer.

(a)



(b)

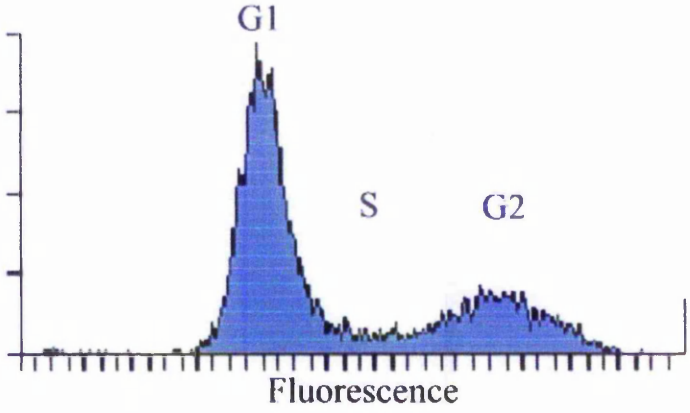


Figure 2.11: Cell-cycle and propidium iodide analysis of cells

The cell cycle can be represented by the schematic above **(a)**. The first gap phase (G1) is followed by a period of DNA synthesis (S) during which time the DNA content of the cell increases. The cell then enters a second gap phase (G2) and at this point contains double the original amount of DNA. Following mitosis (M), the cell returns to G1 and the original DNA content is restored. The fluorescence of a cell following PI staining is proportional to the DNA content of that cell and therefore reflects the cell's position in the cell cycle. Flow cytometry of a population of cells following PI staining gives a histogram that illustrates the proportion of cells in each phase of the cell cycle **(b)**. Cells undergoing apoptosis / necrosis are characterized by DNA degradation, producing a pre-G1 peak (ie. to the left of G1).

Cell-cycle analysis of infected cell-lines

Infected cells were further characterised by cell-cycle analysis using propidium iodide (PI) staining and flow cytometry. Propidium iodide is a fluorescent stain that binds to nucleotides. The fluorescence of a cell following PI staining is proportional to the DNA content of that cell, and this can be measured by flow cytometry. As DNA content reflects the cell's position in the cell cycle, flow cytometry of a population of cells following PI staining can be used to produce a histogram that illustrates the proportion of cells in each phase of the cell cycle. Figure 2.11 illustrates the use of PI staining for the analysis of cell-cycle dynamics.

Cells were cultured at 10^5 cells/well in 6-well plates. Cells were infected with DISC-β-gal as described previously, and negative controls were mock infected with media alone. The cells were incubated at 37°C for five days with cells being collected from 2 infected and 2 control wells at 24-hour time intervals. Cells were washed twice in PBS, fixed in cold 70% ethanol for 30 minutes and then washed twice more in PBS + 0.1% BSA before being resuspended in 1ml PBS + 0.1% BSA. 10µl RNAse (10mg/ml) was added to each sample and mixed, followed by 20µl propidium iodide (2.5mg/ml). The samples were incubated at 37°C for 20 minutes before being placed on ice and analysed by flow cytometry with excitation at 488nm using an Epics XL-MCL flow cytometer (Beckman-Coulter, USA). Mathematical analysis of the cell-cycle data obtained was performed using commercial software packages: WinMDI 2.8 (Scripps Institute, La Jolla, CA) and Cylchred 1.0.2 (Cardiff, UK). The latter is based on algorithms by Watson *et al* (Watson *et al.* 1987) and Ormerod *et al* (Ormerod *et al.* 1987) with modifications by Ormerod (1991) and Hoy (1996-99).

Infection of human prostate cancer xenografts with DISC-β-gal

Xenografts derived from the DU145 and PC3 prostate cancer cell-lines were induced by subcutaneous injection of 5 x 10^6 cells in the flanks of Balb/c nude mice (Harland, UK). Mice were observed for the development of tumours. Xenografts were injected with 70μl of DISC-β-gal containing a total of 1 x 10^7 pfu. The mice were killed 24 hours later, and the xenografts excised and fixed in 0.25% glutaraldehyde at room temperature for 24 hours. Following fixation, the tumours were transfected and stained with X-gal solution for 30 minutes at 37°C. A section of tumour was analysed using electron microscopy for the detection of intracellular viral particles.

Electron microscopy of prostate cancer xenografts

Solid tissues were fixed in 2.5% glutaraldehyde in cacodylate buffer at pH 7.4 for 24 hrs and secondary fixed in 1% osmium tetroxide. Further processing and imaging was performed as described above.

Infection of ex-vivo *human prostate cancer samples derived from surgical specimens*

The applicability of DISC virus based intra-tumoural therapy to human prostate cancer was further evaluated by investigating gene expression in clinical prostate cancer specimens following infection with DISC. Specimens were obtained from patients with known prostate cancer undergoing transurethral resection of the prostate (TURP) at Nottingham City Hospital. Informed consent was obtained from all patients. The tumour samples were transported on ice in a prostate-specific tissue culture medium, Pr-EGM. The external surface of each prostate chip was trimmed by sharp dissection to remove charred tissue resulting from diathermy and samples of approximately equal sizes produced. These samples were washed twice using PBS

and placed in wells of a 48-well plate. 300µl media was then added, containing 10^8 pfu of either DISC-ß-gal, DISC-hGMCSF or no virus. After 24 hours at 37°C, the media was removed and the samples washed 3 times with fresh media. Half of the tumour samples were transferred to a new 48 well-plate and stained for ß-galactosidase activity, following the same protocol used for staining cells in tissue culture, as described above. 300µl media was added to the remaining specimens, which were incubated again at 37°C. The media was removed from these samples and replaced with fresh media daily. ELISA assays for human GM-CSF were performed on media samples using a DuoSet human GM-CSF ELISA kit (R+D Systems, Abingdon UK).

To ascertain whether the observed protein production could be attributed to the expression of viral genes following infection, or alternatively to the absorption of protein-containing media by the tissue specimens during the infection period, a negative control experiment was designed. Half the prostate tissue samples were subjected to two cycles of freezing and thawing by immersion in liquid nitrogen in order to kill the prostate cancer cells whilst minimising significant structural changes. These were subsequently exposed to live virus using the protocol described above. Any protein production observed in the dead specimens was attributed to passive protein absorption rather than viral infection.

Construction of a DISC-HSV based multi-epitope test vaccine for in vivo study

Contents

Overview

It is intended to produce DISC-HSV-based multi-epitope vaccines based on novel prostate cancer epitopes. DISC-HSV has been used extensively as a vector for gene delivery (Ali *et al.* 2000; Ali *et al.* 2002), but the application of this virus as a vector for the delivery of multi-epitope vaccines is untested. This study therefore aims to establish the ability of DISC-HSV to deliver such vaccines using well-known immunogenic epitopes. The importance of both class-I and class-II immune responses for effective vaccination has been discussed in the Introduction chapter. Two test vaccines were therefore designed, comprising class-I and class-II epitopes from p53 and HEL respectively.

This chapter describes the development and construction of multi-epitope viral vaccines using class-I and class-II test epitopes. Firstly, the epitopes to be included were identified, and their DNA nucleotide sequences determined using GenBank® database sources. A multiepitope construct was then designed, consisting of the epitope DNA sequences flanked by appropriate restriction and ligation sites and additional DNA included to enhance intracellular processing of the encoded epitope peptides; for example, an ER targeting sequence (Anton *et al.* 1997; Ishioka *et al.* 1999). The assembly of DNA constructs was then performed using a PCR method.

The class-I and class-II DNA constructs were proliferated in a prokaryotic vector (TOPO) before being transferred to a eukaryotic shuttle vector (PIMJ28). This was used to transfer the construct DNA into the DISC-HSV backbone. This was followed by the propagation of virus in tissue culture and estimation of the viral titre.

Introduction

For any given tumour cell type, it is likely that there are a limited number of immune epitopes that can be effectively targeted by the immune system. By creating a vaccine that directs the immune response against these but not other epitopes, the effectiveness of immunotherapy may be improved. However, strategies that use a single epitope might only be effective against a proportion of tumours expressing a certain tumour antigen and risk the problems of immune evasion through antigen loss, for example. Multiepitope vaccines aim to direct the immune system against a number of specific tumour targets simultaneously, thus limiting the ability of tumours to evade the immune response and maximising their response rates.

The use of a multi-epitope vaccination strategy is proposed for prostate cancer. A preliminary step in the design of such a vaccine is the optimisation of a suitable delivery vector. Although DISC-HSV has a proven efficacy as a gene delivery system and as a vaccine component (Rees *et al.* 2002), its effectiveness as a delivery system for a multi-epitope vaccine has not been tested. It is anticipated that certain features of DISC-HSV might render it particularly suitable for this purpose, not least the size of the viral genome, its inherent immunogenicity(Rees *et al.* 2002), and its safety and side-effect profile (McLean *et al.* 1994; Hickling *et al.* 1998).

Vaccinia has been extensively used as a delivery vector for multi-epitope vaccines (An *et al.* 1996; Anton *et al.* 1997; Woodberry *et al.* 1999), but adenovirus (Toes *et al.* 1997) and plasmid (Whitton *et al.* 1993; Loirat *et al.* 2000) vectors have also been used. Although effective and easy to manufacture, the use of vaccinia as a delivery system is attended by a risk of complications including encephalopathy (Behbehani

1983), potentially affecting over 300 per million vaccinees (Gurvich 1992). This problem might be anticipated to be more significant in immunocompromised hosts, such as those with advanced cancers. The usefulness of vaccinia and adenovirus may also be limited because immune responses induced against the vector could reduce the efficacy of subsequent vaccine doses, or compete with responses against the intended tumour targets (An and Whitton 1999). Vaccine DNA delivered by plasmid alone may be inherently less immunogenic than that delivered by a viral vector, and variable results have been published (Corr *et al.* 1997).

This chapter describes the construction of two DISC-HSV-based vaccines, one employing class-I epitopes and the other employing class-II epitopes. It is intended to establish the viability of this viral vector for the delivery of multi-epitope vaccines with a view to the development of a novel prostate cancer vaccine.

Identification of epitopes and vaccine design

Class-I construct

The identification of class-I CTL epitopes can be approached using a variety of techniques, as described in the introduction. Strong binding of several p53 peptides to HLA-A2 has been demonstrated (Houbiers *et al.* 1993; Nijman *et al.* 1994), and some of these peptides have been used to generate CTL *in vitro* in either human (Gnjatic *et al.* 1998; Chikamatsu *et al.* 1999) or transgenic mouse (Theobald *et al.* 1995; Theobald *et al.* 1997; Theobald *et al.* 1998) models. Selected epitopes derived from p53 are shown in table 3.1.

Although these p53-specific CTL were able to destroy peptide labelled target cells, only three peptides (149-157, 217-225 and 264-272) produced CTL capable of killing tumour cells, indicating that these peptides are presented as a result of endogenous processing (Ropke *et al.* 1996; Gnjatic *et al.* 1998; Chikamatsu *et al.* 1999; McArdle *et al.* 2000). These three epitopes were therefore chosen for inclusion in the class-I multi-epitope vaccine construct.

In addition to these three epitopes, p53.65-73 was also included. Although lysis of antigen bearing tumour cells has not been verified, this epitope has consistently exhibited very strong HLA-A2 binding affinity and efficient CTL induction. Although p53.187-197 has shown similarly promising properties in many studies, it has been found to be tolerated by HLA-A2 transgenic mice (Theobald *et al.* 1997), which negated its usefulness for this study. The selected p53 peptides are illustrated in figure 3.1, which also shows the nucleotide sequences of the DNA that encodes them.

p53 peptide	HLA-A2 binding	CTL induction	endogenous processing
24-32	✓		
25-35	✓	✓	
65-73	✓	✓	
65-74	✓	✓	
66-74	✓		
69-76	✓		
78-86	✓		
122-130	✓		
129-137	✓	✓	
149-157	✓	✓	✓
187-197	✓	✓	
210-218	✓		
217-225	✓	✓	✓
263-272	✓	✓	
264-272	✓	✓	✓
322-330	✓		

Table 3.1: Properties of known HLA-A2 p53 epitopes

(a)

MEEPQSDPSV EPPLSQETFS DLWKLLPENN VLSPLPSQAM
DDLMLSPDDI EQWFTEDPGP DEAPRMPEAA PPVAPAPAAP
TPAAPAPAPS WPLSSSVPSQ KTYQGSYGFR LGFLHSGTAK
SVTCTYSPAL NKMFCQLAKT CPVQLWVDST PPPGTRVRAM
AIYKQSQHMT EVVRRCPHHE RCSDSDGLAP PQHLIRVEGN
LRVEYLDDRN TFRHSVVVPY EPPEVGSDCT TIHYNYMCNS
SCMGGMNRRP ILTIITLEDS SGNLLGRNSF EVRVCACPGR
DRRTEEENLR KKGEPHHELP PGSTKRALPN NTSSSPQPKK
KPLDGEYFTL QIRGRERFEM FRELNEALEL KDAQAGKEPG
GSRAHSSHLK SKKGQSTSRH KKLMFKTEGP DSD

(b)

p53.65-73	RMPEAAPPV	→	CGT	ATG	CCT	GAA	GCT	GCT	CCT	CCT	GTT
p53.149-157	STPPPGTRV	→	AGT	ACG	CCT	CCA	CCG	GGT	ACT	AGA	GTG
p53.217-225	VVPYEPPEV	→	GTG	GTG	CCT	TAC	GAG	CCT	CCA	GAA	GTA
p53.264-272	LLGRNSFEV	→	CTG	CTA	GGA	CGA	AAC	TCG	TTC	GAG	GTC

Figure 3.1: Selected class-I epitopes derived from mutant p53.

(a) The amino-acid sequence of mutant human p53 is given (Accession number P04637). The positions of several class-I epitopes are highlighted. **(b)** shows these epitopes and the corresponding nucleotide sequences of the DNA that encodes them.

A well-established murine class-I epitope derived from the human adenovirus E1B $H2D^6$ protein was also included in order to provide a positive control for subsequent testing of CTL activation in a murine MHC-transgenic model system.

Following cellular infection with virus and the translation of virally encoded peptides, the efficacy of the vaccine is dependent on the successful cellular processing of epitope peptides and their cell-surface presentation by MHC molecules. It has been postulated that the insertion of triple alanine (AAA) nucleotides between adjacent minigenes may facilitate the processing of the resultant epitopes by preventing disruption by unfavourable flanking residues (Del Val *et al.* 1991; Eisenlohr *et al.* 1992; Anton *et al.* 1997), although other reports suggest that this is an unnecessary

refinement (Whitton and Oldstone 1989; Whitton *et al.* 1993; An and Whitton 1997). Our understanding of the processing of minigene-encoded oligopeptides remains incomplete. Although successful immunisation is possible without flanking residues, there exists the possibility of increasing the efficiency of epitope presentation through the inclusion of flanking residues. Epitopes in this class-I vaccine have therefore been separated by AAA spacers.

The intracellular processing of the encoded peptides can be further influenced by the introduction of additional DNA segments. Endoplasmic reticulum (ER) processing is essential for class-I epitope presentation and it has been shown that entry into the ER can be facilitated by a targeting sequence (Anderson *et al.* 1991; Ciernik *et al.* 1996; Fu *et al.* 1998), resulting in improved immune responses against test peptides (Anton *et al.* 1997; Ishioka *et al.* 1999). This gene has therefore been included in the vaccine design. As an alternative to the use of an ER targeting sequence, the fusion of ubiquitin and minigenes was also shown to improve class-I proteosomal processing and thus the effectiveness of the vaccine (Rodriguez *et al.* 1998), but this strategy was rejected as its role in multi-epitope vaccination is not well documented.

DNA vaccines must also comprise sequences that optimise the translation of their genes and maximise the expression of vaccine-encoded peptides. Most eukaryotic mRNAs contain a short recognition sequence that greatly facilitate the initial binding of mRNA to the small subunit of the ribosome. The consensus sequence for initiation of translation in vertebrates (also called Kozak sequence) is (A/G)XXATGG (Kozak 1981; Kozak 1987), where X represents a pyrimidine. The inclusion of a Kozak sequence at the 5' end of the vaccine may significantly improve expression levels of the subsequent DNA sequences, its inclusion has been shown to significantly enhance

the immunogenicity of a DNA multi-epitope vaccine (An *et al.* 2000). The Kozak translation initiation sequence has therefore incorporated into the vaccine in this study. Similarly, a "stop" sequence is required to demarcate the end of the gene. The triplet codes for stop sequences in humans are TAA, TGA and TAG.

Finally, restriction enzyme binding sites have also been included in the vaccine construct design. These are necessary for the insertion of the multi-epitope DNA into a cloning vector plasmid, and subsequently the transfer to a shuttle-vector plasmid and ultimately to DISC-HSV. The final vaccine design is illustrated in figure 3.2.

(a) Nucleotide sequence

AAGCTTCGCCCTTT.....ACC ATG GCG GCC CCC GGC GCC CGG CGA
CCG CTG CTC CTG CTG CTG CTG GCA GGC CTT GCA CAT GGC GCC
TCA GCA CTC TTT GAG GAT CTA **GCT GCT GCT** CGT ATG CCT GAA
GCT GCT CCT CCT GTT **GCT GCT GCT** AGT ACG CCT CCA CCG GGT
ACT AGA GTG **GCT GCT GCT** GTG GTG CCT TAC GAG CCT CCA GAA
GTA **GCT GCT GCT** CTG CTA GGA CGA AAC TCG TTC GAG GTC **GCT**
GCT GCT GTC AAC ATT CGA AAC TGC TGT TAC ATA **GCT GCT GCT**
TAA GAA TTC GGATA

(b) Amino acid translation

AAPGARRPLLLLLLAGLAHGASALFEDL **AAA** RMPEAAPPV **AAA** STPPPGTRV **AAA**
VVPYEPPEV **AAA** LLGRNSFEV **AAA** VNIRNCCYI **AAA**

(c) Vaccine components

HindIII restriction site . . . non-coding sequence . . . KOZAK/Start . . . ER targeting . . . **AAA** . . . p53 A2.1 epitopes (65-73; 149-157; 217-225; 264-272) . . . **AAA** . . . E1B $H2D^6$ . . . **AAA** . . . **stop** . . . $ECoR_1$ restriction site

Figure 3.2: p53 multi-epitope class-I vaccine construct.

This figure illustrates the constituent parts of the DNA construct that is to be created using PCR. **(a)** illustrated the nucleotide sequence of the DNA vaccine. The amino acid translation of this genetic code is shown in **(b)**. Finally, **(c)** shows an explanation of the functions of each of these components.

Class-II construct

A number of murine MHC class-II epitopes derived from HEL have been identified. Peptides produced by tryptic digestion of HEL were presented on paraformaldehyde-fixed macrophages and tested for their ability to stimulate HEL-specific, I-A^k restricted T-cell hybridomas (Allen *et al.* 1984). The ability of these epitopes to bind I-A^k and stimulate class-II immune responses has been confirmed in further studies (Johnson *et al.* 1989).

The immunological properties of bovine pancreatic RNAse-A have been extensively studied. Tryptic fragments of this protein were used to determine its immunogenic determinant using T-cell hybridomas (Lorenz *et al.* 1988). Synthetic peptides were constructed, and the essential immunostimulatory portion of the protein was further localised.

These class-II epitopes have been used extensively in experimental systems for many years and their properties have been described in detail. They therefore represent useful positive control peptides for inclusion in this multi-epitope vaccine. The selected epitopes are illustrated in figures 3.3 and 3.4, which also illustrate the nucleotide sequences of the DNA that encodes them.

(a)

KVFGRCELAA AMKRHGLDNY RGYSLGNWVC AAKFESNFNT
QATNRNTDGS TDYGILQINS RFWCNDGRTP GSRNLCNIPC
SALLSSDITA SVNCAKKIVS DGNGMNAWVA WRNRCKGTDV
QAWIRGCRL

(b)

HEL.46-61		AAC ACC GAT GGG AGT ACC GAC TAC
NTDGSTDYGILQINSR	→	GGA ATC CTA CAG ATC AAC AGC CGC
HEL.116-129		AAG GGC ACC GAC GTC CAG GCG TGG
KGTDVQAWIRGCRL	→	ATC AGA GGC TGC CGG CTG

Figure 3.3: HEL amino-acid sequence.

(a) The amino-acid sequence for hen egg-white lysozyme (HEL) (Accession number 1065031). The positions of two class-II epitopes are highlighted. **(b)** shows these epitopes and the corresponding nucleotide sequences of the DNA that encodes them.

(a)

KETAAAKFER QHMDSSTSAA SSSNYCNQMM KSRNLTKDRC
KPVNTFVHES LADVQAVCSQ KNVACKNGQT NCYQSYSTMS
ITDCRETGSS KYPNCAYKTT QANKHIIVAC EGNPYVPVHF
DASV

(b)

RNAse-A.43-56		GTG AAC ACC TTT GTG CAC GAG TCC
VNTFVHESLADVQA	→	CTG GCT GAT GTC CAG GCG

Figure 3.4: Bovine RNAse-A amino-acid sequence.

(a) The amino-acid sequence for Bovine RNAse-A (Accession number 3212647). The position of the class-II epitope is highlighted. **(b)** shows this epitope and the corresponding nucleotide sequence of the DNA that encodes it.

The intracellular processing of class-II epitopes can be enhanced by the inclusion of genes that alter their intracellular processing. There are two principal pathways by which antigens may enter the endosomal and lysosomal compartments, and ultimately undergo MHC class-II presentation. The process of phagocytosis or endocytosis of exogenous proteins by APC has been described in the Introduction chapter. Additionally, a pathway for the presentation of endogenously synthesized membrane proteins by APC has been described, and cytoplasmic proteins have been induced to enter this pathway experimentally using compartment specific peptide sequences. The cytoplasmic domain of lysosomal-associated membrane protein (LAMP-1) is one such "targeting sequence", and the linking of genes encoding antigen and LAMP-1 has been shown to enhance the class-II presentation of antigen to $CD4^+$ cells *in vitro* (Wu *et al.* 1995; Thomson *et al.* 1998) and thus increase vaccine potency.

In the design of the class-II multiepitope vaccine, it was necessary to place an ER targeting sequence at the N-terminus of the protein in order to facilitate entry into the endoplasmic reticulum, as described above in the construction of the class-I p53 vaccine. In order to subsequently reroute the peptides into the MHC class-II processing pathway, the LAMP-1 sequence was also included at the C-terminus of the protein, thus targeting the peptides into the endosomal and lysosomal compartments. Appropriate restriction enzyme sites, and a Kozak and stop sequence were also included as described above for the class-I p53 construct. The final class-II vaccine design is illustrated in figure 3.5.

(a) Nucleotide sequence

AAGCTTCGCCCTTT.....ACC ATG GCG GCC CCC GGC GCC CGG CGA
CCG CTG CTC CTG CTG CTG CTG GCA GGC CTT GCA CAT GGC GCC
TCA GCA CTC TTT GAG GAT CTA AAC ACC GAT GGG AGT ACC GAC
TAC GGA ATC CTA CAG ATC AAC AGC CGC.AAG GGC ACC GAC GTC
CAG GCG TGG ATC AGA GGC TGC CGG CTG.GTG AAC ACC TTT GTG
CAC GAG TCC CTG GCT GAT GTC CAG GCG.CTT AAC AAC ATG TTG
ATC CCC ATT GCT GTG GGC GGT GCC CTG GCA GGG CTG GTC CTC
ATC GTC CTC ATT GCC TAC CTC ATT GGC AGG AAG AGG AGT CAC
GCC GGC **TAT CAG ACC ATC** TAG TCT AGA

(b) Amino acid translation

AAPGARRPLLLLLLAGLAHGASALFEDL NTDGSTDYGILQINSR
KGTDVQAWIRGCRL VNTFVHESLADVQA
LNNMLIPIIVGAALAGLVLIVLIAYLIGRKRSHAG**YQTI**

(c) Vaccine components

HindIII restriction site . . . non-coding sequence . . . KOZAK/Start . . .
ER-targeting . . . HEL class II epitopes (46-61; 116-129) . . . RNAase-A class
II epitope (43-56) . . . LAMP-1 . . . **stop** . . . Xba1 restriction site

Figure 3.5: Class-II multi-epitope vaccine construct.

This figure illustrates the constituent parts of the DNA construct that is to be created using PCR. **(a)** illustrated the nucleotide sequence of the DNA vaccine. The amino acid translation of this genetic code is shown in **(b)**. Finally, **(c)** shows an explanation of the functions of each of these components.

Results

The construction of the class-I and class-II multi-epitope vaccines was undertaken with the assistance of Dr. Shahid Mian.

The processes involved in the assembly of the class-I and class-II vaccines were similar. Selected results illustrating the development of the two vaccines have been included below; each is presented as a representation of the construction of both viruses. Thus, each stage of vaccine assembly has been illustrated using either the class-I or the class-II vaccine, but not (for the sake of clarity) both.

Construction of multi-epitope DNA constructs by PCR

Multiple overlapping oligonucleotide primers were serially ligated using PCR. Successful addition of DNA was confirmed at each stage by electrophoresis of PCR products through a 1.2% agarose gel. Transillumination with ultraviolet light allowed the visualisation of ethidium bromide-stained DNA. The sizes of the PCR products were estimated by comparison with a DNA ladder (standardised oligonucleotides of known size) that was run on the same gel. This allowed comparison with the anticipated size of the product in each case.

Figure 3.6 shows a diagnostic 1.2% agarose gel containing ethidium bromide on which all nine sequential PCR products obtained during the construction of the class-II vaccine have been run. The gradual increase in the sizes of the DNA strands can be seen from right to left, indicating the addition of the appropriate amount of DNA at each stage. The class-I minigene was constructed using the same process of sequential PCR reactions.

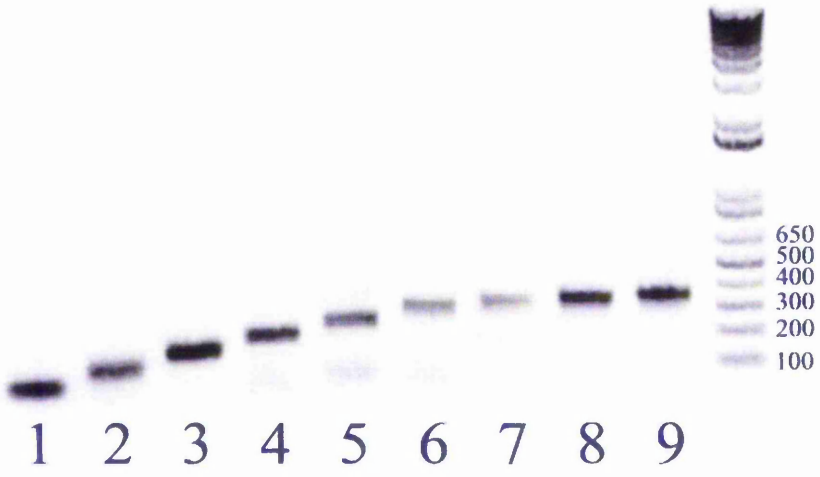


Figure 3.6: Diagnostic gel illustrating construction of class-II multiepitope DNA using sequential PCR.

1.2% agarose gel illustrating the sequential addition of oligonucleotides by PCR amplification. A total of nine PCR reactions were performed (from right to left). The left-most column contains a DNA ladder, allowing the approximate size of each PCR product to be estimated. The size of each component of the ladder is given in base-pairs.

Insertion of PCR-product into the Zero blunt TOPO cloning vector

The Zero blunt-II TOPO PCR cloning kit was used to insert the vaccine construct into the TOPO plasmid genome, and transformation into *E.Coli* proceeded immediately. Twenty individual bacterial colonies grown on agar plates were transferred into separate tubes of LB culture media.

To establish whether the PCR product had been successfully inserted into the TOPO plasmid, genomic DNA was extracted from these 20 bacterial clones so that a sequencing reaction could be undertaken. EcoR1 restriction digestion was performed on these "miniprep" DNA samples to remove the insert DNA from the plasmid. The

digestion products were run on a 0.8% agarose gel to detect the presence of inserts of the correct size (see figure 3.7), and a sequencing reaction was performed on those samples. The sequence of the clone 8 insert was identical to that of the multi-epitope construct (data not shown).

Clone 8 bacteria were single-colony purified to maximise the proportion of bacteria containing the desired plasmid DNA. A large scale "Maxiprep" preparation of bacterial DNA was then made, and the presence of the multi-epitope construct confirmed using the same method as above (results not shown).

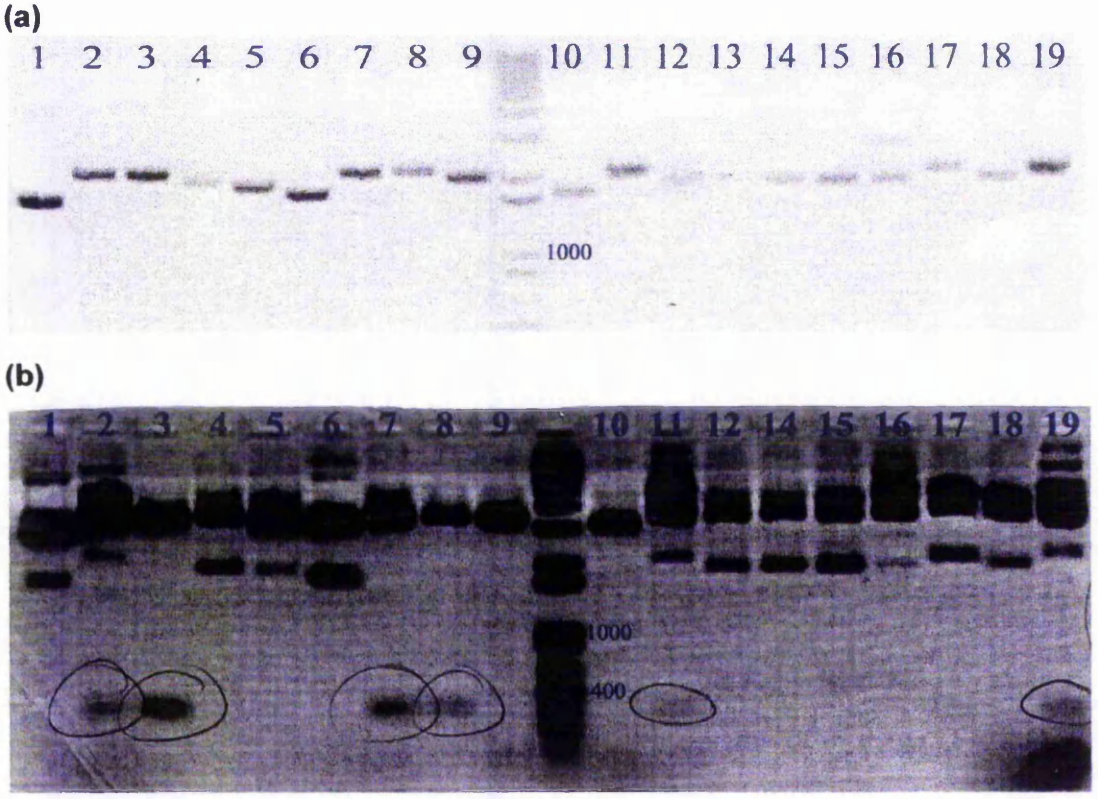


Figure 3.7: Diagnostic gels of TOPO plasmid extracted from E.Coli minipreps.
Diagnostic agarose gel showing DNA extracted from *E.coli* clones **(a)**. All except #13 show plasmid DNA. A further gel has been run on the same DNA samples following enzyme digestion with EcoR1 **(b)**. Six clones (2, 3, 7, 8, 11 and 19) contained an insert approximating the size of the class-II construct.

Insertion of multi-epitope construct DNA into shuttle vector PIMJ28

Having produced a large scale preparation of plasmid DNA and confirmed the correct DNA sequence, the multi-epitope construct was transferred from the TOPO cloning vector into the PIMJ28 viral shuttle vector. This was achieved by restriction digestion of both plasmids using Hind-III and Xba-I enzymes followed by ligation of the excised construct DNA with the decircularised PIMJ28 plasmid. Both purified DNA samples as well as the resulting ligated DNA were run on a 0.8% agarose diagnostic gel, as shown in figure 3.8.

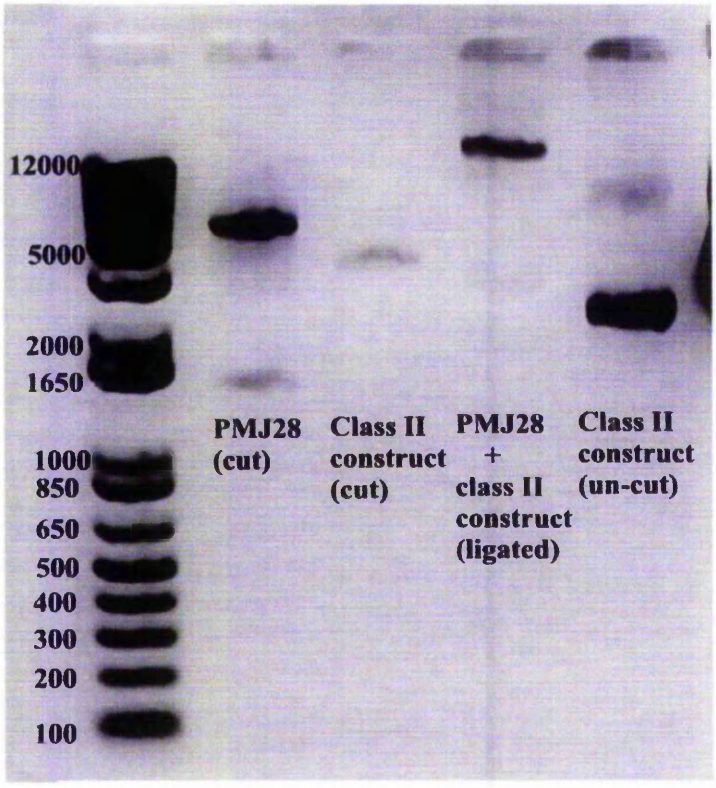


Figure 3.8: Insertion of multiepitope construct into PIMJ28 plasmid.

The above gel shows the multi-epitope construct DNA before (un-cut) and after (cut) extraction from the TOPO plasmid using Xba1 and HindIII enzyme digestion. The class-II insert is too faint to see clearly on this gel, although the residual TOPO plasmid can be distinguished. Partial enzyme digested PIMJ28 DNA is also shown (labelled as cut), as is the DNA resulting from the ligation of construct and PIMJ28.

The PIMJ28/multiepitope construct DNA was subsequently transformed into *E.Coli* by electroparation. The resultant cultures were single colony purified and analysed by gel electrophoresis and DNA sequencing as before. Figure 3.9 shows DNA isolated from *E.Coli*, confirming the presence of plasmid DNA. No restriction digest was performed. A sequencing reaction confirmed the presence of a correct vaccine construct insert (data not shown).

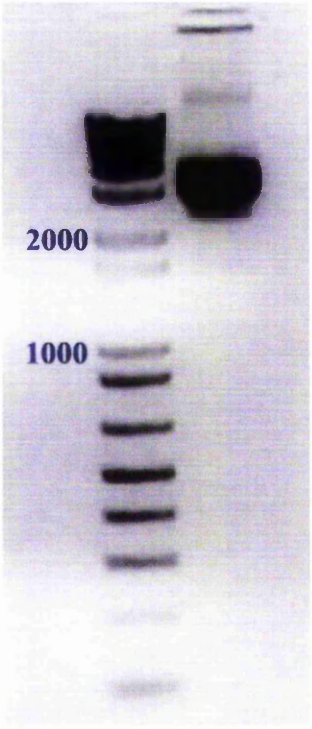


Figure 3.9: Diagnostic agarose gel confirming presence of PIMJ28 plasmid DNA. DNA extracted from E.Coli was run on a 1.2% agarose gel, confirming the presence of plasmid DNA.

Insertion of vaccine DNA into DISC-HSV viral backbone

The multi-epitope construct was transferred from the PIMJ28 plasmid into the DISC-HSV genome by restriction digestion of both plasmid and viral DNA followed by DNA ligation. Gel purification of the PIMJ28 DNA was performed after Pac1 digestion (see figure 3.10). This was followed by ligation of the virus and plasmid

DNA strands using T4 DNA ligase. A control ligation, using DISC-HSV DNA alone, was also performed to ensure that a functional virus could be produced in the absence of insert DNA. Successful ligation was confirmed by running the products on a 1.2% agarose gel (see figure 3.11).

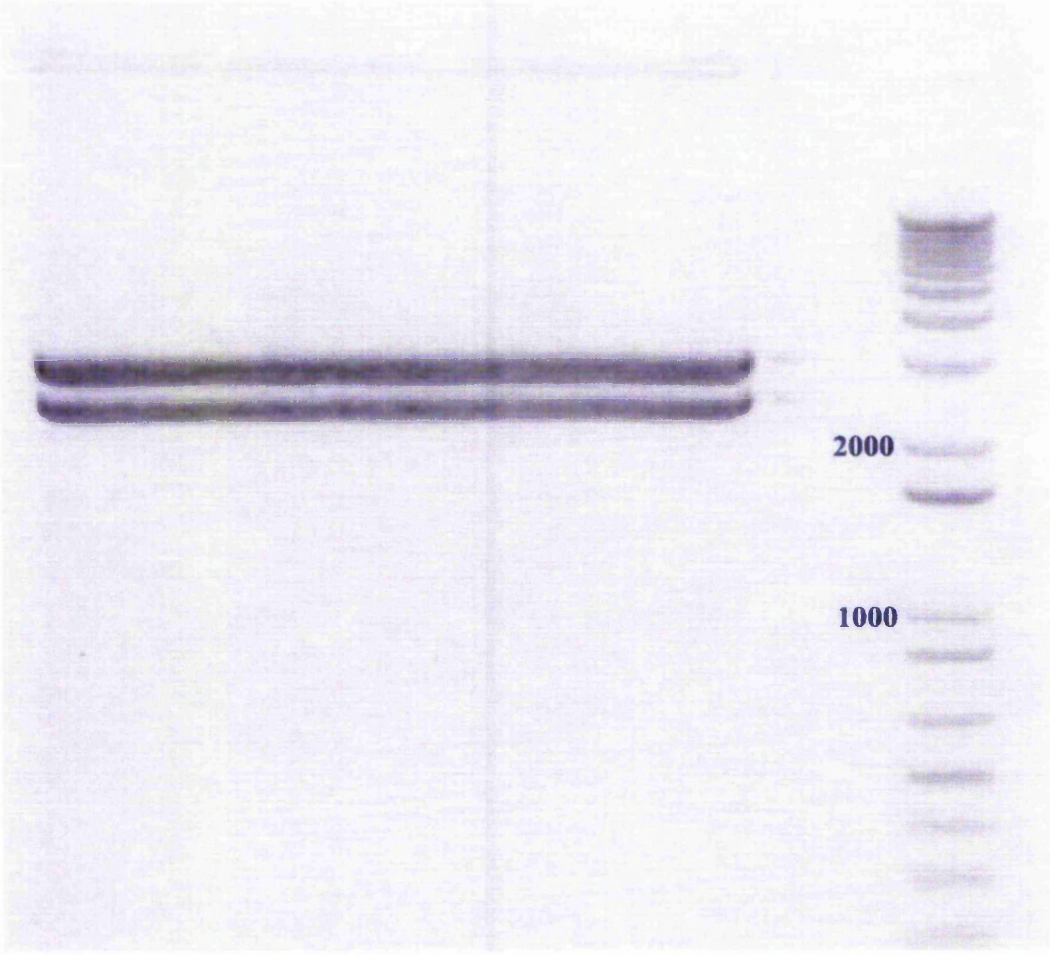


Figure 3.10: Gel purification of PIMJ28 plasmid following Pac1 enzyme digestion.
The PIMJ28 plasmid containing the class-II multi-epitope vaccine construct has been digested using the Pac1 restriction enzyme in preparation for ligation into the DISC-HSV genome. The slower (lower) band contains the insert and is cut out. The rest, containing the plasmid DNA, is discarded. Other chemical contaminants are also removed by this process. The large size of the band containing the construct is attributable to the addition of plasmid DNA (between the digestion sites and the insert position).

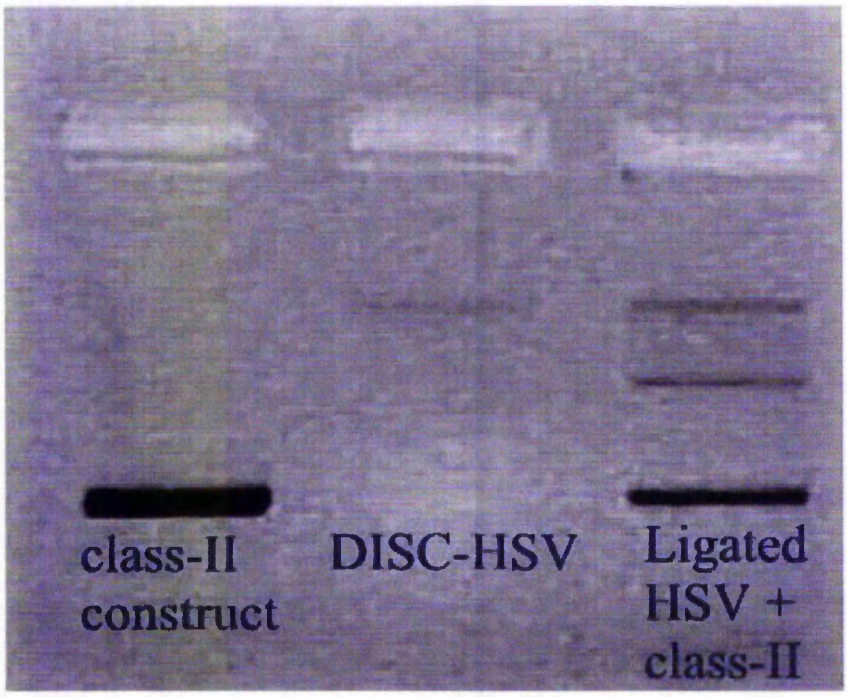


Figure 3.11: Diagnostic agarose gel illustrating successful ligation of DISC-HSV and class-II multi-epitope construct.

The class-II construct DNA is shown on the left. The much larger DISC-HSV DNA is adjacent to it. Following ligation (right-hand well), bands corresponding to both the virus and class-II construct can be seen, along with a new band of intermediate size. Although larger than the other two, this runs through the gel more quickly than the DISC-HSV DNA because the linear configuration of DISC-HSV prior to ligation causes it to run more slowly. Following ligation, the virus is recircularised, explaining its position in relation to the other bands.

CR2 cells were transfected with the ligated DNA (DISC-HSV plus vaccine construct or control). The cells were observed for cytopathic effects (CPE), indicating successful viral infection, over the subsequent 7 days (see figure 3.12). When CPE was observed, aliquots of culture medium taken from the infected wells were used to infect further wells, and thus propagate the virus further.

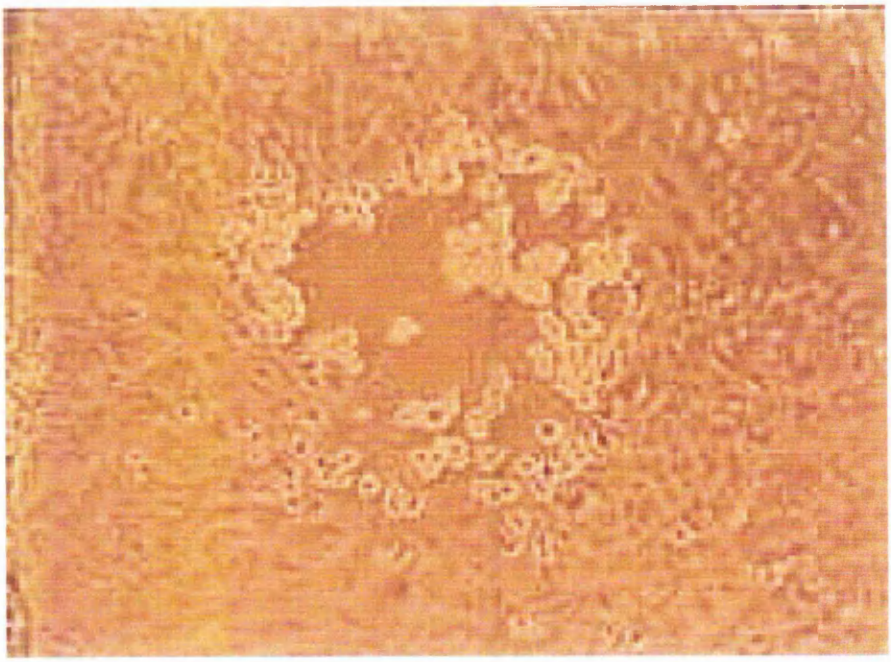


Figure 3.12: Cytopathic Effect (CPE) of HSV infection on CR2 cells in culture.

Cells on day five exhibit characteristic rounding, with loss of adherence producing punched out areas surrounded by normal, confluent cells.

Following the establishment of viral infection, multiple single-colony purifications of virus-infected CR2 cells were performed producing viral clones. After each round of subcloning, viral DNA was isolated so that the presence of insert (vaccine construct) DNA could be confirmed. PCR was performed on DNA extracted from DISC-HSV clones. The PIMJ28 primers produce a product comprising 351 base-pairs derived from the original plasmid, plus the size of the multi-epitope construct. The total length of the p53 class-I construct was 318 bases, and that of the HEL class-II construct was 366 bases. Figure 3.13 shows PCR products obtained from a representative sample of 17 clones containing the class-I multi-epitope construct, confirming inserts of the correct size from two clones (numbers 9 and 13).

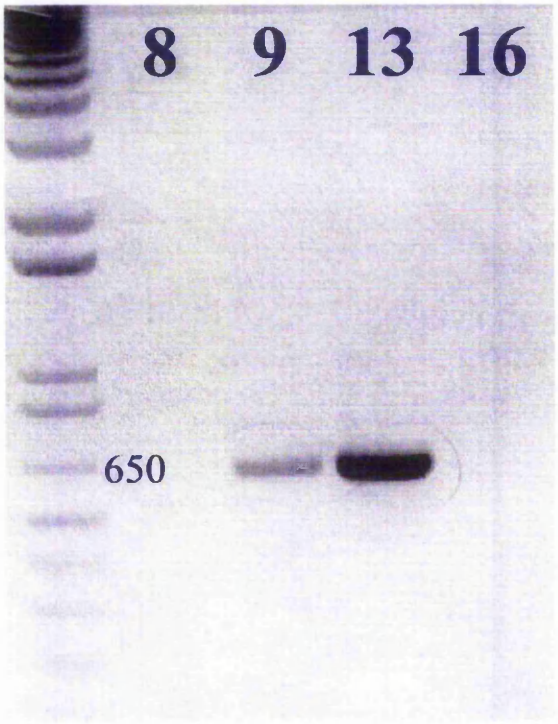
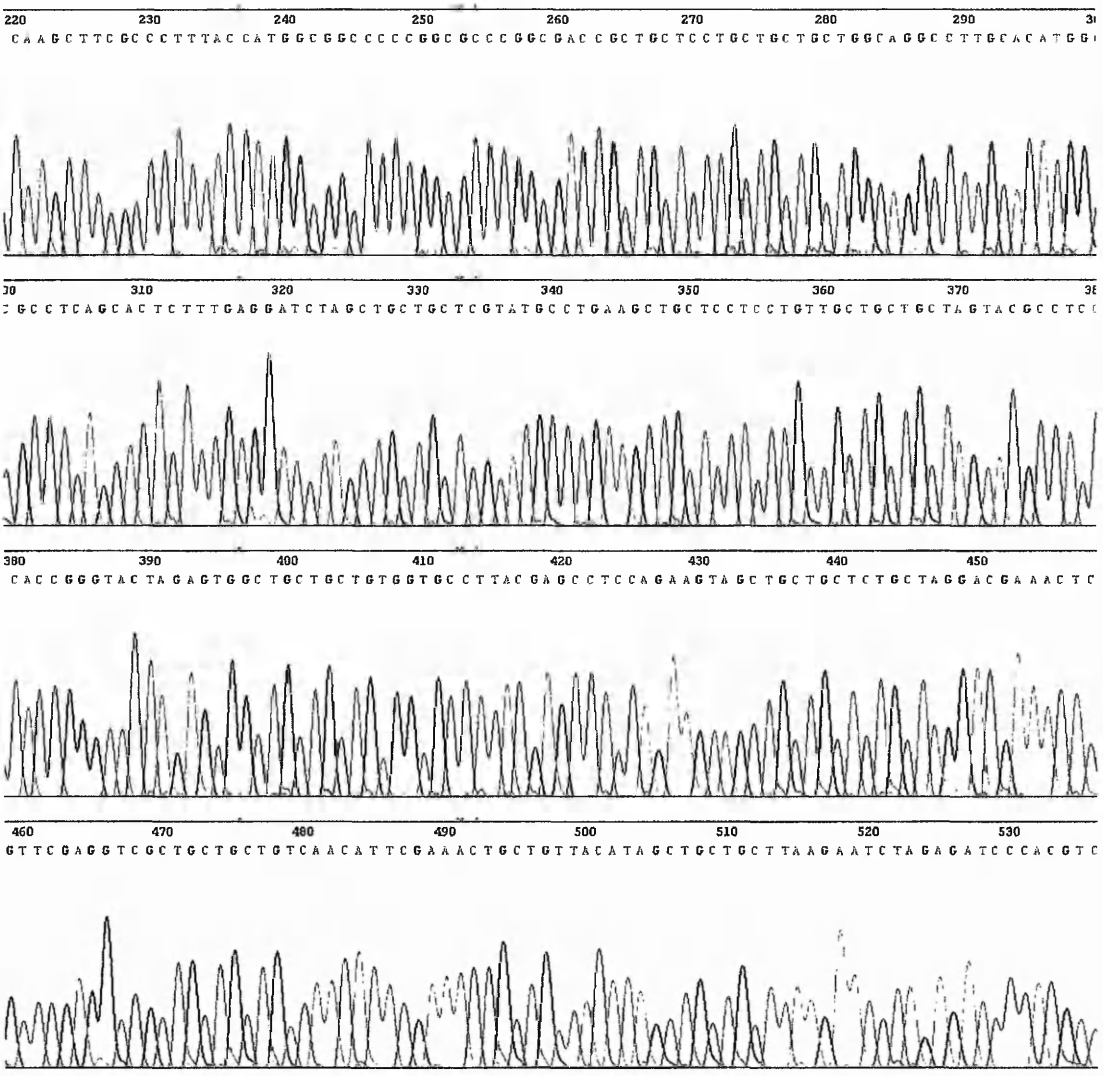


Figure 3.13: Detection of multi-epitope construct with DISC-HSV genome using PCR. DNA was extracted from 17 clones of CR2 cells infected with DISC-HSV. PCR using PIMJ28 primers was performed to determine the size of DNA insert for comparison with the expected size of the multi-epitope construct DNA. No inserts were detected in many of clones. This gel shows a selection, including clones 9 and 13, for which a strong band can be seen corresponding to a size of approximately 650 base pairs. The other 2 clones shown here do not have any insert, suggesting the failure of ligation of DISC-HSV with the PIMJ28 plasmid in these cases.

The PCR-amplified DNA from clones 9 and 13 (containing correct-sized inserts) was then sequenced using PIMJ28 primers. Figure 3.14 shows the result of a sequencing gel performed on clone 13, confirming the correct sequence of the class-I multi-epitope construct.



Sequencing results: ACCATGGCGGCCCCCGGCGCCCGGCGACCGCTGCTCCTGCTGCTGCT
Target sequence: ACCATGGCGGCCCCCGGCGCCCGGCGACCGCTGCTCCTGCTGCTGCT

GGCAGGCCTTGCACATGGCGCCTCAGCACTCTTTGAGGATCTAGCTGCTGCTCGTATGCCTGAAGCTGC
GGCAGGCCTTGCACATGGCGCCTCAGCACTCTTTGAGGATCTAGCTGCTGCTCGTATGCCTGAAGCTGC

TCCTCCTGTTGCTGCTGCTAGTACGCCTCCACCGGGTACTAGAGTGGCTGCTGCTGTGGTGCCTTACGA
TCCTCCTGTTGCTGCTGCTAGTACGCCTCCACCGGGTACTAGAGTGGCTGCTGCTGTGGTGCCTTACGA

GCCTCCAGAAGTAGCTGCTGCTCTGCTAGGACGAAACTCGTTCGAGGTCGCTGCTGCTGTCAACATTCG
GCCTCCAGAAGTAGCTGCTGCTCTGCTAGGACGAAACTCGTTCGAGGTCGCTGCTGCTGTCAACATTCG

AAACTGCTGTTACATAGCTGCTGCTTAAGAATTC
AAACTGCTGTTACATAGCTGCTGCTTAAGAATTC

Figure 3.14: Sequence reaction confirming insertion of class-I multiepitope DNA into DISC-HSV vector.

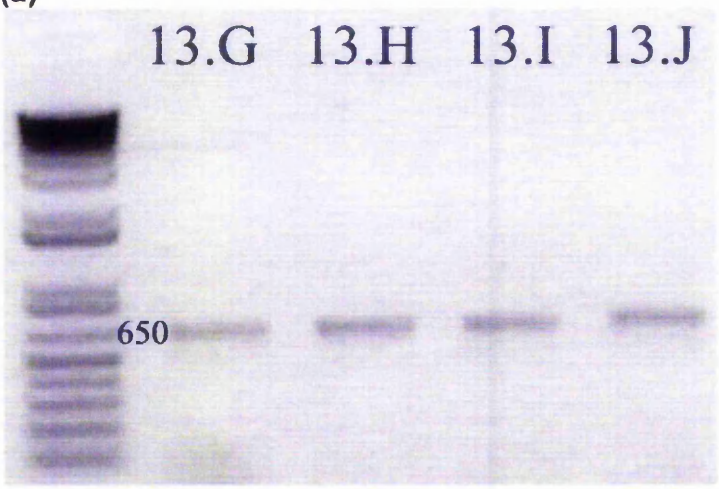
Graphical representation of DNA sequencing gel (CHROMAS software package). A comparison of the DNA sequence obtained with the intended sequence is also shown.

Single-colony purification of clone 13 proceeded. This process was repeated three times, with the presence of the correct DNA insert being confirmed at each stage using the procedures described above. Figure 3.15 shows the results of PCR analysis of subclones following the second and third purification steps; all subclones contained an insert of the correct size. A sequencing gel confirmed the correct nucleotide sequence of the insert DNA. Subclone denominated 13.B.a was selected and used to create a large-scale preparation of virus, by infection and propagation in bulk cultures of CR2 cells.

Virus was then harvested by lysing infected CR2 cells by ultrasonication and titration of the virus was performed, as described in the Materials and Methods chapter.

The creation of both class-I and class-II constructs and their insertion into the DISC-HSV genome was performed using the same procedures, as described above. Representative results from both sets of experiments have been illustrated here. Equivalent results were obtained for both class-I and class-II constructs although, for clarity, not all gels and sequencing reactions have been included. A sequencing gel performed on DNA derived from DISC-HSV following subcloning of the class-II multi-epitope construct is shown in figure 3.16: the successful incorporation of the insert is shown into the viral genome is confirmed.

(a)



(b)

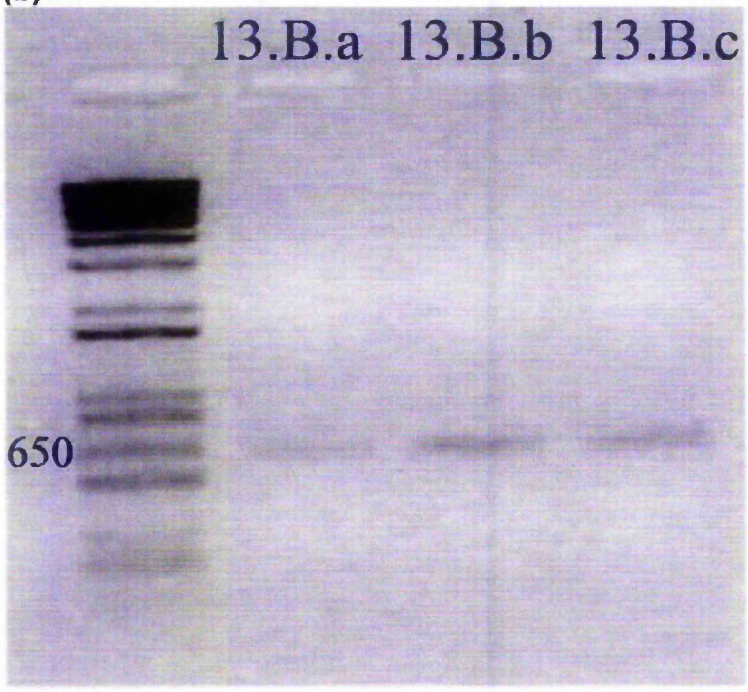
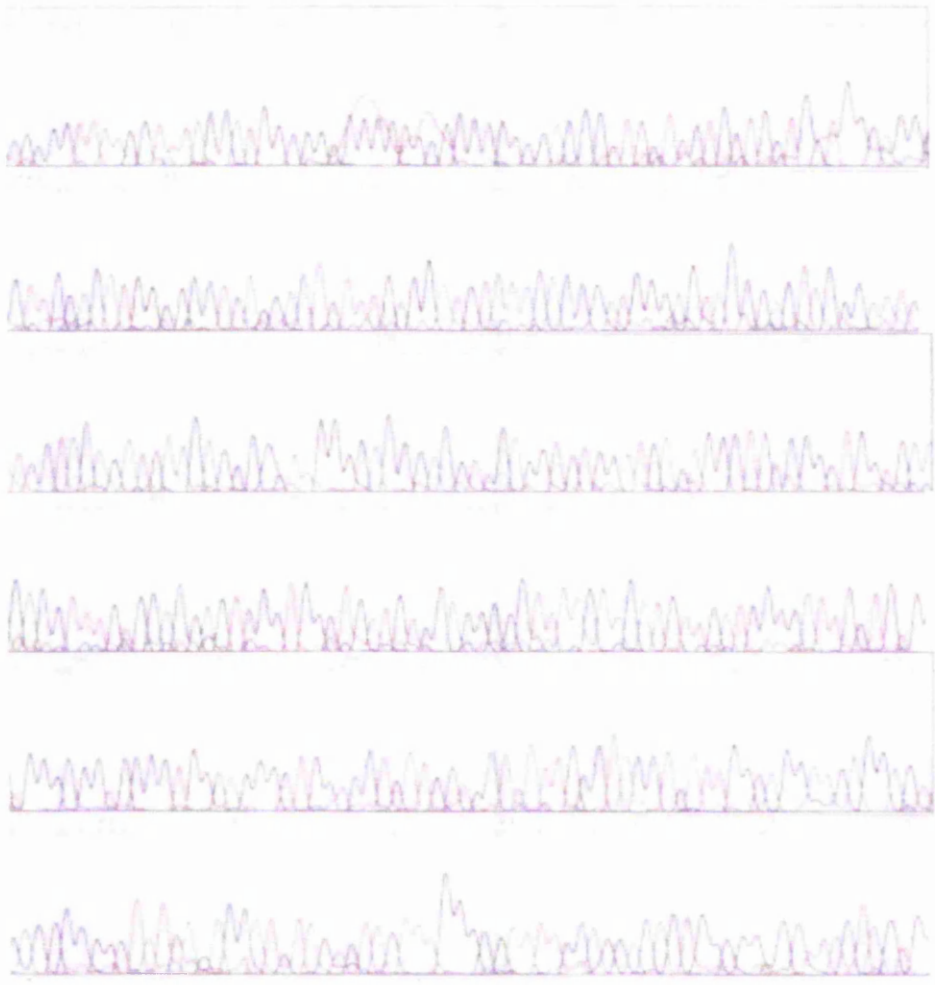


Figure 3.15: PCR-identification of multi-epitope inserts following clonal purification. Single-colony purification of Clone 13 (known to contain an insert with the correct sequence) was performed. Isolation of viral DNA and PCR amplification of the insert was performed as before. All sub-clones (labeled 13.A to 13.J) contained an insert of appropriate size. A representative sample of 4 are shown **(a)**. Following a second stage of subcloning (labeled 13.B.a to 13.C.c), the presence of the insert was again confirmed (representative 3 out of 6 shown) **(b).**



Sequencing results: ACCATGGCGGCCCCCGGCGCCCGGCGACCGCTGCTCCTGCTGCTG
Target sequence: ACCATGGCGGCCCCCGGCGCCCGGCGACCGCTGCTCCTGCTGCTG

CTGGCAGGCCTTGCACATGGCGCCTCAGCACTCTTTGAGGATCTAAACACCGATGGGAGTACCGACTAC
CTGGCAGGCCTTGCACATGGCGCCTCAGCACTCTTTGAGGATCTAAACACCGATGGGAGTACCGACTAC

GGAATCCTACAGATCAACAGCCGCAAGGGCACCGACGTCCAGGCGTGGATCAGAGGCTGCCGGCTGGTG
GGAATCCTACAGATCAACAGCCGCAAGGGCACCGACGTCCAGGCGTGGATCAGAGGCTGCCGGCTGGTG

AACACCTTTGTGCACGAGTCCCTGGCTGATGTCCAGGCG.CTTAACAACATGTTGATCCCCATTGCTGT
AACACCTTTGTGCACGAGTCCCTGGCTGATGTCCAGGCG.CTTAACAACATGTTGATCCCCATTGCTGT

GGGCGGTGCCCTGGCAGGGCTGGTCCTCATCGTCCTCATTGCCTACCTCATTGGCAGGAAGAGGAGTCA
GGGCGGTGCCCTGGCAGGGCTGGTCCTCATCGTCCTCATTGCCTACCTCATTGGCAGGAAGAGGAGTCA

CGCCGGCTATCAGACCATCTAG
CGCCGGCTATCAGACCATCTAG

Figure 3.16: Sequence reaction confirming successful insertion of class-II multiepitope DNA into DISC-HSV vector.

Graphical representation of DNA sequencing gel (CHROMAS software package). A comparison of the DNA sequence obtained with the intended sequence is also shown.

Discussion

The concept of minigene vaccination was developed by Whitton and Oldstone (Whitton and Oldstone 1989). They showed that short a DNA segment (minigene) encoding a known peptide epitope derived from lymphocytic choriomeningitis virus (LCMV) could be incorporated into a recombinant vaccine and produce protective immunity against the virus (Klavinskis *et al.* 1990). Similar principals have also been applied to bacterial immunisation (An *et al.* 1996) and cancer immunotherapy (Nestle *et al.* 1998; Rosenberg *et al.* 1998).

The minigene approach may confer certain advantages over other immunisation strategies. Vaccines that deliver entire antigen proteins may induce an immune response against a single immunodominant epitope only, with subdominant epitopes being ignored. An immune response directed at a narrow repertoire of epitopes may allow cancer cells to evade immune destruction through antigenic variation and immune tolerance (Maryanski *et al.* 1982; Sercarz *et al.* 1993); and selection of subdominant epitopes has been proposed as a means of overcoming this type of immune evasion. Furthermore, the generation of peptide sequences that are poorly immunogenic but which outcompete other more immunogenic peptides has been suggested as a means by which viruses might escape CTL attack (Bertoletti *et al.* 1994). The minigene approach abrogates this problem.

There is also evidence that the cytosolic expression of epitopes may be greatly enhanced when delivered as minigenes rather than embedded within a longer protein (Anton *et al.* 1997). Epitopes were detected in the cytosol at a concentration of 55,000 copies per cell following minigene delivery, compared to just 30 to 1,800 copies per cell following administration of the full-length protein.

As well as targeting optimal epitopes and subdominant epitopes, the minigene approach has the capacity to avoid sequences that are known to be targets for pre-existing T_H- or B-cell responses. It has been suggested that the inclusion of such sequences in the vaccine might inhibit effective CTL responses (Apostolopoulos *et al.* 1998; Qin *et al.* 1998), possibly by adversely influencing the dominance of either T_H-1 or T_H-2 responses (Fernando *et al.* 1998).

The use of epitopes rather than the entire parent proteins has the additional advantage of avoiding the potential dangers of delivering genes and proteins that are implicated in neoplastic transformation. Mutant p53, ras, bcr/abl and HER2/neu are examples of oncoproteins whose cancer specificity makes them attractive targets for immunotherapy, but which would be hazardous if their entire functional gene was delivered in a recombinant viral vaccine.

Multi-epitope vaccination strategies aim to deliver several immune epitopes derived from one or more antigens simultaneously. This technique was developed using a vaccinia virus to deliver multiple epitopes derived from lymphocytic choriomeningitis virus (LCMV) (Whitton *et al.* 1993); minigenes encoding different epitope peptides were joined in a "string of beads" fashion. The delivery of multiple epitopes allowed vaccination against a number of antigenic targets simultaneously where the limited capacity of the vaccinia vector would have prevented the delivery of several whole antigens. Multi-epitope vaccines have been shown to induce CTL responses in transgenic mice against HIV (Woodberry *et al.* 1999) and hepatitis B epitopes (Loirat *et al.* 2000).

The multi-epitope approach has also been applied to bacterial vaccination, and to vaccination against multiple organisms simultaneously (An and Whitton 1997). Successful processing and presentation of all encoded epitopes has been confirmed (Thomson *et al.* 1995), and the simultaneous presentation of multiple epitopes does not appear to be attended by problems of epitope suppression. However, the emergence of dominant epitopes has been reported in multi-epitope vaccines, highlighting the importance of epitope selection and vaccination protocols (Palmowski *et al.* 2002). Class-I and class-II epitopes have been delivered in the same vaccine construct, and were shown to be both expressed and recognised by T-cell hybridomas (Thomson *et al.* 1995).

Anti-tumour responses have been observed following multi-epitope vaccinations in melanoma (Mateo *et al.* 1999), and a vaccine containing human T-cell epitopes from PSA has been used to produce CTL responses both *in vitro* and *in vivo* using transgenic mice (Correale *et al.* 1998). There is evidence that this approach can provide superior immune protection against tumour challenge than that observed with irradiated whole-cell vaccines (Toes *et al.* 1997).

Induction of immunity against preselected T-cell epitopes has been shown to induce protective anti-tumour immunity (Minev *et al.* 1994), but can also induce tolerance and therefore promote tumour growth (Toes *et al.* 1996; Toes *et al.* 1996). It has been proposed that the simultaneous delivery of a number of epitopes derived from one or more antigens might overcome this difficulty (Toes *et al.* 1997).

The diversity of MHC phenotypes can limit many target-specific vaccines to relatively small populations. A multi-epitope vaccine with the ability to raise CTL responses restricted by multiple HLA alleles would clearly be desirable, and although

the majority of epitope identification has been concentrated on the most common phenotypes, such as HLA-A2, this remains a theoretical possibility. Immunisation against multiple MHC alleles has been shown to be practical and effective in transgenic mice (Whitton *et al.* 1993).

In this study, vaccine construction was by serial addition of DNA using PCR. Pfu DNA Polymerase was used in this application. In addition to catalysing the polymerisation of nucleotides into duplex DNA in the 5′ to 3′ direction, this enzyme also possesses 3′ to 5′ exonuclease (proof-reading) activity, allowing excision of base misinsertions that may occur during polymerisation. In comparison to other DNA polymerases (such as Taq), Pfu DNA Polymerase therefore exhibits a very low error rate. As single base errors would be highly detrimental in this study, such high-fidelity synthesis was deemed essential for these experiments.

For most applications, PCR is an extremely robust procedure. However, particular DNA regions occasionally prove difficult to amplify by PCR, requiring optimisation of the reaction conditions. For example, DNA rich in guanine (G) and cytosine (C), or which forms secondary structures can result in poor yield of the expected PCR product. Relatively little alteration of the PCR conditions was required in the above reactions although the addition of dimethylsulphoxide (DMSO) was useful in improving the yield and specificity. DMSO facilitates strand separation by disrupting base pairing (Frackman *et al.* 1998).

The addition of the final 24 bases to the class-II HEL vaccine proved problematic. Despite alterations to the DMSO and magnesium concentrations of the PCR reaction, the final primer addition was unsuccessful. This was also independent of the annealing temperature of the reaction. It was concluded that the primer was forming a

secondary DNA structure that prevented annealing and extension, and three shorter primers were therefore constructed that comprised the same nucleotide sequence, as shown below in figure 3.17. The addition of much shorter DNA sequences allowed the reactions to proceed without difficulty.

Original primer	TAA CCG TCC TTC TCC TCA...GTG CGG CCG ATA GTC TGG TAG ATC
Alternative primers	
primer #1	TAA CCG TCC TTC TCC TCA...GTG CGG CCG ATA
primer #2	TCC TCA GTG CGG CCG ATA...GTC TGG TAG
primer #3	CCG ATA GTC TGG TAG ATC...AGA TCT

Figure 3.17: Alternative primers designed to overcome failure of PCR reaction.
The addition of the final primer to the class-II construct presented problems. Failure of the PCR reaction necessitated the addition of the final nucleotides in three smaller instalments. The alternative primers are given, along with the original, which had failed.

PCR products resulting from pfu DNA polymerase reactions are "blunt" ended, in contradistinction to products produced by taq polymerase reactions, which characteristically add a single deoxyadenosine (A) to the 3' ends of the resulting PCR products (Campbell 1996). The zero blunt-II TOPO PCR cloning kit was therefore used to insert the vaccine constructs into the TOPO plasmid (see figure 3.18).

The TOPO vector contains ampicillin and kanamycin resistance markers, a β-galactosidase reporter gene, T7 promoter and EcoR-I restriction sites flanking the PCR insertion site. The ampicillin and kanamycin resistance inserts allow for selection of bacterial colonies that take up the vector plasmid following transformation. The T7 promoter region in the vector allows for sequencing of the DNA sequence of the insert. The EcoR I sites flank the insertion position enabling the insert to be easily removed by EcoR I restriction enzymes. The appendix contains a detailed "map" of this plasmid.

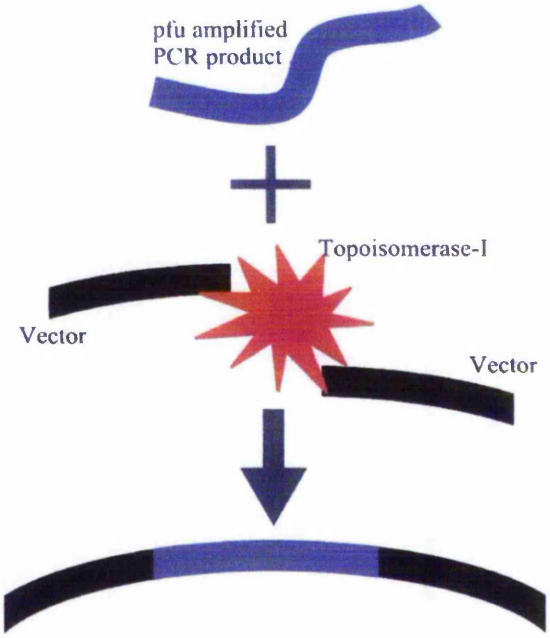


Figure 3.18: pCR® Blunt-II TOPO vector.

The multiepitope DNA construct was transferred from the TOPO plasmid into the PIMJ28 plasmid vector in order to facilitate the insertion of the multiepitope DNA into DISC-HSV. PIMJ28 contains additional DNA essential for the successful expression of the multiepitope construct, and is illustrated schematically in figure 3.19. The HindIII and Xba1 restriction sites are shown, indicating the position at which the DNA insert was included.

An ampicillin-resistance gene is included, allowing selection of *E.Coli* containing the plasmid. RNAII serves as a primer for plasmid DNA replication, and its inclusion results in an increase in plasmid copy number. These two components are only necessary during the propagation of plasmid in the bacterial host, and are subsequently removed during the Pac1 digestion step immediately preceding insertion into the DISC-HSV DNA backbone.

Other components of PIMJ28 are carried along with the insert DNA during ligation with DISC-HSV, including upstream and downstream CMV promoters. A polyadenylation sequence, derived from the SV40 virus facilitates the expression of the virally encoded DNA. Maturation of messenger RNAs by polyadenylation protects them from exonuclease activity and is also important for transcription termination, for export of the mRNA from the nucleus, and for translation. Enhanced green fluorescent protein (eGFP) is a reporter gene, allowing detection of plasmid/viral DNA expression by cells. The long terminal repeat (LTR) from Rous sarcoma virus (RSV) is a powerful viral transcription promoter.

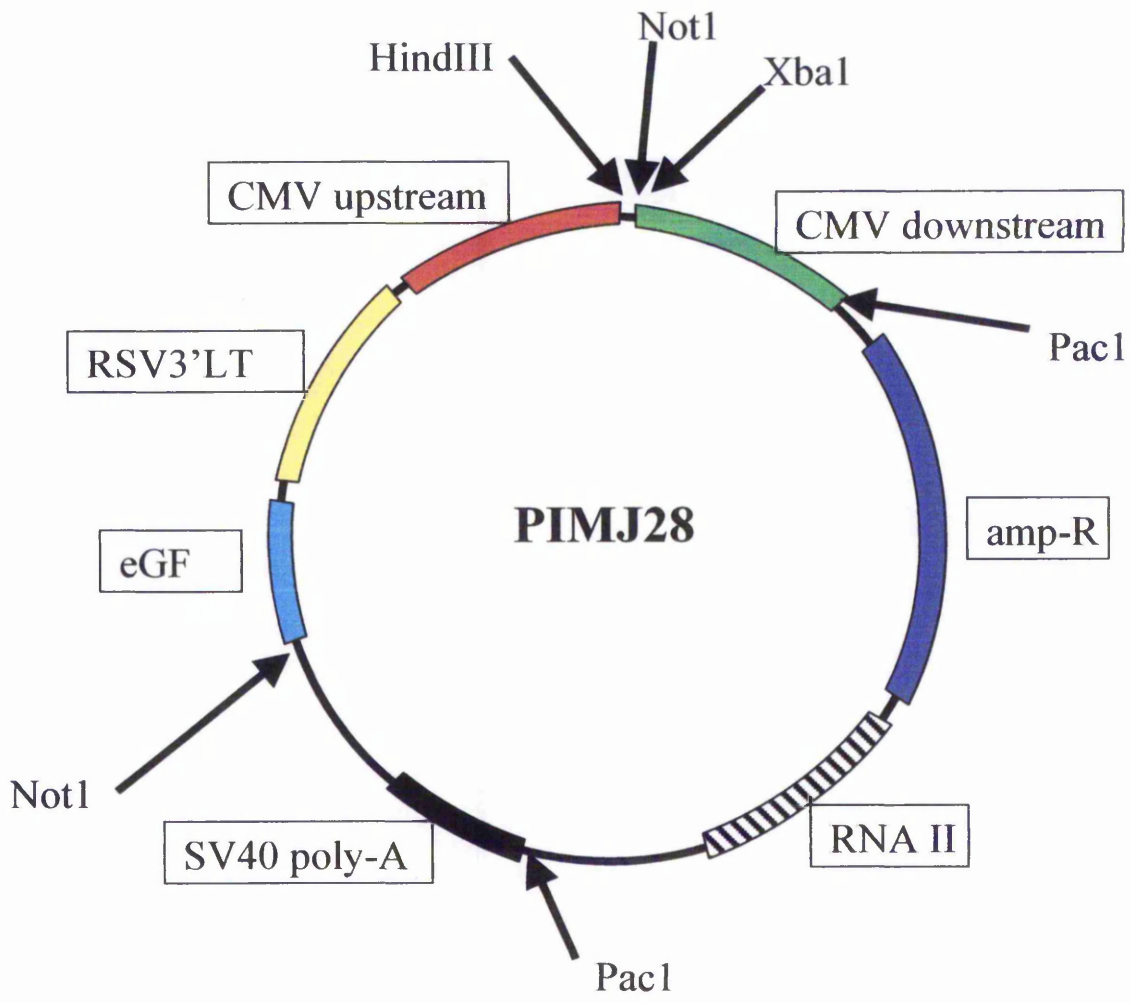


Figure 3.19: Plasmid map for PIMJ28 shuttle vector.

Arrows indicate the positions of restriction enzyme sites. Other components include an ampicillin resistance gene (Amp-R), a primer for plasmid DNA replication (RNA II), a polyadenylation sequence (SV40 poly-A), the long terminal repeat from Rous sarcoma virus (RSV3'LTR) and CMV promoters. See text for full details.

Difficulties were experienced in sequencing the insert DNA derived from DISC-HSV. Repeated failures of the sequencing reaction were attributed to contamination of the template DNA. Template DNA was obtained from PCR amplification of the insert in the DISC-HSV genome, raising the possibility of residual primers, dNTPs etc. in the

final DNA solution. A variety of methods of DNA clean-up were therefore evaluated, including the gel purification and phenol-chloroform methods described previously. The latter appeared to give the optimum results, and allowed confirmation of the correct DNA sequence of the multi-epitope insert. Attempts to sequence the insert directly from the virus DNA failed, probably due to the size of the viral genome. Folding of large DNA strands may inhibit the binding of primers in certain positions.

The construction of both class-I and class-II test vaccines was ultimately successful, with propagation of DISC-HSV containing the construct DNA and confirmation of the correct nucleotide sequences. Although these vaccines had not been tested during the time frame of this study, further work will continue along these lines. Studies in transgenic mice will allow the ability of the viruses to stimulate epitope-specific immune responses *in vivo* to be investigated. These would follow a protocol similar to that described in the earlier chapter.

Once these experiments have been satisfactorily conducted, it will be possible to design and construct similar vaccines based on prostate cancer epitopes, including those described in the preceding chapters, such as PAP.135. Although further studies are required, this research provides the basis for the development of novel immunotherapy strategies against prostate cancer.

Identification of prostate specific Cytotoxic T-Lymphocyte (CTL) epitopes

Contents

Overview

The various vaccination strategies used in cancer immunotherapy have been discussed (see chapter 1). Vaccines directed at specific immune epitopes, whether in the form of dendritic cell vaccines (Burch *et al.* 2000), peptide vaccines (Slovin *et al.* 1999) or DNA vaccines (Mincheff *et al.* 2000), all require the identification of antigenic epitopes capable of stimulating the proliferation of cytotoxic T-lymphocytes (CTL) that are able to destroy antigen bearing cancer cells.

The use of approaches that direct the immune response to CTL epitopes rather than complete antigens may confer certain advantages. The immune response can be focussed towards epitopes that are known to be capable of stimulating CTL-mediated cytotoxicity (Disis *et al.* 1996; Kim *et al.* 1998). Epitopes derived from proteins implicated in neoplastic transformation can be used safely (Moss *et al.* 1996), whereas the administration of the entire parent protein would be potentially hazardous. The identification of CTL epitopes is also important in the development of multi-epitope vaccines, which allow multiple antigens to be targeted simultaneously (Thomson *et al.* 1996; Kawashima *et al.* 1998).

These experiments aimed to identify peptides derived from known prostate cancer associated proteins that could successfully bind to HLA-A2 molecules, and therefore act as potential targets for CD8+ cytotoxic T-lymphocytes. The identification of suitable target antigens is based on cancer and tissue specificity. Candidate HLA-A2*0201 binding motifs derived from these antigens were identified using internet-based epitope prediction algorithms (Rammensee *et al.* 1999), which analyse peptides for the presence of certain amino acid residues that favour MHC binding (Rammensee

et al. 1993; Ruppert *et al.* 1993). The immunogenicity of these predicted motifs was then ascertained experimentally.

The immunogenicity of CTL epitopes largely correlates with their ability to bind MHC molecules, with most strong CTL inducers being strong binders (Sette *et al.* 1994). MHC binding affinity was determined by the coincubation of putative class-I epitopes with TAP-deficient T2 cells (*vide infra*); thus, avid HLA-A2 binding peptides were selected for further study.

Introduction

Methods for the identification of epitopes derived from cancer-associated antigens have been described in chapter 1 ("Introduction"). These include peptide elution, lymphocyte proliferation assays using tumour cDNA libraries and peptide sequence screening. This chapter describes the use of the latter to identify potential CTL epitopes derived from prostate cancer antigens.

Mathematical algorithms can be used to identify peptides derived from a given antigen that have a strong predicted binding affinity for MHC class-I molecules (Rammensee *et al.* 1999). Analysis of peptides that are known to bind MHC with high affinity has identified patterns within their amino acid sequences. MHC class-I binding peptides are typically 8-10 amino acids in length, and the presence of certain amino acids in key "anchor" positions has a significant influence on binding affinity. The amino acids that act as strong anchor residues for some of the more common human class-I haplotypes are given in table 4.1. The amino acids occupying other positions have further influences.

Epitopes capable of binding HLA-A2 are of particular interest, as this haplotype is the most common haplotype in humans and occurs in over 50% of individuals. Therefore, vaccines using such targets are most likely to be effective in a large proportion of patients.

HLA allele	Amino-acid position			References
	2	3	9/10	
A1	**TMS** AS	**DE**	**Y**	(DiBrino *et al.* 1994; Kubo *et al.* 1994)
A2	**LM** IVAT		**LIV** AM	(Rammensee *et al.* 1993; Ruppert *et al.* 1993; Kast *et al.* 1994; Kubo *et al.* 1994)
A3	**LMIVSATF** CGD		KRY A	(DiBrino *et al.* 1994; Kubo *et al.* 1994)
A11	**MLVSATGN** CDF		**K** R	(Kubo *et al.* 1994)
A24	**YF** M		**FLIW**	(Kubo *et al.* 1994)

Table 4.1: Anchor residues favouring HLA class-I binding

Testing MHC binding affinity: T2 assay

In 1985, Salter *et al* described a lymphoblastoid cell-line, designated 174.CEM.T2 (also known as T2) that exhibited a deficiency in MHC class-I expression on the cell surface despite synthesizing normal HLA-A2 heavy chains and $ß_2$-microglobulin (Salter *et al.* 1985; Salter and Cresswell 1986). T2 cells lack the TAP-1 and TAP-2 genes located within the MHC class-II region of chromosome 6, which encode the Transport Associated Proteins (TAP) necessary for the transport of oligopeptides from the cytosol into the endoplasmic reticulum, and it is known that TAP-1 and TAP-2 are essential for the processing and presentation of endogenous MHC class-I peptides on the cell-surface, as described in chapter 1 ("Introduction"). Hence, the amount of MHC class-I molecules expressed on the surface of T2 cells is very low because

empty complexes (ie. MHC class-I/ ß$_2$-microglobulin) presented on the cell-surface are unstable and rapidly recycled.

Although endogenous peptides are not presented by T2 cells, if an exogenous peptide with high HLA-A2 binding affinity is added to cells in culture then it may bind to empty complexes and stabilise them on the cell-surface (Cerundolo *et al.* 1990) (Anderson *et al.* 1991). This phenomenon can be detected and quantitated by immunostaining of T2 cells for the presence of HLA-A2 and analysis by flow cytometry. In this manner, T2 cells have been used in a numerous assays to determine the binding affinities of motifs from a variety of peptides (Nijman *et al.* 1993; Drijfhout *et al.* 1995).

An alternative method employs purified MHC class-I molecules to ascertain the binding affinities of test peptides. MHC class-I can be isolated from cell lysates using immunoaffinity columns. Radio-labelled peptides are then incubated with MHC class-I and β_2-microglobulin in order to allow binding to occur (Olsen *et al.* 1994). The relative affinity of binding can be ascertained using a gamma-counter. Similar techniques have employed various other methods of peptide labelling (eg. FITC or antibody labelling). The T2-binding assay described here enjoys the advantage of being methodologically simpler, and is a robust and reproducible assay for the identification of strong MHC class-I binding peptides.

Results

Identification of candidate prostate cancer target antigens

Potential target antigens were identified using a number of criteria. Consistent expression of antigen by prostate cancer tissue was vital. Prostate specificity was also deemed to be essential, as damage to normal tissues expressing a target antigen would limit the clinical tolerability of any vaccine. However, expression of antigen in tissues non-essential for life (eg. breast, testis), exclusively female tissues (eg. placenta, ovary) or other cancers (eg. breast carcinoma) were considered to be acceptable. The upregulation of antigen expression in prostate cancer and its expression in all grades and stages of the disease were highly desirable.

Table 4.2 shows candidate antigens for prostate cancer immunotherapy, some of which were rejected on the basis of the criteria described above, or because of insufficient available data. Four promising antigens have been identified from this analysis of the literature: Prostate Specific Membrane Antigen (PSMA), Prostate Specific Antigen (PSA), Prostatic Acid Phosphatase (PAP) and Prostate Stem-Cell Antigen (PSCA). These are described in more detail below. Other promising antigens, including Prostein and trp-p8, have only been discovered since the current study was completed, but would otherwise have also been included in this list.

Antigen	Expression pattern	References
KSA/ EpCAM	Prostate cancer and PIN	(Poczatek *et al.* 1999)
	Metastatic prostate cancer	(Zhang et al. 1998)
	Luminal borders of normal secretary epithelia	(Zhang et al. 1998; Zhang et al. 1998)
MUC-1	Prostate cancer	(Poczatek *et al.* 1999)
	Metastatic prostate cancer	(Zhang et al. 1998)
	Luminal borders of normal secretary epithelia	(Zhang *et al.* 1998; Zhang *et al.* 1998)
MUC-2	Prostate cancer	(Poczatek *et al.* 1999)
	Metastatic prostate cancer	(Zhang et al. 1998)
	Luminal borders of normal secretary epithelia	(Zhang et al. 1998; Zhang et al. 1998)
PAGE-4	Prostate cancer	(Brinkmann *et al.* 1998)
	Testes, female tissues	(Brinkmann *et al.* 1998)
PAGE-1 /GAGE-7	Prostate cancer	(Chen *et al.* 1998)
	Testes	(Chen *et al.* 1998)
GM-2	Prostate cancer	(Zhang *et al.* 1997; Zhang *et al.* 1998)
	Grey matter of brain	(Zhang *et al.* 1997; Zhang *et al.* 1998)
	Luminal borders of normal secretary epithelia	(Zhang *et al.* 1997; Zhang *et al.* 1998)
Tn, sTN, TF	Prostate cancer	(Zhang *et al.* 1997; Zhang *et al.* 1998)
	Other epithelial cancers	(Zhang *et al.* 1997; Zhang *et al.* 1998)
	Luminal borders of normal secretary epithelia	(Zhang *et al.* 1997; Zhang *et al.* 1998)
HGF/ SF Rc	Prostate cancer	(Di Renzo *et al.* 1991; Pisters *et al.* 1995)
	Liver, stomach, pancreas, GIT	(Di Renzo *et al.* 1991; Prat *et al.* 1991)
5E10	Prostate cancer	(Rokhlin *et al.* 1998)
	No normal tissues?	(Rokhlin *et al.* 1998)
PCTA-1	Prostate cancer	(Su *et al.* 1996)
bcl-2	Prostate cancer	(Colombel *et al.* 1993)
NY-ESO-1	Prostate cancer	(Chen *et al.* 1997; Fossa *et al.* 2004)
	Upregulated in hormone refractory prostate ca.	(Fossa *et al.* 2004)
	Oesophageal SCC	(Chen *et al.* 1997)
	No normal tissues (N-blot)	(Chen *et al.* 1997)
KLK4	Prostate cancer + normal prostate	(Obiezu and Diamandis 2000; Korkmaz *et al.* 2001; Obiezu *et al.* 2002)
	Placenta	(Obiezu and Diamandis 2000)
	Ovarian cancer	(Obiezu *et al.* 2001)
trp-p8	Normal prostate, prostate cancer	(Tsavaler *et al.* 2001; Kiessling *et al.* 2003)
	Other tumours (colon, breast,lung, skin)	(Tsavaler *et al.* 2001; Kiessling *et al.* 2003)
	Some other normal tissues (but low levels)	(Tsavaler *et al.* 2001)
Prostein	Normal prostate and prostate cancer	(Xu *et al.* 2001)
	Other normal tissues negative (RT-PCR, immunohistochemistry, N-blot)	(Xu *et al.* 2001)

Table 4.2: Candidate target antigens for prostate cancer immunotherapy

Antigen	Expression pattern	References
PSMA	Prostate cancer	(Horoszewicz *et al.* 1987; Israeli *et al.* 1994; Zhang *et al.* 1998; Zhang *et al.* 1998)
	Metastatic tissues	(Zhang *et al.* 1998)
	Not normal tissues	(Israeli *et al.* 1994; Zhang *et al.* 1998)
	Vascular endothelium of non-prostatic tumours	(Liu *et al.* 1997)
	Trace amounts in brain, salivary gland, small intestine, kidney, breast carcinoma, TCC	(Horoszewicz *et al.* 1987; Troyer *et al.* 1995; Uria *et al.* 1997; Dumas *et al.* 1999; Gala *et al.* 2000)
PSCA	Prostate cancer (androgen dependent & independent) & PIN	(Reiter *et al.* 1998) (Ross *et al.* 2002)
	Metastatic tissue	(Gu *et al.* 2000) (Ross *et al.* 2002)
	Small amounts in kidney, placenta, GI tract	(Gu *et al.* 2000) (Reiter *et al.* 1998; Bahrenberg *et al.* 2000; Ross *et al.* 2002)
	Pancreas and pancreatic carcinoma	(Argani *et al.* 2001; McCarthy *et al.* 2003)
	Urothelium and TCC	(Bahrenberg *et al.* 2000; Amara *et al.* 2001; Ross *et al.* 2002)
PSA	Prostate cancer	(Ford *et al.* 1985; Nelson *et al.* 1998)
	Breast and breast cancer	(Levesque *et al.* 1995; Yu *et al.* 1996)
	Various cancer cell-lines (ovary, lung, colon, kidney, etc.)	(Levesque *et al.* 1995; Smith *et al.* 1995; Yu *et al.* 1995)
	Trace amounts in cystitis cystica, neutrophils, kidney, urachus, periurethral glands	(Epstein 1993)
PAP	Prostate cancer	(Hoyhtya *et al.* 1987; Lam *et al.* 1989; Nelson *et al.* 1998)
	Not normal tissues	(Hoyhtya *et al.* 1987; Solin *et al.* 1990; Nelson *et al.* 1998)
	Trace amounts in kidney, bone-marrow, breast	(Lam *et al.* 1989)

(Table 4.2: Candidate target antigens for prostate cancer immunotherapy – continued)

Immunohistochemical analysis has demonstrated that PSMA expression is largely restricted to normal and malignant prostate epithelia (Lopes *et al.* 1990; Zhang *et al.*; Zhang *et al.* 1998). The tissue specificity of PSMA was first examined using immunohistochemistry of a variety of normal and malignant tissues. Staining was observed in a variety of prostate specimens, including 11 localised and metastatic cancers (Horoszewicz *et al.* 1987). None of the 26 various non-prostatic tumours nor 120 out of 122 specimens from 28 different normal organs were reactive. Positive

staining has been seen in 2/14 normal kidney specimens (Horoszewicz *et al.* 1987), and in duodenal epithelium, renal tubular epithelium, colonic ganglion cells and benign breast epithelium (Chang *et al.* 1999). Further immunohistochemical evidence of tissue specificity was provided by Zhang, who reported no staining in a large panel of normal and malignant non-prostatic tissues (Zhang *et al.* 1998; Zhang *et al.* 1998), while confirming expression in prostate cancers, including metastases. Although the monoclonal antibody developed by Lui *et al* showed affinity for the vascular endothelium of some non-prostatic tumours (Liu *et al.* 1997), no normal tissues were reactive.

Ribonuclease protection analysis (RPA) has also demonstrated that the expression of PSMA mRNA is prostate specific in human tissues (Israeli *et al.* 1994), and the PSMA glycoprotein was not detected in the majority of non-prostate tissues examined by Western blotting (Troyer *et al.* 1995). However, a low yet significant amount was detected in normal salivary gland, brain and small intestine, raising the possibility of some extra-prostatic expression of the protein. The pattern of staining in normal tissue shown in the latter paper was markedly different to that seen in prostate. Although this could be explained by post-translational modifications to the PSMA protein or atypical glycosylation, it is also highly possible that it is due to non-specific binding by the detection antibody used.

Further evidence of extra-prostatic expression has been found in breast carcinoma (Uria *et al.* 1997), normal and malignant renal tissue (Dumas *et al.* 1999) and transitional cell carcinoma of the bladder (Gala *et al.* 2000) using RT-PCR. PSMA mRNA has also been detected in a variety of normal tissues, including heart, liver, lung, kidney, spleen, and thyroid gland, using RT-PCR (Renneberg *et al.* 1999),

although this data is not quantitative and has not been related to protein expression. Overall, extra-prostatic expression of PSMA in normal male tissues is very limited, especially when compared to expression in prostate cancer, and appears only to be detectable using highly sensitive RT-PCR. The possibility of false positive results and primer non-specificity in these circumstances must be considered.

PSCA was first identified using representational difference analysis (RDA), a PCR-based subtractive hybridisation technique (Reiter *et al.* 1998). mRNA expression was highly up-regulated in both androgen-dependent and -independent prostate cancer xenografts, and this was confirmed using Northern blotting and RT-PCR. A large panel of normal tissues did not express PSCA, although low levels (<1% of that seen in prostate) were expressed in placenta, kidney and small intestine. These findings were confirmed by immunohistochemical analysis with intense staining in clinical specimens of localised and metastatic prostate cancers (Gu *et al.* 2000). Less intense staining was also detected in urothelium, placental trophoblasts, renal collecting ducts and neuroendocrine cells in the stomach and colon, although other normal tissues were negative. Immunostaining of prostate cancer and PIN in a further study was accompanied with staining of normal urothelium (Ross *et al.* 2002). Urothelial expression of PSCA has also been detected using immunohistochemistry and Northern blotting (Amara *et al.* 2001), including expression in transitional cell carcinoma. Serological analysis of gene expression (SAGE) and Northern blotting were also used to detect PSCA in normal urothelium, and epithelia of the oesophagus and stomach (Bahrenberg *et al.* 2000). Subsequent studies have detected PSCA in pancreas and pancreatic carcinoma using SAGE, immunohistochemistry (Argani *et al.* 2001; McCarthy *et al.* 2003) and RT-PCR (Argani *et al.* 2001).

PSA is known to be expressed in prostate tissues and is upregulated in prostate cancer and PIN (Ford *et al.* 1985; Epstein 1993; Nelson *et al.* 1998). Expression has also been detected in breast and breast cancer using RT-PCR (Yu *et al.* 1996) and highly sensitive protein immunofluorometric assays (Levesque *et al.* 1995; Yu *et al.* 1996) (Bodey *et al.* 1997), as well as a variety of other tumour types, including colon, liver, kidney, adrenal and parotid (Levesque *et al.* 1995; Yu *et al.* 1995). A variety of tumour cell-lines including ovary, lung, and leukaemia have been shown to contain PSA mRNA using nested RT-PCR (Smith *et al.* 1995).

Immunostaining for other normal tissues has been reported to be negative (Ford *et al.* 1985), and EST analysis has also confirmed prostate specificity (Nelson *et al.* 1998). Later studies using immunohistochemistry showed some positive staining in salivary glands, pancreas (Elgamal *et al.* 1996), cystitis cystica, neutrophils, kidney, urachus, and periurethral glands, but at levels far less than seen in prostate (Epstein 1993). RT-PCR demonstrated PSA mRNA in thyroid tissue in one study (Magklara *et al.* 2000). Although PSA is not entirely prostate specific, its expression in normal male tissues is negligible and it remains a promising target for immunotherapy.

PAP is strongly expressed in prostate and prostate cancer as determined by immunohistochemistry (Hoyhtya *et al.* 1987; Lam *et al.* 1989) and EST analysis (Nelson *et al.* 1998). Trace immunostaining was seen in a small proportion of bone marrow, breast and kidney specimens (Lam *et al.* 1989). However, studies failed to demonstrate PAP in other non-prostatic sites (Hoyhtya *et al.* 1987; Nelson *et al.* 1998), and prostate specificity has been confirmed using Northern blotting (Solin *et al.* 1990). This protein is one of the most promising targets for prostate

immunotherapy due to its prostate specificity and consistent expression in over 80% of cancer specimens (Ljung *et al.* 1997).

Identification of known class-I epitopes

Previous studies have identified a number of HLA-A2*0201 restricted CTL epitopes derived from PSMA, PAP, PSCA and PSA. Table 4.3 illustrates class-I epitopes derived from these prostate cancer antigens that have been published in peer-review journals.

Antigen	Epitope		Reference
PSMA	347-356:	HSTNGVTRIY	(Corman *et al.* 1998)
	557-566:	ETYELVEKFY	(Corman *et al.* 1998)
	4-12:	LLHETDSAV	(Tjoa *et al.* 1996)
	711-719:	ALFDIESKV	(Tjoa *et al.* 1996)
	27-35:	VLAGGFFLL	(Lu and Celis 2002)
PAP	6-14:	LLLARAASL	(Peshwa *et al.* 1998)
	299-307:	ALDVYNGLL	(Peshwa *et al.* 1998)
	30-38:	VLAKELKFV	(Peshwa *et al.* 1998)
PSCA	7-15:	ALLMAGLAL	(Dannull *et al.* 2000; Matsueda *et al.* 2004)
	14-22:	ALQPGTALL	(Dannull *et al.* 2000; Kiessling *et al.* 2002)
	21-30:	LLCYSCKAQV	(Matsueda *et al.* 2004)
	115-123:	LLLWGPGQL	(Dannull *et al.* 2000)
	105-113:	AILALLPAL	(Kiessling *et al.* 2002)
PSA	170-178:	KLQCVDLHV	(Correale *et al.* 1997; Xue *et al.* 1997)
	165-174:	FLTPKKLQCV	(Correale *et al.* 1997)
	178-187:	VISNDVCAQV	(Correale *et al.* 1997)
	186-194:	QVHPQKVTK	(Correale *et al.* 1998)
	92-101:	VSHSFPHPLY	(Corman *et al.* 1998)

Table 4.3: Published class-I epitopes derived from prostate cancer antigens

The positions of epitopes within the parent protein are given.

Identification of novel epitopes: MHC binding prediction algorithms

Through analysis of the positions of amino acids within a peptide sequence, it is possible to predict which oligopeptides derived from a longer protein will bind MHC molecules most efficiently (Rammensee *et al.* 1993; Ruppert *et al.* 1993). Mathematical algorithms have been developed that ascribe a relative numerical value to any given peptide that reflects that peptide's likely affinity for the MHC type under investigation (Rammensee *et al.* 1999). Such algorithms were applied to selected prostate cancer antigens in order to identify possible class-I binding epitopes for the more common MHC haplotypes.

Table 4.4 shows a selection of HLA-A2*0201 class-I epitopes derived from prostate cancer antigens predicted by two algorithms: SYFPEITHI and bimas. Each gives results in arbitrary units, and so direct comparisons between the two are not possible. Instead, for each database, the relative binding affinities of the oligopeptides should be examined. Epitopes that scored well on both algorithms (PSMA.168, PAP.135, PAP.284), or which scored particularly highly on one or the other (PSMA.34, PAP.15) were selected for further study. The epitopes PAP.30 and PAP.299 have been investigated previously (Peshwa *et al.* 1998), and were also included. Figure 4.1 shows the complete amino acid sequences of PSA, PSMA, PAP and PSCA, and illustrates the positions of selected predicted epitopes within the parent protein.

Antigen	GenBank® accession number	Epitope	Start Position	Predicted binding affinity	
				SYFPEITHI	bimas
PSMA	AAC83972.1	LLHETDSAV	4	25	485
		VLAGGFFLL	27	27	400
		LLGFLFGWFI	34	-	2780
		GMPEGDLVYV	168	27	2605
		LLQERGVAYI	441	28	920
		LMYSLVHNL	469	27	-
		KLGSGNDFEV	514	-	1412
		MMNDQLMFL	663	24	1360
PAP	AAA60022.1	ALASCFCFFC	15	-	602
		VLAKELKFV	30	26	1496
		TLMSRMTNL	112	21	182
		ALFPPEGVSI	122	27	-
		ILLWQPIPV	135	24	438
		GLHGQDLFGI	196	26	139
		IMYSAHDTTV	284	23	273
		ALDVYNGLL	299	23	-
PSCA	AAC39607.1	VLLALLMAGL	4	26	309
		LLCYSCKAQV	21	23	118
		QLGEQCWTA	43	-	153
		ALLPALGLL	108	30	-
PSA	AAA60192.1	VLVHPQWVL	53	22	112
		VILLGRHSL	72	26	-
		ALPERPSLYT	235	19	736

Table 4.4: Candidate class-I epitopes based on predicted HLA-A2 binding affinities.

(a)
MWNLLHETDS AVATARRPRW LCAGALVLAG GFFLLGFLFG WFIKSSNEAT
NITPKHNMKA FLDELKAENI KKFLHNFTQI PHLAGTEQNF QLAKQIQSQW
KEFGLDSVEL AHYDVLLSYP NKTHPNYISI INEDGNEIFN TSLFEPPPPG
YENVSDIVPP FSAFSPQGMP EGDLVYVNYA RTEDFFKLER DMKINCSGKI
VIARYGKVFR GNKVKNAQLA GAKGVILYSD PADYFAPGVK SYPDGWNLPG
GGVQRGNILN LNGAGDPLTP GYPANEYAYR RGIAEAVGLP SIPVHPIGYY
DAQKLLEKMG GSAPPDSSWR GSLKVPYNVG PGFTGNFSTQ KVKMHIHSTN
EVTRIYNVIG TLRGAVEPDR YVILGGHRDS WVFGGIDPQS GAAVVHEIVR
SFGTLKKEGW RPRRTILFAS WDAEEFGLLG STEWAEENSR LLQERGVAYI
NADSSIEGNY TLRVDCTPLM YSLVHNLTKE LKSPDEGFEG KSLYESWTKK
SPSPEFSGMP RISKLGSGND FEVFFQRLGI ASGRARYTKN WETNKFSGYP
LYHSVYETYE LVEKFYDPMF KYHLTVAQVR GGMVFELANS IVLPFDCRDY
AVVLRKYADK IYSISMKHPQ EMKTYSVSFD SLFSAVKNFT EIASKFSERL
QDFDKSNPIV LRMMNDQLMF LERAFIDPLG LPDRPFYRHV IYAPSSHNKY
AGESFPGIYD ALFDIESKVD PSKAWGEVKR QIYVAAFTVQ AAAETLSEVA

(b)
MRAAPLLLAR AASLALASCF CFFCWLDRSV LAKELKFVTL VFRHGDRSPI
DTFPTDPIKE SSWPQGFGQL TQLGMEQHYE LGEYIRKRYR KFLNDSYKHE
QVYIRSTDVD RTLMSRMTNL AALFPPEGVS IWNPILLWQP IPVHTVPLSE
DQLLYLPFRN CPRFQELESE TLKSEEFQKR LHPYKDFIAT LGKLSGLHGQ
DLFGIWSKVY DPLYSESVHN FTLPSWATED TMTKLRELSE LSLLSLYGIH
KQKEKSRLQG GVLVNEILNH MKRATQIPSY KKLIMYSAHD TTVTGLQMAL
DVYNGLLPPY ASCHLTELYF EKGEYFVEMY YRNETQHEPY PLMLPGCSPS
CPLERFAELV GPVIPQDWST EVMTTNSHQG TEDSTD

(c)
MKAVLLALLM AGLALQPGTA LLCYSCKAQV SNEDCLQVEN CTQLGEQCWT
ARIRAVGLLT VISKGCSLNC VDDSQDYYVG KKNITCCDTD LCNASGAHAL
QPAAAILALL PALGLLLWGP GQL

(d)
MWVPVVFLTL SVTWIGAAPL ILSRIVGGWE CEKHSQPWQV LVASRGRAVC
GGVLVHPQWV LTAAHCIRNK SVILLGRHSL FHPEDTGQVF QVSHSFPHPL
YDMSLLKNRF LRPGDDSSHD LMLLRLSEPA ELTDAVKVMD LPTQEPALGT
TCYASGWGSI EPEEFLTPKK LQCVDLHVIS NDVCAQVHPQ KVTKFMLCAG
RWTGGKSTCS GDSGGPLVCN GVLQGITSWG SEPCALPERP SLYTKVVHYR
KWIKDTIVAN P

Figure 4.1: Sequences of selected prostate cancer antigens.

Amino acid sequences of four prostate cancer antigens are given. The positions of candidate class-I epitope peptides are illustrated. (a) PSMA (GenBank™ accession number AAC83972.1). (b) PAP (GenBank™ accession number AAA60022.1). (c) PSCA (GenBank™ accession number AAC39607.1). (d) PSA (GenBank™ accession number AAA60192.1).

Homology search for candidate epitopes

All predicted epitopes were cross-checked for homology with other known human proteins to ensure that the peptides derived from prostate cancer antigens did not coexist within other proteins, potentially impairing the prostate-specificity of the epitopes chosen. Each of the selected epitopes was analysed using the BlastiMer tool provided on the website of the National Center for Biotechnology Information (EpiVax, Providence, RI). Any sequence that was similar to a peptide component of another protein in the human proteome (accessible and published to date) was excluded from the study set.

For each of the candidate epitopes used in this research, no homology with any other protein was detected (data not shown). A similar search was performed to exclude the cross-reactivity of candidate peptides with murine proteins. Again, no homology was detected.

Identification of novel epitopes: MHC binding assay

The ability of test peptides to bind to HLA-A2 molecules was ascertained using a T2 binding assay. T2 cells are TAP deficient and therefore unable to present endogenously processed peptides on the cell-surface in conjunction with MHC. Empty MHC molecules are inherently unstable, but their rapid recycling from the cell-surface may be prevented by binding to suitable peptides present in the extracellular milieu. Therefore, T2 cells were incubated with increasing concentrations of peptide, allowing time for MHC binding to take place. Successful binding was measured by immunostaining for the presence of HLA-A2 molecules on the cell surface. The fluorescent intensity of each cell is proportional to the amount of

antibody bound to the cell, which in turn is related to the amount of HLA-A2 stabilised on the cell surface by the test peptide. Flow cytometry was used to quantitate the fluorescence and hence the HLA binding affinity of test peptides, as well as positive and negative controls.

The relative binding affinities (equivalent to the mean fluorescence ratio) for the peptides tested are illustrated in figure 4.2. Each value represents the mean of five separate experiments. Error bars indicate 95% confidence intervals as determined by Student's t-test. Increased staining for HLA-A2 was detected on cells incubated with the positive control peptides (Flu.57 and gp100.154) and four other test peptides: three derived from prostate acid phosphatase (PAP.30, PAP.135, PAP284) and one derived from prostate specific membrane antigen (PSMA.168).

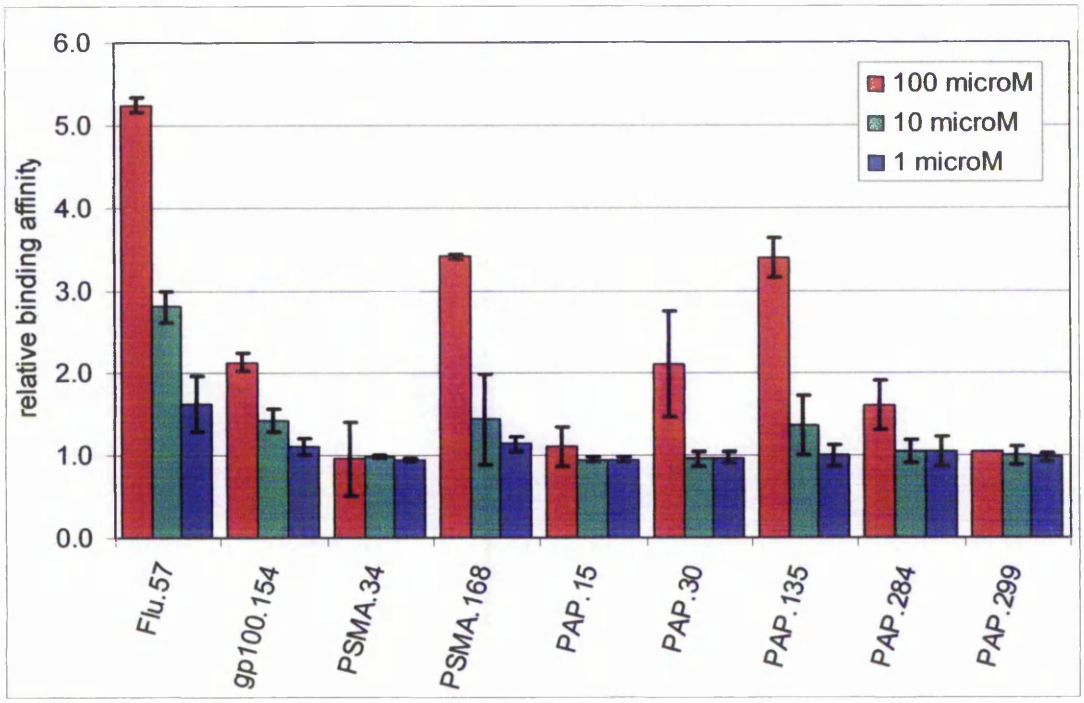


Figure 4.2: Relative HLA-A2 binding affinities of selected prostate antigen-derived peptides.

A T2 binding assay was performed to ascertain the binding affinities of test peptides derived from PAP and PSMA with HLA-A2. Flu.58 and gp100.154 peptides were included as positive controls. T2 cells were incubated in 1, 10 or 100 μM of peptide overnight prior to immunostaining for the presence of HLA-A2 on the cell-surface and analysis by flow cytometry. Moderate binding of peptide to HLA-A2 can be inferred from relative binding affinities of 1.5 to 3.0. Affinities of over 3.0 indicate strong binding. Error bars indicate 95% confidence intervals (Student's t-test).

Discussion

The activation of immune effector cells capable of recognising and destroying prostate cancer cells can be achieved through various mechanisms, and has applications in all stages of the disease. Clinical studies employing whole-cell vaccines in prostate cancer patients have achieved only limited success (Simons *et al.* 1999; Hrouda *et al.* 2000; Eaton *et al.* 2002), possibly because of the simultaneous presentation of large numbers of unselected antigenic determinants to the immune system. Immune targeting using a limited number of epitopes, specifically selected for their ability to induce CTL-mediated tumour lysis, represents an alternative approach; and vaccines based on either APC pulsed with MHC class I peptides (Murphy *et al.* 1999; Murphy *et al.* 1999; Small *et al.* 2000) or peptides alone (Meidenbauer *et al.* 2000) have been investigated with promising preliminary results. However, to date few MHC class I and class II peptides derived from prostate specific proteins have been identified.

Evidence of gene or protein expression by cell populations may be provided by a variety of techniques, including immunohistochemistry, PCR, and northern, Southern, and western blotting. It was concluded from the available data that PSA, PSMA, PAP and PSCA were the proteins that most closely fit the selection criteria. These antigens are upregulated in prostate cancer and expressed in both metastatic and androgen independent cancers. Furthermore, tissue specificity is good, although some limited extraprostatic expression was noted for PSMA and PSA.

PSCA expression in colon, stomach and pancreas may limit its usefulness as an immune target. However, this protein has been included in this report as its extraprostatic expression pattern was not known at the time of these studies and the available data suggested prostate specificity. Other antigens, including some from table 4 may also prove to be suitable target antigens, but insufficient data is currently available on these compounds to support their inclusion in this research.

Studies have indicated the potential for PAP as an immune target both in animal models (Fong *et al.* 1997) and in humans (Fong *et al.* 2001). Furthermore, PAP derived peptides have been used to induce antigen-specific CTL responses in human studies (Peshwa *et al.* 1998). MHC class II epitopes have also been identified that are able to stimulate human T_H responses in vitro (McNeel *et al.* 2001), although the precise haplotype of the MHC molecule to which $CD4^+$ T-cells proliferated was not clearly defined. HLA-A2*0201 is the most common HLA class-I phenotype, and therefore peptides derived from cancer specific antigens that are able to stimulate a CTL reaction though presentation by HLA-A2*0201 represent potentially useful therapeutic agents in cancer immunotherapy.

A number of promising HLA-A2 dependent epitopes derived from PSMA, PSCA, PSA and PAP were identified using prediction algorithms. The algorithms applied by SYFPEITHI and bimas often give conflicting results, as each gives a different degree of importance to certain structural features. Oligopeptides known to act as CTL epitopes or exhibiting strong HLA-A2 binding in previous studies often achieved high scores on both algorithms. For example, the positive control Flu.58 peptide was predicted to be a strong HLA-A2 binder by both algorithms, as were PSMA.168 and PAP.30. The gp100.154 peptide was predicted to bind strongly to HLA.A2 by the

bimas algorithm, but less so by SYFPEITHI, while the PAP.299 peptide scored well on SYFPEITHI but not bimas. This confirms the validity of both methods, and illustrates the merit of using both systems for epitope prediction.

The immunogenicity of CTL epitopes largely correlates with their ability to bind MHC molecules, with most strong CTL inducers being strong binders (Sette *et al.* 1994). The coincubation of putative class-I epitopes with TAP-deficient T2 cells allowed the selection of strong HLA-A2 binding peptides to be selected for further study. This T2 assay identified a number of high affinity peptides derived from the PAP and PSMA antigens. In terms of the relative binding affinity, or mean fluorescence ratio (MFR), peptides with a ratio below 1.5 can be considered to have little or no binding affinity for HLA-A2. Ratios between 1.5 and 3 were considered moderate affinity binders, and a ratio over 3 represented a strong binding affinity for the test peptide (Nijman *et al.* 1993).

The binding affinities of PAP.299 and PAP.30 for HLA-A2 have been described previously (Peshwa *et al.* 1998) following a similar methodology. PAP.299 was also shown to generate peptide-specific $CD8^+$ CTL from the peripheral blood of healthy volunteers. These CTL were able to lyse both peptide-pulsed targets as well as LNCaP, a prostate cancer cell-line known to constitutively express PAP. The ability of PAP.30 to stimulate CTL was not presented, although its binding affinity was similar to that of PAP.299.

Moderate HLA-A2 binding was confirmed for PAP.30 in this study with a MFR of 2.1. Interestingly, we were unable to reproduce the strong HLA-A2 binding described for PAP.299, which may be attributable to small differences in the assay protocol or peptide manufacture. For example, the method described by Peshwa *et al* employs

Hybridoma Serum Free Media (HSFM) rather than RPMI for the T2 assay. Also the method used to dissolve the test peptides, although not described in the Peshwa paper, may have been different, and influenced the binding affinity measured.

Two further PAP derived peptides, PAP.135 and PAP.284 exhibited HLA-A2 binding in this study. PAP.284 demonstrated weak to moderate affinity, with an MFR of 1.6. However, strong binding was observed for PAP.135, with an MFR of 3.4. This was significantly greater than the MFR for the previously reported peptide PAP.30 and was commensurate with the high prediction scores obtained from both algorithms. A PSMA derived peptide PSMA.168, which also scored well using both prediction protocols, showed strong binding to HLA-A2 in the T2 assay. An MFR of 3.3 was obtained. The peptides PSMA.168, PAP.135 and PAP.284 have not been previously reported as HLA-A2 binders and therefore represent novel candidate CTL epitopes.

Although the prediction score for PAP.15 obtained from the bimas algorithm was very high, this peptide exhibited no measurable binding to HLA-A2. Similarly, PSMA.34 scored very highly on bimas and yet did not demonstrably bind to HLA-A2 in this study. Neither peptide scored well using the SYFPEITHI protocol. A comparison of prediction scores and MFR for selected peptides is given in table 4.5.

Other researchers have indicated that co-incubation of T2 cells with both peptide and β_2-microglobulin may increase the avidity of peptide binding (Regner *et al.* 1996; Xue *et al.* 1997; Butterfield *et al.* 2001). Therefore 20μg β_2-microglobulin was also added along with the test peptide in some experiments. This step did not noticeably enhance the performance of the assay for the peptides tested, and was therefore not included in the results analysis (data not shown).

Peptide	SYFPEITHI score	bimas score	measured MFR
flu.58	30	551	5.2
gp100.154	18	316	2.1
PAP.15	-	602	1
PAP.30	26	1496	2.1
PAP.135	24	438	3.4
PAP.284	23	273	1.6
PAP.299	23	1	1
PSMA.34	-	2780	1
PSMA.168	27	2605	3.3

Table 4.5: Predicted and measured HLA-A2 binding affinities of selected peptides

In conclusion, PAP.30, PAP.284, PAP.135 and PSMA.168 have shown reproducible binding to HLA-A2 and merit further study as potential HLA-A2 restricted CTL epitopes. PAP.135 is of particular interest as it is entirely novel, it exhibits strong MHC binding, and it is derived from a highly prostate-specific protein.

Evaluation of CTL epitopes in a transgenic model system

Contents

Overview

Strong HLA-A2-binding peptides were tested for their ability to stimulate a CTL-mediated immune response in HLA-A2.1/K^b transgenic mice, which have been used successfully by many researchers to identify HLA-A2*0201 restricted CTL epitopes (Vitiello *et al.* 1991; Theobald *et al.* 1995; Heukamp *et al.* 2001). CTL cultures were established from peptide-vaccinated mice and an *in vitro* cytotoxicity assay used to determine the ability of CTL to destroy peptide-bearing target cells.

These studies have identified at least one CTL epitope derived from the prostate-specific molecule Prostate Acid Phosphatase (PAP) that shows promise as a target for immunotherapy. Strong HLA-A2 binding was confirmed by T2-binding assay. Following vaccination of HLA-A2 transgenic mice with this test peptide, *ex vivo* CTL cultures were able to destroy peptide bearing target cells, including the human prostate cancer cell-line LNCaP. The effect has been shown to be CTL-mediated by the ability of an anti-HLA-A2 antibody to abrogate the cytotoxicity observed.

Introduction

Chapter 4 described the identification of several peptides that are able to bind to HLA-A2 molecules, and which may therefore be considered as potential epitopes for Cytotoxic T-Lymphocytes (CTL). These experiments aimed to assess the ability of these peptides to induce the proliferation of CTL capable of killing target cells in a peptide specific manner *in vivo*. This was determined initially in an animal model using HLA-A2.1/K^b transgenic C57 black mice. Similar systems have been used successfully by other authors to determine CTL epitope motifs (Vitiello *et al.* 1991; Theobald *et al.* 1995; Heukamp *et al.* 2001).

Lymphocytes, harvested from the spleens of immunised mice and matured *in vitro*, were tested in chromium-release cytotoxicity assays using peptide-pulsed RMAS cells or tumour cell-lines as targets. RMAS/HLA-A2 cells, like T2, are unable to express endogenous peptides on their cell surface. Whereas T2 cells express human MHC class-I molecules, these RMAS have been transfected with a transgenic class-I (HLA-A2.1/K^b) allowing the murine CD8 molecule on the murine CD8+ T-lymphocytes to interact with the syngeneic α-3 domain of hybrid MHC class-I molecule (Vitiello *et al.* 1991). Thus, RMAS provide a more appropriate target cell for cytotoxicity testing in this transgenic model.

In order to characterise the nature of the immune stimulation, the release of the cytokines IL-5 and IFN-γ by cells during maturation was measured using ELISA and the expression of CD4 and CD8 by cells was determined using immunostaining and flow cytometry.

Results

A transgenic mouse model was employed to test the immunogenicity of putative CTL epitopes identified by T2 binding experiments. HLA-A2.1/K^b mice were immunised with test peptides in formulation with a class-II hepatitis-B virus peptide and incomplete Freund's adjuvant (IFA). The mouse spleens were removed and processed to extract splenocytes, which were then allowed to grow in culture in the presence of the test peptide for 5 days.

A 4-hour chromium-release assay was then performed in which target peptide-bearing cells were pulsed with 1.85MBq ^{51}chromium and then co-cultured with effector splenocytes at known ratios of cells. Quantitation of the emission of gamma-radiation by aliquots of culture medium taken from these co-cultures allowed cell lysis to be estimated by using the formula described in the Materials and Methods chapter.

Cytotoxicity in response to known CTL epitopes (positive controls)

The Flu.58 epitope was used as a positive control to confirm the viability of the transgenic system and to optimise the protocols for immunisation, splenocyte culture and cytotoxicity assay. Typical cytotoxicity results are shown in figure 5.1. Splenocyte cultures derived from peptide immunised animals exhibited cytotoxicity against peptide-pulsed RMAS target cells. RMAS cells pulsed with an irrelevant peptide (gp100 in these experiments) were not lysed.

Overall, chromium-release assays demonstrated cytotoxicity in 38% (6/16) of animals following peptide immunisation with flu.58, with a mean of 26% lysis. These data confirmed the ability of peptide immunised animals to produce effector cells capable of destroying RMAS target cells in a peptide specific manner.

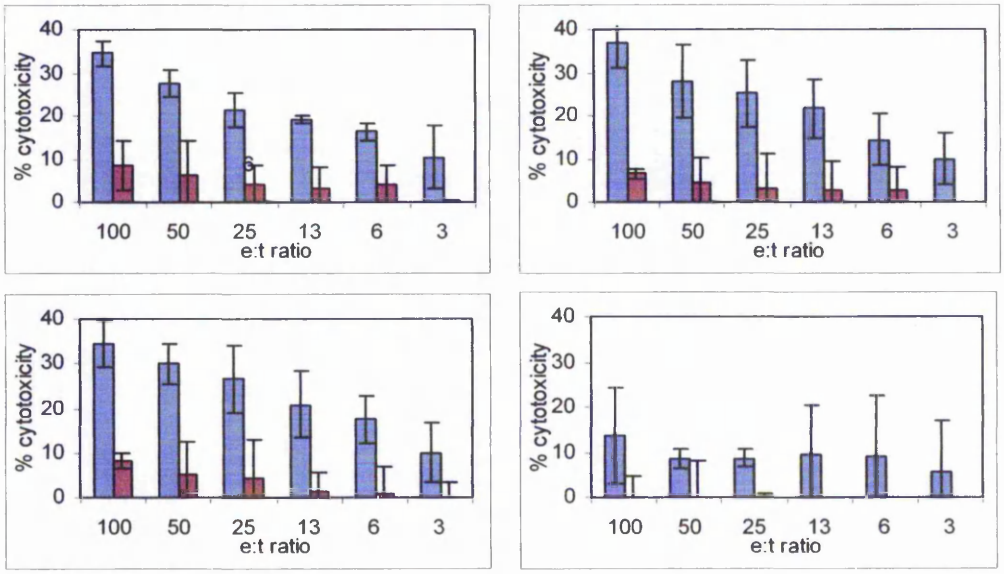


Figure 5.1: Chromium-release assay: immunisation with Flu.58 peptide.

RMAS target cells were pulsed with either Flu.58 (blue bars) or a negative control peptide (gp100.154) (red bars). Significant peptide-specific target lysis was observed in 6/16 mice immunised with Flu.58 (typical results for 4 mice are shown). Error bars indicate 95% confidence intervals (Student's t-test).

In a second set of animals, gp100.154 immunisations were undertaken. In all experiments, lymphocyte cultures produced significant lysis of gp100.154-bearing targets, with a mean of 34% cytotoxicity. Furthermore, effector cells derived from non-immunised (naïve) controls were unable to destroy target cells (see figure 5.2).

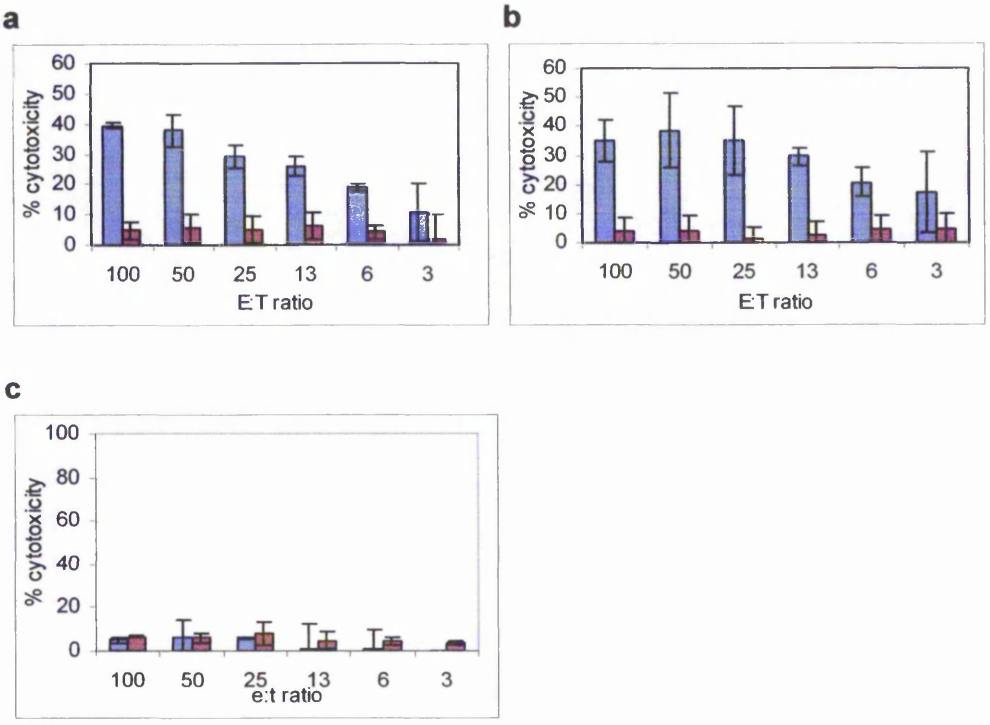


Figure 5.2: Chromium-release assay: immunisation with gp100.154 peptide.

RMAS target cells were pulsed with either gp100.154 (blue bars) or a negative control peptide (Flu.58) (red bars). Significant peptide-specific target lysis was observed in 18 mice immunised with gp100.154 (typical results for 2 mice are shown: **a**, **b**). A naïve (control) mouse produced no significant target lysis (**c**), indicating the requirement for *in vivo* immunisation. Error bars indicate 95% confidence intervals (Student's t-test).

Effector cells from the same gp100.154-immunised animals were also able to destroy FM3 cells, which express gp100 endogenously, whereas LCL-BM control cells were not affected (see Figure 5.3). Results shown are typical of 18 animals and 4 separate experiments.

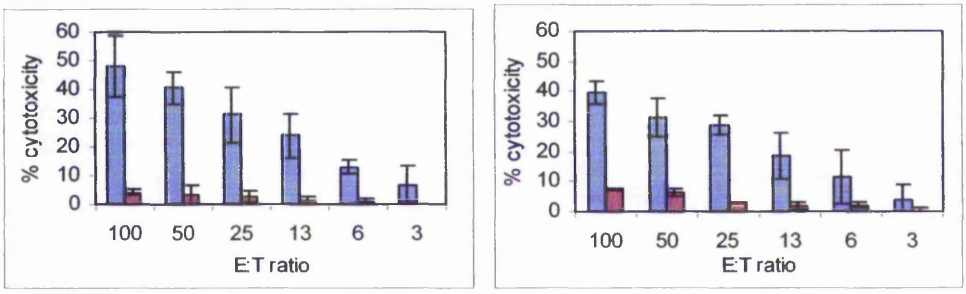


Figure 5.3: Chromium-release assay: immunisation with gp100.154 peptide.

FM3 cells, which endogenously express gp100, were used as targets (blue bars) and LCL-BM cells were employed as control targets (red bars). Significant peptide-specific target lysis was observed in 18 mice immunised with gp100.154 (typical results for 2 mice are shown). Error bars indicate 95% confidence intervals (Student's t-test).

Peptide immunisation using peptides derived from prostate antigens

Figure 5.4 shows typical cytotoxicity results following immunisation with PAP.30: peptide-specific target lysis was demonstrated against PAP.30-pulsed RMAS targets in 57% (8/14) of animals over 3 separate experiments, with a mean of 33% cell lysis.

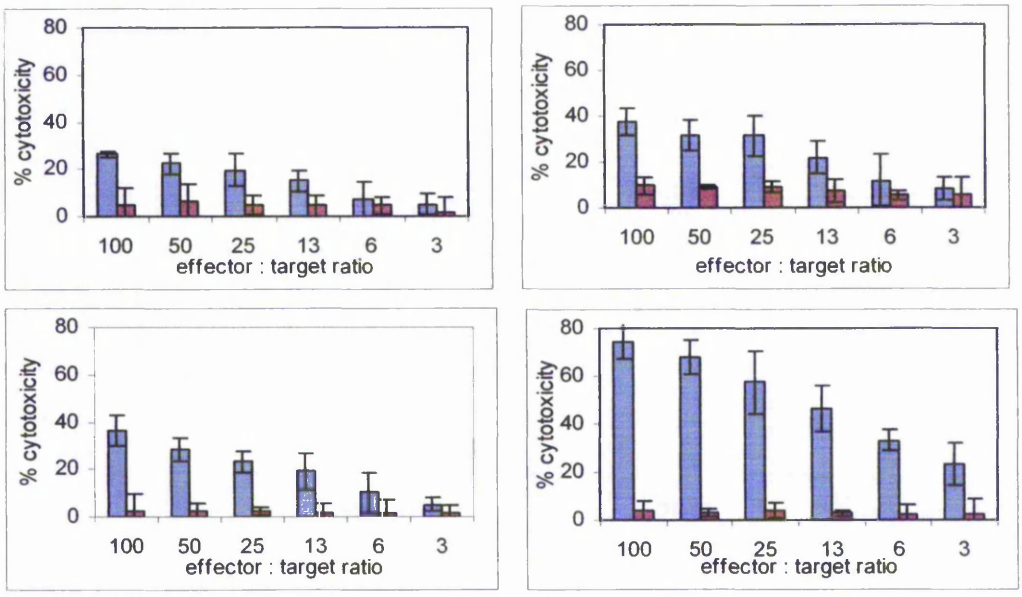


Figure 5.4: Chromium-release assay: immunisation with PAP.30 peptide.

RMAS target cells were pulsed with either PAP.30 (blue bars) or a negative control peptide (Flu.58) (red bars). Significant peptide-specific target lysis was observed in 8/14 mice immunised with PAP.30 (typical results for 4 mice are shown). Error bars indicate 95% confidence intervals (Student's t-test).

PAP.135 is an entirely novel peptide: no HLA binding or cytotoxicity studies have previously been published. Over a total of 5 separate experiments, chromium-release assays demonstrated a mean of 56% peptide-specific cytotoxicity in 96% (25/26) animals immunised with the PAP.135 peptide. Typical results are shown in figure 5.5. Effector cells derived from non-immunised animals produced low levels of lysis of PAP.135 target cells, but significantly less than immunised animals. In four separate experiments, an average of just 17% lysis was observed.

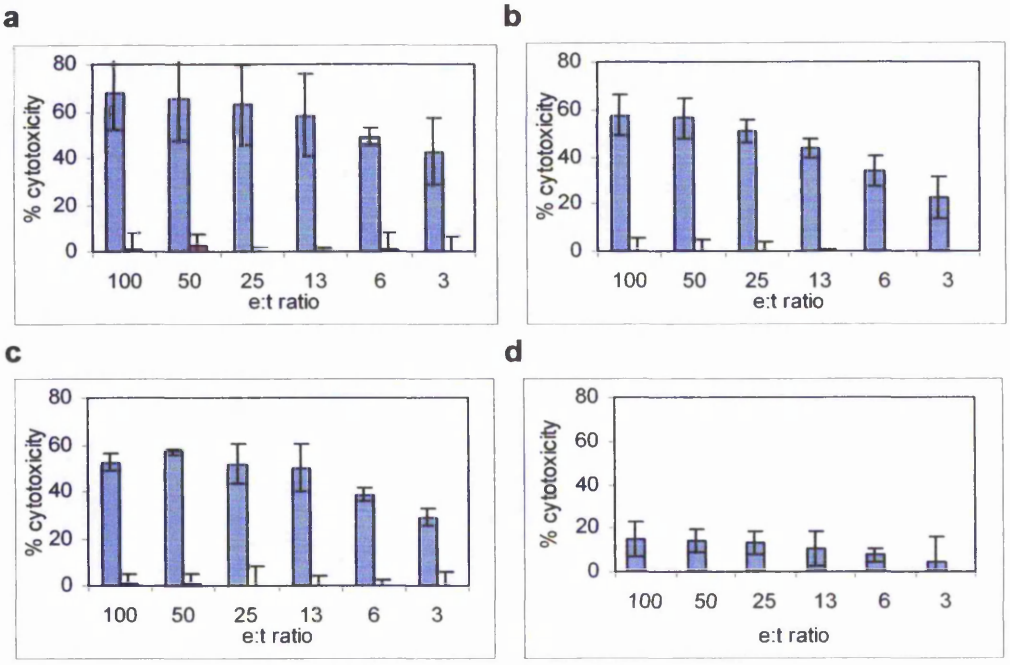


Figure 5.5: Chromium-release assay: PAP.135 peptide.

RMAS target cells were pulsed with either PAP.135 (blue bars) or a negative control peptide (Flu.58) (red bars). Significant peptide-specific target lysis was observed in 25/26 mice immunised with PAP.135 (typical results for 3 mice are shown: **a**,**b**,**c**). A naïve (control) mouse produced minimal target lysis (**d**), indicating the requirement for *in vivo* immunisation. Error bars indicate 95% confidence intervals (Student's t-test).

The ability of splenocyte cultures derived from PAP.135 immunised animals to destroy human prostate cancer cells was investigated using the LNCaP cell-line, which is known to express both HLA-A2 and PAP. In three separate experiments, lysis of LNCaP target cells was exhibited by 93% (14/15) splenocyte cultures with a mean cytotoxicity of 20%. Again, negligible cytotoxicity was produced by effectors from non-immunised animals. Typical results are shown in figure 5.6.

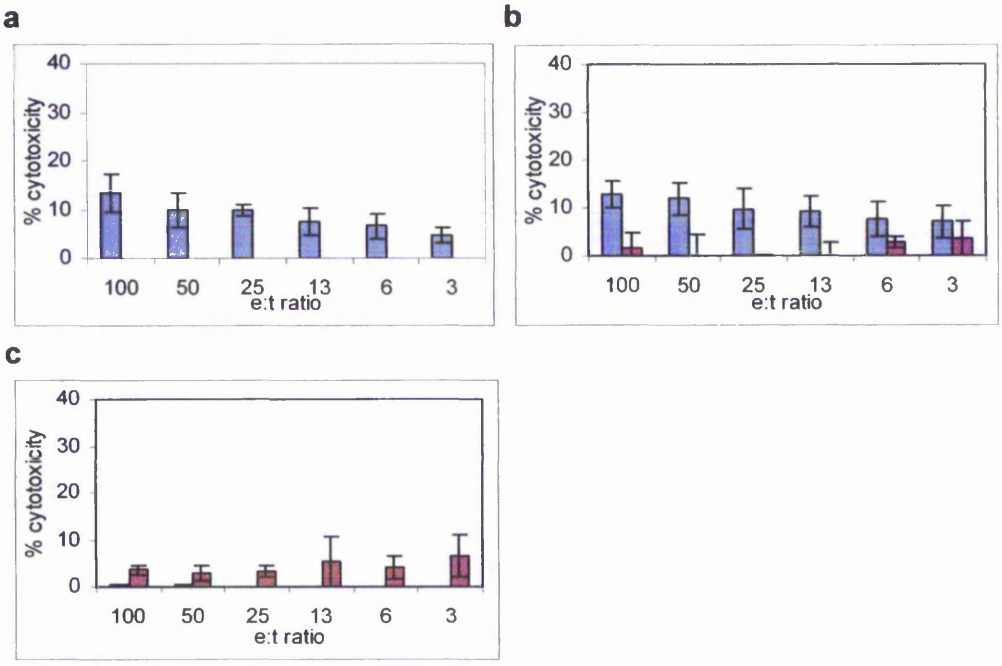


Figure 5.6: Chromium-release assay: immunisation with PAP.135 peptide.

LNCaP cells, which endogenously express HLA-A2 and PAP, were also used as targets (blue bars); LCL-BM cells, which express HLA-A2 but not PAP, were employed as control targets (red bars). Significant peptide-specific target lysis was observed in 14/15 mice (typical results for 2 mice are shown: **a**,**b**). A naïve (control) mouse produced no target lysis (**c**), indicating the requirement for *in vivo* immunisation. Error bars indicate 95% confidence intervals (Student's t-test).

HLA-A2 dependence was tested using a monoclonal antibody with the ability to block the α-1 and α-2 domains of the HLA-A2 molecule. The addition of this antibody to the effector/target co-culture during chromium-release assay inhibited the lysis of peptide-bearing targets. In two experiments, the cytotoxicity was reduced from 40% to 8% (see figure 5.7).

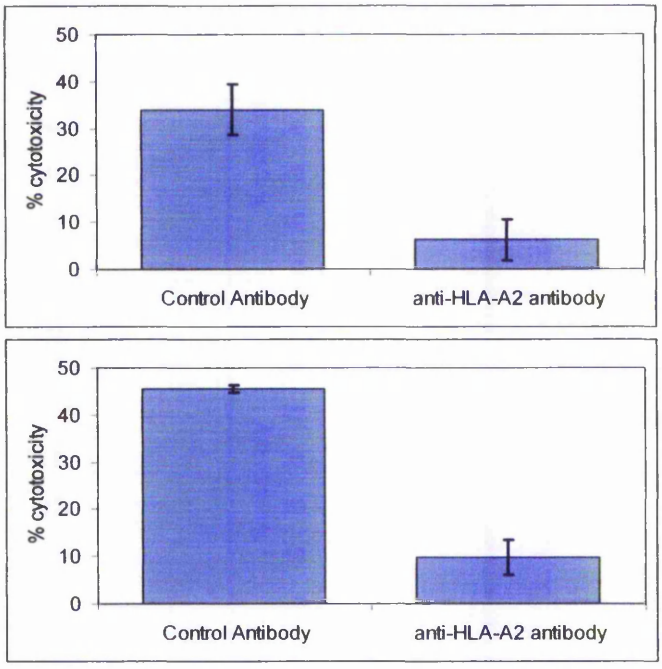


Figure 5.7: Chromium-release assay: PAP.135 peptide.

Addition of an anti-HLA-A2 antibody effectively blocked the lysis of target cells (2 mice) indicating that lysis is MHC class-I dependent. Error bars indicate 95% confidence intervals (Student's t-test).

Characterisation of splenocyte cultures by flow cytometry

Further characterisation of effector cells was carried out using flow cytometric analysis following immunostaining for CD4 and CD8. CD4 was expressed by 14.7% of splenocytes (95% C.I. = 13.3-16.0, Student's t-test) on day 0, which increased to 27.7% (95% C.I. = 26.3-29.0, Student's t-test) on day 5. Expression of CD8 increased from 10.5% (95% C.I. = 8.7-12.3, Student's t-test) on day 0 to 54.3% (95% C.I. = 52.5-56.2, Student's t-test) on day 5. See figure 5.8. The development of CD4 and CD8 expression by naïve mice was identical to that by immunised mice. The splenocytes derived from six mice were analysed.

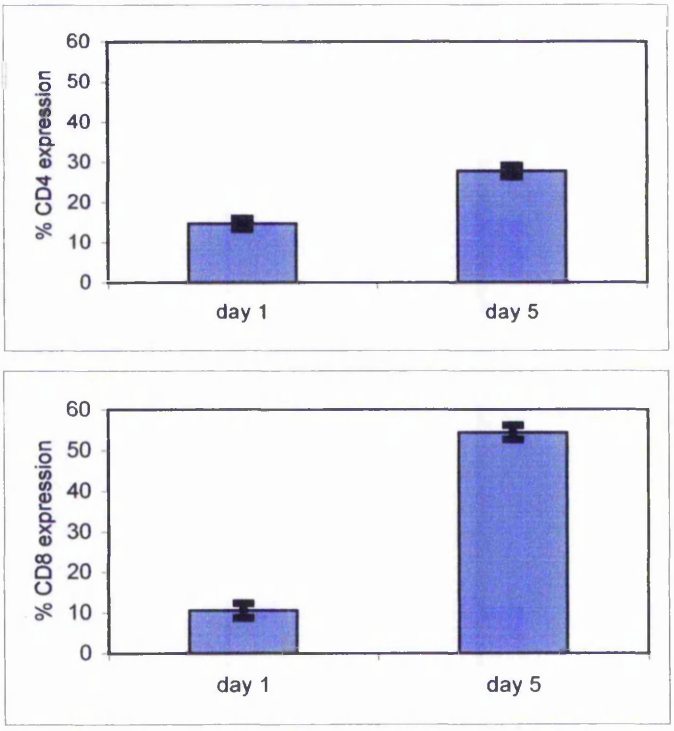


Figure 5.8: CD4 and CD8 expression by splenocyte cultures.

Flow cytometry was used to quantitate the expression of CD4 and CD8 by splenocytes during *in vitro* stimulation with peptide. The proportion of cells exhibiting CD4 and CD8 expression on day 1 and day 5 of cell culture is shown. Error bars indicate 95% confidence intervals (Student's t-test).

Cytokine production by splenocyte cultures

In order to characterise the type of immune activation occurring within splenocyte cultures, the IL-5 and IFN-γ content of media samples removed from tissue cultures were measured using an ELISA (see figure 5.9). Only minimal IFN-γ release was detected after 2 days' culture. After 5 days, significant levels of IFN-γ could be detected in splenocyte cultures derived from immunised mice containing test peptide. Much lower levels of IFN-γ were measured in cultures containing an irrelevant peptide. Splenocyte culture derived from naïve mice produced significant levels of IFN-γ to both peptides (having had prior exposure to neither). Thus, IFN-γ production is augmented in a peptide-specific manner in immunised mice, but not in naïve mice.

Insignificant amounts of IL-5 were detected at day 2 and day 5 in all media samples tested (results not shown).

(a)

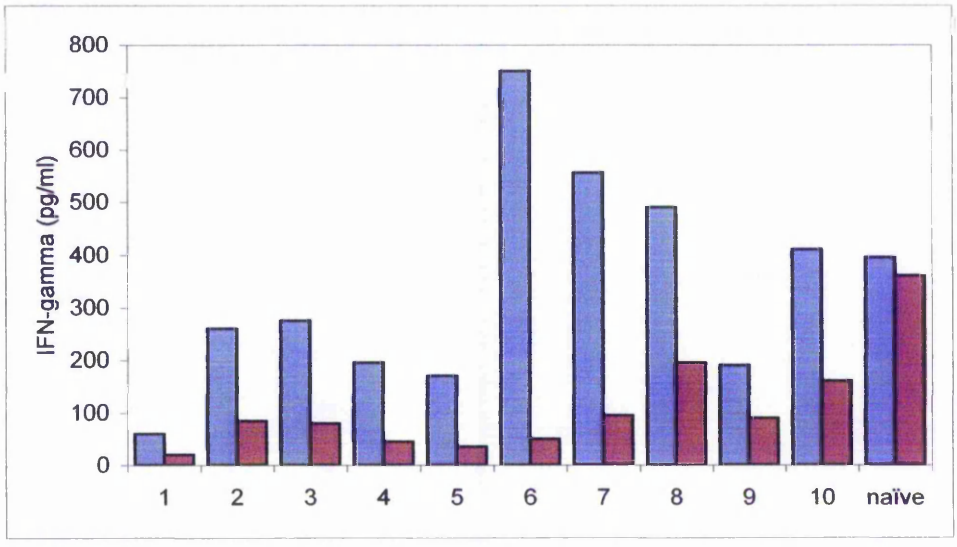


Figure 5.9: IFN-γ production by splenocyte cultures.

See caption on following page.

(b)

Exp. No.	% cytotoxicity at 50:1 ratio using RMAS targets pulsed with		IFN-γ released (pg/ml) by splenocytes cultured with	
	PAP.135	flu.58	PAP.135	flu.58
1	25	3	60	20
2	57	1	260	85
3	68	2	275	80
4	30	7	195	45
5	69	7	170	35
6	65	9	750	50
7	56	4	556	95
8	66	1	490	195
9	63	4	190	90
10	65	5	410	160
naive	13	11	395	360

Figure 5.9: IFN-γ production by splenocyte cultures.

An ELISA was performed on media samples taken from splenocyte cultures derived from 10 PAP.135-immunised mice. At day 2, levels of IFN-γ were very low or undetectable (data not shown). The bar chart above **(a)** shows IFN-γ levels on day 5 taken from these 10 splenocyte cultures, incubated with either PAP.135 (blue) or an irrelevant MHC class-I peptide (red). IFN-γ production was significantly greater with PAP.135 compared with the control peptide. Splenocytes derived from a non-immunised (naïve) mouse and subsequently incubated with PAP.135 or the control peptide also produced IFN-γ by day 5, but in a non-peptide-specific manner. The table **(b)** illustrates the same data, and also shows the corresponding cytotoxicity produced by CTL cultures.

Discussion

HLA-A2.1/K^b transgenic mice have been used successfully by many researchers to identify HLA-A2*0201 restricted CTL epitopes (Vitiello *et al.* 1991; Theobald *et al.* 1995; Heukamp *et al.* 2001). In these studies, peptide immunisation in combination with a generic MHC class-II helper epitope derived from hepatitis B virus delivered in emulsion with IFA produced peptide-specific CTL proliferation as demonstrated by subsequent *in vitro* target lysis assay.

Reproducible target-specific cytotoxicity was observed following immunisation with the known MHC class-I epitope peptides flu.58 and gp100.154. Although significant cell-lysis was demonstrated using flu.58, this was seen in only 38% of animals. This may be attributable to a degree of degeneration of the peptide during storage, or alternatively, overstimulation of the splenocyte cultures due to the potent immunogenicity of this peptide. Cytotoxicity following gp100.154 was more consistent, with 100% response rate. Furthermore, the ability of splenocytes to lyse endogenously processed and presented peptide on FM3 cells was also confirmed.

Although data have been published which confirm the HLA-A2 binding affinity of PAP.30 (Peshwa *et al.* 1998), the ability of this peptide to induce cytotoxic cellular responses *in vivo* has not been established. In these experiments, over half the animals tested (8/14) produced cytotoxic effects against PAP.30-labelled RMAS targets in a series of 3 experiments. Targets labelled with an irrelevant class-I epitope were not lysed, confirming the peptide specificity of this effect.

The ability of effector cells to lyse the PAP expressing prostate cancer cell-line LNCaP could not be established. Technical problems maintaining the LNCaP cell-line meant that viable target cells were unavailable for chromium-release assays. Further experiments will be required in order to obtain this important data before this part of the research can continue.

CTL cultures derived from PAP.135-immunised animals were able to lyse around 60% of PAP.135-labelled target cells. This effect was reproducible, with 25 out of 26 animals demonstrating similar levels of lysis in five separate experiments. The peptide specificity of CTL cultures derived from immunised mice was demonstrated by the failure of effector lymphocytes to produce target lysis in the absence of PAP.135: lysis of target cells pulsed with test peptide was significantly greater than lysis of similar target cells pulsed with an irrelevant MHC class-I epitope.

Effector lymphocytes derived from non-immunised (naïve) mice were unable to lyse target cells, indicating the necessity of vaccination to stimulate a significant immune response against the test peptide and the efficacy of *in vivo* peptide immunisation to produce a CTL mediated immune response in this animal model system.

The above data demonstrate the ability of PAP.135 to induce peptide-specific immune responses against peptide-loaded target cells. However, their efficacy as immunotherapeutic agents is dependent on their ability to induce cytotoxicity of antigen bearing tumour cells. The degree of target peptide presentation on tumour cells is dependent on levels of antigen expression and, significantly, endogenous processing of antigen. CTL cultures derived from PAP.135-immunised mice were able to recognise and lyse the human prostate cancer cell-line LNCaP, which is HLA-A2 positive and constitutively expresses PAP (Horoszewicz *et al.* 1983), indicating

that CTLs can recognise endogenously synthesised and processed PAP in the context of HLA-A2 expressed on the surface of human prostate cancer cells. This represents a much closer approximation of the possible effects of immunisation *in vivo* and is promising for the effectiveness of PAP.135 as a target for immunotherapy.

Because the effector cells used in the above assays were derived from splenocyte cultures consisting of a mixture of immune cell types, it is possible that the observed cytotoxic effects were not mediated by CTL. The lysis of target cells by CTL cultures was reduced by 80% in the presence of an HLA-A2 blocking antibody. A control antibody had no effect on the lysis of target cells. This indicates that the mechanism of target cell lysis is via CD8+ CTL.

Splenocytes were shown to release significant levels of IFN-γ during incubation (range 190 to 750 pg/ml). Splenocytes from non-immunized (naïve) mice also released IFN-γ, but in a peptide-independent manner. The Introduction provides a more detailed discussion of cytokine responses to types of immune activation. Interferon-gamma (IFN-γ) production has been shown to be indicative of CTL activation and a T_H-1 immune response. IL-5 production, however, is typical of T_H-2 responses and in the context of these studies such a response would be undesirable.

The proportion of cells expressing CD4 and CD8 molecules increased significantly during *in vitro* culture. 14.7% of cells exhibited CD4 expression on day 0 of culture, and this increased significantly to 27.7% by day 5. The increase in CD8 expression was even more marked with 10.5% of cells showing positive staining on day 0, and 54.3% on day 5. These findings are in keeping with the maturation of lymphocytes during *in vitro* stimulation. These data verify that mature lymphocytes can be produced using the processes described herein and that CD8+ lymphocytes are the

predominant cell-type. They also support the other evidence presented, which indicate that the target-specific lysis produced by splenocyte cultures is due to the action of CTL. The development of CD4 and CD8 expression by splenocytes derived from naïve mice was identical to that by splenocytes derived from immunised mice. The failure of these splenocytes subsequently to produce cytotoxicity against target cells indicates that the lymphocytes produced were not peptide-specific.

Optimisation of the *in vitro* maturation process was required in the initial stages of the study. In an attempt to enhance the efficiency of lymphocyte maturation, a protocol was designed in which splenocytes from immunised mice were incubated with APC that had been previously activated *in vitro*. Non-immunised (naïve) mice were killed and the splenocytes harvested as above. These cells were incubated with lipopolysaccharide (LPS) and dextran sulphate for 3 days, producing mature antigen-presenting B-cells (LPS-blasts). LPS is a bacterial component that initiates a signal transduction cascade leading to activation of antigen presenting cells (Chen *et al.* 1992). The immunised mice were killed and their splenocytes isolated. These splenocytes were then incubated with peptide-pulsed LPS-blasts derived from the naïve mice (see Appendix 2 for a summary of this protocol). This method was ultimately abandoned in favour of the simpler and ultimately more efficient protocol described above, in which the splenocytes from immunised animals were incubated with the test peptide without any APC maturation step.

The animals used in this study were HLA-A2.1/K^b transgenic C57 black mice. Such mice have chimeric class-I molecules, consisting of human α-1 and α-2 chains, but murine α-3. This facilitates the interaction of the peripheral murine $CD8^+$ T-lymphocytes with the transgenic MHC molecule. Although this system has been used

successfully by numerous workers for the study of class-I responses (Vitiello *et al.* 1991; Theobald *et al.* 1995; Heukamp *et al.* 2001), there have been reported difficulties related to the preferential development of mouse (H2-restricted) CTL responses rather than human responses (Barra *et al.* 1989; Epstein *et al.* 1989). For this reason, transgenic mice devoid of H2 class-I molecules were developed by disruption of the murine H2 and β_2-microglobulin genes. Such mice express the chimeric class-I molecule (human α-1 and α-2 chains, and murine α-3) covalently linked to the human β_2-microglobulin chain, which is denominated HHD (Pascolo *et al.* 1997). This system has yielded highly favourable results (Firat *et al.* 1999; Carmon *et al.* 2002; Alves *et al.* 2003). Although the sensitivity of this newer HHD system is undoubtedly more sensitive for the identification of human class-I epitopes, the validity of the HLA-A2.1/K^b system has not been questioned.

Collectively, these data illustrate the potent ability of the PAP.135 epitope to bind HLA-A2 and stimulate a cell-mediated immune response *in vivo*, and support the hypothesis that PAP.135 is an important potential epitope for use in cellular immunotherapy. It is strongly and reproducibly immunogenic in this transgenic model system, and lysis of LNCaP cells by PAP.135-specific CTL has been confirmed, demonstrating that this epitope is presented following endogenous synthesis and processing by human prostate cancer cells.

As this peptide is immunogenic and exhibits high affinity for HLA-A2, it is possible that potentially reactive CTL in human systems may become clonally deleted or rendered anergic as a result of natural mechanisms of "self-tolerance", thus reducing the efficacy of PAP.135 immunisation. Further testing in human model systems is therefore indicated.

Activation of naïve human PBMC by MHC class-I peptides

Contents

Overview

This chapter describes the further examination of the immunogenicity of putative CTL epitopes in an *in vitro* human lymphocyte culture system. Peripheral blood mononuclear cells (PBMC) were isolated from blood taken from known HLA-A2 donors and treated to stimulate differentiation into CTL and/or DC with appropriate cytokine exposure. CTLs were subsequently primed with a test epitope peptide and their ability to lyse peptide-pulsed target cells was determined using a standard chromium-release assay.

PAP.135-primed CTL exhibited target-specific lysis of PAP.135-bearing targets. Although further work is necessary to confirm these early findings, these data suggest that PAP.135 is a suitable target for immunotherapy against prostate cancer.

Introduction

This chapter aims to assess the suitability of epitopes derived from prostate cancer-specific antigens for immunotherapy strategies. The preceding two chapters have described the identification or putative MHC class-I epitopes and their testing in a transgenic murine model. Strong HLA-A2 binding has been confirmed, particularly for the peptides PAP.30 and PAP.135. The ability of these peptides to excite a CTL response *in vivo* has also been demonstrated. The following experiments will ascertain whether the PAP.30 and PAP.135 peptides are capable of inducing a target-specific CTL response in a human system.

The aim of immunotherapy strategies is to overcome immune tolerance to cancer cells. Although the emergence of immunogenic neo-antigens is recognised in many tumour types, the immune system usually fails to be activated. It is hoped that vaccination with lymphocyte epitopes may provide a strong enough stimulus to overcome tolerance and activate a CTL-mediated immune attack against antigen-bearing cancer cells. However, a prerequisite of such an approach is the existence of T-lymphocytes with the ability to recognise the vaccine epitope. It is theoretically possible for epitopes to be immunogenic in an HLA-A2-transgenic mouse, but be unable to activate CTL in humans.

Immune tolerance of cancer antigens is thought to be acquired during cancer development, and a number of mechanisms have been implicated; these have been discussed in the Introduction chapter. The induction of immune tolerance also occurs during normal foetal development and is responsible for the removal of lymphocytes reactive against self-antigens and hence the prevention of autoimmunity. Self-reactive

T-lymphocytes may be destroyed (clonal deletion) or rendered inactive (clonal anergy) during their development in the foetal thymus (Ramsdell and Fowlkes 1990). If lymphocytes reactive against a certain epitope had been clonally deleted in the thymus, then vaccination strategies would not be able to overcome the immune tolerance. As PAP is expressed in normal prostate tissue as well as prostate cancer, it is a true "self-protein" and may be particularly susceptible to the clonal deletion of highly reactive T-cells in this manner. This possibility cannot be investigated using transgenic animal models (as human PAP is a truly foreign protein in these animals) and a human system is therefore required to ensure the presence of potential epitope-reactive precursors in the human T-cell repertoire.

The testing of potential CTL epitopes in a human system has the additional advantage of being a closer approximation to clinical situations. When tested with a panel of epitopes, transgenic models may suggest a hierarchy of immunogenicity that does not correlate with the strengths of responses observed in human experiments using the same epitopes (Bharadwaj *et al.* 2001). Thus, epitopes that elicit strong immune responses in animal studies may not produce adequate CTL responses in humans, and *vice versa.*

The method described for the stimulation of CTL *in vitro* has been developed from a technique devised by Plebanski *et al* (Plebanski *et al.* 1995) and others (Sallusto and Lanzavecchia 1994). It has been used successfully to generate CTL clones specific for p53 epitopes (McArdle *et al.* 2000). Similar protocols have identified lymphoma antigens (Sallusto and Lanzavecchia 1994), viral epitopes (Peshwa *et al.* 1996; Bharadwaj *et al.* 2001) and PAP epitopes (Peshwa *et al.* 1998).

Results

The ability of prostate antigen epitopes to stimulate anti-tumour immunity was investigated using an *in vitro* human model. PBMC were isolated from the blood of healthy HLA-A2 positive adult donors and used to generate DC and CTL by *in vitro* maturation and stimulation with peptides. A standard chromium-release assay was used to quantitate the lysis of T2 target cells by effector cells. The cytotoxicity of effectors against targets bearing the relevant test peptide was compared with the cytotoxicity against targets bearing irrelevant control peptides.

Each lymphocyte culture underwent two cycles of stimulation with peptide, and a chromium-release assay was performed after each. Each well of the 96-well plates contains an individual clone of CTL, and it is therefore inappropriate to express the cytotoxicity results as an average of all wells. Each well containing CTL mixed with targets pulsed with the relevant peptide has, instead, been compared with the corresponding well on the control plate (containing the same CTL clones mixed with targets pulsed with the irrelevant peptide).

In order to facilitate the comparison of large numbers of wells, the results of all experiments have been expressed as three-dimensional bar charts. Each bar represents the difference between the percentage cytotoxicity of an individual test well and the percentage cytotoxicity of its corresponding control well. An example is shown below.

Cytotoxicity of gp100 CTL + gp100 targets	**Cytotoxicity of gp100 CTL + Flu.58 targets**	**Difference**
57	14	33

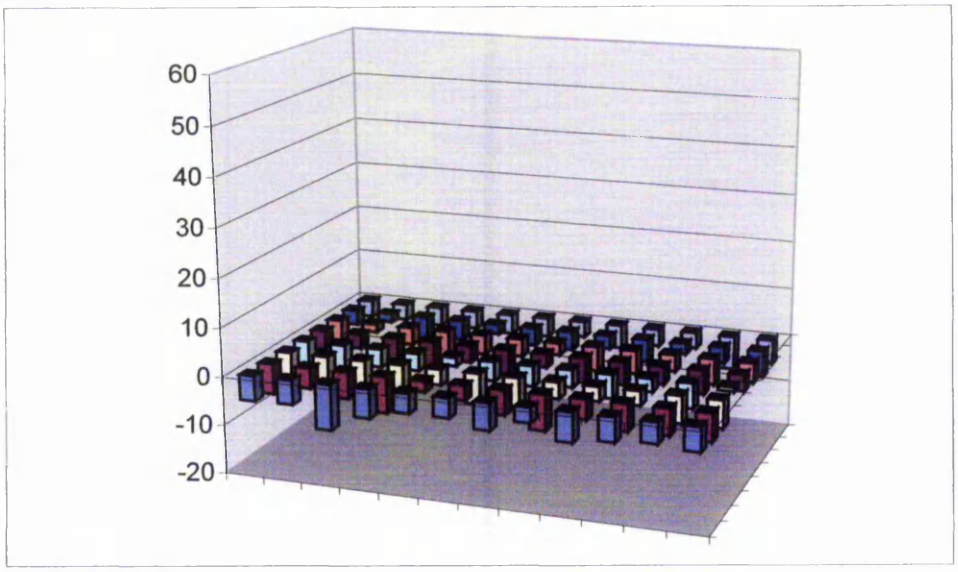
Proof of principle using known CTL epitopes as positive controls

Known HLA-A2 CTL epitopes derived from the influenza virus matrix protein M1 and gp100 were used as positive controls. The immunogenicity of both have been established in previous studies (Man *et al.* 1994; Kirkin *et al.* 1999), and their ability to excite an immune response following immunisation in a transgenic model system has been confirmed in this thesis (see previous chapter). PBMC derived from the blood of HLA-A2 healthy volunteers were stimulated with peptide *in vitro* and matured by incubation with suitable cytokines. The ability of PBMC-derived CTL to lyse T2 targets pulsed either with the test peptide or an irrelevant control peptide was determined using a chromium-release assay.

Figures 6.1 and 6.2 illustrate the cytotoxicity of flu.58-stimulated CTL in two independent experiments. The upper charts (a) show cytotoxicity after the first stimulation with peptide, with bars representing lysis of flu.58-targets relative to control-targets. The cytotoxicity following the second round of peptide stimulation is shown in the lower charts (b).

In both experiments, after one round of stimulation, a small amount of target lysis was elicited in a minority of wells (figures 6.1a and 6.2a), with many other wells exhibiting less lysis of flu.58 targets than controls (negative bars). After the second round of stimulation, no significant peptide-specific lysis could be demonstrated in the first experiment (figure 6.1b). In the other, significantly more lysis was produced against peptide-specific targets than control targets (figure 6.2b).

(a)



(b)

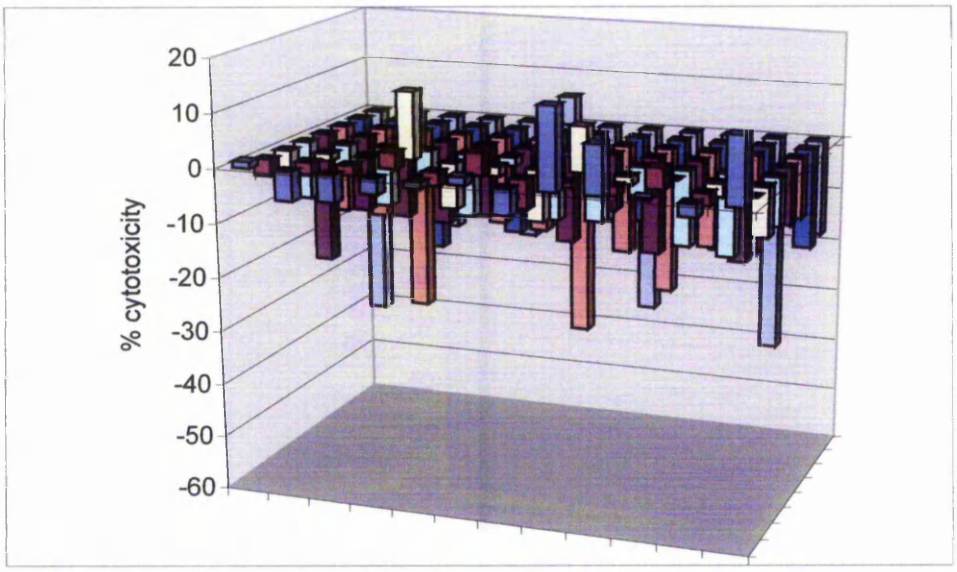


Figure 6.1: Chromium-release assays (human PBMC): flu.58

Effectors derived from human PBMC and stimulated *in vitro* with flu.58 were incubated with T2 targets bearing either flu.58 or an irrelevant control. Cytotoxicity was measured by chromium-release assay. Relative cytotoxicity of effector cells against peptide-bearing is shown. **(a)** after first stimulation with peptide. **(b)** after second stimulation with peptide.

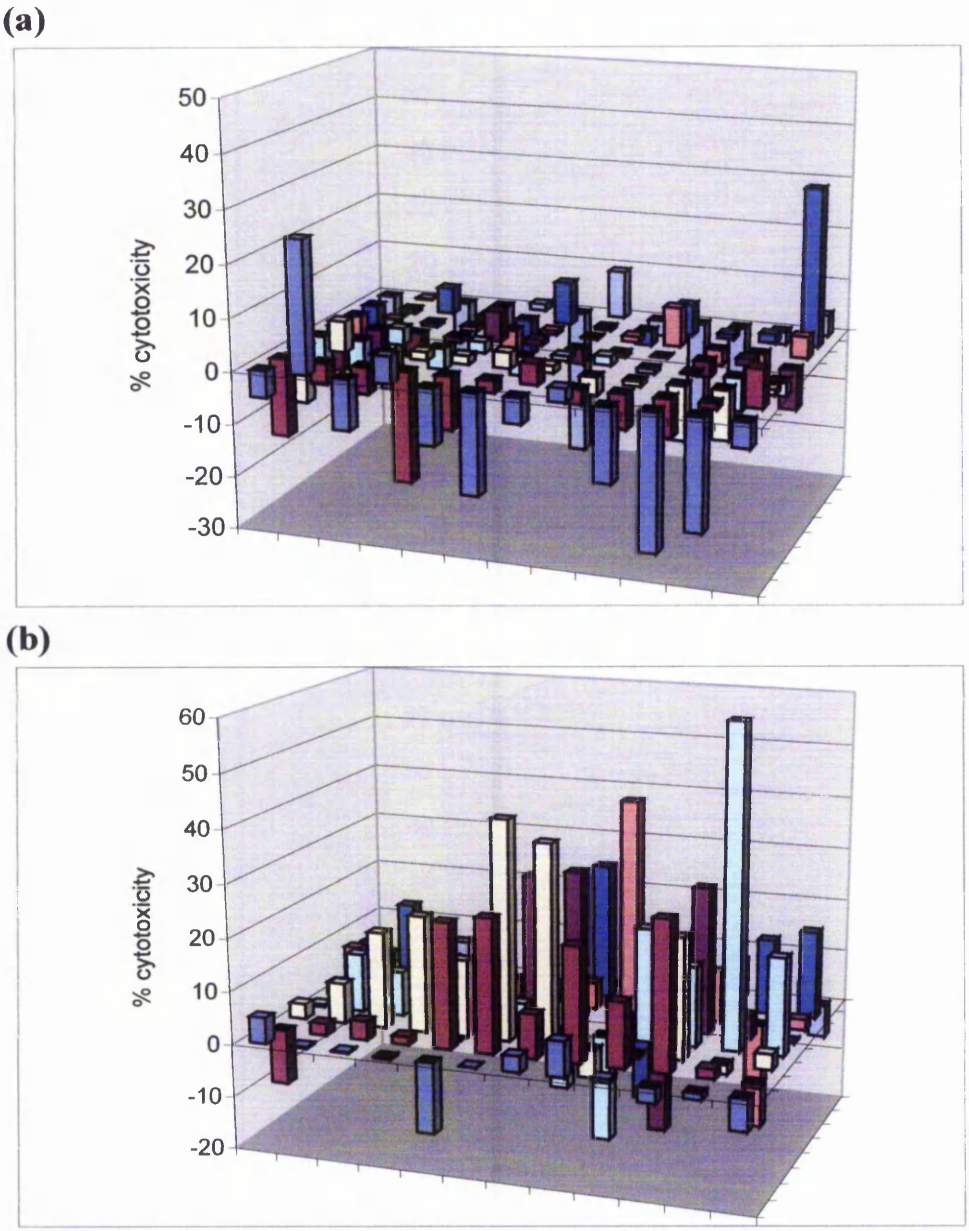


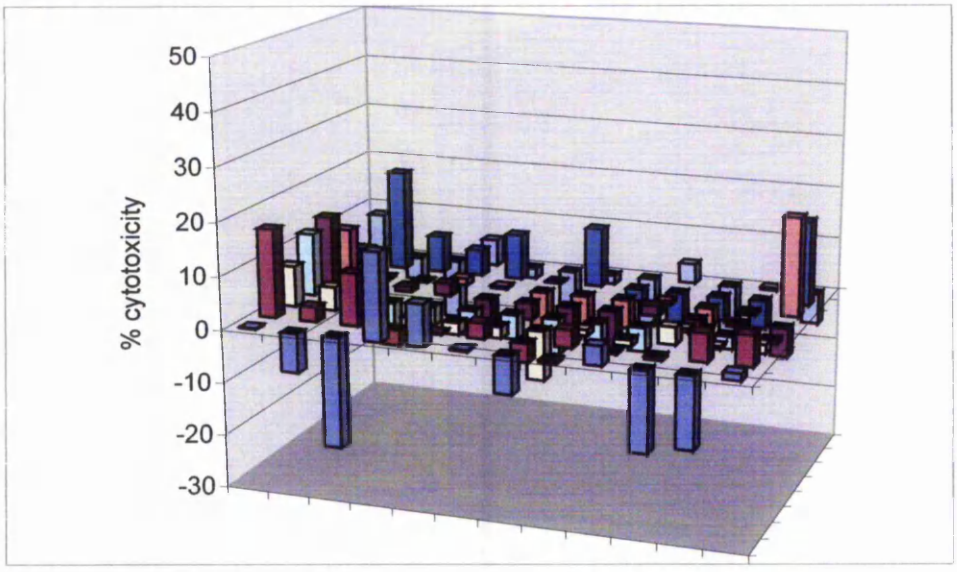
Figure 6.2: Chromium-release assays (human PBMC): flu.58

Effectors derived from human PBMC and stimulated *in vitro* with flu.58 were incubated with T2 targets bearing either flu.58 or an irrelevant control. Cytotoxicity was measured by chromium-release assay. Relative cytotoxicity of effector cells against peptide-bearing is shown. **(a)** after first stimulation with peptide. **(b)** after second stimulation with peptide.

The results of similar experiments using the gp100.154 peptide are illustrated in figures 6.3 and 6.4. Again, two independent experiments were performed. After the first peptide stimulation, a variable and inconsistent amount of target-specific lysis was measured in one experiment (figure 6.3a). The second experiment produced significantly greater lysis of peptide-specific targets than controls in many of the wells (figure 6.4a).

After a second stimulation with peptide, the lysis of peptide-specific targets was greatly augmented in experiment one, with the majority of wells producing significantly increased cytotoxicity against gp100.154 targets compared to controls (figure 6.3b). A large proportion of wells exhibited little or no peptide-specific lysis in the second experiment (figure 6.4b). However, there was significant, specific cytotoxicity in a small number of wells.

(a)



(b)

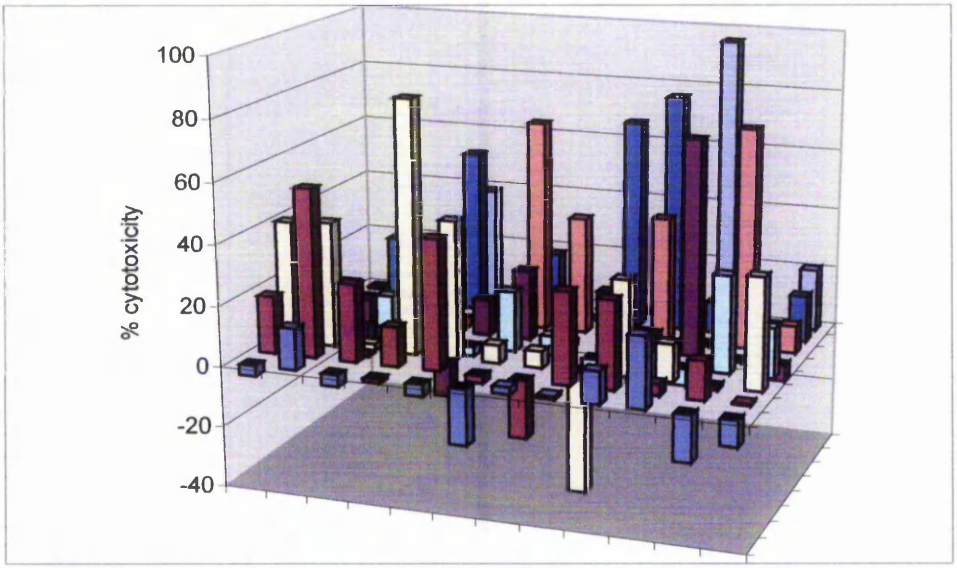
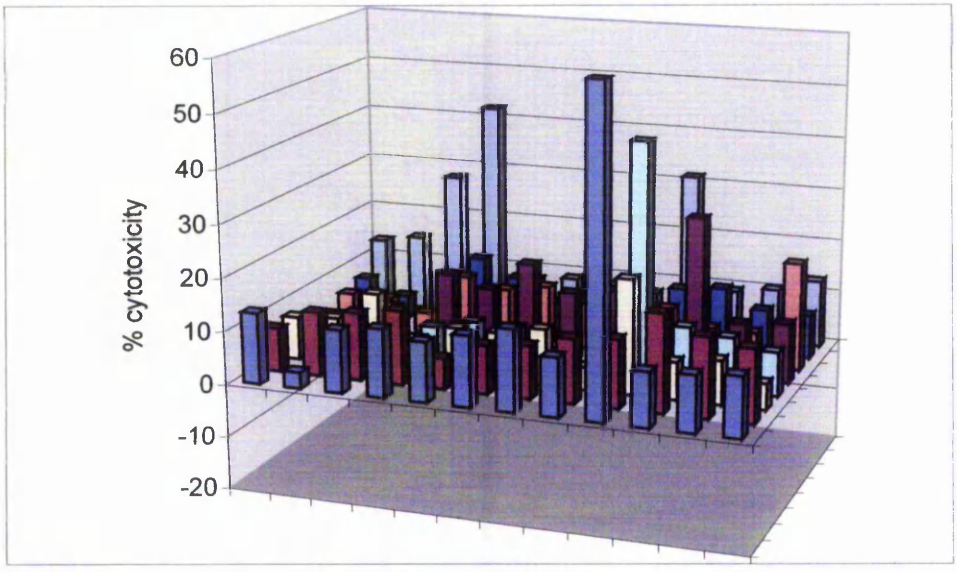


Figure 6.3: Chromium-release assays (human PBMC): gp100.154

Effectors derived from human PBMC and stimulated *in vitro* with gp100.154 were incubated with T2 targets bearing either gp100.154 or an irrelevant control. Cytotoxicity was measured by chromium-release assay. Relative cytotoxicity of effector cells against peptide-bearing targets is shown. **(a)** after first stimulation with peptide. **(b)** after second stimulation with peptide. Little specific cytotoxicity was observed after one round of stimulation, but lysis was significantly augmented after the second round of stimulation.

(a)



(b)

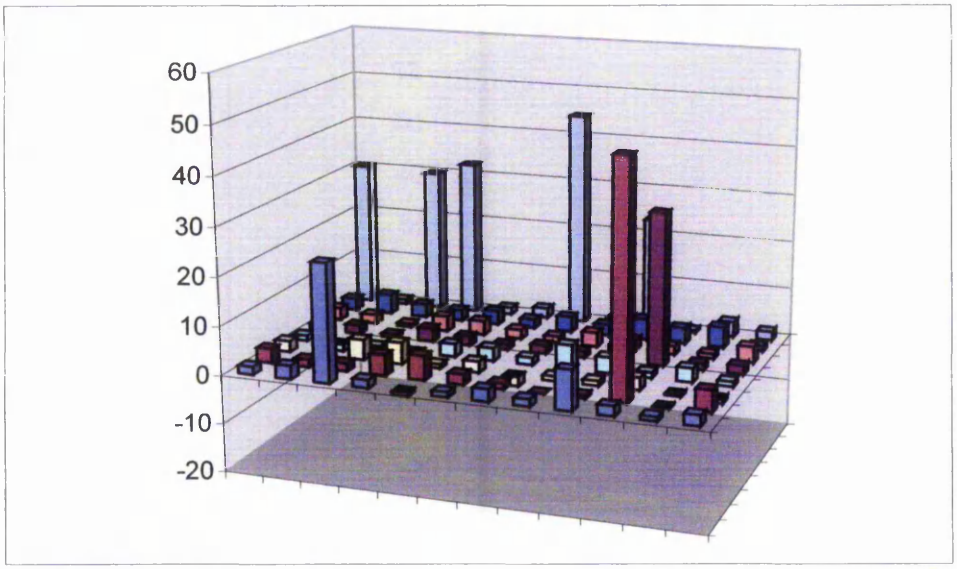


Figure 6.4: Chromium-release assays (human PBMC): gp100.154

Effectors derived from human PBMC and stimulated *in vitro* with gp100.154 were incubated with T2 targets bearing either gp100.154 or an irrelevant control. Cytotoxicity was measured by chromium-release assay. Relative cytotoxicity of effector cells against peptide-bearing targets is shown. **(a)** after first stimulation with peptide. **(b)** after second stimulation with peptide. Significantly greater lysis of peptide-specific targets than controls was observed in many wells after one round of stimulation. A large proportion of wells exhibited little or no peptide-specific lysis after the second round of stimulation; however, there was significant specific cytotoxicity in a small number of wells.

Cytotoxicity using test peptides derived from prostate antigens

The same system was employed to test the cytotoxicity of two PAP-derived epitopes identified using the transgenic model, PAP.30 and PAP.135. Again, two independent experiments were performed for each peptide.

Following stimulation with PAP.30, no peptide-specific target lysis could be detected in any wells in the first experiment. In the second experiment, a small number of test wells exhibited significantly increased cytotoxicity against relevant targets compared to controls (see figures 6.5 and 6.6). Unfortunately, a second round of peptide stimulation could not be performed due to loss of effector cell cultures after the first cytotoxicity assay in both experiments. No further data are therefore available.

Following the first stimulation with PAP.135, a small number of test wells exhibited increased cytotoxicity against relevant targets in both experiments (figures 6.7a, 6.8a). However, many wells also exhibited negative relative cytotoxicity, and these results should therefore be interpreted with caution. After a second round of stimulation with PAP.135, a significant and consistent pattern of peptide-specific target lysis was observed in experiment one (figure 6.7b). Although many wells in the second experiment showed no relative cytotoxicity, a small number again exhibited significant lysis (figure 6.8b).

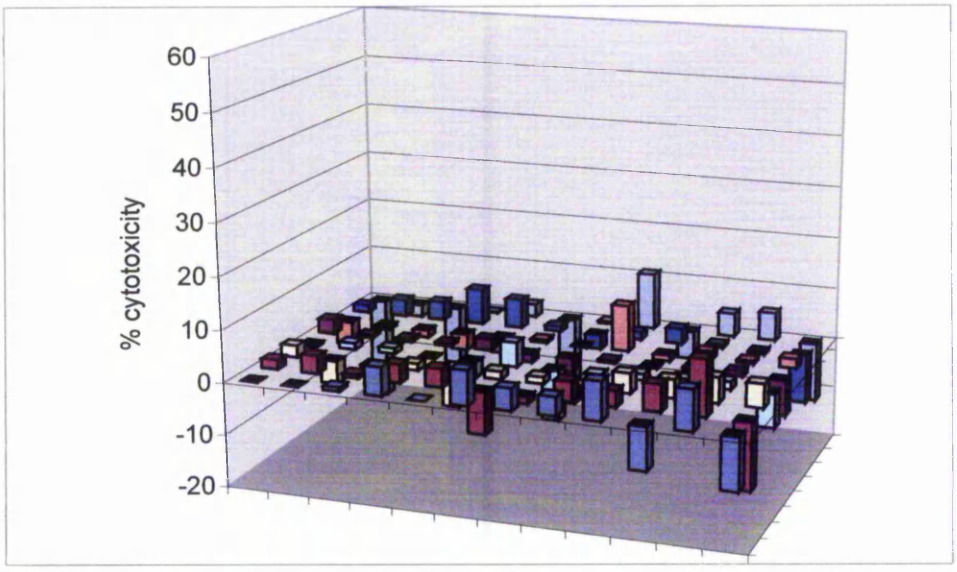


Figure 6.5: Chromium-release assays (human PBMC): PAP.30

Effectors derived from human PBMC and stimulated *in vitro* with PAP.30 were incubated with T2 targets bearing either PAP.30 or an irrelevant control. Cytotoxicity was measured by chromium-release assay. Relative cytotoxicity of effector cells against peptide-bearing targets after a single stimulation with peptide is shown. Due to loss of effector cell cultures after the first cytotoxicity assay, no further results are available.

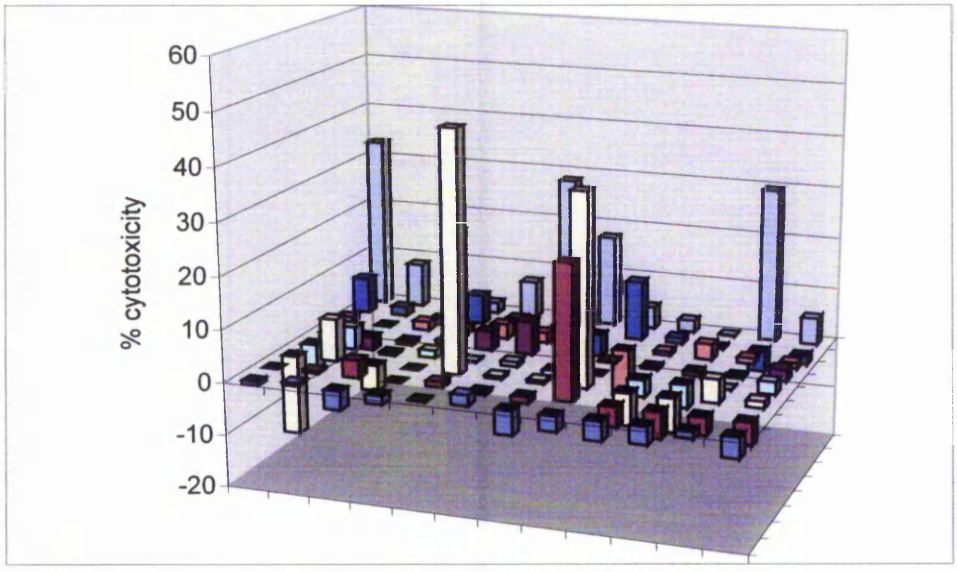
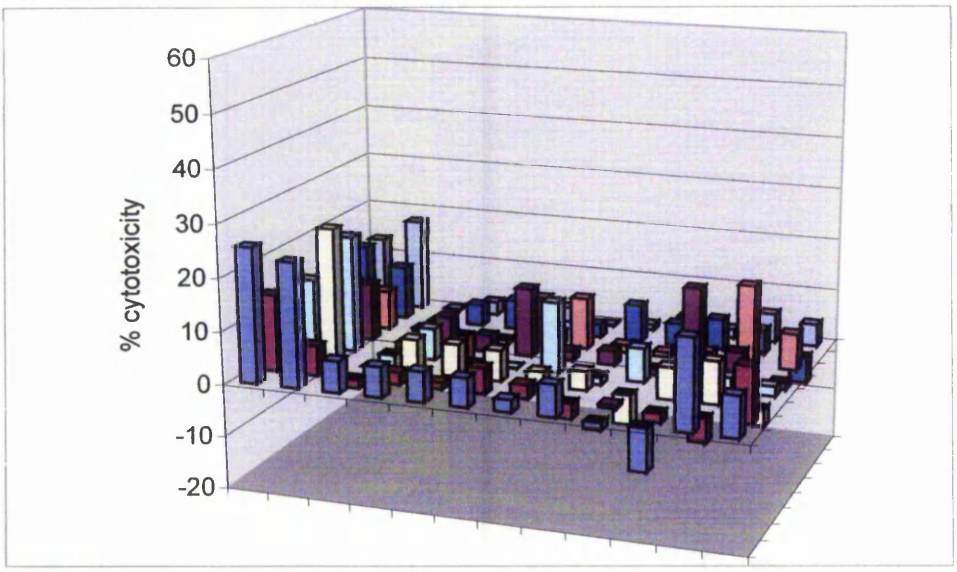


Figure 6.6: Chromium-release assays (human PBMC): PAP.30

A second experiment is shown, with results similar to figure 6.5.

(a)



(b)

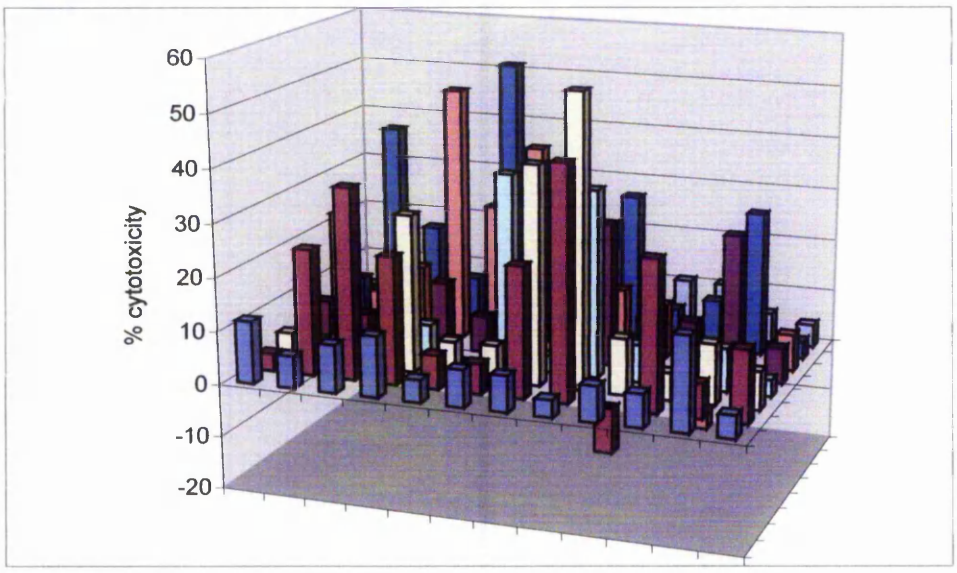
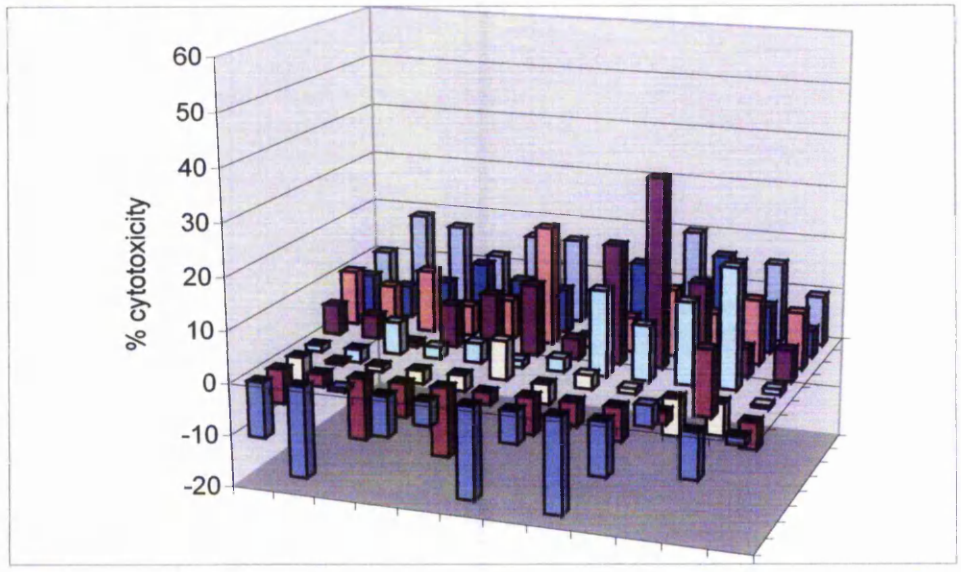


Figure 6.7: Chromium-release assays (human PBMC): PAP.135

Effectors derived from human PBMC and stimulated *in vitro* with PAP.135 were incubated with T2 targets bearing either PAP.135 or an irrelevant control. Cytotoxicity was measured by chromium-release assay. Relative cytotoxicity of effector cells against peptide-bearing targets is shown. Some peptide-specific lysis was seen after the first round of stimulation with peptide **(a)**, but much more marked cytotoxicity was observed after a second round of stimulation **(b)**.

(a)



(b)

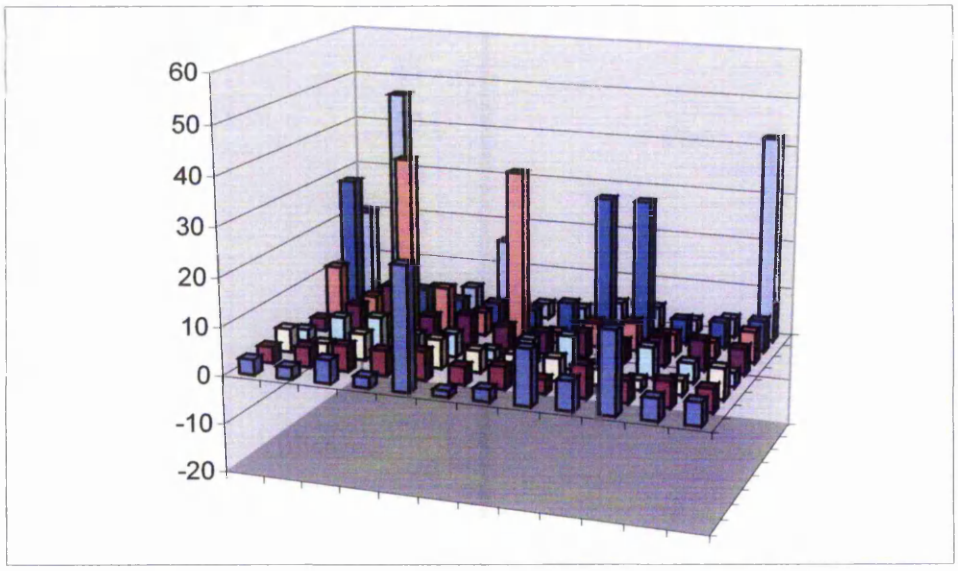


Figure 6.8: Chromium-release assays (human PBMC): PAP.135

Effectors derived from human PBMC and stimulated *in vitro* with PAP.135 were incubated with T2 targets bearing either PAP.135 or an irrelevant control. Cytotoxicity was measured by chromium-release assay. Relative cytotoxicity of effector cells against peptide-bearing targets is shown. Variable peptide-specific lysis was seen after the first round of stimulation with peptide **(a)**. Significant levels of cytotoxicity were observed in a small proportion of wells after the second round of stimulation **(b)**.

Discussion

The investigation of the abilities of test peptides to induce CTL proliferation in a human system is an essential step in epitope discovery. Peptides that exhibit immunogenicity in transgenic model systems may ultimately prove to be ineffective in human systems if immune tolerance to these epitopes is too strong. Epitopes capable of producing potent immune stimulation may be particularly prone to such effects, and anergy or clonal deletion of lymphocytes reactive against antigen epitopes may be induced by natural mechanisms intended to protect against autoimmune effects. The testing of putative epitopes in human model systems is therefore essential.

The methods described here employ PBMC to generate presenting cells and effector cells and has been used successfully by others (Sallusto and Lanzavecchia 1994; Passoni *et al.* 2002). Monocytes were isolated from the blood of healthy HLA-A2 positive donors and used to generate antigen presenting cells and lymphocytes, which were stimulated *in vitro* against a variety of target peptides. The ability of activated lymphocytes to lyse peptide bearing T2 target cells was determined by a chromium-release assay.

Cytotoxicity against flu.58 bearing targets was disappointing, and may be explained by deterioration of the peptide stocks in storage or overstimulation of effector cells *in vitro* causing cell death prior to the cytotoxicity assay. The results of cytotoxicity testing in the transgenic mouse model using this peptide were similarly disappointing (see previous chapter), and the mechanisms involved in both are likely to be related. Cytotoxicity against gp100.154 was more robust and confirmed the validity of the

system. Relatively low levels of cytotoxicity were observed after a single stimulation with peptide-pulsed APC, but a greater degree of target-specific lysis was produced after a second stimulation.

Although the ability of peptide PAP.30 to bind HLA-A2 has been reported (Peshwa *et al.* 1998), this is the first study to address the ability of this peptide to stimulate an immune response against peptide-bearing target cells. Results from the transgenic mouse model were promising, with moderate levels of cytotoxicity produced against RMAS target cells. In this human model system, PAP.30 produced no significant cytotoxicity against T2 targets after a single round of stimulation in two experiments (see figures 6.5, 6.6). Unfortunately, the effector cells were subsequently lost due to plate infection in both experiments, and no data for the second rounds of stimulation could be obtained. It is therefore impossible to draw meaningful conclusions about the immunogenicity of this peptide in the human system until these experiments are repeated with further rounds of *in vitro* stimulations.

The peptide PAP.135 produced excellent levels of cytotoxicity in the transgenic model system, raising concerns that CTL clones against such an immunogenic peptide might be clonally deleted or rendered anergic in humans. In the first experiment utilising human PBMC, relatively low levels of target lysis were observed in the first cytotoxicity assay (figure 6.7a), whereas quite marked lysis was produced after the second round of stimulation (figure 6.7b). In the second experiment, moderate lysis of targets was observed in some wells after the first stimulation (figure 6.8a). After the second stimulation, many wells remained quiescent, while significant cytotoxicity was observed in a small number (12/96) of wells (figure 6.8b). It is possible that a small number of CTL clones are able to mount a strong immune response against PAP.135.

It is also possible that overstimulation may have led to the death of highly reactive lymphocyte clones. In future experiments, it will be helpful to clonally expand CTL taken from those wells exhibiting cytotoxicity in an attempt to produce lysis in a greater number of wells.

Similar epitope discovery techniques have been applied to PAP by other workers. Peshwa *et al* examined a panel of peptides selected using an HLA-A2*0201 binding motif screening algorithm (Peshwa *et al.* 1998). Two "moderate" affinity peptides (PAP.6 and PAP.30, using our nomenclature) and one "strong" affinity peptide (PAP.299) were identified. PAP-specific CTL were generated by *in vitro* stimulation with DC loaded with PAP.299, but cytotoxicity data were not reported for the other two peptides. An HLA-A2*2402-restricted PAP peptide, PAP.213 has been tested using HLA binding assays and *in vitro* stimulation of CTL derived from the PBMC of both normal and prostate cancer patients (Inoue *et al.* 2001). Tumour specific CTL were successfully generated.

Others have reported significant increases in cytotoxicity after multiple stimulations (Passoni *et al.* 2002), and three or four rounds of stimulation with APC are often performed before cytotoxicity assays are performed. Unfortunately, in this study, this was not possible due to availability of donated blood and time constraints, although future work will address this issue.

The cytotoxicity results of individual test wells were presented in this study. Ideally, further rounds of restimulation would have allowed the selection and subcloning of lymphocytes from wells that exhibited strong cytotoxicity. It is likely that this would have improved the uniformity of the levels of cytotoxicity results, as observed in other

studies (Plebanski *et al.* 1995), and also allowed the effects of HLA-A2 blocking antibody and effector cell concentration to be investigated.

Because of the lack of availability of donated HLA-A2 positive blood, time constraints and technical problems including plate infection, these data are incomplete. Before any definite conclusions can be drawn about the immunogenicity of the peptides in the human system, these experiments should be repeated and extended as indicated above.

However, advances have been made in the optimisation of these protocols and the preliminary data is promising with regard to at least one of these novel prostate cancer epitopes. This is the first study to examine the abilities of PAP.30 and PAP.135 to stimulate cell-mediated immune responses in either transgenic or human systems. Target-specific CTL were generated in response to *in vitro* stimulation with APC loaded with PAP.135, supporting the use of PAP.135 as an immune target in vaccination strategies against prostate cancer.

DISC-HSV as an intratumoural delivery system for prostate cancer immunotherapy

Contents

DISC-HSV as an intratumoural delivery system for prostate cancer immunotherapy

Overview

As alluded to in the Introduction, whole-cell vaccines transduced with the GMCSF cytokine have shown promise in prostate cancer immunotherapy. The transfection of tumour cells with GMCSF, either *in vivo* (Ali *et al.* 2000) or *in vitro* (Sanda *et al.* 1994), render these cells capable of provoking anti-tumour immune responses. In pre-clinical studies, it has previously been shown that irradiated colorectal tumour cells infected with DISC-HSV expression cassettes for mGMCSF can induce both prophylactic and therapeutic protection against a lethal challenge of tumour cells via the activation of $CD4^+$ and $CD8^+$ T-cells (Ali *et al.* 2000). The results obtained from the colorectal model suggest that this approach could ultimately be applied to the treatment of other tumour systems.

To establish the feasibility of applying such a methodology in human prostate cancer, it is necessary to examine the ability of the virus to infect prostate cancer tissue and translate the cytokine gene inserted into its genome. To test the hypothesis that prostate cancer cells are permissive to DISC-HSV infection, a series of experiments were designed to confirm successful entry of the virus into the cell nucleus, and the subsequent generation of the proteins encoded by genes incorporated into the viral genome. The production of virally encoded proteins following infection was quantitated over time, and related to the effects of viral infection on the metabolic and proliferative functions of the prostate cancer cells.

In these studies, two DISC-HSV viruses have been used to determine their ability to infect prostate cancer cell-lines and solid prostrate tumours. DISC-HSV carrying genes for β-galactosidase (β-gal) or human GMCSF were used to infect DU145, LNCaP and PC3 human prostate cancer cells *in vitro*. Xenograft models of DU145

and PC3 raised on nude mice were infected with DISC-β-gal *in vivo*. Cells and tissue were monitored for reporter (β-gal) gene expression and the production of hGMCSF following infection with the appropriate viruses, and the changes in gene expression over time after infection were monitored. Successful infection of cells and tissues was confirmed by electron microscopy. In addition, the metabolic activity and cell-cycle dynamics of prostate cancer cells following DISC-HSV infection were measured in order to explain changes in gene expression over time.

Expression of virally encoded genes, including GMCSF, was maintained for up to 3 days. Loss of gene expression at this time could not be explained by cell-death, and cellular metabolism did not appear to be significantly abrogated. These data demonstrate the ability of DISC-HSV to infect prostate cancer and express virally encoded GMCSF at significant levels for several days. This supports the hypothesis that immune therapy using HSV-DISC containing the GMCSF gene is a potentially useful treatment for prostate cancer treatment.

Introduction

Creating specific immunological responses against unique or tumour-associated antigens (TAA) derived from prostate cancer cells would undoubtedly represent a significant step forward in the development of anti-tumour vaccines against this disease. In order to achieve this goal a number of alternative methodologies have been developed to facilitate the selected targeting of prostate cancer cells by the immune system. These strategies have ranged from specific approaches seeking to raise T-cell responses ($CD8^+$) against well-defined MHC class I epitopes (Peshwa *et al.* 1998; Murphy *et al.* 1999), to "broad range" techniques stimulating T-cell activity against unidentified cellular antigens (e.g. "whole cell" vaccines (Simons *et al.* 1999; Hrouda *et al.* 2000; Eaton *et al.* 2002)).

The development of generalised approaches, designed to promote immunity to both patient specific and universal antigens, would be extremely beneficial for several reasons. Firstly, the implementation of therapeutic protocols would not require an extensive analysis of antigen expression patterns within the tumour, and secondly, there would no longer be the necessity to haplotype patients prior to vaccination; the immune response would be initiated against peptides derived from TAA restricted by the patients' MHC antigens.

Promoting $CD8^+$ and $CD4^+$ T-cell responses has been shown to be important in order to achieve tumour regression, and may be elicited through the transient expression of inflammatory cytokines such as IL-2, IL-12, IFN-γ and most notably GMCSF (Watanabe *et al.* 1989; Fearon *et al.* 1990; Dranoff *et al.* 1993; Toda *et al.* 1998; Ali *et al.* 2000; Ali *et al.* 2002) via the activation of professional antigen presenting cells

(APC) such as dendritic cells (DC). The ability to promote T-cell activation in response to specific antigens is also contingent upon the simultaneous expression of co-stimulatory molecules such as B7.1/B7.2, OX-40L and CD40 by APC through direct interaction with cognate receptors (e.g. CD28) upon the T-cell surface (Weinberg 2002), and evidence supports the contention that expression of co-stimulatory molecules by circulating APC can be stimulated by inflammatory cytokines such as GMCSF (Markowicz and Engleman 1990; Caux *et al.* 1992).

Viruses have proved to be extremely versatile delivery vehicles for the transient/stable transduction of genes into target cells (Ali *et al.* 1994). In addition, viruses are extremely adept at stimulating immune activation by providing "danger signals" which enhance the activity of $CD4^+$ and $CD8^+$ cells via DC activation (Matzinger 1994).

The use of a Disabled Infectious Single Cycle (DISC) herpes simplex virus (HSV) as an effective vector for delivery and *in vivo* expression of both IL-2 and murine GMCSF in a variety of tumour cell types (e.g. sarcoma and melanoma) has been reported (Ali *et al.* 2000; Rees *et al.* 2002). Furthermore, experimental data have suggested that irradiated colorectal tumour cells (CT-26) infected with DISC-HSV and transiently expressing GMCSF can provide up to 80% protection from rechallenge with the same tumour (Todryk *et al.* 1999). Depletion of either T-helper or T-cytotoxic subsets resulted in the abrogation of the therapeutic effect, suggesting that immune activation was dependent upon a co-ordinated synergism between $CD4^+$ and $CD8^+$ T-cell populations (Todryk *et al.* 1999; Ali *et al.* 2000).

Based upon this pre-clinical data, DISC-hGMCSF was entered into a phase I clinical trial for the treatment of malignant melanoma and its efficacy for therapy is currently under evaluation.

DISC-HSV exhibits various properties that support its application for gene transduction in cancer immunotherapy: the size of the viral genome; its infectivity; its adjuvant effect; and its proven safety in humans (see also chapter 7). This preliminary study explores the possibility of DISC-HSV-based therapies for prostate cancer by examining the ability of this viral transduction system to infect prostate cancer cells and cause expression of virally encoded genes.

Results

DISC-β-gal infection of prostate cancer cell lines

To establish whether DISC-HSV could be considered as a candidate vector system for the delivery and expression of cytokine genes into human prostate cancer, DU-145, LNCaP and PC-3 cells were infected with HSV expression cassettes containing the β-galactosidase reporter gene. Virus to cell ratios of 1, 5, 10, 20 and 50 to 1 were utilised to optimise infection conditions for each cell line and the number of positively infected cells (blue) ascertained by direct counting under light microscopy (figure 7.1 a,b,c). The maximum level of infection attained was approximately 90% using ratios of 10:1, 20:1 and 20:1 for PC-3, LNCaP and DU-145 respectively. These conditions were used for all subsequent *in vitro* infections using these cell-lines.

The successful infection of prostate cancer cell-lines following incubation with DISC-HSV was confirmed by electron microscopy. The micrographs shown in figure 7.2 demonstrate viral particles within the nucleus, indicating cellular infection by DISC-HSV.

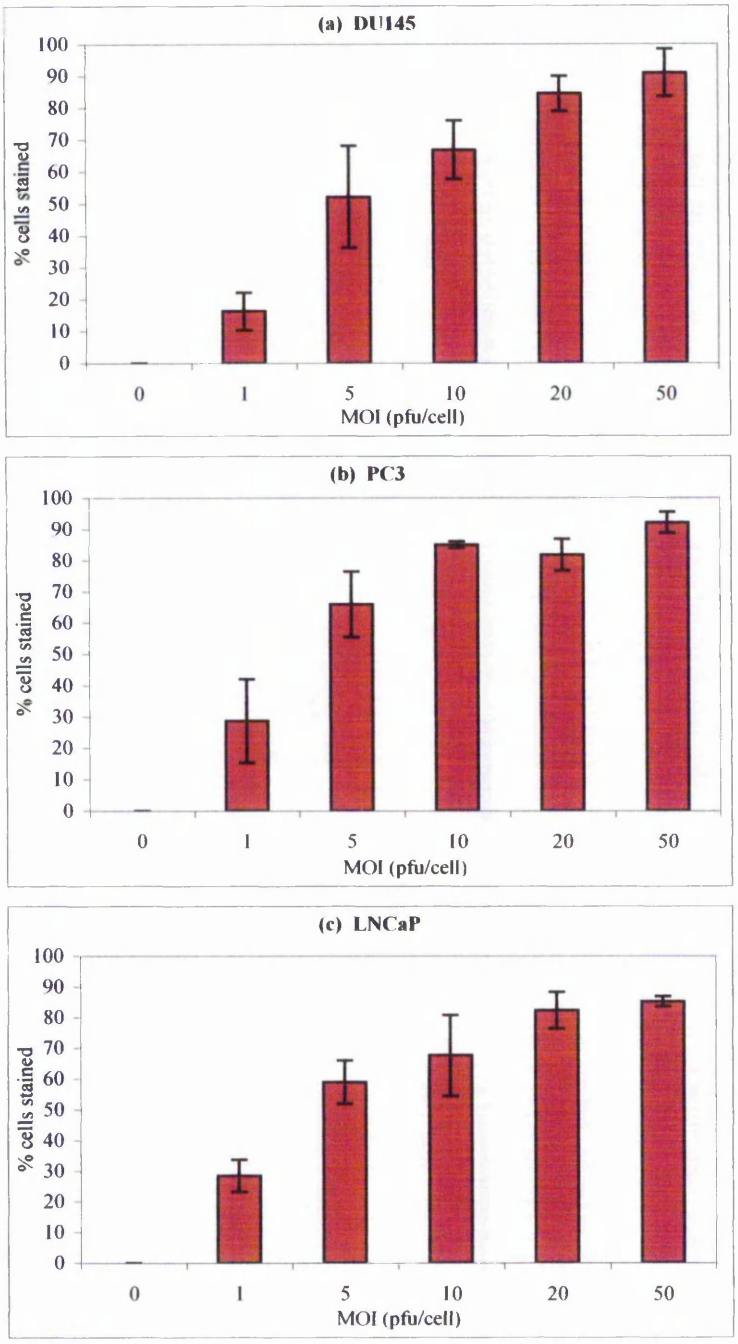


Figure 7.1: Staining of DU145, PC3 and LNCaP cell-lines for β-galactosidase following infection with DISC-β-gal at increasing doses.

Cells were infected with DISC-β-gal. Between 1 and 50 pfu of virus were used per cell. Cells were fixed and stained for β-galactosidase activity 1 hour after infection. The maximum level of infection attained was approximately 90% using ratios of 10:1, 20:1 and 20:1 for PC-3, LNCaP and DU-145 respectively. Error bars indicate 95% confidence intervals (Student's t-test).

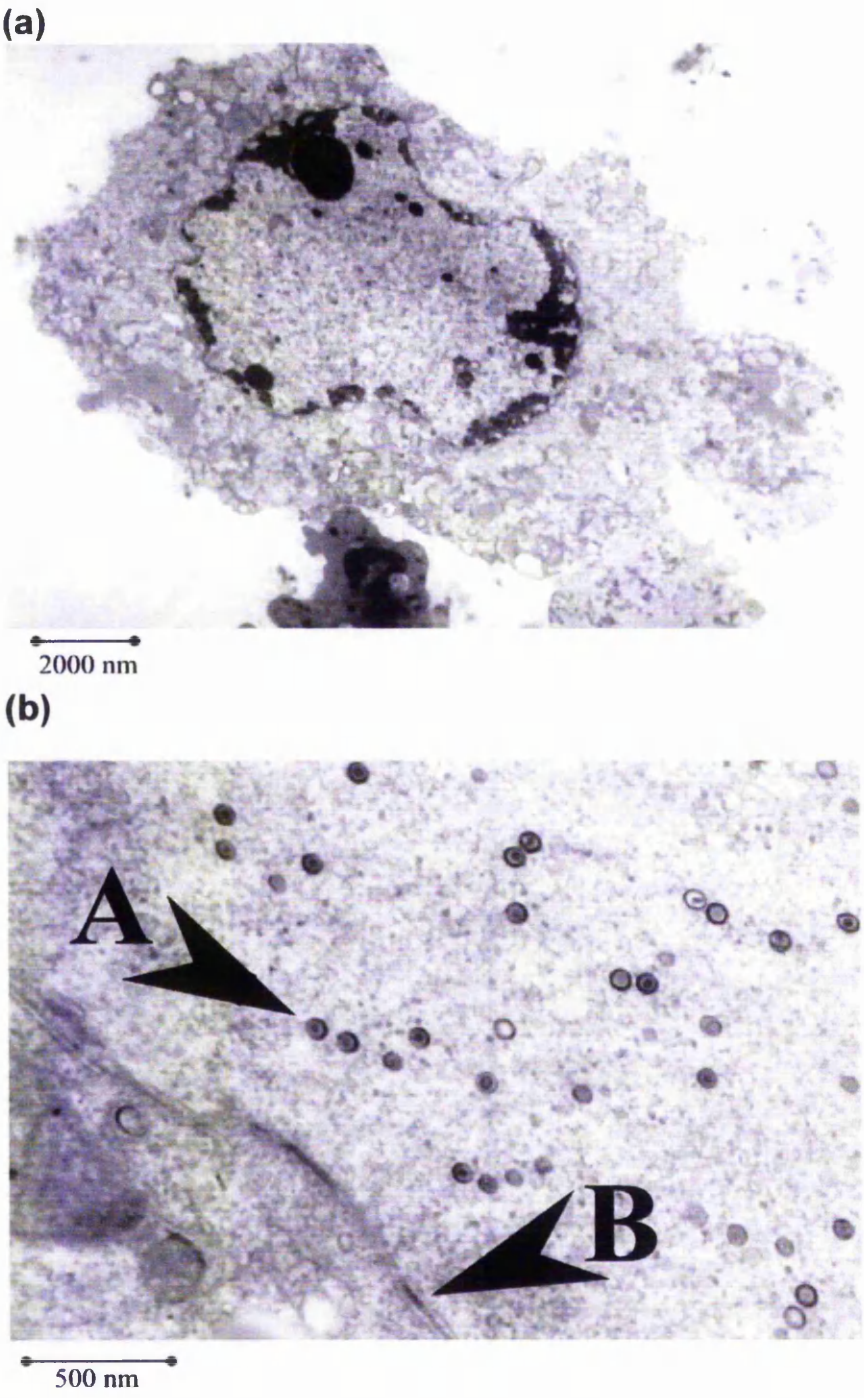


Figure 7.2: Electron micrographs of DU145 cells following infection with DISC-β-gal. DU145 cells were infected with DISC-β-gal and analysed by electron microscopy (a). The high power electron micrograph (b) illustrates the presence of viral particles within the cell nucleus (arrow A). The nuclear membrane (arrow B) is also indicated.

ß-gal activity in infected cell-lines was quantitated using a standard assay over a five-day period following infection. The total protein content of the cells was also measured. Enzyme activity per unit of total cellular protein was undetectable on day 0 following infection, but increased significantly to 6 and 11 U/mg for PC3 and DU145 respectively by day 1 (figure 7.3). There was no significant change in ß-gal activity by day 5. Taking total protein as a surrogate marker for cell mass, these data indicate that the cellular levels of β-galactosidase following infection reach a maximum level within 24 hours, and that β-galactosidase levels are maintained at a steady concentration for at least 5 days.

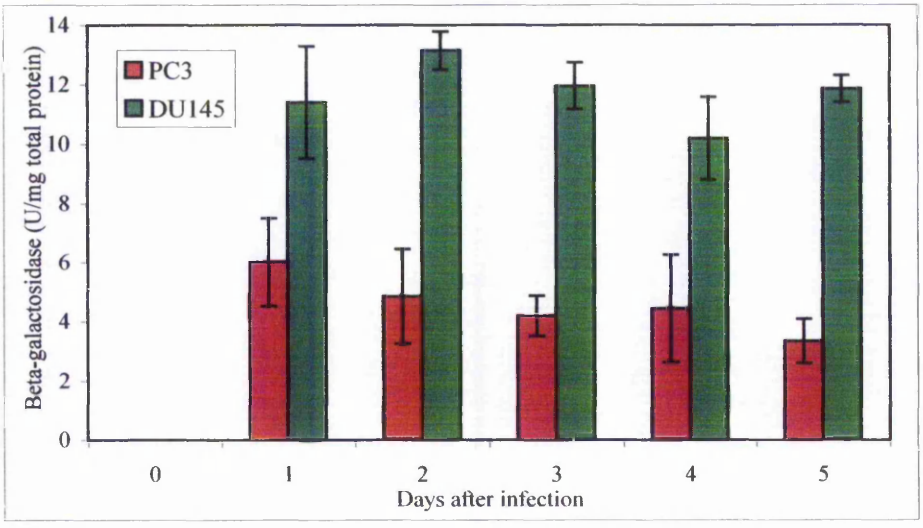


Figure 7.3: β-galactosidase activity of cells following infection with DISC-β-gal.
The β-galactosidase activity of infected cells was measured using a standard kit assay. The total protein content of the cell samples was also measured. The β-galactosidase activity per unit mass of total protein was calculated. Error bars indicate 95% confidence intervals (Student's t-test)

GMCSF production by prostate cancer cell lines

DU-145, LNCaP and PC-3 were tested for their ability to express GMCSF following infection with DISC-hGMCSF. The level of GMCSF produced was monitored every 24 hours over a five day period and compared directly to cells either mock infected or infected with DISC-HSV expressing the control gene ß-galactosidase (figure 7.4 a,b,c).

Figure 7.4b represents the data obtained with PC-3 cells. At 24 hours post infection there was a significant increase in the amount of GMCSF released (520 ng GMCSF/ml/10^6 cells/24 hours) compared with either cells alone (16 ng GMCSF/ml/10^6 cells/24 hours) or cells infected with the DISC-ß-gal virus(14 ng GMCSF/ml/10^6 cells/24 hours). The increase above control values remained significant until day five at which point the values dropped to control levels; however, it was noted that by day three, the rate of cytokine production had fallen to approximately one third recorded on day one.

LNCaP cells had significantly higher levels of GMCSF production for all five days compared to control values (Figure 7.4c), and by day three the rate of production had again fallen to approximately one third. For DU-145, the rate of cytokine production was significantly increased for the first three days as noted with PC-3 and LNCaP, after which the rate of production had fallen to approximately one fifth of the value noted at one day post-infection (Figure 7.4a). It is apparent that all three cell lines express hGMCSF above control levels following infection with DISC-hGMCSF, and that the rate of production is significant until at least day three under *in vitro* conditions.

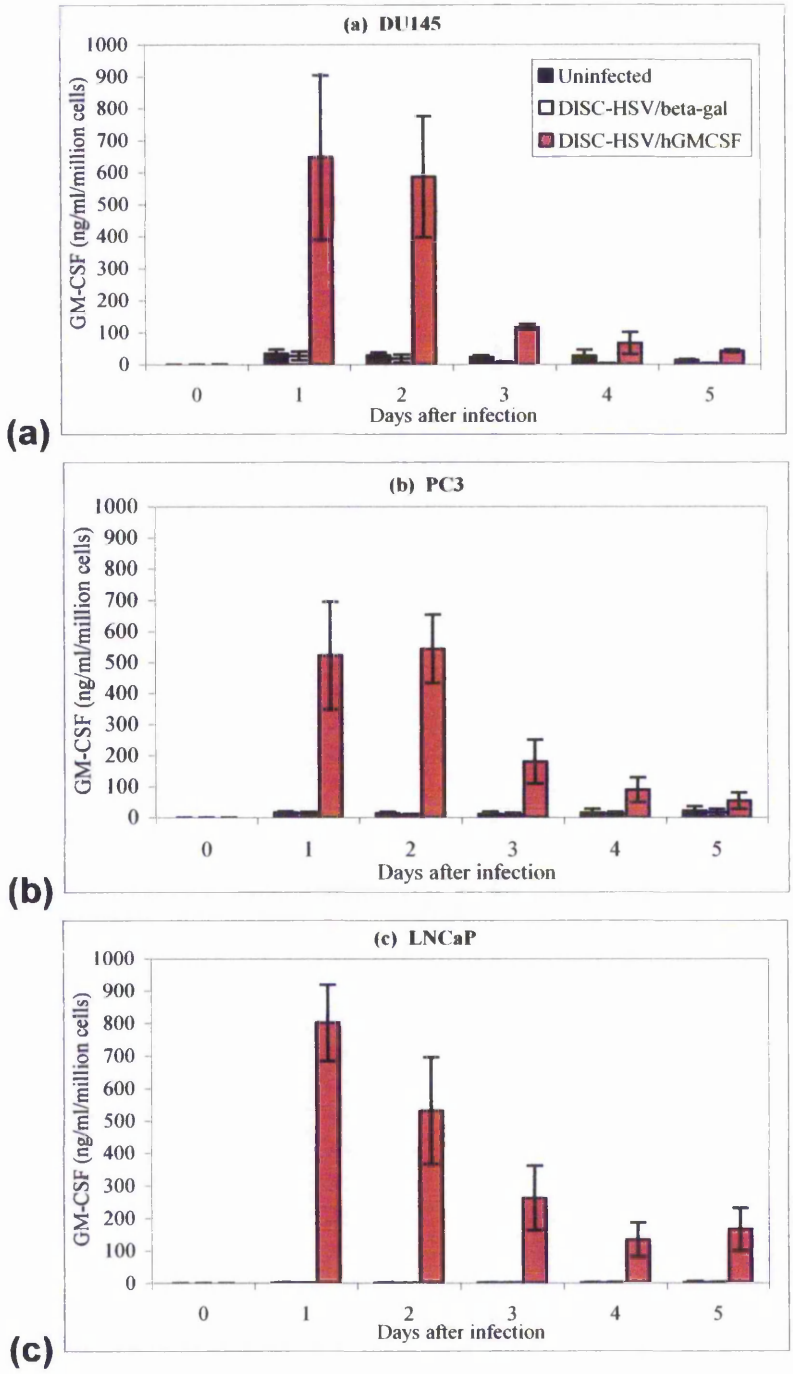


Figure 7.4: Release of hGM-CSF by DU145, PC3 and LNCaP cell-lines following DISC-HSV infection.

The release of hGM-CSF by cells into culture media was assayed by ELISA, with samples taken at daily time points following infection. Cells infected with DISC-hGM-CSF released significantly larger amounts of hGM-CSF than either non-infected controls, or cells infected with an irrelevant virus (DISC-β-gal). Error bars indicate 95% confidence intervals (t-test).

Cell viability following DISC-HSV infection

To ascertain whether the decrease in GMCSF production was attributable to an overall decrease in cell viability, direct counting of trypan blue stained cells collected at daily intervals after DISC-HSV infection was undertaken. Control cells continued to proliferate following mock infection (data not shown), while infected cells showed reduced proliferative activity; however, there was no significant reduction in cell viability for infected populations over 96 hours (Figure 7.5 a,b,c).

The preservation of cell viability was confirmed by flow cytometry following propidium iodide staining of infected cells, which allowed the effects of viral infection on the cell-cycle to be measured. This technique is described in the Material and Methods chapter. Cell-cycle analysis was performed 24 hours after infection and every 24 hours thereafter for seven days.

For all three cell-lines, DISC-β-gal infection produced a significant decrease in the number of cells in G1 compared to non-infected controls (see figure 7.6). This effect was evident on the first day after infection and was maintained until day 7. Infected cells also exhibited an increase in the number of cells in S phase compared to controls. Both these changes were significant (Student's t-test, $p<0.05$). There were no significant changes in cell fractions in G2 for any cell-line, and no significant accumulation of cells in the "pre-G1" peak could be demonstrated.

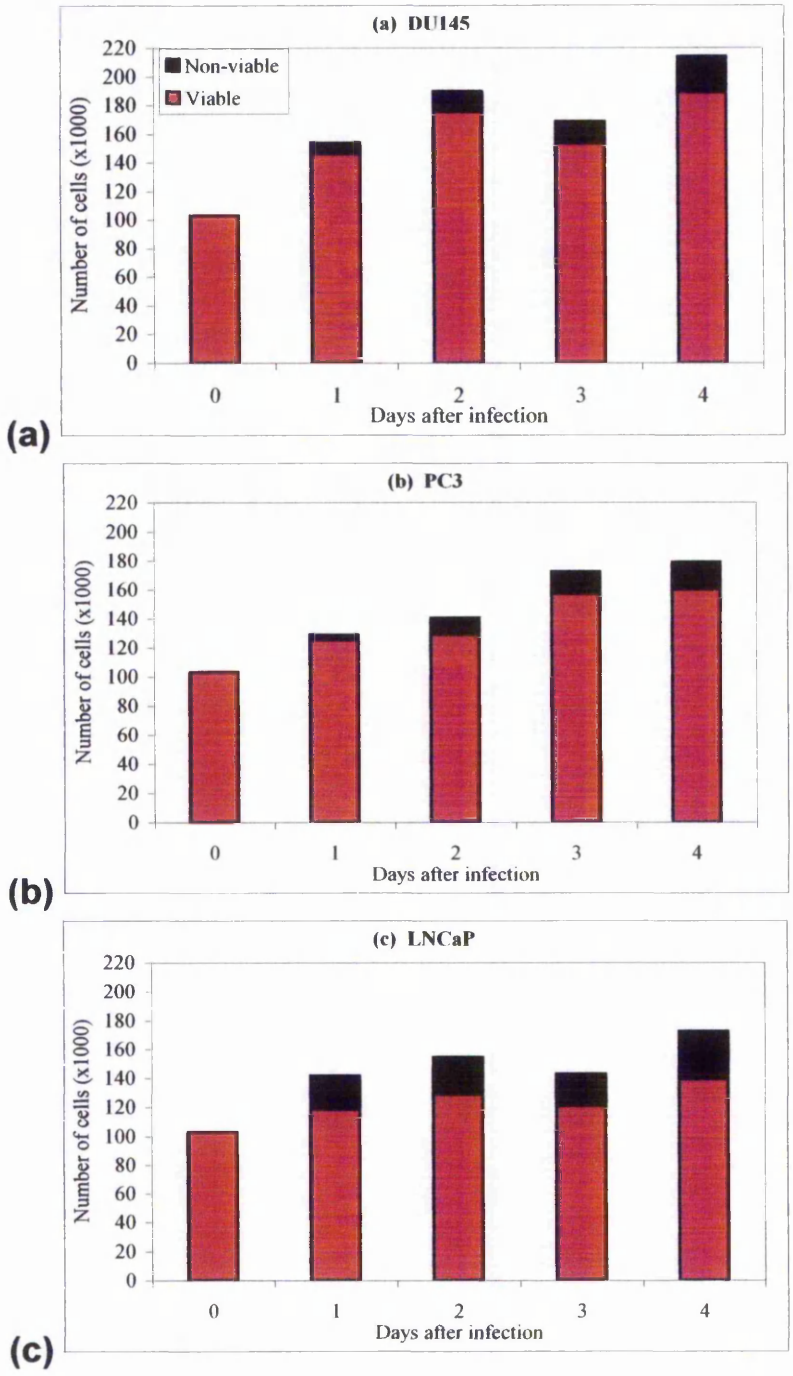


Figure 7.5: Proliferation and cell viability of DU145, PC3 and LNCaP cell-lines following infection with DISC-HSV.

Cells were infected, and counted at daily intervals. The proportion of non-viable cells was determined by staining with 0.1% trypan blue. There were no significant reductions in either viable cells or total cell numbers for any cell line.

(a)

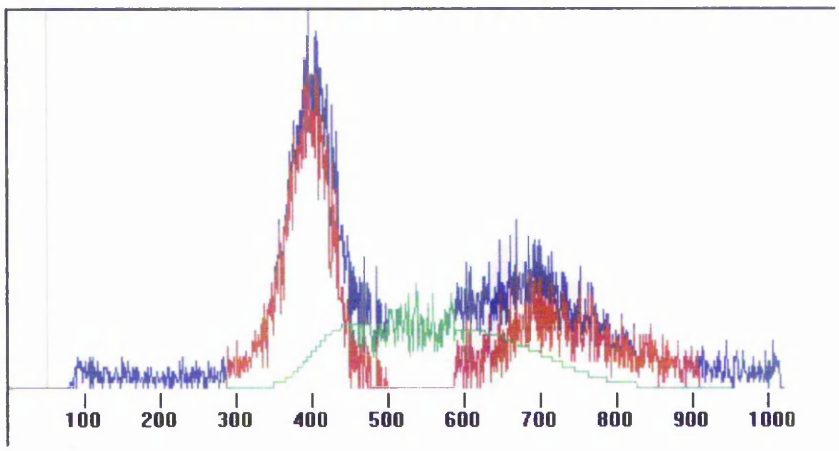
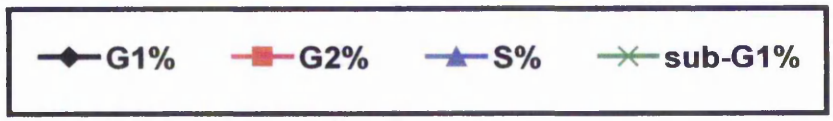


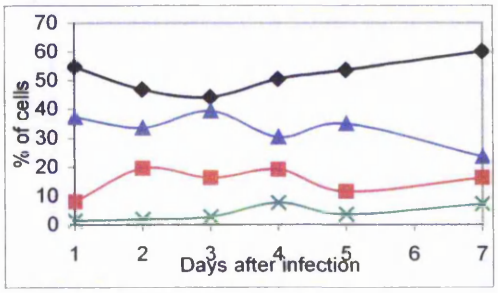
Figure 7.6: Cell-cycle analysis of infected cell-lines.

Flow cytometry following propidium iodide staining was used to characterize cell-cycle dynamics of prostate cancer cell-lines following infection with DISC-β-gal. The data generated were analysed using the Cylchred software package. A typical cell-cycle histogram is shown in **(a)**. The blue line indicates the total number of cells exhibiting a given DNA content (fluorescence). The red and green lines indicate the estimation of G1, S and G2 phases as calculated by the Cylchred software. Graphs **(b, overleaf)** illustrate the proportion of cells occupying each phase of the cell-cycle at different time-points following infection. Non-infected controls are shown for comparison.

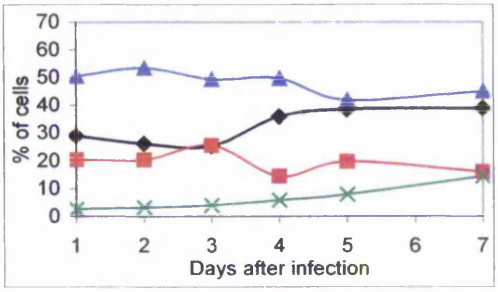
(b)



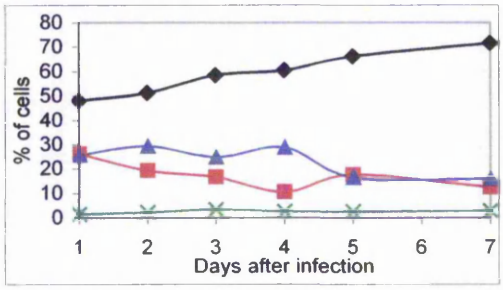
DU145 **control**



infected



PC3 **control**



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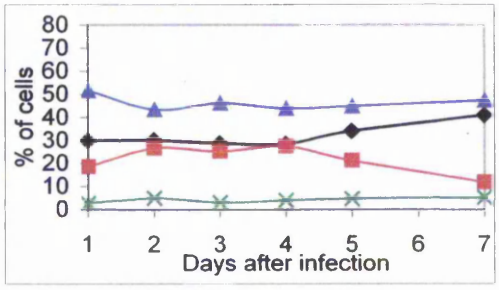
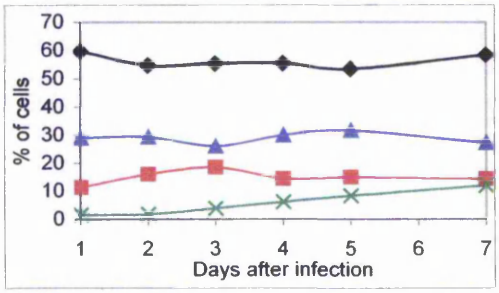
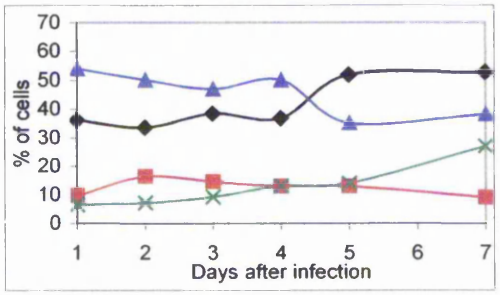


LNCaP **control**



infected



Cell metabolic activity following DISC-HSV infection

To determine whether the decrease in GMCSF production could be attributed to an overall reduction in metabolic activity following viral infection, an electron coupling assay (MTS assay) was conducted to measure the relative amounts of NADH/NADPH within control and virally infected cells. To control for any possible effect that GMCSF expression might have upon cellular metabolism, cells were also monitored for metabolic activity following infection with DISC-β-gal.

Figures 7.7 a,b and c represent the data obtained with each cell line infected with either DISC-β-gal or DISC-hGMCSF. DU-145 cells infected with either virus did not show any significant reduction in metabolic activity over a 72 hour period compared to control cells (figure 7.7a). PC-3 cells did not show significant reductions in metabolic activity for either DISC-GMCSF or DISC-β-gal infected cells (figure 7.7b). There was a significant reduction in the metabolic activity of infected LNCaP cells, by approximately 20% and 30% for DISC-GMCSF and DISC-β-gal infected cells respectively (figure 7.7c).

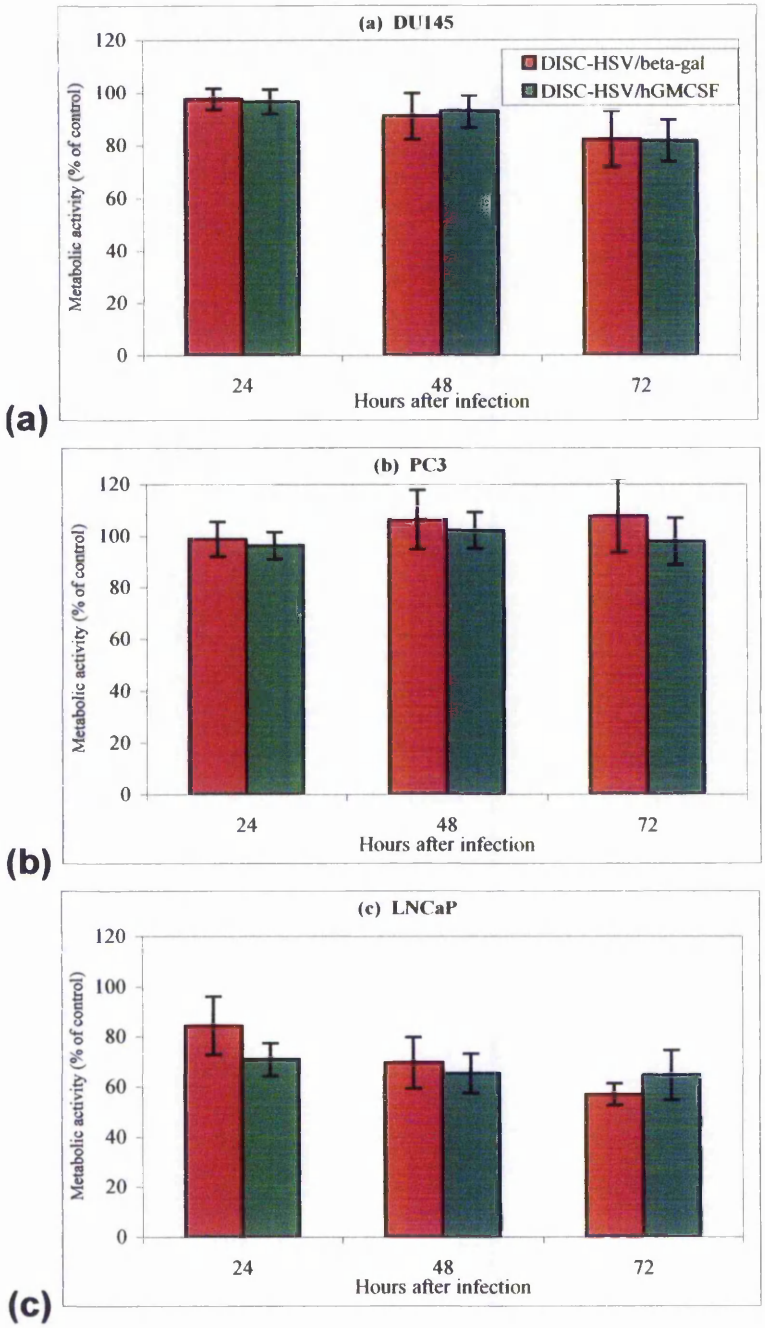


Figure 7.7: Metabolic activity of DU145, PC3 and LNCaP cell-lines following DISC-HSV infection.

The metabolic activity was measured using a NADH/NADPH electron-coupling assay. The metabolic activity of DISC-β-gal and DISC-hGMCSF infected cells is expressed as a percentage of the activity of non-infected controls at each time point. There was no significant decrease in the metabolic activity of either DU145 or PC3 cells, and LNCaP cells exhibited a moderate 20-30% decrease over 3 days. Error bars indicate 95% confidence intervals (t-test).

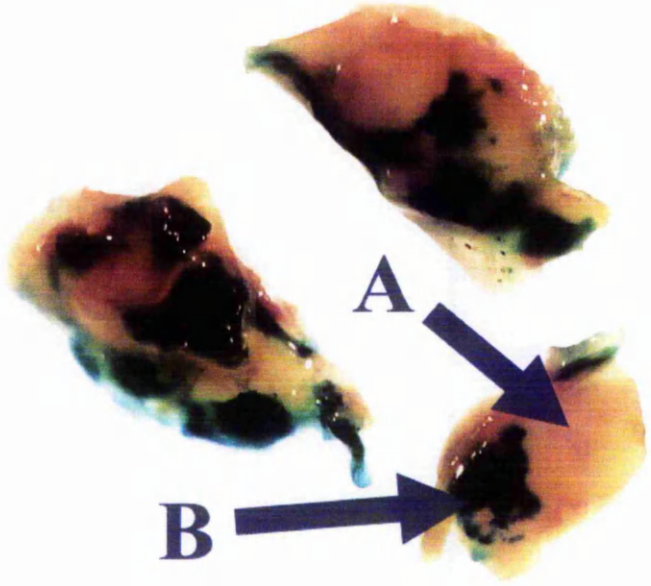
Infection of human prostate cancer xenografts

The introduction of cytokine/immune modulating genes into tumour tissues using DISC-HSV may best be facilitated by the administration of the virus by direct intratumoural injection. To ascertain whether DISC-HSV vectors were capable of infecting prostate cancer tissue, human xenografts of PC-3 and DU-145 were established in athymic "nude" mice, and DISC-ß-gal virus injected directly into the tumour. Twenty-four hours following injection, the tumours were excised and stained for β-galactosidase activity prior to electron-microscopic examination.

DISC-ß-gal was utilised for this study as areas containing enzyme activity can be readily detected (by X-gal staining), facilitating the localisation of prostate cancer cells that have been infected by the virus. Figure 7.8a shows a representative staining pattern obtained with DU-145 xenograft following intra-tumoural injection with DISC-HSV and areas either devoid (arrow A) or containing ß-gal activity (arrow B) can be readily identified. Using positively stained areas as a guide, tissue sections were prepared for examination under electron microscopy.

Figure 7.8b shows a representative micrograph of DU-145 prostate cancer xenografts infected with DISC-ß-gal in which non-enveloped viral particles can be clearly distinguished within the nucleus of infected prostate cells (arrow A). These particles have the same structure and dimensions as non-enveloped particles identified from pure stock cultures of DISC-ß-gal (data not shown). Arrow B indicates the boundary of the nuclear membrane and arrow C delineates the intercellular space. Based upon morphological appearances (intact intracellular membranes and organelles), these cells appear to be viable and undamaged following injection procedure. Similar results were also obtained for PC3 tumours (Figure 7.9 a,b).

(a)



(b)

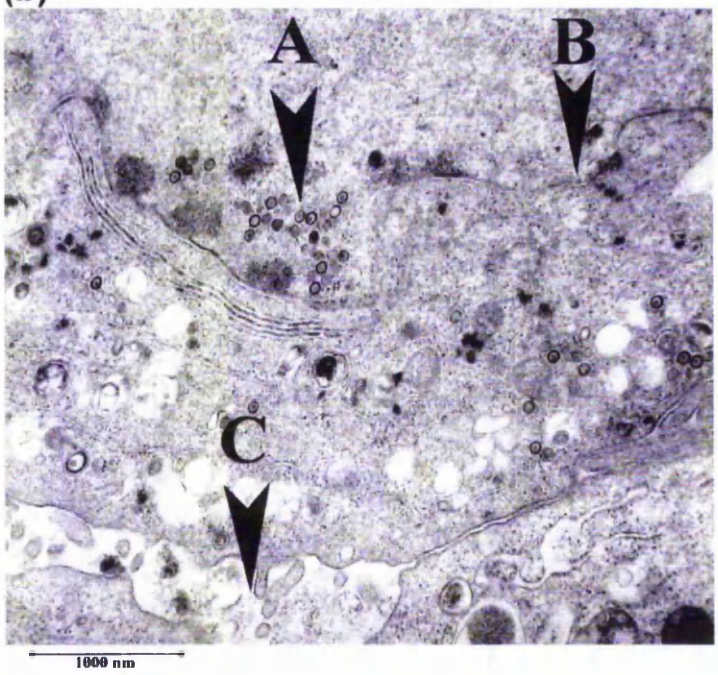


Figure 7.8: DU145 xenograft following infection with DISC-β-gal.

(a) shows staining of xenograft tumour using X-gal following infection. Unstained tissue appears pale (arrow A) while dark areas (arrow B) indicate positive staining for the β-galactosidase reporter gene. (b) shows an electron micrograph of the same tumour, with viral particles seen within the cell nucleus (arrow A). The nuclear membrane (arrow B) and intercellular space (arrow C) are also indicated.

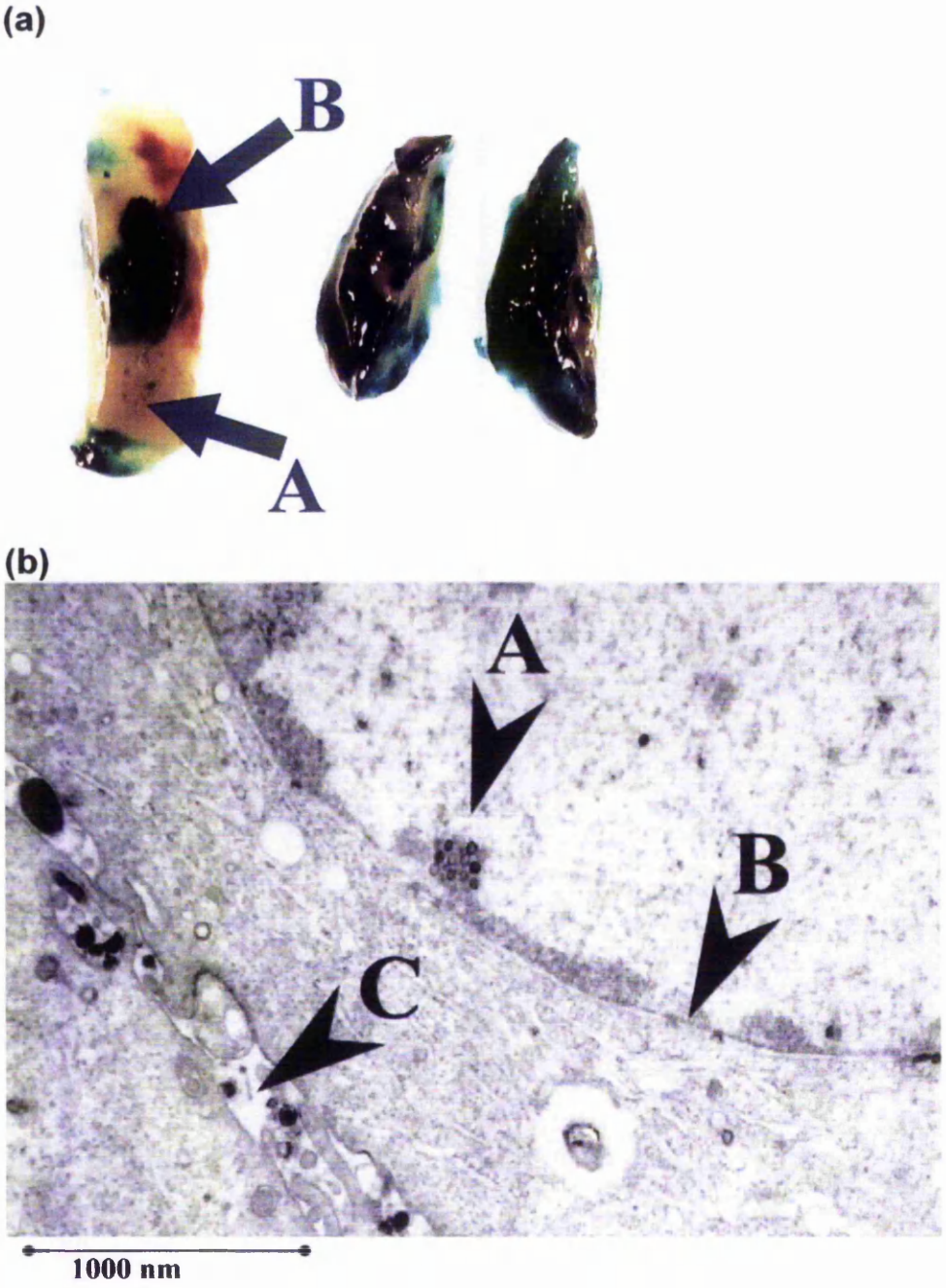


Figure 7.9: PC3 xenograft following infection with DISC-β-gal.

(a) shows staining of xenograft tumour using X-gal following infection. Unstained tissue appears pale (arrow A) while dark areas (arrow B) indicate positive staining for the β-galactosidase reporter gene. (b) show an electron micrograph of the same tumour, with viral particles seen within the cell nucleus (arrow A). The nuclear membrane (arrow B) and intercellular space (arrow C) are also indicated.

Infection of* ex vivo *human prostate cancer samples

The ability of DISC-HSV vectors to infect prostate cancer tissues was further evaluated by incubating samples of prostate cancer tissue obtained from surgical specimens with virus containing the reporter gene β-gal. Following excision of charred and necrotic tissue, prostate samples were placed in 48-well plates and covered with 300μl media containing 10^8 pfu of test virus. After 24 hours, the samples were washed and stained for β-gal expression. A negative control was provided by staining tissues processed in an identical manner, but incubated in media containing DISC-hGMCSF or media alone. Figure 7.10 illustrates the resulting staining patterns produced. Samples incubated with DISC-β-gal show marked blue staining, illustrating strong β-galactosidase activity within the tissue. In contrast, control samples, either incubated with DISC-hGMCSF or media alone, show no staining.

In order to ascertain whether the observed β-galactosidase activity could be correctly attributed to viral infection and gene expression by prostate cancer cells, a further control experiment was performed. Prostate cancer samples were exposed to two cycles of freeze-thaw using liquid nitrogen in order to obtain structurally similar samples of dead tissue. Following infection with virus, the samples were stained for β-galactosidase activity as before. Figure 7.10b shows that the tissue samples exhibit high levels of β-galactosidase activity, comparable to that of living tissues. It is therefore impossible to rule out that the observed activity may be at least partly due to the passive absorption of the β-galactosidase enzyme by the tissues during incubation rather than the infection of prostate cancer cells and subsequent expression of virally encoded genes.

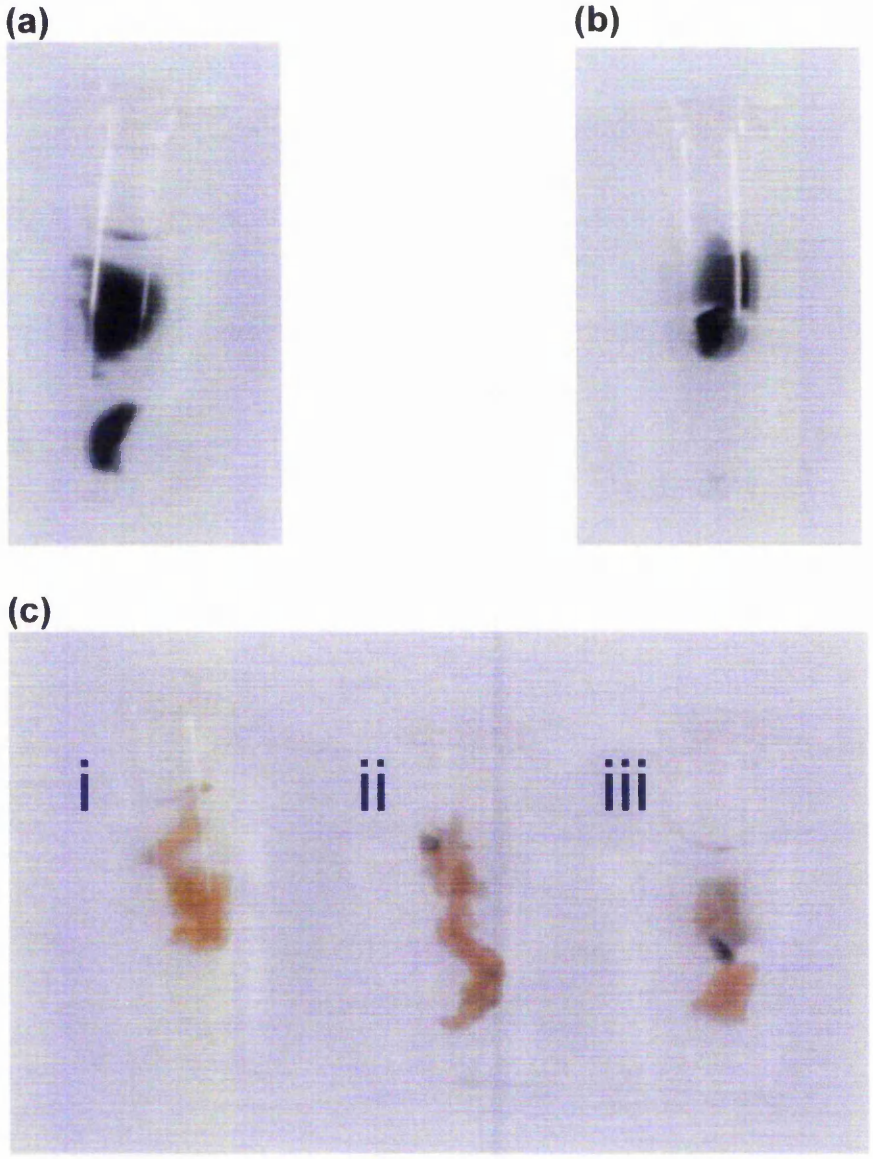


Figure 7.10: Infection of ex-vivo human prostate cancer samples with DISC-β-gal.

(a) shows staining of human prostate tumour using X-gal following infection with DISC-β-gal. Tissues appear dark, indicating positive staining for the β-galactosidase reporter gene. (b) shows staining of human prostate tumour following freeze-thaw cycles prior to DISC-β-gal infection, with similar appearances. It is likely that staining is due to the presence of β-galactosidase in solution in the virus stocks, which may be passively absorbed by the tissues in these experiments. Infection of these tissues by DISC-β-gal cannot be inferred. (c) shows negative controls, including non-infected tissue (i) and tissue infected with DISC-hGMCSF (ii and iii).

Discussion

Clinical studies of prostate cancer patients have suggested that immune targeting is possible using either peptide vaccines (Meidenbauer *et al.* 2000), or a combinatorial approach involving APC pulsed with MHC class I peptides from well-defined prostate cancer associated antigens (Murphy *et al.* 1999; Murphy *et al.* 1999; Small *et al.* 2000). Successful stimulation of an immune response against such antigens would enable anti-tumour strategies to be developed that are not contingent upon the direct manipulation of prostate tissue; however, antigen-specific treatments have the disadvantage of a narrower shared antigenic spectrum with the host's tumour, and are often restricted to a particular MHC phenotype.

Strategies which have the potential to augment the immune response against multiple unknown as well as known cancer antigens present in an individual patient's tumour are likely to be more universally applicable. Autologous whole-cell vaccine approaches employ such a strategy, but have been limited by the technical difficulties in producing sufficient numbers of cells for effective vaccination protocols (Simons *et al.* 1999). Allogeneic whole-cell vaccines (Hrouda *et al.* 2000; Eaton *et al.* 2002) have also enjoyed only limited success, possibly because of the limited antigenic homology between the host tumour and the vaccine, or the need to HLA match the vaccine to the patient.

Previous reports have demonstrated that DISC-mGMCSF is capable of inducing prophylactic immunity against a lethal challenge of tumour cells in murine models of melanoma, renal carcinoma and sarcoma, and that the effect was contingent upon the synergistic action of both $CD4^+$ and $CD8^+$ T-cell populations (Ali *et al.* 2000). Further studies have indicated that direct intra-tumoural injection with DISC-mGMCSF

vectors into murine colorectal (CT26) tumours induced the regression of pre-established tumours in at least 40% of animals (Todryk *et al.* 1999). CT26 cells are known to express the glycoprotein gp70, and following intra-tumoural injection with DISC-mGMCSF, $CD8^+$ CTL capable of recognising specific peptides from this antigen were generated (Ali *et al.* 2002). The translation of this approach to other tumour systems would be of great interest, especially those tumours for which intra-tumoural vaccine delivery would be feasible, such as prostate cancer.

The validity of such a strategy in prostate cancer is supported by early successes using an adenovirus vector expressing IL-2 as an intratumoural agent in prostate cancer patients. A phase I trial in prostate cancer patients confirmed the feasibility of the treatment, which was well tolerated. CD8+ T-lymphocytes were identified in subsequent pathology specimens, and a decline in PSA levels was elicited in patients receiving a low dose of the agent (Trudel *et al.* 2003).

To establish whether DISC-hGMCSF might ultimately be considered as a therapeutic treatment for hormone refractory prostate cancer it was necessary to ascertain whether DISC-hGMCSF could reproducibly infect prostate cancer cells and express hGMCSF to levels significantly higher than background. Using reporter gene expression cassettes it has been shown that DISC-HSV is able to infect greater than 90% of DU-145, LNCaP and PC-3 cells under *in vitro* conditions.

All three cell lines were able to produce significantly raised levels of hGMCSF for at least 72 hours following infection compared to either non-infected or infected control cells. Electron microscopy has confirmed the presence of intracellular particles following infection. These data provide the first evidence of the ability of DISC-HSV to infect human prostate cancer cells and the possible utility of this virus as a gene delivery system in human prostate cancer.

The subsequent decrease in cytokine expression (to approximately one third of the value obtained for the 24 hour time point by 72 hours) could not be attributed to virally induced death as cells infected with DISC-HSV continued to remain viable for at least 7 days post infection. In addition there was no evidence to suggest that the decrease in hGMCSF production by day 3 could be attributed to an overall reduction in the metabolic activity of cells (as measured by NADH/NADPH levels); the metabolic activity for DU-145 and PC-3 were comparable to non-infected control cells, and the small but significant decrease in metabolic activity (30%) noted for LNCaP cells following DISC-β-gal infection would not be sufficient to explain the reduction in cytokine production.

Cell-cycle analysis using propidium iodide staining of infected cells failed to demonstrate a significant accumulation of cells in the "pre-G1" peak, suggesting that infected cells do not undergo apoptosis or necrosis during the first 7 days after infection with DISC-HSV. Cell accumulation in pre-G1 is typical of apoptosis or necrosis, as these processes result in a decrease in the quantity of cellular DNA. These findings are consistent with the cell viability and metabolism data described above, and are in agreement with other similar studies of DISC-HSV infection (Dilloo *et al.* 1997).

Cell-cycle analysis demonstrated an increase in the proportion of cells in S-phase accompanied by a reduction of cells in G1, indicating an increase in cellular DNA following infection. Similar results have been observed following DISC-HSV infection of RENCA cells (Ali *et al.* 2000), and a possible cell-cycle block was postulated. These results would support the theory of an S-G2 block following DISC-HSV infection. Detailed studies of cell-cycle arrest in non-modified HSV have demonstrated a block in the progression to S-phase, probably mediated through immediate-early (IE) genes such as ICP0 and ICP27 (Flemington 2001). The effect of this may have been to confer an evolutionary advantage by reducing the competition from the cellular DNA replicatory apparatus for nucleotides. It remains uncertain to what extent the genetic modification of DISC-HSV may have altered the cell-cycle dynamics of the host cell, although DISC-HSV has deletions of key IE genes (Samaniego *et al.* 1998), which may explain its distinctive effects on the cell-cycle.

It is possible that the observed reduction in gene expression is a result of non-integration of the virus (with consequent loss as the cells divide) rather than the lytic activity of the viral genes. Cells that are non-permissive for expression of cytotoxic "late-gene" products (such as lymphoid cells) or are resistant to their effects may not exhibit toxic effects. Herpes simplex viruses are able to replicate only during a lytic cycle (Skaliter and Lehman 1994). In the latent form, such viruses exist extrachromosomally and will be unable to multiply as the cells divide, thus becoming progressively diluted with time.

The transient nature of the expression of virally encoded genes is a potential disadvantage of DISC-HSV as a vector system for gene therapy. The application described here aims to modify tumour cells to generate immunomodulatory

molecules, and the most important requirement is therefore the rapid and efficient expression of the viral genome, rather than persistent expression. The results of related studies using DISC-HSV indicate that even transient expression of encoded GMCSF can induce anti-tumour immunity against malignant human haemopoietic cells (Dilloo *et al.* 1997).

To determine whether DISC-HSV has the potential to infect prostate cancer tissue following direct intra-tumoural injection (and thus model clinical scenarios for introducing the virus into prostate tissue), human xenograft models were established in congenitally athymic (nu^+/nu^+) mice using DU-145 and PC3 tumour cells, and injected with DISC-ß-gal reporter constructs. X-gal treatment of excised solid tumours produced blue staining of tissues consistent with β-galactosidase activity in infected cells.

Using X-gal staining to identify areas of ß-galactosidase activity, tissue sections were analysed by electron microscopy for evidence of DISC-HSV infection. DISC-HSV particles were clearly identified within prostate cancer cell nuclei. The data obtained from both xenograft models suggest that virus can penetrate prostate cancer cells following direct intra-tumoural injection. Based upon the evidence obtained from these studies, it is suggested that DISC-HSV could be introduced directly into the tumours of prostate cancer patients by direct intra-tumoural injection.

In contrast, the results obtained from the infection of surgical prostate tissue specimens were disappointingly inconclusive. Although high levels of staining were observed following the exposure of tissue samples to the virus, similarly intense staining was also obtained when non-viable (freeze-thaw treated) tissues were treated in an identical manner. One cannot exclude the possibility that passive absorption of

β-galactosidase protein present in the virus media could be the source of the positive staining observed, rather than active expression of viral genes. Confirmation of intracellular viral particles, as seen in the xenograft experiments, was not possible due to the degree of histological disruption caused by intraoperative cautery and post-operative ischaemic necrosis of the excised tissues. Improvements in tissue collection, transport and culture protocols may be helpful in resolving these problems and allow more meaningful results to be obtained using such surgical specimens in the future.

A wide variety of viruses have been used as vectors in immunotherapy applications, including adenovirus and vaccinia. DISC-HSV may have a number of advantages over other viral vector systems (Rees *et al.* 2002), not least its ability to infect a wide variety of target cell types with an infection rate of approaching 100%. The considerable size of the virus also provides a significant benefit, as it allows the possibility of transducing large foreign DNA fragments into the HSV genome.

The data from these studies have suggested that DISC-HSV is a candidate vector system for the delivery and expression of inflammatory cytokines such as hGMCSF in human prostate cancer (Parkinson *et al.* 2003). It is important to extend these studies to establish whether the observed therapeutic effect in colorectal and sarcoma tumour systems can be reproduced in murine models of prostate cancer and thus whether DISC-GMCSF can be used as an immunogene therapy in prostate cancer patients. DISC-HSV has safely been used in humans in clinical trials as an anti-HSV vaccine (Hickling *et al.* 1998). Further safety data specific for the DISC-hGMCSF virus will be required prior to its use as a therapeutic vaccine in human clinical trials.

General Discussion

The recognition of the need for the development of new treatments for all stages of prostate cancer is universal. The management of systemic disease is particularly problematic because, although hormone treatment usually provides medium term palliation, the development of hormone independence is almost inevitable, and alternative treatments have drawbacks that limit their usefulness.

This thesis has described the development of two novel approaches to prostate cancer therapy, both employing a viral vector system to deliver a gene, the product of which can stimulate an anti-tumour immune response.

The intra-tumoural delivery of GMCSF has stimulated anti-cancer immunity against a variety of tumour types, in both model systems (Ali *et al.* 2002) and humans (Simons *et al.* 1999). Viral delivery of the GMCSF gene is an attractive method of generating suitable concentrations of the cytokine within target tissues. The application of such treatment in prostate cancer may be favoured because of the relative ease of access to the prostate gland, facilitating intra-tumoural injection, but is dependent on the ability of the virus to infect prostate cells and initiate expression of virally encoded genes.

The ability of the DISC-HSV viral vector to infect prostate cancer cells has been confirmed in these studies, both *in vitro* and *in vivo*. Inasmuch as the expression of virally encoded gene products has been demonstrated, with significant levels of GMCSF expression being maintained for up to five days in prostate cancer cell-lines, the feasibility of intra-tumoural injections of DISC-GMCSF in prostate cancer is confirmed. The clinical application of DISC-GMCSF injections will be further dependent on the results of clinical trials. The use of DISC-HSV has been shown to be

safe in humans (Rees *et al.* 2002), yet further tolerability studies will be required before the use of the genetically modified DISC-GMCSF virus can be approved.

Animal models of prostate cancer are unlikely to be useful in further predicting the clinical efficacy of DISC-GMCSF in humans. The Dunning and Lobund-Wistar rat models of prostate cancer have been used by several workers, but are limited by long tumour latency and the variability of tumour progression (Bostwick *et al.* 2000), and although they have been useful in the study of cancer development, they are not ideal models for the study of vaccine therapies. Canine models of prostate cancer are attractive, especially as the size of the animal facilitates easy access to the prostate gland. However, dog colonies are prohibitively expensive to maintain, and the variable and prolonged course of prostate cancers in these animals has limited their usefulness (Waters *et al.* 1998). Mouse models include xenografts raised in immune deficient (SCID or nude) animals and transgenic systems (Abate-Shen and Shen 2002). Immune depleted animals with human prostate cancer xenografts will not be able to mount an immune response in response to DISC-GMCSF and so cannot be used to test its clinical efficacy. Transgenic models, such as the TRAMP and LADY strains would require the use of a virus encoding the GMCSF specific for that species. This would be possible in mice, and a DISC-HSV carrying the murine GMCSF gene has already been developed (Ali *et al.* 2002), but the feasibility of such an approach would be limited by the difficulty in access to the gland.

The development of systemic immunotherapy for prostate cancer, targeting cancer-specific epitopes, is attractive owing to the abundance of antigens that are prostate-restricted and overexpressed in cancer. A variety of vaccine strategies have been proposed, employing PAP, PSMA, PSA and others as antigenic targets. A prerequisite

for the development of such treatments is the identification of suitable epitopes, which must be capable of stimulating the proliferation of cytotoxic T-lymphocytes that can recognise and destroy cancer cells bearing the same antigen.

This thesis described the identification of novel epitopes derived from prostate-cancer specific antigens. Epitopes, predicted on the basis of their amino-acid structure, have been tested for their ability to bind HLA-A2, suggesting their ability to act as immune targets. The binding of the PAP.135 epitope is significantly stronger than many prostate epitopes described in earlier studies. The ability of this epitope, and others, to stimulate an immune reaction when administered as a peptide vaccine has been determined using a transgenic animal model. Vaccination produced cytotoxic T-lymphocytes with the ability to destroy target cells in an epitope-specific manner, and this was shown to be CD8+ dependent. Thus, the PAP.135 epitope may be proposed as a potential target for inclusion in vaccination strategies against prostate cancer.

Concern remains that PAP.135-reactive T-lymphocyte clones would have been clonally deleted in human males, owing to prolonged exposure of the immune system to this antigen in the absence of danger signals (see Introduction). It is arguable that strongly immunogenic epitopes would be more susceptible to clonal deletion or other mechanisms of immune tolerance, and the immunogenicity of PAP.135 must therefore be confirmed in human studies. Early data have been presented that suggests that PAP.135 can stimulate an immune response in humans, and produces activation of naïve PBMC derived from the blood of healthy donors. These data are limited and require confirmation and further assessment. As outlined in the relevant chapter, further restimulations of *in vitro* PBMC cultures with the test peptide are often required to elicit strong cytotoxicity. The clonal expansion of cell cultures that show

strong cytotoxic effects would also be desirable, and both these steps will be undertaken in the next phase of these studies. Further useful data may be obtained by using blood donated by patients with advanced prostate cancer, which would more closely mimic clinical scenarios.

The goal of these experiments will be to establish a panel of prostate cancer specific epitopes that can be employed in vaccine therapies. It is hoped that such epitopes may be included in a multi-epitope construct and delivered by means of the DISC-HSV system, as outlined in chapter three. The design and assembly of multi-epitope vaccine constructs has been described. This preparatory work has been undertaken so that the efficacy of a multi-epitope vaccine comprising DISC-HSV can be assessed. The class-I and class-II vaccines will be administered to transgenic mice as intradermal injections at the base of the tail. The stimulation of cytotoxic T-cell responses will be assessed in a similar manner to that described in this thesis for the assessment of immune responses to peptide vaccination.

Ultimately, the multi-epitope vaccine would be the subject of phase I and phase II clinical trials. Although such trials would almost certainly enrol patients with either hormone-refractory or locally advanced prostate cancer, it would be envisaged that such a vaccine could have application in all stages of the disease. It is theoretically possible that a vaccine could be used prophylactically in healthy individuals, but considerable work remains before this can be achieved.

In order to further develop the concepts described in this thesis, work needs to continue in a number of directions. The development of the multi-epitope constructs requires the testing of these vaccines, firstly in a transgenic model system similar to that employed in chapter five. Ideally, peptide vaccination should be undertaken

initially, before moving on to vaccination with the multi-epitope/DISC-HSV construct. Cytotoxicity against peptide bearing target cells would be tested in the case of the class-I construct, whereas lymphocyte proliferation (e.g. using a thymidine uptake assay) would be used to assess the response to the class-II peptides. If successful, work would then be able to progress to the construction of a multi-epitope vaccine comprising prostate cancer epitopes, such as PAP.135.

Work directed at the identification of prostate cancer antigens has yielded at least one strongly immunogenic class-I epitope, PAP.135. A single target epitope is unlikely to provide a universally effective prostate cancer vaccine for reasons alluded to elsewhere; not least, the heterogenicity of cancers and the downregulation of peptide expression by selection pressure. Further peptides are therefore required. The PSMA.168 and PAP.284 peptides exhibited promising MHC class-I binding, as determined by T2 binding assay, but further testing of these peptides in the transgenic mouse system was not undertaken due to the extensive work on PAP.135. These peptides should be tested further, in the same manner as PAP.135. Further examination of prostate associated antigens using epitope prediction algorithms should also be undertaken, and newly emerging peptides such as prostein and trp-p8 should be analysed.

The PAP.135 peptide has shown considerable promise in these experiments. Further testing in human model systems is required prior to its use in clinical trials. The protocol described in chapter six requires modification to facilitate multiple *in vitro* restimulations of lymphocyte cultures in order to improve the reproducibility of the results. Ethical approval should be sought to sanction the donation of blood by healthy volunteers and prostate cancer patients to establish whether the PAP.135 peptide can

be immunogenic in several HLA-A2 positive humans. The possibility of clonal deletion and/or tolerance in some subjects can thus be examined.

Proof of principal for the use of DISC-GMCSF in prostate cancer has been established by the experiments described in chapter seven. The obstacle to the establishment of phase-I clinical trials with this agent is the requirement for further safety data with respect to its use in humans. Although DISC-HSV has undergone extensive safety testing, the further-modified DISC-GMCSF construct would require additional assessment prior to application for the appropriate testing license. Unfortunately, all research relating to the DISC-HSV vector is under the control of the pharmaceutical company that owns the rights to the virus and the intellectual properties relating to it. At the present time, no further research is being sanctioned in this area owing to the financial priorities of the firm.

Appendices

Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid (Aspartate)
Cys	C	Cysteine
Gln	Q	Glutamine
Glu	E	Glutamic acid (Glutamate)
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine
Asx	B	Aspartic acid or Asparagine
Glx	Z	Glutamine or Glutamic acid

Appendix 1: Nomenclature for amino-acids

The abbreviations and symbols used for amino-acids are given, following the conventions of the Joint Commission on Biochemical Nomenclature (1984).

Prepare 3 dilutions of test peptides: 500 μM, 50 μM and 5 μM

T2 cells are grown in suspension using RPMI + 10% FCS
Harvest T2 cells by washing twice with serum free RPMI
Count cells are resuspend at 4 x 10^6 cells / ml
Seed 40 microlitres cells (160,000) into U-bottomed 96 well-plate
3 wells per test peptide
Add 10 microlitres peptide at each concentration (500, 50 and 5 microM)
final concentrations of peptides: 100, 10, 1 microM
Incubate at 37°C overnight (>18 hours)

Harvest cells in 200 microlitres PBS + 0.1% BSA
Transfer to flow cytometry test tubes
Wash 2ml PBS + 0.1% BSA and discard supernatant
Resuspend in residual PBS + 0.1% BSA
Add primary antibody (MA2.1)– 20 microlitres
Incubate 30 minutes on ice
Wash in 2ml PBS + 0.1% BSA
Add secondary antibody to all tubes – 100 microlitres of 1/100 dilution
Wash twice in 2ml PBS + 0.1% BSA
Fix in Isoton solution
Analyse by flow-cytometry – excitation at 488nm

Appendix 2.1: Summary of MHC binding assay protocol.

Day 0	Peptide immunization is given as 100µl bolus at base of tail
Day 7	Kill immunised mice, remove spleen. Disect away peri-splenic fat. Transport on ice in transport media Spleen is gently macerated and flushed with "CTL culture media". A fine bore needle is loaded onto a 10ml syringe filled with media. The spleen capsule is punctured, and media is forced through the spleen, washing out interstitial cells and some debris. Spleen is digested. Remaining spleen tissue is diced and immersed in 1ml Digestion Mix in a 15ml centrifuge tube, and incubated at 37° for 1 hour. After digestion, solid matter is further broken down by pipetting. Digested material containing cells is washed in CTL media. Flushed and digested cells centrifuged at 1500 rpm for 5 minutes Flushed and digested cells combined in 5 ml CTL media. Cells are counted and resuspended at $5x10^6$ cells/ml Cells are resuspended in 5 ml CTL media. A 15µl aliquot is mixed into 45µl white-cell counting solution. A 15µl aliquot of this mixture is mixed with 45µl of trypan blue, and the resultant cell suspension is counted using a haemocytometer. The correct concentration is attained by addition of CTL media to the original stock. 1ml (5×10^6) cells are added to 15 wells of a 24 well plate. Target peptide is diluted to 20µg/ml in CTL media Enough for 12 wells for each splenocyte population. In addition, an irrelevent class-I peptide should be added to 3 wells for each splenocyte population. 1ml peptide solution is added to each well. Incubate at 37°C for 5 days
Day 12	Perform Chromium-release cytotoxicity assay

Appendix 2.2: Summary of the protocol used for the maturation of CTL.

Preparation of targets

Place 3-4 x 10^6 target cells in a 15ml centrifuge tube.
eg. RMAS, T2 cells, LCL-BM cells are used as targets.

Centrifuge at 1500 rpm for 5 minutes and wash in 5 ml of complete media.

Discard the supernatant, and resuspend the pellet in 1ml.

Add 100µg peptide + 20µg ß-2-microglobulin
($ß_2$-m not needed for RMAS)

Incubate at 37°C overnight.
early experiments pulsed only on day of assay, with inferior results

Wash cells, and resuspend in residual media

Add 1.85 - 3.7 MBq ^{51}Cr to the cells and incubate at 37°C for 1 hour.
During this hour, prepare the effector cells derived from the immmunised mice.

Preparation of effector cells

Harvest cells into a single universal container (for each test peptide).

Wash in CTL media (spin 1500 rpm for 5 minutes).

Count and resuspend at 5 x 10^6 / ml or 2 x 10^6 / ml.
Cells are resuspended in 1 ml CTL media. A 15µl aliquot is mixed into 45µl white-cell counting solution. A 15µl aliquot of this mixture is mixed with 45µl of trypan blue, and the resultant cell suspension is counted using a haemocytometer. The correct concentration is attained by addition of CTL media to the original stock.

Add 200µl of cells to 3 adjacent wells of the 1st and 7th columns of a 96 well plate.
This should be done for each peptide to be tested. Use a non-sterile round-bottomed plate.

Perform serial 50% dilutions across the following 5 columns.
Add 100µl media to each well in columns 2, 3, 4, 5 and 6. Remove 100µl from column 1 and add to column 2. Repeat up to column 6. Remove and discard 100µl from column 6. Add 100µl media to each well in columns 8,9,10,11 and 12. Remove 100µl from column 7 and add to column 8. Repeat up to column 12.

Place at 37°C.

Chromium-release assay

After 1 hour, add 10 ml RPMI media to the target cells, and spin to a pellet

Resuspend the targets in the residual media, and wash again in RPMI.

Resuspend in 2ml complete media and allow to "recover" for 1 hour

Wash in RPMI and resuspend in 1ml. Count targets.

Add 100µl target cells at 5 x 10^4 per ml to each well containing effector cells.
This produces a ratio of 100:1 (effector:target) in columns 1 and 7, decreasing in subsequent wells.

Each target cell type requires 3 maximum release wells & 3 spontaneous release cells.
Spontaneous release is obtained by adding media without effector cells. Maximum release is obtained by lysing all target cells in the well using 1% SDS (a detergent)

Incubate for 4 hours at 37°C.

Remove 50µl from each well and transfer to corresponding wells of Lumaplate

Dry overnight in drying cabinet.

Count plates on TopCount gamma counter.

Appendix 2.3: Summary of the protocol used for ^{51}Chromium-release cytotoxicity assay.

Day –8 Isolate human PBMC from donated blood
venesection of 120ml blood
heparinise (10µl per ml blood @ 1000 U/ml)
dilute blood 1:1 with PBS
15 ml blood layered onto 7 ml "Lymphop
spin 400g, NO BRAKE, and at ROOM-TEMPERATURE, 35 minutes
collect interphase into pre-chilled univers
fill universal with cold PBS
spin 600g, with brake, 4°C, 15 minutes
combine all tubes, and wash again with cold PBS
resuspend in 4ml RPMI + 10% FCS

Need $2x10^6$ DC per peptide
Prepare DC from human PBMC
count (use acetic acid 0.6%) → $10x10^6$/well in 5 ml RPMI + 10% FCS
allow to adhere in 6-well plates for 2 hours at 37°C
remove non-adherent cells and media (freeze for later use, to –270°C)
replace with 5ml 10% RPMI
+ 500 U/ml IL-4
+ 1000 U/ml GMCSF
incubate at 37°

Day –2 Mature DC by replating
recover cells (wash adherent cells into media gently) + spin

Need ~$1x10^6$ DC per pepide
plate at $0.5x10^6$ / well in 1ml 10% RPMI (24 well-plate)
+ 500 U/ml IL-4
+ 1000U/ml GMCSF
+ 10ng/ml TNF-α
+ 50µg/ml test peptide(s)
excess DC can be frozen for later use
incubate at 37°C

Appendix 2.4: Protocol for human PBMC isolation.

Day 0	Generate CTL from PBMC, and stimulate using DC
	recover DC into media
	spin and resuspend in 1 ml X-VIVO-15 media
	+ 10μg/ml test peptide(s)
	place at 37° until required
Need	
$4x10^6$ CTL	use same donor(s) as above: thaw and wash 3 vials as previously
+ $4x10^5$ DC	count --> $4x10^6$ / ml (in X-VIVO-15)
per peptide	500μl per well of 24 well plates (2-3 wells per peptide)
	+ 10ng/ml IL-7
	+ 100pg/ml IL-12
	add 500μl DC at $4x10^5$/ml [CTL:DC = 10:1]
	incubate at 37°
Day 3	add IL-2 @ 20 U/ml to CTL/DC culture
	ie. 20U per well delivered in 20μl media
Need	Set-up new DC culture for 2nd round of stimulation
$5x10^5$ DC	protocol as previous (above) – use only 2 vials of PBMC
Day 8	Mature 2nd DC culture by replating
	see "day –2" above

Appendix 2.5: Summary of protocol for the stimulation of CTL using DC in culture.

Day 10	Restimulate CTL using 2nd DC culture
	harvest CTL cells gently into media and spin down
Need	deplete CD4+ cells:
$1x10^6$ CTL	count cells
+$1x10^5$ DC	use dynabeads at 2.4 x cell-number – wash beads x 2 in PBS+2% FCS
per peptide	mix beads and cells in PBS + 2% FCS on ice
	shake at 4°C for 30 minutes
	discard magnetised cells
	wash remaining cells and count → 1 or 2 $x10^5$ cells/ml
	need 10 ml total
	add 100µl/well of round-bottommed 96 well-plate
	harvest DC (2nd culture) as before
	→ 1 ml X-VIVO-15
	+ 10µg/ml peptide (and keep at 37° until needed)
	resuspend at 1 or 2 $x10^4$/ml
	add 100µl to CTL in 96 well-plate
	+ 10ng/ml IL-7
	+ 100pg/ml IL-12
Day 13	add IL-2 @ 20U/ml (final conc.) to CTL/DC culture

Appendix 2.6: Summary of protocol for the second stimulation of CTL by DC in culture.

Day 16

Prepare (T2) targets
need 2000 targets per well
= $0.2x10^6$ per plate (minimum)
Add 40µg peptide
+ 12.5µg $ß_2$-m in 2 ml total
37^o overnight

Day 17

Cytotoxicity assay
ensure correct volume (200µl per well) still present
"top up" if necessary
mix media in wells gently
50µl from each well transfered towells on each of 2 new plates
add 30 x #targets (ie 60,000) of K562 (leukaemic cell-line) in 50µl
[acts to reduce non-specific lysis of targets]
add 2000 ^{51}Cr labelled targets per well in 100µl
cytotoxicity assay using 4 hour ^{51}Cr release
transfer 50µl from each well to lumaplate
dry overnight, before counting
replace 100µl media + 200U/ml IL-2 to original plates (100U/ml final)

Appendix 2.7: Protocol for cytotoxicity assay.

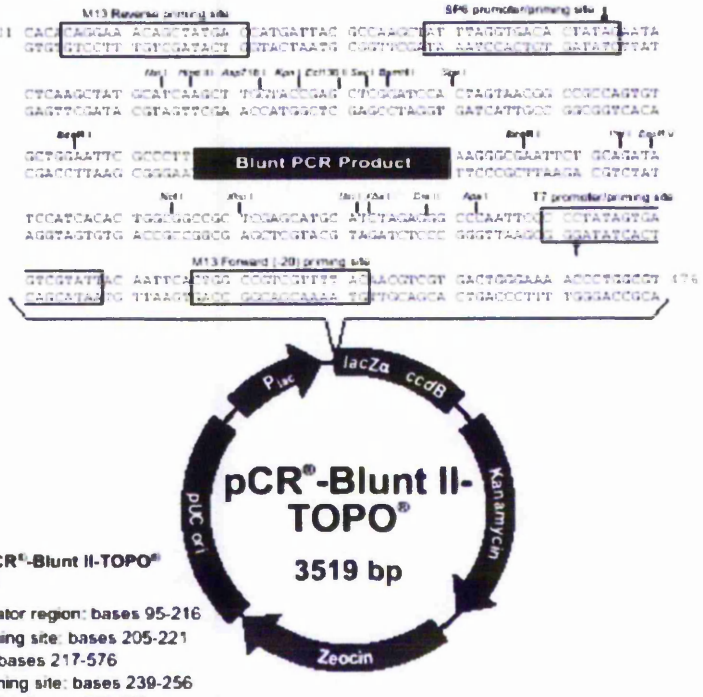
Day 24	Expand stock CTL cultures thaw autologous PBMC (or DC) + allogenic (HLA mismatch) cells wash x 2 resuspend 1ml 10% RPMI add 8μg mitomycin-C 37°C 2 hours wash x 2 X-VIVO-15 peptide pulse autologous cells (1 hour 10μg/ml) add $6x10^3$ autologous + $4x10^3$ allogeneic cells per well in 50μl add IL-7 10ng/ml final conc → 50ng/ml in 50μl IL-12 100pg/ml final conc → 500pg/ml in 50μl
Day 26	add IL-2 @ 20 U/ml final conc.
Day 31	Repeat cytotoxicity assay as described above

Appendix 2.8: Protocol for further stimulations of CTL using human PBMC or DC.

Map of pCR®-Blunt II-TOPO®

pCR®-Blunt II-TOPO® Map

The map below shows the features of pCR®-Blunt II-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. The arrows indicate the start of transcription for the T7 and SP6 polymerases. **The complete sequence of the vector is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 19).**



Comments for pCR®-Blunt II-TOPO® 3519 nucleotides

lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
LacZ-alpha ORF: bases 217-576
SP6 promoter priming site: bases 239-256
Multiple Cloning Site: bases 269-399
TOPO®-Cloning site: bases 336-337
T7 promoter priming site: bases 406-425
M13 (-20) Forward priming site: bases 433-448
Fusion joint: bases 577-585
ccdB lethal gene ORF: bases 586-888
kan gene: bases 1099-2031
kan promoter: bases 1099-1236
Kanamycin resistance gene ORF: bases 1237-2031
Zeocin resistance ORF: bases 2238-2612
pUC origin: bases 2724-3397

Appendix 3: Plasmid map of Zero Blunt-II TOPO

Day 0	Peptide immunization is given as 100µl bolus at base of tail
Day 4	Kill naïve (non-immunised) mice, and remove spleen. Transport on ice in transport media. Spleen is gently macerated and flushed with "CTL culture media". A fine bore needle is loaded onto a 10ml syringe filled with media. The spleen capsule is punctured, and media is forced through the spleen, washing out interstitial cells and some debris. Cells are centrifuged at 1500 rpm for 5 minutes Cells are counted and resuspended at 1.5 x 10^6 cells / ml CTL media Cells are resuspended in 5 ml CTL media. A 15µl aliquot is mixed into 45µl white-cell counting solution. A 10µl aliquot of this mixture is mixed with 45µl of trypan blue, and the resultant cell suspension is counted using a haemocytometer. The correct concentration is attained by addition of CTL media to the original stock. Cells are stimulated with dextran sulphate and LPS Add 7µg/ml dextran sulphate and 25µg/ml LPS to the cell suspension Incubate at 37°C in T75 tissue culture flask (layed flat) for 3 days. Incubation with LPS and dextran sulphate will facilitate the differentiation of blast cells derived from the spleen into antigen presenting B-cells.
Day 7	Spin LPS blasts (see day 7) at 1000 rpm (no brake) for 10 minutes Resuspend in 10 ml CTL media. Wash x 2 in CTL media (spin 1000 rpm 5 minutes) Resuspend at 1 x 10^6 cells / ml Target peptide is added to cells at 20 µg / ml. One or several target peptides may be required. Peptide is added to cells at 10µg/ml (final concentration) to stimulate any peptide-specific CTL present in the lymphocyte sample. If more than one peptide is to be tested, then blast cells must be divided into more than one sample at this point. 1 x 10^6 cells are added to wells of a 24 well plate (1 ml per well). Kill immunised mice, and remove spleen. Transport on ice in transport media. Spleen is gently macerated and flushed *gently* with CTL media. A fine bore needle is loaded onto a 10ml syringe filled with media. The spleen capsule is punctured, and media is forced through the spleen, washing out interstitial cells and some debris. Cells are centrifuged at 1000 rpm (no brake) for 10 minutes. Cells are counted, and resuspended at 4 x 10^6 cells / ml CTL media Cells are resuspended in 5 ml CTL media. A 10µl aliquot is mixed into 45µl white-cell counting solution. A 15µl aliquot of this mixture is mixed with 45µl of trypan blue, and the resultant cell suspension is counted using a haemocytometer. The correct concentration is attained by addition of CTL media to the original stock. 4 x 10^6 cells (1ml) are added to the wells containing LPS blasts. The final concnetration is 5x10^6 cells per well, with 10µg/ml peptide. Incubate at 37°C for 5 days.
Day 12	Perform chromium-release cytotoxicity assay.

Appendix 4: Summary of alternative protocol for the maturation of CTL using LPS blasts

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Appendix 5: Publications and presentations arising from this research

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