The Generation and *In Vitro* Characterisation of EBV B-LCL x Melanoma Hybrid Cells as Potential Candidates in Tumour Immunotherapy.

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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For my Mum and Dad,

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with love

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Abbreviations

Ab	Antibody
APC	Antigen presenting cell
$\beta_2 m$	Beta 2 microglobulin
BCG	Bacille Calmette-Guerin
BL	Burkitt's lymphoma
B-LCL	B lymphoblastoid cell line
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster designation
cDNA	Complementary DNA
СНО	Chinese hamster ovary (cells)
Ci	Curie
Cl.	Clone
cpm	Counts per minute
⁵¹ Cr	⁵¹ Chromium
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
Da	Daltons
DC	Dendritic cell
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxiribonuclease
dNTP	Deoxynucleoside 5'-triphosphates
DTT	Dithiothreitol
E:T	Effector to target ratio
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EBV B-LCL	Epstein-Barr virus transformed B lymphoblastoid cell line
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid

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EGTA	Diaminoethane tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
F(ab') ₂	Fragment of antibody (containing 2 antigen-combining sites)
FBS	Foetal bovine serum
FcR	Fc binding receptor
FIT-C	Fluorescein isothyocyanate
GM-CSF	Granulocyte/macrophage colony-stimulating factor
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HPLC	High-pressure liquid chromatography
HPV	Human papilloma virus
HSA	Heat stable antigen
HSP	Heat shock protein
IAP	Integrin-associated protein
ICAM	Intracellular adhesion molecule
IFN-	Interferon-
Ig	Immunoglobulin
Ii	Invariant chain
IL-	Interleukin-
kDa	Kilo dalton
LAK	Lymphokine-activated killer (cell)
LB	Luria-Bertoni
LFA	Leukocyte function antigen
LMP	Latent membrane protein
Lmp	Low molecular weight protein
Μφ	Macrophage
mAb	Monoclonal antibody
MAGE	Melanoma antigen
MgCl ₂	Magnesium chloride
MHC	Major histocompatibility complex
Mit-C	Mitomycin-C
MLR	Mixed lymphocyte reaction
MMB	Mini Macs Buffer

mRNA	Messenger RNA
MTLC	Mixed tumour lymphocyte culture
MW	Molecular weight
Na ₃ VO ₄	Sodium orthovandidate
NaCl	Sodium chloride
NaF	Sodium flouride
NaOH	Sodium hydroxide
NK	Natural killer (cell)
NP-40	Igepal CA 630 ('Nonidet P40')
NPC	Nasopharygeal carcinoma
OD	Optical density
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
RPE	Phyco-erythrin
rpm	Revolutions per minute
RPMI	Rosswell Park Memorial Institute (medium)
RT	Room temperature
RT-PCR	Reverse transcription PCR
SD	Standard deviation
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLAM	Signalling lymphocyte activation molecule
SSP	Sequence specific primer
TAE	Tris-acetate (buffer)
ТАР	Transporter associated with antigen processing
TBE	Tris-borate-EDTA buffer
TcR	T cell receptor
TEMED	N,N,N',N'-5tetramethylethylenediamine
TGF	Transforming growth factor

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Th	T helper (cell)
TNF	Tumour necrosis factor
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
Triton X-100	T-Octylphenoxypolyethoxyethanol
TRP	Tyrosinase-related protein
Tween 20	Polyoxyethylene-sorbitan monolaurate
U	Units
UV	Ultra violet
VCAM	Vascular cell adhesion molecule
VLA	Very late activation antigen

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Abstract

The Generation and *In Vitro* Characterisation of EBV B-LCL x Melanoma Hybrid Cells as Potential Candidates in Tumour Immunotherapy

by Adele Cywinski

Melanoma cells express antigens which are recognised by cytotoxic T lymphocytes (CTL) *in vitro* and *in vivo*. However, the T cell control of tumour growth *in vivo* is not adequate, as tumour growth and progression often occurs. In order to stimulate an effective anti-tumour response, it was hypothesised that fusion of Epstein-Barr virus transformed B lymphoblastoid cells (EBV B-LCL) with tumour cells would produce hybrid cells which expressed the relevant tumour-antigens and were more potent immunogens.

In this study, human derived melanoma cell lines (518.A2, Gerl43 and DAUV) were fused with EBV B-LCL (HMy2) using polyethylene glycol (PEG) as the fusogen. PEG treated cells were grown in double chemical selection medium to select for hybrid cells generated between the two different parent cell lines. Uncloned and cloned sublines derived from the PEG treated cells had inherited the HLA types from both parent cell lines except for HMy2 x DAUV which contained only the melanoma parent HLA alleles and was not proven to be a true hybrid in this study. The cells derived from the fusion of EBV B-LCL with EBV negative melanoma cell lines retained the EBV genome, except for one clone (HMy2 x 518.A2 clone 4) which was EBV negative and HMy2 x DAUV. The hybrid cells containing the EBV genome expressed only EBNA 1 EBV latent protein rather than the full range of EBV latent proteins, typical of EBV B-LCL.

Although the hybrid cells had a dominant tumour cell phenotype and did not express the co-stimulatory ligand molecules CD80 or CD86, they showed a significant increase in their ability to stimulate primary allogeneic T cell responses *in vitro*, as compared with the parent melanoma cells. The MHC class I(+), MHC class II(-) hybrid cells (518.A2 x HMy2) were able to directly stimulate separated CD8+ T cells, but not CD4+ T cells, whereas the MHC class I(+), MHC class II(+) hybrid cells (HMy2 x Gerl43) were able to directly stimulate both CD4+ and CD8+ T cell subsets. The T cell response was independent of CD80/CD86 interaction with CD28/CTLA-4, as the response could not be blocked with CTLA-4 Ig. Bystander non-T cell co-operation enhanced the T cell responses to the hybrid cells, by providing CD80/CD86 dependent co-stimulatory signals. Genetic modification of Gerl43 and the HMy2 x Gerl43 hybrid cells to express CD80 significantly increased the level of direct CD3+ T cell proliferation, compared with the non-transfected cells.

The HMy2 x 518.A2 and HMy2 x Gerl43 hybrid cells retained expression of tumour-associated antigens, as determined by RT-PCR, and were antigenically stable *in vitro*. HMy2 x 518.A2 clone 2 expressed tumour-associated antigens at greater levels than the parent melanoma cells, and was able to present MAGE 1 and MAGE 3 antigens to HLA class I-restricted, antigen specific CTL clones with greater efficiency than the parent melanoma cell line. In addition, HMy2 x 518.A2 clone 2 was recognised by two anti-tyrosinase specific CTL clones, despite the expression of this antigen not being detected by RT-PCR. Levels of transporter associated with antigen processing (TAP) and low molecular weight protein (Lmp) gene products were upregulated by both the parent melanoma and hybrid cells after they been cultured in interferon-gamma (IFN- γ).

Overall, the data presented in this study supports the idea that APC x tumour cell hybrids have potential as candidates in tumour immunotherapy.

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Antigen Expression, Processing and Presentation

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Introduction

Introduction

1.1 General Introduction to Tumour Immunology and Immunotherapy

The idea of manipulating the immune system in the treatment of cancer has been in existence for many years. Improved understandings in the relationship between the cancer cell and the immune system has renewed scientists' optimism and paved the way for the development of effective immunotherapies for cancer. Evidence from clinical studies has shown that a tumour-specific immune response can develop in many cancer patients, but why this response is ineffective and ultimately fails to protect the host remains unclear.

1.2 The Importance of a T Cell Response in Tumour Immunology

The role of the immune system in the control of tumour growth has become increasingly clear. Clinical data has shown tumours are often infiltrated by lymphocytes (TIL) which are mostly T cells. In primary tumours, this infiltrate is often associated with a better prognosis (Clark, 1991), suggesting that T cells can control tumour growth. However, tumour progression is often associated with the secretion of immune suppressive factors, including IL-10 (Chen *et al.*, 1994b; Lattime *et al.*, 1995) or transforming growth factor (TGF)- β (Moretti *et al.*, 1997; Schmid *et al.*, 1995) and tumours are often associated with down regulation of HLA class I presentation function, suggesting escape from immune response (reviewed by Browning and Dunnion, 1997). Such strong circumstantial evidence suggests many tumours have developed elaborate strategies to prevent an effective immune response from being initiated, or evasion from an established immune response.

Cytotoxic T lymphocytes (CTLs) infiltrating murine and human tumours have been expanded *in vitro* and shown to selectively lyse autologous tumour, and in response to stimulation, excrete various cytokines including interferon- γ (IFN- γ) and tumour necrosis factor (TNF) (Muul *et al.*, 1987; Topalian *et al.*, 1989; Barth *et al.*, 1991). Tumour specific CTL have been identified in patients with a range of tumours including melanoma (Herin *et al.*, 1987; Knuth *et al.*, 1989), ovarian (Ioannides *et al.*, 1991), head and neck cancer (Yasumura *et al.*, 1994) and renal cell carcinoma (Finke *et al.*, 1992). HLA class I restricted antigenic peptide epitopes expressed by tumours and recognised by autologous CTL *in vitro* have been identified and are well characterised at the molecular level (Boon *et al.*, 1994). The use of characterised tumour antigens *in vivo* has shown the potential to mediate tumour rejection (Ressing et al., 1996). In vitro, TILs have been expanded in the presence of interleukin (IL)-2 and with autologous tumour stimulation, and adoptively transferred into animals and humans to treat large tumour burdens (Greenberg et al., 1991; Rosenberg et al., 1994). High doses of IL-2 administered systemically to patients with metastatic melanoma has been shown to induce tumour regression (Rosenberg et al., 1988), and in some patients peptide-based vaccines have been shown to induce tumour regression (Marchand et al., 1995; Nestle et al., 1998), inferring the in vivo relevance of T cell mediated anti-tumour responses. Also, cell based vaccines have been used in clinical trials. Morton et al. immunised melanoma patients with an allogeneic tumour cell vaccine consisting of three different tumour cell lines and administered with BCG (Morton et al., 1993). An increased five year survival rate of 23% compared to 6-10% for matched historical controls was reported for stage IV patients and a five year survival rate of 34% was observed for stage III patients compared with 16% for historical controls (Morton et al., 1993). More recently, autologous DC x renal cell carcinoma hybrid cells have been used in a clinical study with 41% of patients responding positively to hybrid cell vaccination (Kugler et al., 2000). This data supports the view that immunotherapy for the treatment of cancer has potential in a clinical setting.

T cell receptor repertoire analyses have shown the presence of clonal T cells in the TIL population. Mackensen *et al.* showed the presence of clonally expanded T cells in a regressing primary melanoma lesion, suggesting that clonally expanded T cells are involved in tumour regression (Mackensen *et al.*, 1993). Vitiligo-like depigmentation in patients responding in tumour immunotherapy is positively correlated with tumour regression in melanoma and indicates T cells recognising melanoma cells may also destroy normal melanocytes. In addition partial and complete spontaneous tumour regressions, despite being rare, have been reported and have been associated with an increased number of T cells (Halliday *et al.*, 1995). However, the presence of the disease suggests that the *in vivo* tumour-specific T cell response is inadequate to clear tumours.

1.3 T Cell Responses

T cells respond to antigenic peptides, the products of degraded endogenous or exogenous protein, presented in the context of major histocompatibility complex (MHC) gene products on the cell surface. Antigenic peptides are recognised only by T cells bearing an antigen-specific receptor. By virtue of their T cell receptors and cell surface markers, T cells can be categorised into subsets. The various T cell subsets differ in regard to the type of antigens they recognise, their effector functions and their distribution in the host.

1.3.1 T Lymphocyte Development

T lymphocytes (T cells) can be roughly categorised into two main subsets, CD4+ and CD8+ T cells. In most cases, the TcR is composed of an α and a β chain, although a small fraction (approximately 5-10% of blood T cells) use a $\gamma\delta$ heterodimer instead (Ferrick *et al.*, 1989). Three gene segments (variable [V], joining [J] and constant [C] segments) encode for each TcR chain. For the β chain, an additional gene segment is involved (diversity [D]). The TcR is clonally distributed and recognises specific antigen/'self' (autologous) MHC complexes. The CD4 or CD8 co-receptors bind with MHC class II or class I molecules respectively (Davis and Bjorkmann, 1988). Cells expressing 'non-self' (allogeneic) MHC molecules are recognised as foreign and are deleted. In addition, the TcR is complexed with CD3, a non-clonotypic signalling complex. CD3 is composed of three different chains (γ , δ and ε) and the ζ homodimer (Terhorst *et al.*, 1995).

T cells develop from bone marrow stem cells which migrate to the thymus and commit to the T lineage in response to signals from the microenvironment (Shortman and Wu, 1996). The earliest T cell precursors are CD4-CD8- double negative in phenotype. The cells proliferate extensively to become CD4+CD8+ double positive immature T cell precursors. During T cell maturation in the thymus, TcR gene segments rearrange to produce a single, transcriptional unit encoding for a receptor chain. Also, the addition or deletion of nucleotides at junctions between the V/J and J/D gene segments provides more sequence diversity and ensures a limitless repertoire of unique TcR (Davis, 1990). In the thymus the T cell precursors undergo a period of development where T cells that bind 'self'-MHC with low affinity are positively selected and T cells recognising 'self'-MHC with high affinity are deleted (negative selection) (reviewed in Goldwrath and Bevan, 1999). T cells which recognise MHC class I molecules are committed to the CD8 lineage whereas those which recognise MHC class II molecules are committed to the CD4+ lineage. T cells leave the thymus as immunocompetent cells, expressing high levels of CD3 in association with the TcR.

On encounter with specific antigen presented in the context of autologous MHC molecules, naïve T cells require co-stimulatory signals mediated by a number of receptor-

ligand interactions (section 1.5) in order to proliferate and clonally expand. Cells develop effector function and a small number of cells which have experienced antigen join the memory T cell pool. The response to a newly encountered antigen is slow due to a lack of antigen specific T cells. However, memory cells respond rapidly and vigorously to a second encounter with the same antigen.

1.3.2 T Cell Categories

The majority of T cells (>90% of peripheral blood lymphocytes) express the $\alpha\beta$ TcR, and can be further divided on the basis of expression of CD4 or CD8. In general, CD4+ T cells have 'helper' function (Th cells) and regulate the immune response through cytokine secretion, although a small proportion of CD4+ T cells have direct cytotoxic activity (CD4+ CTL) (Browning *et al.*, 1990). CD8+ T cells have direct cytotoxic activity (CTL) and also release cytokines. CTL therefore also have an immunoregulatory role.

CD4+ T helper cells (Th) can be divided into Th0, Th1 and Th2 subsets, depending on the range of cytokines secreted (Mosmann and Coffman, 1989). Th1 cells typically produce IL-2, IFN- γ and TNF- α and Th2 cells typically produce IL-4, IL-5, IL-6 and IL-10. Th1 cells activate CTLs and inflammatory and delayed hypersensitivity reactions, and are therefore important in combating intracellular viral, bacterial and parasitic infections. Th2 cells drive B cell proliferation and antibody (Ab) mediated responses. Th0 cells have cytokine profiles intermediate between Th1 and Th2 cells. Factors which influence the differentiation into Th1 or Th2 cells include the balance of cytokines evoked by the antigen, antigen concentration and the interaction of co-stimulatory molecules on the antigen presenting cell (APC) and the T cell. IL-12 is a potent stimulator of IFN- γ production and can stimulate a Th1 type response. IL-4 stimulates a Th2 type response.

CD8+ cytotoxic T lymphocytes (CTL) can be classified as Tc1 and Tc2 subsets, depending on the cytokine profile release by the cell (Kemeny *et al.*, 1994). Those which produce Th2 type cytokines (Tc2) are associated with regulatory and suppressor function. Tc1 produce Th1 type cytokines, including IL-2, IFN- γ and TNF- α . CTLs contain perforin which inserts into the target cell membrane and forms pores, and granzymes that activate the caspase pathway which leads to apoptosis thus destroying the cell. CTLs can also cause target cells to apoptose through the interaction of FAS ligand (FAS-L) with the FAS receptor expressed on the target cell.

Memory CD4+ and CD8+ T cells persist in the T cell pool long after antigen depletion. Cells which express the high-molecular weight isoform of the leukocyte tyrosine phosphatase CD45 (CD45RA) are considered to have naïve cell characteristics, and in contrast those cells with the low molecular weight isoform of CD45 (CD45RO) are considered to be activated or memory T cells (Janeway, 1992). This is based on the observation that CD45RO+ cells, but not CD45RA+ cells can respond vigorously in culture to previously encountered antigen. In addition, CD45RO+ T cells also circulate throughout the body, including epithelial surfaces, whereas CD45RA+ T cells circulate almost exclusively through the lymphoid tissue (Mackay, 1993). Although CD45RO and CD45RA expression has been used to distinguish memory T cells from naïve T cells respectively, CD4+ T cells which express both forms of the CD45 isomer have been detected (Hamann et al., 1996) and CD4+T cells with a memory phenotype have also been shown to revert to the CD45RA+ positive phenotype (Hargreaves and Bell, 1997). Recently, two types of memory T cells have been identified (Jacob and Baltimore, 1999). The two different cell types differ in migration pathways and mediate the memory response in different ways. Effector memory T cells migrate through peripheral tissues such as the mucosa and skin, whereas central memory T cells probably migrate through the lymph nodes (Sallusto et al., 1999). Effector memory T cells provide immediate protection at sites such as the epithelial surfaces, whereas the central memory cells can rapidly differentiate into effector cells upon exposure to antigen. In order to separate effector and memory T cell populations, a newly identified chemokine receptor, CCR7, has been used (Sallusto et al., 1999). CCR7+ memory T cells lack effector function and revert to a CCR7- state after antigen stimulation. CCR7- memory T cells have the characteristics of effector T cells and produce cytokines such as IFN-y, IL-4 and IL-5 or they express perforin granules.

Gamma-Delta ($\gamma\delta$) T cells express a unique $\gamma\delta$ TcR in association with the CD3 complex but do not usually express a CD4 or CD8 co-receptor. The TcR can exist as γ or δ monomers or the two chains can be disulphide linked. There are two different forms of the $\gamma\delta$ receptor, C γ 1 and C γ 2. Only C γ 2 can exist as a dimer. Most adult peripheral blood $\gamma\delta$ T cells express the C γ 1 receptor. $\gamma\delta$ T cells are found abundantly in various epithelia, including the intestinal epithelium, epidermis, uterus and tongue but form only a small proportion of T cells in the thymus and secondary lymphoid organs. After activation with IL-2, most C γ 1 T cells mediate non-MHC restricted cytotoxicity. Antigens recognised by

 $\gamma\delta$ T cells include exogenous antigens such as mycobacteria, heat shock proteins (HSP) or non-classical MHC molecules including CD1. In response to bacteria or parasites, $\gamma\delta$ T cells can produce Th1 or Th2 type cytokines respectively. They act earlier than $\alpha\beta$ T cells and may thus play a central role in determining the balance of the Th1/Th2 response.

1.4 Antigen Processing and Presentation

1.4.1 The Antigen Presenting Cell

MHC class I molecules are expressed on virtually all somatic cells. Those which do not express MHC class I molecules include hepatocytes, erythrocytes and retinal cells. In contrast, the constitutive expression of MHC class II molecules is limited to a small group of cells termed professional APCs. Professional APCs, which include B cells, macrophages (Mø), dendritic cells (DC), and thymic epithelial cells (Glimcher and Kara, 1992), are the cells which are most effective at stimulating T cells. Professional APCs are able to process endogenous and exogenous antigen, express peptide-MHC complexes on the cell surface and deliver T cell co-stimulatory signals to allow effective interaction with, and modulation of, T cell activation. The level of expression of MHC class II by professional APCs is influenced by the developmental state of the cell and external stimuli, including a number of cytokines. Receptor-mediated endocytosis, phagocytosis and micropinocytosis all assist in antigen uptake by APCs. The efficiency of antigen uptake affects the level of level of antigen expression by MHC class II molecules. MHC class II expression can also be induced on a wide range of non-lymphoid cells types including those of epithelial and endothelial origin in response to cytokine exposure. Nonprofessional APC can express MHC class I and MHC class II molecules but lack the ability to deliver co-stimulatory signals to T cells, so are only recognised by previously activated T cells.

M ϕ are professional phagocytic cells. Myeloid progenitors in the bone marrow differentiate into promonocytes which circulate in the blood as blood monocytes. Once the monocytes reach various organs, they differentiate into M ϕ s. The Fc receptor (FcR) is used by M ϕ s to non-specifically endocytose Ab bound antigen. M ϕ s can bind to certain carbohydrates of the microbial wall, or to immunoglobulin (Ig)G and complement which can bind microbes.

DCs are the most potent APC (reviewed in Steinman, 1991). These cells are able to capture, process and present exogenous antigen more efficiently than Møs and in

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addition, elicit strong T cell responses. DCs are located at the skin and are thought to derive directly from bone marrow precursors. Upon non-specific antigen capture, DCs migrate to the lymphoid organs in order to increase the likelihood of interacting with T cells with the appropriate antigen-specific receptors. DCs can use their IgG FcR or mannose receptor to bind antigen. Once DCs have endocytosed antigen, there is a down regulation in their endocytic activity, and an upregulation of surface MHC class II and co-stimulatory molecule expression. DCs have been described as the only APC capable of stimulating naïve T cells and the potent immune response which is elicited by DCs is able to break neonatal tolerance (Ridge *et al.*, 1996).

B cells develop from bone marrow precursors. B cells express cell surface markers, including CD19 and CD20 which identify them. The cells also express a unique receptor (surface immunoglobulin [sIg]) allowing the capture of exogenous, specific native antigen, internalise it and degrade it for antigen presentation to T cells. B cells can also non-specifically take up exogenous antigen. Due to the sIg receptor which is effective for antigen binding, antigen endocytosis and antigen targeting to the appropriate processing compartments, antigen-specific B cells present antigen to T cells at lower antigen concentrations than do non-specific B cells. The presentation of antigen by a B cell results in antigen-specific CD4+ T cell recruitment, which in turn permits B cell proliferation and differentiation into an Ab secreting plasma cell.

1.4.2 Antigen Processing Along the MHC Class I Pathway

CD8+ T cells recognise antigenic peptides (generated mostly from degraded intracellular proteins, including viral proteins) presented in the context of MHC class I molecules by APCs. Self-compartmentalising proteases (proteasomes) in the cytosol degrade proteins into peptides which are then transported to the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). In the ER, newly synthesised MHC class I molecules associate with antigenic peptides by successive interactions with the ER chaperones, calnexin and calreticulin, and tapasin. Once the MHC class I/antigen complex is formed, it is transported to the cell surface for presentation to antigen-specific CD8+ T cells. In this way, cells can be constantly monitored by the immune system.

1.4.2.1 The Proteasome

Intracellular proteins, including viral proteins, are constantly degraded into short peptides for presentation in association with MHC class I molecules on the cell surface (Townsend *et al.*, 1985). The dominant responsible protease is the proteasome (Rock *et al.*, 1994). The proteasome is a large complex organelle with a barrel-shaped 20S core particle containing multi-catalytic proteases that protects the cytoplasmic environment of the cell from uncontrolled protein degradation. The 20S proteasome is composed of 4 rings (Groll *et al.*, 1997). The outer rings are formed from seven distinct α subunits, having structural and regulatory properties. The inner rings are formed from seven distinct β subunits and have catalytic sites. The endo-peptidase activity has broad specificity and bonds on the carboxyl side of basic, hydrophobic and acidic residues can be degraded ATP-independently (Goldberg and Rock, 1992).

Typically, additional proteins associate with the 20S core in mammalian cells, producing a larger 26S structure where protein degradation occurs in an ATP dependent manner. The majority of *in vitro* studies by immunologists have used the 20S proteasome, as it is easier to obtain and work with. However, most intracellular protein degradation is mediated by the 26S proteasome, and recent studies have shown the range of peptides generated by the 20S and 26S proteasomes differ (Kisselev *et al.*, 1999).

The 20S proteasome associates with a 19S complex (PA700), which has polyubiquitin sites for binding ubiquitin marked proteins and may assist in the unfolding of potential degradation substrates, to form the 26S proteasome (Ma *et al.*, 1994; Coux *et al.*, 1996). In addition, the 20S proteasome can associate with the PA28 accessory complex, an 11S complex, whose expression is IFN- γ inducible. The mechanism by which PA28 modulates the activity of the proteasome remains unclear. It enhances the peptidolytic activity of the proteasome without having hydrolytic activity of its own and promotes more efficient antigen recognition (Dick *et al.*, 1996) and proteasome activity.

1.4.2.2 Low Molecular Mass Proteins (Lmp) 2 and Lmp 7 of 20S Proteasomes

IFN- γ induces the expression of two proteasome β -subunits that are preferentially incorporated into the proteasome and modify its activity. Lmp 2 and Lmp 7 proteins are β subunits which replace the house keeping proteins MB1 and δ in newly synthesised proteasomes (Belich *et al.*, 1994). The majority of cells synthesise Lmp 2 and Lmp 7 at low levels. IFN- γ upregulates their expression and downregulates the expression of their homologues (Akiyama *et al.*, 1994). Lmp 2 and Lmp 7 proteins alter the specificity of the proteases, increasing the rate of peptide cleavage after hydrophobic and basic residues and decreasing the rate of cleavage after acidic residues (Driscoll *et al.*, 1993; Gaczunska *et al.*, 1993). Thus, the availability of specific groups of peptides, with the characteristics of those which are preferably bound by MHC class I molecules, may be increased by Lmp protein expression. Cells are able to process and present antigen when Lmp 2 and Lmp 7 are not expressed, although the endopeptidase specificity changes. Two genes located in the MHC class II region on chromosome 6 in humans encode for Lmp 2 and Lmp 7 proteins (Glynn *et al.*, 1991).

Recently, a third proteasome subunit, MECL-1 (Lmp 10) has been identified and found to replace proteasome subunit Z (Groettrup *et al.*, 1996). Unlike Lmp 2 and Lmp 7, the gene encoding for LMP 10 is not located in the MHC region. The proteasome complex containing Lmp 2, Lmp 7 and MECL-1 is referred to as an 'immunoproteasome' due to its capacity to enhance the expression of MHC class I molecules on the cell surface and antigen presentation to the immune system.

1.4.2.3 Protein Ubiquitination

The typical way for endogenous proteins to enter the MHC class I processing pathway is through the covalent association with ubiquitin (reviewed in Spataro *et al.*, 1998). Ubiquitin is a 76 amino acid protein which is added to the target protein via the interaction of three enzymes, E1, E2 and E3. The enzyme E1 activates ubiquitin. E2 subsequently transfers the protein onto the target which is then ligated by the enzyme E3. Ubiquitin links to specific lysine residues in the target protein and polyubiquitinated proteins arise through the binding of ubiquitin onto already conjugated ubiquitin molecules. Polyubiquitinated proteins are preferentially degraded by the 26S proteasome. Ubiquitin serves to unfold the protein and to target the protein to the proteasome complex.

Other factors which influence the degradation of proteins include the presence of PEST sequences which are rich in proline, glutamine, serine and threonine (Rogers *et al.*, 1986) or the presence of sequences referred to as destruction boxes that confer cell cycle-specific degradation (Glotzer *et al.*, 1991). In addition, N-terminal amino acid residues also influence the rate of degradation of proteins and the efficiency of epitope presentation to CTL (Varshavsky, 1992). Altering the N-terminal amino acid of an antigen can destabilise a protein and enhance its presentation to antigen specific CTL, or stabilise a

protein and reduce its level of presentation to antigen specific CTL (Townsend et al., 1988).

1.4.2.4 Transporter Associated with Antigen Processing (TAP)

Peptides generated in the proteasome are translocated to the lumen of the ER for binding with MHC class I molecules by the heterodimeric transporter associated with antigen processing (TAP) in an ATP dependent manner (Androlewicz *et al.*, 1993; Neefjes *et al.*, 1993). The proteins are members of the ATP Binding Cassette (ABC) transporter family (Trowsdale *et al.*, 1990), having two hydrophobic domains containing many transmembrane regions, and two hydrophilic domains which are cytosolic and contain two ATP binding sites. ATP hydrolysis is required to translocate peptides from proteasome into the ER, but not to load peptides onto the TAP complex. The peptides generated by the proteasome and translocated by TAP to the ER may be longer than most MHC class Iassociated peptides, suggesting additional proteases (other than the proteasomes) may be involved in the generation of some antigenic peptide epitopes. Trimming of peptides may take place in the cytosol or the ER, although the identity of the proteases involved is unknown.

The TAP transporter is composed of two heterologous polypeptides (TAP 1 and TAP 2) which are non-covalently associated. There is more than one allelic form of TAP 2. TAP 2a and TAP 2b exist in the population at frequencies of 80% and 20% respectively, and have not been associated with any functional differences (Kelly *et al.*, 1992; Powis *et al.*, 1992). The TAP proteins are encoded by genes found in a tight cluster with the Lmp genes in the HLA region (Monaco, 1992). A shared bi-directional promoter co-ordinates the expression of Lmp 2 and TAP 1 (Wright *et al.*, 1995). Like both the Lmp proteins, the TAP proteins are also IFN- γ inducible. However, as a consequence of TAP deficiency, cells express low levels of surface of MHC class I molecules and are unable to present antigen efficiently on the cell surface. (Cerundolo *et al.*, 1990; Salter and Cresswell, 1986) though not every MHC antigen is affected equally (Keating *et al.*, 1995). Due to the presence of signal-peptidases resident in the ER, HLA-A2 molecules can bind degraded signal sequences, allowing TAP independent expression at the cell surface (Wei and Cresswell, 1992).

1.4.2.5 MHC Class I

The three classical MHC class I molecules (HLA-A, HLA-B and HLA-C) which present antigen on the cell surface to CD8+ T cells are composed of a transmembrane glycoprotein (heavy chain) (forming the $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains) non-covalently associated with the monomorphic $\beta 2$ microglobulin ($\beta 2m$). Virtually all somatic cells express MHC class I molecules with the exception of hepatocytes, erythrocytes and retinal cells. MHC class I heavy chains are encoded by the HLA complex situated on the short arm of chromosome six. Individuals have two haplotypes, (one is derived maternally and the other is paternally derived), which are co-dominantly expressed. The genes for HLA-A, HLA-B and HLA-C are highly polymorphic. Polymorphism within the population is required to maintain the diversity of peptides expressed by individuals and the population, since polymorphic variants express different peptide antigens and therefore influence the immune response to a given antigen. (Non-classical HLA molecules also encoded in the MHC class I locus include HLA-E, HLA-F and HLA-G.)

1.4.2.6 MHC Class I Molecules and Antigen Binding

Within the ER, the MHC class I heavy chain associates with $\beta 2m$, and this structure is retained in the ER until peptide binding. $\beta 2m$ is a short, 12kDa non-glycosylated protein which interacts non-covalently with the $\alpha 3$ extracellular domain of the heavy chain. This complex is unstable, requiring the binding of an antigenic peptide with the correct length and binding motif, before it can be transported through the Golgi to the cell surface. Peptide loading into the groove of the MHC class I molecule requires TAP (Ortmann *et al.*, 1994; Suh *et al.*, 1994) which introduce peptides from the cytosol to the ER, and the assistance of other proteins (section 1.4.2.7).

The peptide binding specificity of the MHC class I molecule is determined by the structure of the heavy chain. The heavy chain is a 45kDa glycoprotein with three extracellular domains (α 1, α 2 and α 3), a transmembrane region and a short cytoplasmic domain. The α 1 and α 2 extracellular domains form a peptide binding groove. This groove is characterised by a platform of antiparallel β -strands lining the floor and two antiparallel α -helices overlaying the platform. At either end of the peptide binding groove, there are conserved amino acids which restricts the length of the peptides (to eight to ten amino acids) that can bind. Conserved amino acid residues in the peptide binding groove make specific interactions with the peptide antigen, interacting predominantly with the N

and C termini of peptides (Ramensee *et al.*, 1993). Allelic polymorphisms occur in and around the groove, altering the antigenic peptide specificity. This enables different MHC molecules to preferentially bind different peptides. Amino acids which are not required for peptide binding by the antigenic peptides can vary greatly, resulting in the display of a variety of peptides by the individual MHC class I molecule (reviewed in Engelhard, 1994).

1.4.2.7 MHC Class I Binding with Antigen is Associated with Additional Proteins

In addition to TAP, four other proteins are involved in peptide binding to MHC class I molecules in the ER (reviewed by Cresswell et al., 1999). These include calnexin (a transmembrane chaperone), calreticulin (soluble homologue of calnexin), tapasin (a transmembrane glycoprotein) and ERp57 (an ER resident thiol oxidoreductase). In humans, calnexin binds to newly synthesised MHC class I heavy chains, stabilising them before they bind with $\beta 2m$ (Vassilakos *et al.*, 1996). Calreticulin then binds to the MHC heavy chain-\beta2m dimer (Sadasivan et al., 1996) along with additional proteins including ERp57 (Morrice and Powis, 1998), ensuring the correct assembly of the MHC class I heavy chain- β 2m dimer. Tapasin, encoded by a gene located in the MHC, is necessary also for the formation of MHC complexes as it binds simultaneously to the class I and TAP proteins (Grandea et al., 1995), promoting peptide loading onto the class I molecule. Only MHC complexes bound with antigen can be transported across the Golgi apparatus and then to the cell surface for recognition by antigen specific CD8+ T cells. The α 3 domain of the MHC class I molecule has a site for interaction with the CD8 receptor found on CTL. CTL monitor the surface of cells for antigen which bind to their receptor. In this way, virally infected and cells that express mutant gene products, such as tumour cells, are recognised by CTL.

1.4.2.8 Exogenous Antigen in the MHC class I Processing Pathway

In addition to presenting endogenous antigen on the cell surface in the context of MHC class I molecules, APC are able to present exogenous antigen through MHC class I molecules (Rock *et al.*, 1990; Kovacsovics-Bankowski and Rock, 1994). How these exogenous antigens enter the MHC class I processing pathway is still largely unknown (reviewed by Reimann and Schirmbeck, 1999). It is likely the antigens are internalised into endocytic compartments and then transferred into the cytosol in a way which has not yet been determined (Kovacsovics-Bankowski and Rock, 1995; Pfeifer *et al.*, 1993;

Harding and Song, 1994). The antigens are then degraded in the cytosol in the same way as endogenously synthesised proteins, requiring TAP (Kovacsovics-Bankowski and Rock, 1995; Huang *et al.*, 1996b; Albert *et al.*, 1998). However, some exogenous antigens can be processed and presented in a TAP independent manner (Pfiefer *et al.*, 1993; Liu *et al.*, 1997; Song and Harding, 1996; Schoenberger *et al.*, 1998b).

1.4.2.9 Cross Priming

Cross priming is the presentation of exogenous, cell-associated antigen to MHC class I restricted CD8+ T cells. The phenomenon of cross priming was first identified by Bevan (Bevan, 1976) who showed that minor histocompatibility antigens could be transferred to host cells and presented to antigen specific CTLs *in vivo*. In this way antigens expressed in non-haemopoetic cells can be processed and represented (indirectly) by a professional APC. Apoptotic cells, cell debris and tumour cells have acted as sources of antigen for DCs, M\u00e9s and bone marrow derived host APCs respectively (Albert *et al.*, 1998; Kovacsovics-Bankowski and Rock, 1994; Huang *et al.*, 1994).

1.4.3 (Exogenous) Antigen Processing Along the MHC Class II Pathway

Exogenous antigenic peptides are presented to CD4+ T cells in the context of MHC class II molecules on the cell surface, although evidence exists to show that endogenous antigen may also be presented by MHC class II molecules to CD4+ T cells but this is a minority event (Nuchtern *et al.*, 1990; Topalian *et al.*, 1994) (reviewed by Lechler *et al.*, 1996).

1.4.3.1 The Uptake and Degradation of Exogenous Antigen

Exogenously obtained antigen is taken up into the cell by phagocytosis and/or endocytosis. It is channelled through the endosomal/lysosomal pathway where it is progressively broken down into peptides by a variety of hydrolytic enzymes that include proteases. Precisely where peptide loading onto MHC class II molecules occurs, and whether specialised structures are involved, is not well defined. MHC II compartments (MIIC) are structures resembling endosomes and lysosomes where protein degradation may occur. They have lgp/lamp membrane proteins, hydrolases and a low pH, which are markers of late endosomes and lysosomes (Peters *et al.*, 1995; Benaroch *et al.*, 1995; Harding and Geuze 1993) and also include molecules associated with MHC class II, including HLA-DM (Sanderson *et al.*, 1994). The distinction between MHC II compartments and conventional late endosomes and lysosomes in some cells is not clear (Pierre *et al.*, 1996; Kleijmeer *et al.*, 1997) Another compartment where protein degradation is also thought to occur has been termed CIIV (class II vesicle) (Amigorena *et al.*, 1995) and is also thought to be distinct from lysosomes and endosomes (Amigorina *et al.*, 1994; Amigorina *et al.*, 1995; Tulp *et al.*, 1994; Drake *et al.*, 1997; Ferrari *et al.*, 1997). Cell fractionation studies have shown CIIV are physically and functionally distinct from conventional endosomes or lysosomes and are abundant in mature DCs (Pierre and Mellman, 1998).

MHC class II molecules bind peptides preferably of 12-25 amino acids. What is not properly understood is how proteolysis within the endosomal compartments does not degrade proteins down to single amino acids and dipeptides. This may be due to MHC class II molecules immediately exiting the endosome following the binding of the peptide to the groove.

1.4.3.2 MHC class II Molecules

The MHC class II genes, located in the MHC locus, encode the polymorphic HLA-DR, HLA-DP and HLA-DQ molecules. Expression of these genes is tightly regulated at multiple levels (Rohn et al., 1996) and constitutive expression is limited to professional APC only, although IFN- γ can induce MHC class II expression on a range of cell types. MHC class II molecules consist of an α chain and a β chain non-covalently associated. Each chain has two extra cellular domains ($\alpha 1$ and $\alpha 2$ or $\beta 1$ and $\beta 2$), a transmembrane hydrophobic domain and a cytoplasmic tail (Jaraquemada et al., 1990). The peptide binding groove of a single $\alpha\beta$ MHC class II complex is formed by both chains at the top of the dimer between the $\alpha 1$ and the $\beta 1$ domains (Brown *et al.*, 1993). The groove can accommodate peptides of between 12-25 amino acids since, unlike MHC class I molecules, it lacks the conserved regions at the groove termini therefore the groove can open up at each end and interactions between the MHC class II molecule and the peptide form throughout the peptide (reviewed by Engelhard, 1994). This ensures high affinity binding and exposes the unique features of each antigenic epitope. Like MHC class I molecules, there is also variability in range of peptides which can bind to different class II molecules due to sequence differences around the region of the peptide binding groove.

1.4.3.3 Peptide Binding and MHC Class II Molecules

Three $\alpha\beta$ MHC class II complexes associate with a trimeric invariant chain (Ii) chain complex to form a nonameric structure in the ER (Roche *et al.*, 1991). This prevents the $\alpha\beta$ MHC class II dimer loading endogenously synthesised peptides (Roche and Cresswell, 1990). In the ER, the MHC class II bind transiently with proteins including calnexin (Anderson and Cresswell, 1994), GRp94 and ERp72 (Schaiff *et al.*, 1992) and BiP (Bonnerot *et al.*, 1994) which retains the MHC class II molecules in the ER until the dimers are correctly folded. Once correctly folded, the nonameric MHC class II-Ii complexes leave the ER, travelling through the Golgi apparatus and exits by the trans-Golgi network. An N-terminal dileucine signal sequence in the cytoplasmic domain of the Ii chain directs the complex to the endosomal/lysosomal compartments of the cell (Pieters *et al.*, 1993; Odorizzi *et al.*, 1994).

In the endosome, the Ii chain is degraded by an aspartyl protease and then a cysteine protease. This frees the $\alpha\beta$ complexes (Riese *et al.*, 1996), but leaves a short peptide of 15 amino acids (CLIP) inserted in the peptide binding site of the dimer, stabilising the structure (Cresswell, 1996). CLIP binds with low affinity, and in the low acidic environment of the endosome, CLIP dissociates, allowing the binding of peptides to take place. The dissociation of CLIP is catalysed by HLA-DM, a protein which also dissociates peptides of low affinity from the MHC class II peptide binding site. This makes sure that tightly bound peptides replace those that are weakly bound (Kropshofer *et al.*, 1996). A second protein, HLA-DO also stabilises the MHC class II/HLA-DM dimer (Kropshofer *et al.*, 1998). The genes for the non-classical MHC proteins, HLA-DM and HLA-DO, map to the MHC class II region (Kelly *et al.*, 1991) and are located between the genes for TAP and Lmp.

Once the peptide has interacted and bound to the MHC class II molecule, it is then exported and presented on the surface of the cell for recognition by specific CD4+ Th cells. The β 2 domain of the MHC class II molecule has a CD4 binding site. Pinet *et al.* and Zhong *et al.* showed MHC class II molecules may be recycled from the cell surface (Pinet *et al.*, 1995; Zhong *et al.*, 1997) which would explain why MHC class II molecules have a signal sequence which directs them to clathrin coated pits. This also explains why antigen presentation can occur in the absence of protein synthesis or Ii expression.

1.5 T Cell Co-Stimulation

1.5.1 APC and T Cell Interactions

CD4+ and CD8+ T cells play an important role in the generation and regulation of the immune response, in response to antigen expressed on the cell surface in conjunction with MHC class I or MHC class II molecules. In order for naïve T cells to become activated fully, however, they must receive two signals. Signal one is initiated when MHC, complexed with antigen, binds to the antigen specific T cell receptor (TcR)-CD3 complex and its co-receptors (CD4 and CD8) (Davis and Bjorkmann, 1988), providing antigen specificity. Signal two is antigen independent and occurs when other cell surface molecules on the APC interact with their complementary receptors on the T cell. This serves to stabilise the APC-T cell interaction and provide additional co-stimulus which leads to cytokine secretion, cell proliferation and their effector function. Signal one in the absence of signal two has been shown to result in T cell non-responsiveness (Jenkins and Schwartz, 1987), a state termed anergy. In an anergised state, T cells are unable to respond to stimulation with antigen and co-stimulation, being unable to produce IL-2 (Schwartz, 1990).

Various receptor-ligand interactions have been shown to play a role enhancing T cell interaction with APC (Table 1.1). Depending on the receptor-ligand pair, these interactions help to keep the two cells in close proximity and provide co-stimulation signals. After T cell signalling, the association between CD54 (ICAM-1) and CD11a (LFA-1) increases, and stronger adherence between the two cells ensues. The interaction between CD80/CD86 on the APC with CD28 on the T cell has been shown to be the most important interaction in T cell co-stimulation and IL-2 synthesis. The interruption of CD80/CD86 interaction with CD28/CTLA-4 can result in immune suppression and induce T cell anergy (Linsley *et al.*, 1991). Harding *et al.* (Harding, 1992) demonstrated the importance of signalling through CD28 in the murine model by adding anti-CD28 mAb, blocking signal two. The ligation of CD28 overcame T cell anergy, supporting T cell proliferation and IL-2 production (Harding *et al.*, 1992).

1.5.1.1 CD28

CD28 receptor is found on the surface of about 95% CD4+ and about 50% of CD8+ human peripheral T cells and it is expressed on all mouse T cells (Gross *et al.*, 1992). It is a 44kDa homodimeric type I transmembrane glycoprotein composed of two disulphide linked subunits and is a member of the immunoglobulin (Ig) superfamily

T Cell	APC	Reference
CD28 and CTLA-4	CD80 and CD86	June et al., 1990. Boussiotis et al., 1993.
ICOS	Not Known	Hutloff, 1999.
CD40L	CD40	Cayabyab et al., 1994.
CD2	CD58 (LFA-3)	Koyasu <i>et al.</i> , 1990.
CD11a (LFA-1)	CD54 (ICAM-1), CD102 (ICAM-2)	Damle et al., 1992a. Damle et al., 1992b.
	and CD50 (ICAM-3)	de Fougerolles et al., 1994.
CD24 (HSA)	Not Known	Liu et al., 1992.
CD49d/CD29 (VLA-4)	CD106 (VCAM-1)	Damle and Aruffo, 1991.
CD27	CD70	Hintzen et al., 1995.
4-1BB	4-1BB-L	Hurtado et al., 1995.
Ox-40 (CD134)	Ox-40-L	Godfery et al., 1994.
SLAM (CDw150)	SLAM (CDw150)	Cocks et al., 1995.
CD5	CD72	Verwilghen et al., 1993.
Thrombospondin	CD47 (IAP)	Reinhold et al., 1997.

Abbreviations: LFA, leukocyte function antigen; ICAM, intracellular adhesion molecule; HSA, heat stable antigen; VLA, very late activation antigen; VCAM, vascular cell adhesion molecule; IAP, integrin-associated antigen.



(Aruffo and Seed, 1987). The interaction of CD28 with CD80/CD86 has multiple effects. These include the enhanced expression of CD28 (Turka et al., 1990), upregulation of the receptor for IL-2 (Cerdan *et al.*, 1992; Freeman *et al.*, 1995) and the synthesis of various cytokines including IL-2, IFN- γ , TNF- α , granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-3 (Thompson *et al.*, 1989). In addition, the expression of various cell surface receptors including CD40L (de Boer *et al.*, 1993) and CTLA-4 (Lindsten *et al.*, 1993) is upregulated. CD28 ligation also leads to increased expression of various survival factors, such as bcl-xL, which serves to enhance T cell survival and promote T cell clonal expansion by inhibiting apoptosis (Boise *et al.*, 1995).

CD28 signalling complements the signals provided by the TcR to allow the complete activation of T cells. The cytoplasmic domain of CD28 lacks direct enzymatic activity but does contain a phosphorylation site associated with p72^{ITK/EMT} kinase. When CD28 is crosslinked, rapid phosphorylation of p72^{ITK/EMT} occurs (August *et al.*, 1994). In addition, CD28 has been associated with lck tyrosine kinase (Raab *et al.*, 1995) and PI-3 kinase (August and Dupont, 1994). Elucidating the CD28-dependent signal pathway has been complicated by many factors including the state of activation of the T cell and the level of CD28 activation, and influenced by the environment in which the T cell meets antigen (reviewed in Slavik *et al.*, 1999).

1.5.1.2 Cytotoxic T Lymphocyte Antigen #4 (CTLA-4) (CD152)

The structural homologue of CD28 is CTLA-4. Both CD28 and CTLA-4 receptors are 44kDa proteins and members of the Ig superfamily. Both receptors are also homodimeric proteins (Linsley, 1995), with each monomer having a single binding site for CD80 or CD86. However, the receptors have different effects on T cell proliferation. CTLA-4 has a negative effect and down regulates T cell proliferation (Walunas *et al.*, 1994) whereas CD28 positively co-stimulates T cell activation (reviewed by Allison and Krummel, 1995). The receptors also differ in their distribution, temporal expression and binding affinities.

CTLA-4 is not expressed on the surface of naïve T cells, but its expression is upregulated within 24h on the surface of both CD4+ and CD8+ T cells upon T cell activation (Linsley *et al.*, 1992). CTLA-4 also binds CD80 and CD86 with higher affinity than CD28 (Linsley *et al.*, 1995). There is 31% amino acid sequence conservation between the genes encoding for CD28 and CTLA-4. However, conservation between the
cytoplasmic domains is limited. The signal pathway induced by CTLA-4 inhibits or counteracts T cell activation and is still being characterised (reviewed in Slavik *et al.*, 1999). In CTLA-4 knockout mice, the TcR-associated tyrosine kinases (FYN, ZAP70 and LCK) are constitutively activated (Marengere *et al.*, 1996). Furthermore, CTLA-4 associates with a protein tyrosine phosphatase SHP-2, suggesting that by activating this phosphatase, CTLA-4 signalling results in the dephosphorylation of TcR kinases or their substrates (Marengere *et al.*, 1996).

1.5.1.3 CD80 (B7.1) and CD86 (B7.2)

CD80 and CD86, ligands for CD28 and CTLA-4, are members of the B7 family and of the Ig superfamily, and have 25% homology (Freeman *et al.*, 1989; Freeman *et al.*, 1993). CD80 is a 55kDa glycoprotein and CD86 is a 77kDa glycoprotein, having a longer cytoplasmic tail compared with CD80. Each monomer has a single binding site for either CD28 or CTLA-4. The binding of two molecules of either CD80 or CD86 to a homodimer of CTLA-4 or CD28 is required for T cell co-stimulation (Greene *et al.*, 1996). Evidence suggests that CD80 and CD86 bind CTLA-4 and CD28 with similar avidities but with distinct kinetics (Linsley *et al.*, 1994). CD80 and CD86 bind CD28 with low affinities and CTLA-4 with higher affinities . However, CD86 shows a higher rate of dissociation than CD80 (Greene *et al.*, 1996). The binding sites for CD80 and CD86 on the CD28 and CTLA-4 receptors overlap but are non-identical (Ellis *et al.*, 1996).

CD80 and CD86 are expressed on the surface of professional APCs (B cells, monocytes, M ϕ and DCs) (Hathcock *et al.*, 1994). Low levels of CD86 have also been detected on the surface of T cells (Azuma *et al.*, 1993). CD86 is expressed constitutively by monocytes whereas CD80 is induced by IFN- γ , cyclic AMP or MHC class II cross linking (Freedman *et al.*, 1991). Evidence suggests DCs probably express both CD80 and CD86 constitutively (Hart *et al.*, 1993). Resting B cells do not express CD80 and express low levels of CD86. The expression of CD86 is rapidly upregulated by a variety of stimuli, including cross linking surface IgG, CD40 or MHC class II (Leach *et al.*, 1996; Linsley *et al.*, 1996). CD80 expression happens later, 24–48h after stimulation (Boussiotis *et al.*, 1994; Lenschow *et al.*, 1992). Activated B cells, such as Epstein-Barr virus (EBV) transformed B lymphoblastoid cells (EBV B-LCL), constitutively express both CD80 and CD86.

The cytoplasmic domain of CD80 and CD86 are required for T cell co-stimulation (Doty and Clark, 1996). The longer cytoplasmic tail of CD86 contains a phosphorylation site for protein kinase C (Freeman *et al.*, 1993), suggesting CD86 mediated signalling in the APC following activation. Whether CD80 and CD86 have different functions is still under review. A number of studies have shown that CD80 and CD86 co-stimulation may affect Th cell differentiation into Th1 and Th2 subsets. By identifying the range of cytokines secreted from T cells following CD80 or CD86 mediated co-stimulation, data from murine and human studies have shown CD80 co-stimulation may direct the response towards Th1 development whereas CD86 co-stimulation directs the response towards Th2 development (Kuchroo *et al.*, 1995; Freeman *et al.*, 1995; Gajewski, 1996). However, other studies however have shown this not to be the case and Th cell differentiation is likely to involve many receptor-ligand interactions (Levine *et al.*, 1995; Lanier *et al.*, 1995; Van Gool *et al.*, 1996).

1.5.1.4 CD80, CD86 and CD28 Receptor Homologues

A third member of the B7 family, termed B7.3, has been postulated to exist on B cells (Boussiotis et al., 1993) and keratinocytes (Nickoloff et al., 1993). More recently, Swallow et al. (Swallow et al., 1999) and Dong et al. (Dong et al., 1999) have both described the discovery of new co-stimulatory molecules related to the B7 family. By searching a human-expressed sequence tag data base for sequences homologous to CD80 and CD86, Dong et al. found a B7 homologue which they termed B7-H1 (Dong et al., 1999). A B7 homologue was also discovered by Swallow et al. while searching for genes activated by the NF-kB transcription in mouse cells activated with tumour necrosis factor which they termed B7h (Swallow et al., 1999). Whether or not these molecules are distinct or represent the human and murine homologues of the same molecule has not as yet been shown. They both stimulate T cell proliferation but do not bind to the co-receptors CD28 and CTLA-4, although it is thought that B7h may bind to ICOS. ICOS is a newly discovered member of the CD28 family (Hutloff et al., 1999). ICOS expression is induced on T cells shortly after T cell activation, and leads to the production of IL-10. IL-10 can selectively inhibit the upregulation of CD80 and CD86 (Ding et al., 1993) which in turn may inhibit B7 mediated CD28 co-stimulation (Abbas and Sharpe, 1999) and dampen down the immune response. B7-H1 does not bind to ICOS, but also increases the production of IL-10 by T cells. Yoshinaga et al. (Yoshinaga et al., 1999) have since

described a new murine co-stimulatory receptor-ligand pair. The receptor is a homologue of the human ICOS T cell receptor and the ligand, B7RP-1 (B7 related protein-1), has homology to CD80 and CD86. ICOS-B7RP-1 interaction co-stimulates T cell proliferation *in vitro* and *in vivo*. Interaction between the receptor- ligand pair may also play a role in the secondary immune response, since ICOS is not found on resting naïve T cells but is expressed on activated and resting memory T cells. B7RP-1 is found on M\$\$\$\$ and B cells. Since these molecules have only recently been discovered, more work remains to determine their roles.

1.5.2 Trans (Bystander) Co-Stimulation

In the absence of co-stimulatory molecules, antigen specific T cells become anergised on TcR/MHC-peptide engagement (Schwartz, 1990). In vitro studies have shown T cells must receive co-stimulation within 24 hours of TcR ligation to prevent T cell anergy (Gribben et al., 1996). In addition to co-stimulatory molecules and antigen being presented on the same cell to activate T cells (cis co-stimulation), reports of costimulatory signals being provided by third party cells to T cells which recognise antigen on a different cell (trans, remote, or bystander co-stimulation) have been described in both human and murine models. Jenkins et al. have shown co-stimulatory signals, provided by normal allogeneic APCs, could stimulate IL-2 secreting T cell clones in response to antigen (pigeon-fragment 81-104)-Ia molecule complexes on splenocytes that had costimulatory activity damaged by chemical cross-linking (Jenkins et al., 1988). Ding and Shevach subsequently showed trans co-stimulation is as effective as cis co-stimulation by activating murine CD4+ T cells, with anti-CD3 cross-linked by fixed-resting Mo, with trans co-stimulatory signals provided by B7-transfected L cells (Ding and Shevach, 1994). In addition, both CD4 memory and naïve T cells were stimulated effectively. The level of CD80 expression by the third party or bystander cells was shown to be crucial. Smythe et al. (Smythe et al., 1999) have taken advantage of this mechanism for activating T cells by transfecting human fibroblast cells with CD80 or CD86. Transfected cells (providing signal 2) were cultured in vitro with unmodified allogeneic fibroblast cells (providing signal 1) (IFN-y pretreatment induced MHC class II molecule expression), and purified human CD4+ and CD8+ T cells. An important finding from this work was that both CD4+ and CD8+ human T cells were activated at a level comparable to cis co-stimulated T cells under the defined experimental conditions. In addition, they also found the level of CD86

necessary for trans co-stimulation was equivalent to that expressed by human peripheral blood monocytes, and that less than 10% of bystander cells were required for trans costimulation to occur. Gurlo *et al.* have proposed that M ϕ s in inflamed islets of Langerhans provide a form of bystander co-stimulation to β cell-specific CD8+ T cells which enhances antigen-specific activation of cells and CD8+ T cells in insulin-dependent diabetes mellitus (Gurlo *et al.*, 1999). Sun *et al.* (Sun *et al.*, 1996) have suggested a mechanism whereby dying cells release DNA, which activates B cells resulting in the upregulation of their co-stimulatory molecules. In their study, these cells were able to provide trans co-stimulation enabling CD8+ T cells to respond to antigen presented in the context of MHC class I molecules on the surface of separate cells.

There is still controversy over the efficiency of trans co-stimulation. Both Cardoso and co-workers (Cardoso et al., 1997) and Lui and Janeway (Lui and Janeway, 1992) reported that trans co-stimulation was less efficient than cis co-stimulation. The work of Cardoso et al. found CD8+ T cells were stimulated 10-30 fold less efficiently by trans costimulation compared with cis co-stimulation (Cardoso et al., 1997). Lui and Janeway further reported that CD4+ T cells were 80 fold less efficiently stimulated by trans costimulation compared with cis co-stimulation (Lui and Janeway, 1992). Van de Velde et al. (Van de Velde et al., 1993) also found that, although memory (CD45RO) CD4+ T cells were effectively trans co-stimulated, naïve (CD45RA) CD4+ T cells were not. Discrepancies between the results may lie in the different *in vitro* models used. Cardoso et al. used Chinese Hamster Ovary (CHO) cells expressing CD80 to stimulate human CD4+ T cells. Lui and Janeway used monkey COS cells to stimulate murine CD4+ T cells, and Van de Velde et al. used murine 3T6 fibroblasts expressing CD80 to stimulate human CD4+ T cells. It is possible that, despite being transfected with CD80, cells of a different species may express accessory receptors which do not interact with human T cells preventing the transduction of other important co-stimulatory signals.

1.5.3 CD40-CD40L Interactions

CD40 is a cell surface receptor and a member of the TNF receptor superfamily. The receptor has a type I transmembrane protein structure, with an extracellular domain, a transmembrane domain and a cytoplasmic tail. It is expressed on a range of cells including B cells, M ϕ , DCs, haemopoetic progenitors, endothelial cells, epithelial cells and a range of tumours. CD40L (CD154) belongs to the TNF family and is a type II transmembrane

protein. It is expressed on activated, mature T cells but not on resting T cells. CD40L expression has also been detected on mast cells, basophils, eosinophils, B cells, natural killer (NK) cells, M ϕ and DCs under certain conditions (Mach *et al.*, 1997; Pinchuk *et al.*, 1996; Grammer *et al.*, 1995; Carbone *et al.*, 1997). B cells activated through CD40 cross linking proliferate, differentiate and produce Ig. CD40-CD40L interaction also guides B cells through their differentiation programme. This includes the rescue of B cells from apoptosis, B cell differentiation into germinal center cells, and B cell maturation into memory cells. B cells also undergo Ig isotype switching, the specificity of which is determined by cytokines. The functional consequences of CD40 ligation on other cells is reviewed by van Kooten and Banchereau (van Kooten and Banchereau, 2000).

CD40-CD40L interaction is thought to play a role in the priming of a T cell response. Following TcR engagement with MHC complexed with antigen (signal 1), the interaction of receptors expressed on the T cell with their ligands on the APC ensues. CD40L expression is upregulated on T cells after they have cross linked CD3 and received signal 1. The binding of CD40L with CD40 on the APC upregulates CD80, CD86, CD50 and CD58 (Schultze *et al.*, 1995). In addition, MHC class I and class II molecules and TAP expression are also enhanced, for more efficient antigen processing and presentation (Khanna *et al.*, 1997). In some models, T cell priming does not seem to require CD40L. Infection of CD40L knockout mice with lymphocytic choriomenigitis virus, Pichinde virus or vesicular stomatitis virus produced a strong CTL responses, suggesting CD40L independent T cell priming (Borrow *et al.*, 1996; Whitemire *et al.*, 1996; Yang *et al.*, 1996; Oxenius *et al.*, 1996). However, results suggest an impaired memory response forms in CD40L deficient T cell interactions (Borrow *et al.*, 1996). More recently, studies have shown CD40-CD40L interaction can replace the need for T cell help required in the priming of a CTL response to cross-presented antigen (Schoenberger *et al.*, 1998a).

Despite CD40 being first detected in carcinoma cell lines (Paulie *et al.*, 1989; Stamenkovic *et al.*, 1989), the function of CD40 in cancer development remains unclear. Recent studies suggest the effects of CD40-CD40L binding depend on the cell type. CD40-CD40L interaction on human bladder carcinoma cells may inhibit Fas-mediated apoptosis (Jakobson *et al.*, 1998), and promote tumour progression in melanoma and B cell malignancies (Van den Oord *et al.*, 1996; Vyth-Dreese *et al.*, 1995). In contrast, tumour cells of mesenchymal and epithelial origin undergo apoptosis upon CD40 ligation (Hess and Engelmann, 1996). Such diverse effects of CD40 expression by tumour cells may reflect the multiple functional consequences of CD40 activation in a range of cell types.

1.6 Tumour-Associated Antigens

A number of antigens associated with malignancy have been identified (Boon and van der Bruggen, 1996). These antigens presented in the context of MHC class I or MHC class II molecules serve as targets for CD8+ or CD4+ T cells respectively. CTL specific for melanoma antigens have been expanded *in vitro*, and used extensively in tumour-associated antigen identification. Various approaches have been employed in order to determine the epitopes recognised by CTL in tumour patients, including the transfection of genomic or cDNA libraries into COS-7 cells to identify genes encoding the antigen (Boon *et al.*, 1994), and peptide elution from the tumour cell by acid treatment (Cox *et al.*, 1994). A recent technique used in the identification of tumour-associated antigens is to infect APCs with a virus encoding a protein known be expressed in tumours but not normal tissues (except for the testis). CTLs can then be stimulated *in vitro* in order to identify peptide epitopes derived from the protein of interest. This approach may not be effective since immunoproteasomes, which are present in DCs but not in all tumour cells, produce different antigenic peptides to the standard proteasome (Morel *et al.*, 2000).

In humans, the majority of tumour-antigens recognised by CTL have been identified from studying melanoma immune responses *in vitro*. A variety of tumour antigens are recognised by tumour-specific CTL, including non-mutated antigens and mutated antigens. On the basis of their pattern of expression, melanoma-associated antigens can be categorised into three different groups. They include the products of genes which are not expressed in normal adult cells with the exception of immune privileged sites (cancer/testis-specific), the products of genes associated with cell differentiation, and mutated gene products. A number of different peptide epitopes have been identified from the same antigen, presented by the same or different MHC class I molecules. Table 1.2 lists melanoma-associated antigens and various antigenic epitopes identified by CTL recognition.

There are other classes of tumour antigen for other tumour types, including viral antigens associated with oncogenic viruses. For example, the human papilloma virus type 16 (HPV16) nucleoprotein antigen E7 is expressed in 90% of human cervical carcinomas (Ressing *et al.*, 1995). Mucins, large heavily glycosylated molecules expressed and secreted by ductal epithelial cells of various tissues and tumours of the same histological

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Melanoma Antigen	Restriction	Peptide Epitope	Reference
	Element		
Cancer/Testis-Specific		<u>,</u>	
MAGE 1	HLA-A1	EADPTGHSY	Traversari et al., 1992.
	HLA-Cw16	SAYGEPRKL	van der Bruggen et al., 1994b.
	HLA-A3	SLFRAVITK	Chaux et al., 1999a.
	HLA-A28	EVYDGREHSA	Chaux et al., 1999a.
	HLA-B53	DPARYEFLW	Chaux et al., 1999a.
	HLA-Cw2	SAFPTTINF	Chaux et al., 1999a.
	HLA-Cw3	SAYGEPFKL	Chaux et al., 1999a.
MAGE 2	HLA-A2	KMVELVHFL	Visseren et al., 1997.
	HLA-A2	YLQLVFGIEV	Visseren et al., 1997
MAGE 3	HLA-A1	EVDPIGHLY	Gaugler et al., 1994.
	HLA-A2	FLWGPRALV	van der Bruggen et al., 1994a.
	HLA-DR13	AELVHFLLLKYKAR	Chaux et al., 1999b.
	HLA-DR13	LLKYRAREPVTKAE	Chaux et al., 1999b.
MAGE 4	HLA-A2	GVYDGREHTV	Duffour et al., 1999.
MAGE 6	HLA-A3	MVKISGGPR	Zorn and Hercend, 1999.
MAGE 10	HLA-A2.1	GLYDGMEHL	Huang et al., 1999.
BAGE	HLA-Cw16	AARAVFLAL	Boel et al., 1995.
GAGE 1, 2	HLA-Cw6	YRPRPRRY	Van den Eynde, 1995.
RAGE	HLA-B7	SPSSNRIRNT	Gaugler et al., 1996.
LAGE 1	HLA-A2	MLMAQEALAFL*	Aarnoudse et al., 1999.
PRAME	HLA-A24	LYVDSLFFL	Ikeda et al., 1997.
NY-ESO-1	HLA-A2	SLLMWITQC	Jager er al., 1998.
	HLA-A2	SLLMWITQCFL	Jager et al., 1998.
	HLA-A2	QLSLLMWIT	Jager <i>et al.</i> , 1998.

Table 1.2 Melanoma-associated antigens recognised by CTL and CD4+ T cells (continued overleaf).

* Alternative ORF

Mutations are underlined

_Chapter 1

Figure 1.3 cont.....

Melanocyte-specific antigen	8		
MelanA/MART1	HLA-A2	AAGIGILTV	Kawakami et al., 1994.
	HLA-A2	ILTVILGVL	Castelli et al., 1995.
Tyrosinase	HLA-A2	YMNGTMSQV	Wolfel et al., 1994.
	HLA-A2	MLLAVLYCL	Wolfel et al., 1994.
	HLA-B44	SEIWRDIDF	Brichard et al., 1996.
	HLA-A24	AFLPWHRLF	Kang et al., 1995.
	HLA-B35	LPSSADVEF	Morel et al., 1999.
	HLA-DR4	QNILLSNAPLGPQFP	Topalian <i>et al.</i> , 1996.
	HLA-DR4	SYLQDSDPDSFQD	Topalian <i>et al.</i> , 1996.
gp100/Pmel17	HLA-A2	LLDGTATLRL	Kawakami et al., 1995.
	HLA-A2	ITDQVPFSV	Kawakami et al., 1995.
	HLA-A2	VLYRYGSFSV	Kawakami et al., 1995.
	HLA-A2	KTWGQYWQV	Bakker et al., 1995.
			Kawakami et al., 1995.
	HLA-A2	YLEPGPVTA	Cox et al., 1994.
			Kawakami <i>et al.</i> , 1995.
	HLA-DR4	WNRQLYPENTEAQRLD	Halder et al., 1997.
TRP-1 (gp75)	HLA-A31	MSLQRQFLR*	Wang et al., 1996b.
TRP-2	HLA-A31	LLPGGRPR	Wang et al., 1996a.
Tumour-specific mutated			
antigens			
β-catenin	HLA-A24	SYLDSGIH <u>F</u>	Robbins et al., 1996.
MUM-1	HLA-B44	EEKL <u>I</u> VVLF	Coulie et al., 1995.
N-acetylglucosaminyl- transferase-V	HLA-A2	VLPDVFIRC	Guilloux et al., 1996.
p15	HLA-A24	AYGLDFYIL	Robbins et al., 1995.
CDK4	HLA-A2	A <u>C</u> DPHSGHFV	Wolfel et al., 1995.

* Alternative ORF

Mutations are underlined

type, are targets for CTLs in ovarian tumours (Ioannides *et al.*, 1993), myeloma cell (Takahashi *et al.*, 1994), breast and pancreatic adenocarcinomas (Jerome *et al.*, 1993) due to incomplete glycosylation.

1.6.1 Cancer/Testis-Specific Antigens

Antigens which are expressed by a range of tumour types, but not the majority of normal cells, include products of the MAGE gene family. These are non-mutated antigens. Four other closely related families have also been identified, BAGE, GAGE, RAGE and LAGE (Boel *et al.*, 1995; Van den Eynde *et al.*, 1995b; Gaugler *et al.*, 1996; Lethe *et al.*, 1998). Table 1.3 shows the percentage of tumours expressing MAGE, BAGE and GAGE in a variety of histological types.

Tumour Type	MAGE 1	MAGE 3	BAGE	GAGE 1, 2
Melanoma	36	65	22	24
Head and neck cancer	25	48	8	19
Lung carcinoma	34	31	6	19
Mammary carcinoma	18	11	10	9
Colorectal carcinoma	0	16	0	0
Renal carcinoma	0	0	0	0
Lymphoma and leukaemia	0	0	0	0

Table 1.3. Expression of tumour specific, shared antigens (MAGE 1, MAGE 3, BAGE and GAGE 1, 2) in diverse tumour types. (Modified from Van den Eynde et al., 1995a).

1.6.1.1 The MAGE Gene Family

MZ2-E was the first tumour antigen to be identified on a human tumour (van der Bruggen *et al.*, 1991) and was found to be encoded by a new gene, termed MAGE 1. More than twelve MAGE genes have since been identified which share 64-85% nucleotide sequence homology. The MAGE genes are located on the long arm of chromosome X in the Xq28 region. They are expressed in melanomas and in other tumours of differing histological types. These include non-small-cell lung carcinomas (Weynants *et al.*, 1994), bladder cancers (Patard *et al.*, 1995), breast tumours (Brasseur *et al.*, 1992), head and neck carcinomas, gastric and oesophageal cancers, ovarian tumours and sarcomas. Leukaemias, lymphomas and renal carcinomas do not express the MAGE genes. The only normal cells known to express the MAGE genes are the testis and the placenta. However, the testis cells lack HLA class I expression and the cells are also not accessible to be the immune system, so T cell tolerance to these non-mutated antigens is not likely to exist.

The functions of the MAGE proteins are unknown. The hydrophobic regions of the MAGE gene family products are conserved, suggesting the proteins may perform similar functions. The promoters and first few exons of the MAGE genes show great variability, suggesting that a similar protein function could be regulated under different transcriptional controls at specific times and places (Coulie, 1997). Expression of cancer/testis-specific genes is due to the demethylation of the promoter (de Smet *et al.*, 1999). In non-transformed somatic tissues, the cancer/testis-specific genes are silenced by heavy methylation of the promoters.

MAGE 1 antigenic peptides have been characterised and found to be presented through two different HLA restriction molecules – HLA-A1 (Traversari *et al.*, 1992) and HLA-Cw16 (van der Bruggen *et al.*, 1994b). MAGE 3 encodes for three antigenic peptides which are presented through HLA-A1, A2 and B44 (Gaugler *et al.*, 1994; Celis *et al.*, 1994; Van der Bruggen *et al.*, 1994a). MAGE 2 derived antigenic epitopes have been identified using HLA transgenic mice and are presented through HLA-A2 (Visseren *et al.*, 1997). The level of expression of the MAGE antigens is variable between tumour cell lines.

More recently, five more MAGE 1 epitopes have been identified which are presented by HLA-A3, -A28, -B53, -Cw2 and -Cw3 (Chaux *et al.*, 1999a). Monocyte derived DCs were transduced to express MAGE 1 and CD8+ T cells recognising peptide antigens were cloned. An epitope from the MAGE 10 gene which is presented through HLA-A2.1 (Huang *et al.*, 1999), a MAGE 4 peptide presented through HLA-A2 (Duffour *et al.*, 1999) and an epitope from the MAGE 6 gene (Zorn and Hercend, 1999) have also been identified. The CTL clone recognising the MAGE 6 antigenic peptide was identified in a patient showing spontaneous tumour regression.

1.6.1.2 The GAGE, BAGE, RAGE and LAGE Gene Families

Like the MAGE gene products, BAGE (Boel et al., 1995), GAGE (Van den Eynde et al., 1995b) and LAGE (Lethe et al., 1998) gene products are expressed in melanomas and a range of other tumours, including breast cancer, head and neck cancer, bladder tumours and sarcomas, but excluding leukaemias, lymphomas and renal cell carcinomas. These antigens have also been identified on the testis and the placenta only in normal tissues. There are six members of the GAGE family, but only GAGE 1 and 2 have been shown to encode for antigenic peptides. Both genes encode for the same peptide which is presented on HLA-Cw6. BAGE also belongs to a multigene family and encodes for a peptide that is restricted through HLA-Cw1601. The LAGE gene was identified by representational difference analysis to identify genes that are not expressed in the skin (Lethe *et al.*, 1998).

RAGE was identified on a renal cell carcinoma (Gaugler *et al.*, 1996), and has subsequently been identified on other kidney tumours, bladder cancers, melanoma and sarcomas. RAGE is silent in other tissues excepting the retina which is an immune privileged site. The identified antigen is presented through HLA-B7.

1.6.1.3 PRAME

PRAME encodes for an antigenic peptide presented through HLA-A24 (Ikeda *et al.*, 1997). The function of the PRAME gene product at present is unknown. Like MAGE, PRAME is expressed in a range of solid tumours including non-small cell lung carcinoma, sarcomas, renal carcinomas and head and neck cancer, but unlike MAGE, PRAME has also been identified in acute leukaemias. In addition to the testis, PRAME expression in normal tissues includes the adrenal glands, ovaries and endometrium. The level of expression of PRAME is low in normal tissues, except in the endometrium.

1.6.2 Differentiation Antigens

CTLs directed against melanocyte differentiation antigens can recognise both melanomas and melanocytes. The recognition of melanocyte differentiation antigens by CTLs indicates the lack of strong tolerance to this group of non-mutated peptides. This is presumably due to the absence of expression of the melanocyte differentiation antigens in the thymus, therefore reactive T cells are not centrally deleted. Antigens associated with lineage-specific proteins include MelanA/MART1, gp100/Pmel17, gp75 (tyrosinase-related protein [TRP]-1) and tyrosinase.

From the MelanA/MART1 molecule, two overlapping peptide epitopes have been identified which are HLA-A2 restricted (Kawakami *et al.*, 1994b; Castelli *et al.*, 1995). Five HLA-A2 restricted epitopes have been identified from gp100 (Kawakami *et al.*, 1995). An HLA-A31 restricted peptide has been identified which is encoded from an open

reading frame (ORF) different to the normal ORF for the protein gp75. T cells reacting to this epitope also reacted to normal HLA-A31+ melanocytes, thus demonstrating an example in eukaryotes of alternative ORF being used to encode for a protein. The majority of the peptide epitopes from the proteins MelanA/MART1 and gp100 bind with relatively low affinity to HLA-A2 compared with viral epitopes and foreign antigens (Kawakami *et al.*, 1995). The functions of the MelanA/MART1, gp100 and gp75 proteins at present are unknown.

Tyrosinase is an enzyme involved in the production of melanin. The enzyme converts tyrosine into dihydroxyphenylalanine (DOPA) which is an intermediate in melanin synthesis. Other cells which synthesise DOPA do not use tyrosine but instead use an alternative enzyme called tyrosine hydrolase. This enzyme does not have sequence homology to tyrosine. Two antigenic epitopes first identified from tyrosinase were found to bind to HLA-A2 (Brichard *et al.*, 1993; Wolfel *et al.*, 1994). Recent studies have shown the sequence of another naturally occurring antigenic epitope presented by HLA-A2 to a have single amino acid change (Skipper *et al.*, 1996). Deamination of an asparagine residue resulted in the appearance of aspartic acid, indicating a post translational modification. The enzyme responsible has not been identified. Tyrosinase epitopes have now been identified that are expressed through HLA-A24, HLA-B44 and HLA-B35 (Kang *et al.*, 1995; Brichard *et al.*, 1996; Morel *et al.*, 1999).

Since the differentiation antigens are also expressed by normal cells, there has been concern for their safety in therapy. Retinal epithelium also contains some melanocytes, but as yet, there has been no associated problems with the use of differentiation antigens in therapy (Richards *et al.*, 1992), and vitiligo (skin depigmentation) has been associated with good prognosis in melanoma therapy (Bystryn *et al.*, 1987).

1.6.3 Mutated Gene Product Antigens

The products of mutated genes also represent targets for tumour specific CTLs. A point mutation may enable a peptide epitope to bind successfully to the specific HLA molecule but alter T cell recognition, or a point mutations may allow a peptide to bind with HLA molecules which, without a mutation, it would not normally bind. Point mutations are unique to the tumour or restricted to a few, and CTLs recognising unique mutations are likely to be tumour specific (Anichini *et al.*, 1996). Due to their limited expression, the products of mutated genes are unlikely to be of use in widely applicable cancer vaccines.

Mutations of widely expressed genes include β -catenin, MUM-1, and cyclin dependent kinase 4 (CDK4).

 β -catenin is involved in cell surface adhesion mediated by cadherins (Grunwald, 1993) and has been shown to bind to the adenomatous polyposis coli tumour suppresser gene product (Su *et al.*, 1993). Mutations in the β -catenin gene could therefore result in tumour metastasis and tumourigenesis (Robbins and Kawakami, 1996). A point mutation identified in a melanoma cell line resulted in the increased binding of a T cell epitope to HLA-A24. This meant that compared with the normal antigen, a lower concentration of antigen was required for recognition by CTL (Robbins *et al.*, 1996).

A point mutation in CDK4, a proto-onco gene involved in cell cycle regulation, produced a T cell epitope which showed enhanced binding to HLA-A2.1. This also prevented the protein binding to the CDK4 inhibitor $(p16^{INK4a})$ (Wolfel *et al.*, 1995) and indicated this mutation may have a direct role in tumourigenicity. This point mutation was also found in one out of twenty eight melanoma cell lines tested (Robbins and Kawakami, 1996).

MUM-1 antigen was identified as an HLA-B44 restricted tumour-antigen on a melanoma cell line (Coulie *et al.*, 1995). The antigenic peptide was encoded by a sequence located across an exon-intron junction where a point mutation in the intron region was responsible for the epitope. The function of the MUM-1 protein is not known.

1.6.4 Serological Analysis of Tumour Antigens by Recombinant cDNA Expression (SEREX)

Very recently, a new technique based on the systematic analysis of the Ab repertoire in cancer patients has allowed the isolation and identification of new tumour antigens. The results suggest human tumour-associated antigens are able to elicit both a humoral and cellular immune response. SEREX analysis has been applied to a variety of tumour types including renal cancer, lung cancer (Brass *et al.*, 1997; Gure *et al.*, 1998), colon cancer (Scanlan *et al.*, 1998) and melanoma (Chen *et al.*, 1998). Several categories of tumour antigens have been identified using this approach, including antigens expressed in tumours and the testis only, mutational antigens, over-expressed antigens, differentiation antigens and antigens encoded by potential tumour suppresser genes. It has lead to the identification of a large number of novel genes and has also identified tumour specific shared antigens originally identified by CTL analysis, including MAGE and tyrosinase

(Sahin *et al.*, 1995). Other genes encoding for tumour specific shared antigens identified by SEREX analysis include SSX2 in patients with melanoma (Sahin *et al.*, 1995; Tureci *et al.*, 1996) and SCP1 in patients with renal cell carcinoma (Tureci *et al.*, 1998). MAGE 4 has more often been isolated by SEREX than MAGE 1, suggesting MAGE 4 is more immunogenic to the humoral immune system (Chen *et al.*, 1998). The gene CT7 has been identified by SEREX analysis and shown to have partial homology to the MAGE gene family. It is also expressed mainly in the testis and a range of tumour cell types, although trace amounts have also been detected in the liver, kidney, placenta and foetal brain (Chen *et al.*, 1998).

NY-ESO-1 was identified using the SEREX method in oesophageal cancer (Chen *et al.*, 1997). It is also expressed in a range of human tumours but not in normal tissues except for the testis. Approximately 10-40% of a range of tumours tested expressed this antigen, including melanoma. LAGE 1 is highly homologous to NY-ESO-1. CTL analysis has shown this antigen is recognised in the context of HLA-A2 and HLA-A31.

1.6.5 MHC Class II Restricted Melanoma-Associated Antigens

In melanoma, MHC class I restricted antigens are well characterised at the molecular level. In contrast, few MHC class II restricted antigens have been identified. Those which have been identified include non-mutated epitopes derived from the expression of the melanocyte differentiation genes, tyrosinase and gp100. These transmembrane proteins are located within melanosomes, a group of organelles related to the lysosomal organelles. Tyrosinase and gp100 may therefore bind MHC class II molecules directly (Topalian *et al.*, 1996) and serve as targets for CD4+ T cells. Two HLA-DRB1*0401 restricted tyrosinase epitopes have been identified (Topalian *et al.*, 1996).

More recently, two additional MAGE 3 epitopes presented through MHC class II molecules have been identified (Chaux *et al.*, 1999b). A MAGE 3 recombinant protein was loaded onto monocyte derived DCs and used to stimulate autologous CD4+ T cells. Two clones recognising different MAGE 3 epitopes presented through HLA-DR13 were identified. As growing evidence is revealing the critical role thatf CD4+ T cells play in sustaining a long lasting protective anti-tumour immune response and maintaining immunity to tumours (Topalian 1994), greater attention is now being paid to MHC class II restricted antigens and it is likely more MHC class II epitopes will be defined.

1.7 Current Strategies in Tumour Immunotherapy

Despite being antigenic, the majority of tumour cells fail to stimulate the immune system effectively, and grow progressively in immunocompetent hosts (Speiser *et al.*, 1997). The poor immunogenicity of tumour cells has been attributed to one or a combination of reasons. These include poor antigen processing and presenting capabilities which may involve the complete loss of expression or down regulation of one or more HLA molecules, or a lack of co-stimulatory and/or adhesion molecule expression. Immune escape mechanisms in melanoma are reviewed by thor Straten *et al.* (thor Straten *et al.*, 1999).

In order protect the host against tumour growth and progression, a number of therapeutic cancer vaccination strategies are currently under investigation in animal studies and human clinical trials, with the main objective to induce strong tumour-antigen specific, long lasting CTL responses in order to achieve clinical improvement (reviewed in Sinkovics and Horvath, 1999; Chamberlain, 1999). The results of animal studies and clinical trials support the use of immunotherapy for human cancer vaccination. However, depending on the vaccine strategy, there are limitations to each of these approaches at present, including the best route of vaccine administration, which of the identified tumour-antigens is the best choice for immunisation, and which vector delivery system is the most appropriate. Some of the techniques and the results of several studies are described below.

1.7.1 Peptide-Based Vaccines

The identification of tumour-associated antigens recognised by TILs *in vitro* as well as the genes encoding the tumour-associated antigens, has led to the development of a range of peptide-based tumour therapies for the generation of tumour-specific CTLs *in vivo*. Peptides used alone, with adjuvant, or modified to enhance their immunogenicity have been used in tumour therapy. Proteins or peptides can be injected directly or pulsed onto APCs for use in the vaccination of cancer patients. Alternatively, the genes encoding tumour antigens can be used directly for immunisation ('naked' DNA) or by tumour antigen gene incorporation into recombinant viral vectors. Peptide-based strategies are relatively cost-effective for clinical use, although the HLA restricted pattern of expression limits their use to a set of patients expressing the appropriate HLA molecule. Peptide-based techniques are also hampered by the availability of appropriate, defined immunogenic tumour antigens for many tumour and HLA types. Recent concerns have centered on the use of appropriate peptides in tumour therapy (Velders *et al.*, 1998) and it

has been suggested that peptides which elicit both a humoral and CTL response, for example NY-ESO, may be more effective (Jager *et al.*, 1999). It is well known that tumour masses are composed of heterogenous cells. From an immunotherapeutic point of view, immunising against a single antigen or epitope may not be ideal as antigen loss variants can arise thus demonstrating the need for immunisation with a range of immunogenic antigens or peptides (Lehmann *et al.*, 1995; Maeurer *et al.*, 1996).

Clinical trials evaluating peptide-based therapies are under investigation, although few have been published to date with some conflicting results. In one study, three out of twelve HLA-A1, MAGE 3 positive melanoma patients receiving a MAGE 3 peptide showed tumour regression, including one complete response, although no T cell precursors reactive with the peptide or tumour were identified (Marchand et al., 1995). In a separate study, 42% of melanoma patients in a trial receiving modified gp100 peptide (to enhance its HLA binding) along with IL-2 showed objective cancer responses with ten out of twelve patients showing high levels of anti-peptide T cell precursors (Rosenberg et al., 1998). In another study, fifteen patients out of eighteen immunised with a peptide from MelanA/MART1 antigen with incomplete Freund's adjuvant induced MelanA/MART1 specific CTLs but did not show tumour regression (Cormier et al., 1997). Morel et al. have recently suggested the use of peptides which do not require degradation in the proteasome in DCs for tumour therapy, as some melanoma differentiation antigens which are used in anti-tumour vaccination are poorly processed by immunoproteasomes, which are present in DCs, and produce different antigenic peptides to the standard proteasome (Morel et al., 2000).

An additional approach to peptide-based cancer therapy involves loading the peptide onto professional APCs *ex vivo*, for optimum antigen presentation. Peptide loaded APCs should directly prime a CTL response *in vivo*. Animal models have shown DCs pulsed with tumour antigen or peptides *in vitro* can induce immune responses *in vivo* which protect against lethal tumour challenge or regression of established tumours expressing the relevant tumour antigen (Mayordomo *et al.*, 1997; Celluzzi and Falo, 1996). Clinical trials have confirmed the efficacy of this approach in follicular B cell lymphoma, where all patients developed a measurable anti-tumour cellular immune response and with one patient experiencing complete tumour regression (Hsu *et al.*, 1996), and in melanoma, where five out of sixteen patients showed an objective response, including two complete responses (Nestle *et al.*, 1998). Mukherji *et al.* showed the induction of melanoma-specific CTLs *in situ* in patients immunised with autologous synthetic-peptide pulsed APCs

(Mukherji et al., 1995). In a phase I study by Mackenson et al., DCs were pulsed with a mixture of MAGE 1, MAGE 3, tyrosinase, MelanA/MART1 or gp100 peptides (depending on the patient HLA type) (Mackenson et al., 2000). Out of fourteen patients immunised, two showed anti-tumour responses. In order to prevent the growth of tumour-escape variants, APCs have also been loaded with unfractionated peptides eluted from tumour-cell membranes. This also eliminates the need for immunogenic peptide identification. DCs pulsed with peptides eluted from an immunogenic tumour resulted in the eradication of established tumours in all treated mice, and DCs pulsed with peptides from a weakly immunogenic tumour dramatically suppressed the growth of established tumours (Zitvogel et al., 1996). However, mice immunised with DCs genetically engineered to express IL-2 and loaded with peptides from a non-immunogenic tumour did not elicit a protective immune response (Ribas et al., 1999b). Boczkowski et al. have shown DCs pulsed with tumour derived RNA dramatically reduced lung metastases in mice (Boczkowski et al., 1996; Boczkowski et al., 2000), suggesting such an approach may also elicit an effective immune response which is directed against the patient's own unique tumour antigen spectrum.

1.7.2 Vector-Mediated and DNA Vaccines

Viral vectors encoding tumour antigens have also been constructed and used in a clinical setting, and naked DNA encoding tumour antigens have also induced potent antitumour immune responses in animal models (Conry et al., 1995; Condon et al., 1996; Irvine et al., 1996; Park et al., 1999). In a study by Ribas et al. the intramuscular injection of a MelanA/MART1 expression plasmid, or the systemic administration of an adenovirus vector expressing MelanA/MART1, into mice induced partial protection against challenge with a poorly immunogenic murine fibrosarcoma line stably transfected with the MelanA/MART1 gene (Ribas et al., 1997). Complete tumour rejection was observed when murine DCs were transduced directly to express MelanA/MART1 by an adenovirus vector and immunised intravenously into mice, demonstrating the importance of inoculation sites in vaccination strategies (Ribas et al., 1999a). MelanA/MART1 specific CTLs were generated and immunological specificity and memory attained. In a phase I trial, recombinant adenovirus expressing either MelanA/MART1 or gp100 was used to immunise patients with metastatic melanoma. One complete response was observed out of sixteen patients, and other patients experienced partial responses only when the vector was administered with IL-2. High levels of neutralising Ab to the viral vector in the patient's

sera prior to immunisation may have affected the efficacy of the vaccine, indicating a potential problem with adenoviral vectors in human vaccination strategies.

1.7.3 Cell Based Vaccines

Despite the efforts to identify tumour-associated antigens, for the majority of human tumours, the immunologically relevant antigens have not been identified or characterised. In an attempt to over come the poor immunogenicity of tumour cells and the lack of well characterised tumour-antigens, tumour cells have been used as whole cell vaccines in cancer therapy. Using this approach, the immune system can be stimulated against multiple tumour-specific epitopes. Early work using whole cell vaccines included immunising patients with irradiated allogeneic tumours cell mixtures along with a non-specific adjuvant, including bacille Calmette-Guerin (BCG) (Morton *et al.*, 1993) and DETOX (Mitchell *et al.*, 1988). Morton *et al.* immunised stage III and stage IV melanoma patients with a vaccine consisting of three different tumour cell lines administered with BCG (Morton *et al.*, 1993). An increased five year overall survival rate of 23% was observed in stage IV patients who received the vaccine, compared with 6-10% in matched historical controls. An overall survival rate of 34% for stage III patients who received the allogeneic cell vaccine as compared with 16% for matched historical controls was also observed.

Based on the modest success achieved with non-genetically modified whole cell vaccines, tumour cells modified by gene transfection to express cytokines, singly or in combination (Fearon *et al.*, 1990; Golumbek *et al.*, 1991; Dranoff *et al.*, 1993; Cayeux *et al.*, 1995), co-stimulatory molecules (Chen *et al.*, 1992; Townsend and Allison, 1993; Baskar *et al.*, 1993) or MHC class I and II molecules (Restifo *et al.*, 1993b; Ostrand-Rosenberg, 1990; Armstrong *et al.*, 1997) for use as candidate tumour vaccines have been generated (reviewed by Nawrocki and Mackiewicz, 1999). The aim of these approaches is to make the tumour cell resemble, or bypass the need for, professional APC.

1.7.3.1 Cytokine Transfection

IL-2, a cytokine critical for CTL activation and growth, has been transfected into a variety of tumour cells for use in cancer therapy. In a study by Fearon *et al.*, mice rejected colon cancer cells genetically modified to express IL-2. Rejection was mediated by CTLs, by-passing the need for helper T cells (Fearon *et al.*, 1990). Furthermore, animals vaccinated with the gene modified melanoma cells were protected against subsequent

challenge with the non-modified tumour. IL-2 transfected melanoma cells were able to induce a potent and long lasting immune response which protected mice against challenge with the non-modified tumour cells after several months in a study by Zatloukal *et al.* (Zatloukal *et al.*, 1995). However, both CD4+ and CD8+ T cells were required for tumour-specific protection. Maass *et al.*, suggested the potent T cell response induced against IL-2 secreting melanoma cells in mice in their experiments was due to the uptake and re-presentation (cross priming) of tumour-derived antigens by APCs at the vaccination site (Maass *et al.*, 1995).

Dranoff *et al.* (Dranoff *et al.*, 1993) compared the ability of ten different retroviruses encoding potential immunomodulators (IL-2, IL-4, IL-5, IL-6, IFN- γ , TNF- α , GM-CSF, CD2, ICAM1 and IL-1RA) to stimulate systemic anti-tumour immunity in a single tumour murine model using the B16-F10 murine derived melanoma cell line. The results showed GM-CSF transduced cells were the most powerful immune stimulant. Vaccination sites were infiltrated with T cell, DCs, M ϕ s and eosinophils and showed extensive tumour destruction. It was thought GM-CSF may have enhanced APC antigen processing and presenting function enhancing tumour-antigen representation, especially since the melanoma cells did not express MHC class II antigens and the anti-tumour response required both CD4+ and CD8+ T cells.

The gene for IL-4 has been transfected into the renal cell carcinoma cell line Renca, and used in mice vaccination protocols (Golumbek et al., 1991). The results showed that activated Mos were present in the tumour infiltrate and the anti-tumour response depended on both CD8+ and CD4+ T cells. In addition, non-transfected cells injected into a site distant from the initial immunisation site, were also rejected. Since IL-4 is a is important in APC recruitment, it is possible cross priming might have occurred However, transfection of IL-4 into B16 melanoma cells and J558L plasmacytoma cells was found to induce an anti-tumour response which was also capable of rejecting established tumours without requiring T cell immunity and an immune response capable of protecting against tumour challenge was not observed (Tepper et al., 1992). Although Møs infiltrated the tumour area, the anti-tumour response was dependent on eosinophils. Cayeux et al. found a stronger immune response in the same plasmacytoma model when tumour cells were transfected with both IL-4 and CD80 (Cayeux et al., 1996). Immunisation of mice with IL-4 and CD80 transfected tumour cells led to complete tumour rejection and the generation of a stronger systemic immunity compared with single transfectants.

Combination gene therapy may be more effective than single transfections for tumour treatment through the activation of different cell types in the vaccination area.

A number of clinical trials have now been initiated in patients with melanoma. These trials include autologous irradiated melanoma cells transduced to secrete IFN-y (Abdel-Wahab et al., 1997), IL-2 (Stewart et al., 1999), GM-CSF (Soiffer et al., 1998), and allogeneic cells expressing IL-4 (Arienti et al., 1999). Patients receiving autologous IFN-y (Abdel-Wahab et al., 1997) and IL-2 (Stewart et al., 1999) transduced melanoma cells showed weak clinical responses but confirmed the safety of adenoviral vectors for use in gene delivery in humans. However, patients receiving GM-CSF transduced melanoma cells showed potent anti-tumour responses (Soiffer et al., 1998). In the phase I clinical trial conducted by Soiffer et al., all twelve patients with metastatic melanoma who received autologous melanoma cells engineered to secrete GM-CSF showed immunisation sites intensely infiltrated with T cells, DCs, Møs and eosinophils. Extensive tumour destruction was associated with CTLs and Ab responses in eleven out of sixteen patients. Extensive genetic modification of tumour cells for cancer immunotherapy in a clinical setting may prove to be labour intensive and expensive if individual vaccines have to be prepared. In addition, the genetic modification of tumour cells require tumour cell growth in vitro, a major difficulty for most tumour types.

1.7.3.2 CD80 and CD86 Transfection

The majority of tumours lack the expression of important co-stimulatory molecules. A lack of co-stimulation has been shown to anergise T cells (Schwartz, 1993), which is one way in which tumours may be able to escape immune recognition and elimination. Anergised T cells can be activated by the addition of high doses of IL-2 (Essery *et al.*, 1988) or by providing co-stimulation with CD80 (Harding *et al.*, 1992).

The critical role of CD80 and CD86 in tumour rejection has been shown in transfection studies (Guinan *et al.*, 1994). Transfection of tumour cells with either CD80 and/or CD86 to directly activate tumour-specific CTL, has been met with both positive and negative results, depending on the tumour model used. Chen *et al.* (Chen *et al.*, 1992) and Townsend and Allison (Townsend and Allison, 1993) both reported the induction of an anti-tumour response in mice injected with the murine melanoma cell line K1735-M2 modified to express CD80. Despite expressing MHC class I and MHC class II antigens, the response required CD8+ T cells only, suggesting the CD80 modified tumour cells were

able to directly prime CTLs. In addition, the study showed immunised mice were capable of rejecting the wild type (unmodified) tumour. Transfection of CD80 into tumour cells has also been shown to increase the number of T cell epitopes recognised by CD8+ T cells *in vitro* (Johnston *et al.*, 1996). In contrast, the unmodified parent tumour induced a CTL response to only one immunodominant epitope.

CD4+ T cells played a critical role in the immune response when the murine Sal sarcoma cell line (which expresses MHC class I but not MHC class II antigens) was transfected with CD80 and MHC class II (Basker *et al.*, 1993). The mice immunised with CD80 and MHC class II transfected SaI cells were also able to reject tumour challenge with the wild type tumour. In a follow up study, Basker *et al.* showed in T cell depletion studies that CD4+ T cells were important for tumour rejection in naïve animals, and CD8+ T cells were required for the rejection of established tumours, suggesting the effectiveness of the vaccine was due to the activation of both T cell subsets (Basker *et al.*, 1995).

In contrast, other groups have shown that CD80 transfection into tumour cells does not induce an immune response which protects against the wild type tumour challenge. Ramaranthinam et al. showed the murine plasmacytoma cell line J558 (which expresses MHC class I but not MHC class II antigens) transfected to express CD80 failed to induce a response in mice which protected against unmodified parent tumour challenge (Ramaranthinam et al., 1994). Chen et al. further investigated discrepancies in the results using a panel of tumour cell lines transfected with CD80 (Chen et al., 1994a). These cell lines varied in their inherent immunogenicity. The results showed that CD80 transfection was effective in tumour cell lines (RMA, E6B2, P815 and EL4) which were immunogenic, but not in those cell lines (B16, Ag104, MCA101 and MCA102) which were not immunogenic. These results show the effective rejection of tumours engineered to express CD80 is dependent on their inherent immunogenicity. Other factors have also been shown to play a role in the induction of the immune response against CD80 transfected tumour cells, including the level of expression of CD80, and the expression of other cell surface molecules in conjunction with CD80, including ICAM-1 (Wu et al., 1995; Cavallo et al., 1995). Cavallo et al. showed both CD80 and ICAM 1 expression were necessary for the rejection of tumours and for establishing a memory response (Cavallo et al., 1995). Wu et al. showed that the non-immunogenic melanoma cell line B16 expressing high levels of CD80 did not grow in mice (Wu et al., 1995). However, the response did not protect against challenge with the parent melanoma cell line. NK cells and CD8+ T cells were shown to be responsible for the immune rejection of the CD80 transfected cells,

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demonstrating direct killing induced by the non-immunogenic tumours expressing CD80 did not elicit enhanced systemic immunity to the parental tumour. This result is similar to those obtained by Chong et al. in a murine colorectal tumour cell line engineered to express CD80 (Chong et al., 1996). In addition, Chong et al. showed CD80 or CD86 transfected murine melanoma cells showed less systemic anti-tumour immunity in mice than the non-transfected parent melanoma cells (Chong et al., 1996). Other evidence has shown that CD80 transfection in conjunction with cytokine transfection may be therapeutically more efficacious. The expression of both CD80 and IL-2 (Gaken et al., 1997) or CD80 and IL-4 (Cayeux et al., 1996) in murine tumour models, have recently shown enhanced immune protection in mice, as compared with single gene transfections. CD86, the alternative receptor to CD80 for binding with CD28, has also been transfected into a number of tumour cell lines with diverse results. In a recent study, CD86 appeared to be a more potent stimulator of tumour immunity as compared with CD80 in a human renal cell carcinoma cell line (Jung et al., 1999). However, in a separate study, murine colonic adenocarcinoma cells transfected with CD80 or CD86 were rejected equally well in mice (Hodge et al., 1994). The implications of CD80 and CD86 transfection in different tumour types for the development of anti-tumour immune responses requires further investigation.

Collectively, the work from the many studies performed using CD80 transfected cells have shown diverse results, resulting from the complex cellular interactions which occur *in vivo*. The mechanism by which CD80 transfected cells stimulate T cells *in vivo* has recently come under scrutiny. CD80 transfected tumour cells were thought to resemble APCs, and directly stimulate T cells in order to generate an anti-tumour immune response *in vivo*. However, Huang *et al.* have recently shown, using CD80 transfected colonic epithelial tumour cells, that cross priming is dominant over direct CTL priming (Huang *et al.*, 1996a). Only after repeated stimulation with CD80 transfected cells could direct antigen presentation be observed. On the other hand, Cayeux *et al.* demonstrated the potential for both direct priming and cross priming to occur after one immunisation using a different tumour model expressing CD80 and different experimental system (Cayeux *et al.*, 1997). Clearly, further work remains to clarify these issues.

1.7.4 Adoptive T Cell Therapy

Cancer therapy with *ex vivo* cultured tumour reactive T cells has proved to be a promising approach to cancer therapy which does not require identified tumour antigens

(reviewed in Cheever and Chen, 1997; Liebiwitz et al., 1998). Stimulation of TIL with appropriate cytokines (eg IL-2) and autologous tumour cells in vitro can expand cell numbers for adoptive transfer into humans (discussed in section 5.1.1.4). This approach is not always feasible with tumours which are poorly or non-immunogenic as optimal ex vivo T cell expansion requires CD28 mediated co-stimulation (Levine et al., 1995), although artificial culture conditions can overcome this problem. The approach has been used successfully in a number of animal and human studies. In a study by Rosenberg et al. nine out of fifteen patients responded to TIL therapy. Patients showed a decrease of at least 50% in the cross sectional area of tumour masses and there was no disease progression (Rosenberg et al., 1988). The adoptive transfer of EBV specific T cell clones into patients with malignant EBV-associated B-cell lymphoproliferative disorders has shown the approach was safe and successful against virus infected cells (Papadopoulos et al., 1994). The results from various other clinical trials using this strategy in cancer therapy have not been as encouraging. This may be due to the infusion of CTLs recognising only the strongest antigens into cancer patients (Topalian et al., 1990), which does not take into account the great heterogeneity which exists in tumour masses in vivo.

1.7.5 Hybrid Cells as Tumour Vaccines

A more recent approach to tumour immunotherapy is based on the fusion of professional APC with tumour cells. This strategy aims to combine the antigen processing and presenting capabilities of a professional APC with the expression of relevant tumour-associated antigens. Patient specific vaccines can be created, with hybrid cells expressing those antigens specific to the patient's tumour, producing a host response against many tumour-associated antigens (dominant and sub-dominant epitopes) and minimising the risk of antigen loss variants arising *in vivo*. The identification of appropriate tumour antigens for each tumour vaccination does not need to be known and, in theory, the technique eliminates the need for genetic modification.

Guo *et al.* (Guo *et al.*, 1994) were the first group to successfully demonstrate the potential of activated B cells fused with hepatocarcinoma cells to elicit an effective tumour-specific immune response (requiring both CD4+ and CD8+ T cells), which was also able to protect against subsequent challenge with the wild-type tumour cells in the rat model. More recent studies in the murine model have focused on DCs as the professional APC partner for fusion with a range of tumour cell types (Celluzi and Falo, 1998; Gong *et al.*, 1997; Wang *et al.*, 1998a). DCs are the most potent APC (Steinman *et al.*, 1993) and

recent advances have enabled DCs to be cultured at high numbers in vitro (Romani et al., 1994; Inaba et al., 1992).

DC x tumour cells hybrids have been used successfully to immunise against a range of tumours in several animal studies, reducing tumour incidence and significantly prolonging survival time (Wang et al., 1998a; Celluzzi and Falo, 1998; Cao et al., 1999). As the tumour cell partner, various well defined murine cell lines were used. Gong et al. used the MC38 adenocarcinoma cell line, Lespagnard et al. used the methylcholanthreneinduced P815 mastocytoma cells, Celluzzi and Falo and Cao et al. used the B16 melanoma cell line (Gong et al., 1997; Lespagnard et al., 1998; Celluzzi and Falo, 1998; Cao et al., 1999). The responses generated were capable of protecting against both established parent tumours and parent tumour cell challenge (Celluzzi and Falo, 1998; Cao et al., 1999). Results have also shown hybrid cells express tumour-associated antigen and DC derived co-stimulatory molecules (Gong et al., 1997; Lespagnard et al., 1998; Celluzzi and Falo, 1998; Cao et al., 1999). The importance of an anti-tumour T cell response in hybrid cell therapy was shown in these experiments. Tumour-specific CD8+ T cells were induced by the hybrid cells which were capable of lysing unfused parent tumour cells in vitro and in vivo (Celluzzi and Falo, 1998), and the adoptive transfer of activated lymph node draining T cells primed by the DC x tumour cell hybrids was therapeutically effective against established tumours in mice (Wang et al., 1998a). In several experiments, the protective anti-tumour response generated in the mice was dependent on both CD4+ and CD8+ T cells, as depletion of either CD4+ or CD8+ T cells could diminish the therapeutic effects of the hybrid vaccine (Stuhler and Walden, 1994; Gong et al., 1997; Cao et al., 1999).

Recent observations have also shown hybrid cells do not need to be selected at the expense of the unfused parent cell lines following 20% fusion efficiency, minimising the length of time spent in culture (to maintain tumour-antigenic phenotype) and reducing the wait between tumour resection and hybrid cell vaccination in cancer patients (Wang *et al.*, 1998a). However, purified DC x melanoma hybrids induced a more potent immune response which was effective against high dose tumour challenge and a greater percentage of mice remained tumour free (Cao *et al.*, 1999). Furthermore, an additional study has shown tumour cells grown for a short length of time in culture with DCs induce an immune response in mice which is comparable to vaccination with unselected DC x tumour cell hybrids (Celluzzi and Falo, 1998). The mechanism by which DC-tumour cell co-cultures induce an anti-tumour response is not known. Cross priming is one possible mechanism or

DCs in close proximity to the tumour cell may provide a bystander (or remote) costimulatory function.

Slightly modifying the hybrid cell protocol, Cao *et al.* (Cao *et al.*, 1999) transfected murine DCs with an adenoviral vector expressing GM-CSF in addition to fusing them with tumour cells. This approach led to an enhanced immune response compared with the non-transfected DC x melanoma hybrid cells in the murine model. The immune response generated by the non-transfected hybrid cells was completely blocked by *in vivo* depletion of both CD4+ and CD8+ T cells, suggesting both T cell subsets are required for therapeutic anti-tumour immunity. However, the immune response generated by the GM-CSF transfected hybrid cells was only partially blocked by CD4+ and CD8+ T cell depletion *in vivo*. M\$\$\$\$ and neutrophils were found at the vaccinating site, suggesting a role also for non-specific immune cells and also suggesting GM-CSF transfected hybrid cells may have potentially increased therapeutic value.

In order to demonstrate the potential to fuse *ex vivo* derived tumour cells with human DCs, Gong *et al.* in a separate study generated autologous DC x primary human breast carcinoma cells hybrids (Gong *et al.*, 2000). *In vitro* experiments showed the DC x breast carcinoma cells expressed tumour antigens and DC derived co-stimulatory molecules. In addition, the hybrid cells stimulated autologous T cell proliferation and induced CTL activity against autologous breast cells.

The remarkable success of APC x tumour cell hybrids has recently been translated into a clinical setting for the treatment of melanoma and renal cell carcinoma. Clinical trials showing positive results using hybrid cells, have already been initiated in patients with melanoma and renal cell carcinoma (Trefzer *et al.*, 1997; Kugler *et al.*, 1998; Kugler *et al.*, 2000). Trefzer *et al.* reported a complete response in one patient and a stable state for nine months in another five patients with metastatic melanoma using completely autologous B cell x tumour cell hybrids (Trefzer *et al.*, 1997). Kugler *et al.* evaluated the safety, acute and long term toxicity and therapeutic activity of B cell x allogeneic and autologous renal carcinoma cells in patients with metastatic renal cell carcinoma in a phase I trial (Kugler *et al.*, 1998). Of eleven patients receiving the 'autologous' vaccine (autologous tumour cells fused with MHC class I mismatched, MHC class II mismatched activated B lymphocytes), six responded to treatment with two complete and two partial responses. Thirteen patients were vaccinated with the 'allogeneic' cell vaccine (allogeneic tumour cell line fused with MHC class I matched, MHC class II mismatched lymphocytes) and only three responded to treatment. More recently, Kugler *et al.* refined the hybrid vaccine protocol for the treatment of renal cell carcinoma, and used autologous DCs as the fusion partner to the patient's own tumour and observed improved responses in a clinical trial (Kugler *et al.*, 2000). Not only was the vaccine safe, it was also effective with 41% of the patients responding to the vaccine. Following a mean follow-up of thirteen months, four patients showed complete tumour remission, two patients showed partial tumour remission, two patients had a tumour mass reduction of more than 50%, and one patient showed 'mixed' tumour remission, where there was regression of the local recurrence but progression in bone metastases.

In contrast to the promising work performed with B cells and DCs as the APC partner to tumour cells in hybrid cell tumour therapy, Souberbielle and colleagues (Souberbielle *et al.*, 1998) did not observe an enhanced immune response in mice immunised with autologous and allogeneic M ϕ x B6-F10 melanoma hybrid cells. There was no improvement in the survival of mice vaccinated with either the autologous or allogeneic hybrid cell vaccine. This may have been due to the lack of expression of MHC class I and II molecules and the co-stimulatory molecules CD80 and CD86 on the hybrid cell populations used in the vaccination protocols. MHC class I and MHC class II molecules and CD80 but not CD86 were detected by flow cytometric on the cell surface analysis on the allogeneic cell hybrids following IFN- γ pretreatment, but cells cultured in IFN- γ were not tested in the study. These results stress the need for careful analysis of hybrid cells *in vitro* prior to their use in a clinical setting.

1.7.6 EBV B-LCL x Tumour Cell Hybrids

Using a similar approach to Guo *et al.* (Guo *et al.*, 1994) who fused activated B cells with hepatocarcinoma cells for potential use cancer therapy, Dunnion *et al.* fused an EBV B-LCL (which has an activated B cell phenotype) as the APC partner, to a range of human derived haematological tumour cell lines (including erythroleukaemia, T cell leukaemia and promyelocytic leukaemia cell lines) (Dunnion *et al.*, 1999). In this study, the hybrid cells showed an enhanced capacity in their ability to stimulate primary allogeneic T cell responses *in vitro*, as compared with the parent tumour cell lines. The hybrid cell lines showed a marked increase in their capacity to directly stimulate memory (CD45RO+) as well as naïve (CD45RA+) T cells, and both CD4+ and CD8+ T cell subsets. However, not all of the hybrid cells had an enhanced stimulatory phenotype.

Hybrid cells with differing phenotypes (phenotype similar to the EBV B-LCL or similar to the tumour parent) could be produced from a single hybridisation, depending on the parent tumour cell. Those hybrid cells which were non-stimulatory were phenotypically similar to the tumour parent, and did not express the EBV latent proteins typical of EBV B-LCL, thus demonstrating the need for cloning and the careful phenotypic analysis of hybrid cells prior to their use *in vivo*. The generation of hybrid cells with different phenotypes from a single hybridisation between EBV B-LCL and non-lymphoid cells has been reported previously (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1991), although these hybrid cells were not characterised with a view to using them as agents in tumour immunotherapy (section 3.1.4).

1.7.6.1 Why Use EBV B-LCL as the APC Partner in the Hybrid Cells?

EBV B-LCL have certain advantages over DCs for use in hybrid therapy. EBV readily transforms B cells in vitro into EBV B-lymphoblastoid cells (EBV B-LCL). EBV B-LCL can therefore be expanded in vitro prior to fusion, so less patient sample is required. Unlike DCs, EBV B-LCL are easy to grow and are robust in culture. EBV B-LCL display an activated B cell phenotype and express high levels of both MHC class I and class II, co-stimulatory and adhesion molecules on the cell surface, which are necessary for the stimulation of a strong T cell response. EBV B-LCL may have an added advantage, EBV gene products may provide an adjuvant effect and help to amplify the anti-tumour immune response. Only a small number of B cells are required for viral transformation. DCs on the other hand proliferate poorly, if at all, in culture requiring more patient sample to produce enough to make the vaccine. Cao et al. (Cao et al., 1999) reported using large numbers of mice to prepare DCs for fusion and a DC:tumour ratio of 10:1 to achieve 12.7-26.8% fusion rate. In mice administering a haematopoetic growth factor, Flt3L, can increase the numbers of DCs, but the problem of low DC numbers in humans remains. So, even though DCs are the most potent APC cell, they may not be the most practical for use in tumour therapy. Recently, however, publications have described human DC x tumour cell hybrids, indicating this approach may be feasible (Kugler et al., 2000; Gong et al., 2000).

1.7.6.2 Melanoma Cells as the Tumour Partner in the Hybrid Cells

From an immunotherapeutic point of view, melanoma to date remains the best characterised tumour, and there is evidence from clinical trials that immunotherapy for the treatment of melanoma is effective (Rosenberg, 1988; Marchand *et al.*, 1995). Melanoma is easily accessible from patients, and typically melanoma cells can be established as long term cell lines more easily than other tumour types. Studies on melanoma have shown it is also one of the more immunogenic tumours, rarely undergoing spontaneous regression (Halliday *et al.*, 1991) and often being infiltrated with lymphocytes (Clark, 1991). Melanoma-associated antigens recognised by CTLs *in vitro* have been identified and characterised extensively at the molecular level, and since many tumours share the same tumour-associated antigens as melanoma, it is thought that what is learnt from these studies can hopefully be applied in the study of other cancers. Thus, melanoma is the putative tumour model of choice for human studies.

1.7 Aims of this Study

The primary aim of this study was to generate EBV B-LCL x melanoma hybrid cells and to investigate these as potential agents in tumour immunotherapy. The hybrid nature of these cells was to be confirmed through a variety of ways. These included chemical selection of the heterologous hybrid cells formed by fusion of the two different parent cell lines, at the expense of the non-fused parent cell lines or homologous hybrid cells. Furthermore, cloned hybrid cells were to be identified as true hybrids by the analysis of the HLA genotype, cellular phenotype and the presence of the EBV genome.

For APC x tumour cell hybrids to be effective in tumour therapy, they must be more immunostimulatory than the parent tumour cell lines. The second aspect of the work was to investigate the stimulatory potential of the confirmed hybrid cells in an *in vitro* model of a primary allogeneic immune response. The capacity of these cells to stimulate PBMCs, separated T cells and T cell sub-groups *in vitro* was to be assessed and compared with that of both parent cell lines. The role various immunologically important cell surface markers, in particular CD80, as a critical T cell co-stimulatory molecule, was also to be determined.

To stimulate a specific anti-tumour immune response, hybrid cells must express relevant tumour-associated antigens and present these effectively to CTLs. The final aspect of the study was to assess the capacity of the hybrid cells to retain the expression of various tumour-associated antigens and to present these tumour-associated antigens to antigen specific, HLA restricted CTL clones compared with the parent melanoma cell lines. The results of this study should indicate the potential of EBV B-LCL x melanoma hybrid cells as therapeutic cell vaccines in cancer immunotherapy at the pre-clinical level.

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Chapter 2

Materials and Methods

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Cell Culture Reagents

General chemicals (analytical grade) were from the Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). All cell culture media and cell culture reagents were purchased from Gibco BRL (Life Technologies Ltd., Paisley, UK) unless otherwise stated. Plastic cell culture flasks, plastic tubes and cell culture plates were purchased from Nalge Nunc International (Naperville, Illinois, USA) and Corning Costar (High Wycombe, Buckinghamshire, UK). Distilled water, produced with an Elgastat option 2 water purifier (USF Elga Ltd., High Wycombe, Buckinghamshire, UK) and a NANOpure water purifier (Fisher Scientific UK, Loughborough, Leicestershire, UK) were used to prepare solutions.

2.2 Cell Culture

2.2.1 Cell Lines

The origins and characteristics of the cell lines utilised in this study are shown in Table 2.1. The human derived cell lines HMy2 and K562 were maintained in suspension by continuous passage in Rosswell Park Memorial Institute (RPMI) 1640 supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine, penicillin (100U/ml) and streptomycin (100µg/ml) (growth medium). The EBV B-LCL, HMy2, used as the parent APC in the generation of the APC x tumour hybrid cells, was a gift of Prof A Rickinson, Birmingham, UK. This cell line has a HAT sensitive, ouabain resistant phenotype, allowing for the chemical selection of the hybrid cells (Edwards et al., 1982) (section 2.3.3). The melanoma cell lines used as the parent tumour cells in the hybrids, DAUV and Gerl43, the gift of Dr P Coulie, Brussels, Belgium, and 518.A2, a gift from Dr A Murray, Sheffield, UK, were cultured as monolayers in Dulbecco's Modified Eagle's medium (DMEM) supplemented as above (growth medium). Adherent cells were passaged by a brief exposure to trypsin/EDTA, detaching the cells from the cell sheet. Hybrid cell lines were cultured in selective medium consisting of DMEM growth medium containing 2% hypoxanthine aminopterin and thymidine (HAT) and 2.5x10⁻⁶ to 5x10⁻⁷M ouabain (Sigma-Aldrich Company Ltd.) (selective medium). Cellular morphology was captured using a Nikon E900 S digital camera (Nikon UK Ltd., Kingston-upon-Thames, Surrey, UK).

Cell Lines	Origins	Use	Reference
HMy2	EBV transformed B cell line	B LCL parent in hybrid	Edwards et al., 1982
K562	Erythro-leukaemia	NK target in assays	Lozzio and Lozzio, 1975.
DAUV	Melanoma	tumour parent in hybrid	Gift of P Coulie, Brussels
Gerl43	Melanoma	tumour parent in hybrid	Gift of P Coulie, Brussels
518.A2	Melanoma	tumour parent in hybrid	Gift of A Murray, Sheffield

Table 2.1. Origins and characteristics of cell lines used in this study.

2.2.2 Cell Culture Conditions

All cells were maintained in a humidified atmosphere at 37° C and 5% CO₂. In order investigate the effects of interferon-gamma (IFN- γ) (Boehinger Ingelheim Ltd., Bracknell, Berkshire, UK), cells were cultured in the appropriate growth medium containing 100U/ml IFN- γ for a minimum of 3 days prior to analysis.

2.2.3 Cell Freezing and Thawing Conditions

Approximately $5x10^5$ cells were cryopreserved in 1ml of 10% dimethyl sulphoxide (DMSO) in FBS in a 1.5ml cryovial. Bringing the samples gradually to room temperature (RT) thawed the frozen cells. Thawed cells were immediately transferred to 10ml growth medium, pelleted by centrifugation and resuspended in fresh growth medium before being placed under appropriate cell culture conditions for recovery.

2.3 EBV B LCL x Melanoma Hybrid Production

2.3.1 Preparation of Polyethylene Glycol

Cells were fused using polyethylene glycol (PEG) molecular weight 1300-1600 (ICN Biomedicals Inc, Aurora, Ohio, USA) as the chemical fusogen, according to standard methods (Kohler and Milstein, 1975; Kerr *et al.*, 1992). Briefly, serum free medium was added to previously autoclaved PEG to give a 50% (w/v) solution. The pH of the solution at 37°C was adjusted to pH 8.2 measured with a Kent EIL 7020 pH meter (Fisher Scientific UK) using 1.0M sodium hydroxide (NaOH) or 1.0M hydrochloric acid (HCl), then sterile filtered through an acrodisk with a pore diameter of 0.45µm (Gelman Sciences, Northampton, UK).

2.3.2 Cell Hybridisation Procedure

A mixture of 1×10^7 cells of each parent cell line was washed in serum free RPMI 1640 and pelleted by centrifugation. Spent medium was aspirated off the cell pellet, and 1ml of PEG solution was added over a 1 minute period with continuous swirling. The solution was diluted over a 6 minute period with 10ml of serum free RPMI 1640 medium and the cells were then recentrifuged and resuspended in 80cm² tissue culture flasks in DMEM growth medium. As controls, the same procedure was performed on 1×10^7 cells of each parent cell type alone.

2.3.3 Hybrid Selection

After 24 hours, the cells were transferred to DMEM selective medium containing HAT and ouabain (section 2.2.1), allowing the growth of hybrids formed between the two parent cell lines only. Preliminary viability tests were performed first, in order to determine the concentrations of HAT and ouabain required to prevent the growth of the parent cells. HMy2 cells were reported to die in growth medium containing 2% HAT (Edwards et al., 1982) which was tested and confirmed whereas normal cells are able to grow in HAT supplemented medium. Since cells to differ in their sensitivity to ouabain, viability tests with a range of ouabain concentrations were performed in order to predetermine the concentration of ouabain necessary for total killing of parent melanoma cells in the hybrid cell selection process. Parent melanoma cells were cultured in 25cm² tissue culture flasks at starting confluencies of 50% in growth medium containing concentrations of ouabain ranging from 1×10^{-4} to 1×10^{-8} M. Every three days, the spent media was removed and the cell monolayer was washed in PBS. The media was replaced allowing dead cells to be washed off the monolayer and viable cells only able to proliferate. The concentration of ouabain chosen for the selection of hybrid cells was approximately 30% higher than the minimal concentration required to kill the unfused tumour cells within 7-14 days. The concentration of ouabain used in the selective medium to kill the 518.A2 melanoma parent cells was 2.5x10⁻⁶M and that required to kill the Gerl43 and DAUV parent cells was 5x10⁻⁷M. The mutated EBV B-LCL cell line HMy2 was able to grow in growth media supplemented with ouabain at both of these concentrations of ouabain. Growth medium supplemented with both HAT and ouabain prevented the growth of unfused parent cells and cells fused between the same type but allowed the growth of cells fused between the two different cell lines.

2.3.4 Cloning of Cell Hybrids

Cell hybrids were cloned at one cell per well in selective medium after an initial growth in tissue culture flasks. Cells were cloned into 48 well, flat bottomed tissue culture plates and cultured in selective medium. Positive wells from plates with 30% or fewer positive wells were taken as representing clonal populations. Following growth in tissue culture plates, cells were transferred to 25cm² and 80cm² tissue culture flasks and maintained in selection medium throughout the study. Both uncloned ('bulk') cell populations and cloned hybrid cell lines were maintained by serial passage in culture.

2.4 HLA Typing

2.4.1 DNA Extraction

Total genomic DNA was isolated from parent and cloned hybrid cell lines using the Nucleon[®] II Genomic DNA Extraction Kit (Nucleon[®] Biosciences Division, Scotlab Ltd., Coatbridge, Lanarkshire, UK) according to the manufacturer's instructions. Between 3x10⁶ and 1x10⁷ cells were harvested, and vortexed with 2ml of Reagent B [400mM Tris-HCl, 60mM EDTA, 150mM sodium chloride (NaCl), 1% sodium dodecyl sulphate (SDS)], in order to lyse the cells. Samples were deproteinated by shaking the mixture gently by hand with 500µl of sodium perchlorate (5M). The DNA was then extracted by gently shaking the mixtures by hand after the addition of 2ml of chloroform. To each mixture, 300µl of Nucleon[®] resin was added, to bind to unwanted cell impurities, and the samples were then centrifuged at 1300x g for 3 minutes. The upper phase containing DNA from each sample, was transferred to a fresh tube without disturbing the Nucleon[®] resin layer, and the DNA was ethanol precipitated. The DNA was washed in 70% ethanol, air dried and finally resuspended in sterile NANOpure water.

2.4.2 Spectrophotometric Analysis of DNA

Genomic DNA was analysed in ultra violet (UV) grade cuvettes (Fisher Scientific UK) using a PU 8720 UV/VIS Scanning Spectrophotometer (Spectronic Unicam, Cambridge, UK). Optical density (OD)₂₆₀ readings and OD_{260/280} ratios were obtained to determine DNA and protein content. An OD_{260/280} ratio of >1.6 was considered suitable DNA purity for PCR analysis.

2.4.3 Sequence Specific Primer PCR

Genomic DNA from cell hybrids and their parent cell lines was typed for HLA class I (HLA-A and HLA-B) and class II antigens (HLA-DRB1) using a SSP-PCR based system, based on the phototyping method of Bunce and colleagues (Bunce *et al.*, 1995). Sequence specific primer pairs, based on nucleotide sequence polymorphism, were used to determine HLA specificities. Custom synthesised oligonucleotide primers from R&D Systems (Abingdon, Oxon, UK) were used. An internal control reaction giving rise to a 796 base pair (bp) fragment from the third intron of HLA-DRB1 was included in each reaction. For class I and class II HLA typing, identical parameters and protocol were used (Bunce *et al.*, 1995). All reactions were performed in a PTC-100TM Programmable Thermal Controller (Genetic Instrumentation Ltd., Dunmow, Essex, UK).

2.4.4 PCR Reaction Components

To each reaction well containing 5µl of specific primer mixes (for sense and antisense primer mixes, reaction specificities and primer concentrations see Bunce *et al.*, 1995) in a 96 well (theromowell) plate (Corning Costar) a mixture containing 5µl of PCR buffer [174mM Tris Base (pH8.8), 43mM ammonium sulphate, 5.2mM magnesium chloride (MgCl₂), 0.026% (v/v) Tween 20, 520µM of each of the four deoxynucleotides (dNTP) (dATP, dCTP, dGTP, dTCP) (Pharmacia Biotech, St.Albans, Hertfordshire, UK) and 0.26µM of each control primer], 0.1µg of DNA and 0.25 units (U) of BioTaqTM polymerase (Advanced Biotechnologies, Epson, Surrey, UK) made up to a total volume of 8µl with NANOpure water, was added. The mixtures were overlaid with 10µl of mineral oil prior to thermal cycling.

2.4.5 Thermal Cycling Conditions

The following thermal cycling conditions were applied to each reaction: 1 minute at 96°C followed by 5 cycles of 25 seconds at 96°C, 45 seconds at 70°C, 45 seconds at 72°C then 17 cycles of 25 seconds at 96°C, 50 seconds at 65°C, 45 seconds at 72°C and 8 cycles of 25 seconds at 96°C, 60 seconds at 55°C 120 seconds at 72°C.

2.4.6 Visualisation of PCR Products

PCR products were run in a 1.0% agarose gel in 0.5X Tris-borate-EDTA buffer (TBE) [10X TBE buffer: 1.78M Tris, 1.78M boric acid, 40mM EDTA (pH 8.0)]. The products were loaded into the gel with loading buffer [0.25% Orange G, 30% (v/v) glycerol in 0.5X TBE buffer], electrophoresed at 180 volts for approximately 40 minutes and DNA was stained with ethidium bromide (5 μ g/ml). Gels were visualised using a dual intensity UV transilluminator (UVP Ltd., Cambridge, UK) and photographed with a Polaroid MP-4 land camera (Polaroid (UK) Ltd., Wheathampstead, Hertfordshire, UK). Band sizes were determined by comparison with the 1kb ladder (Gibco BRL) and those of the appropriate size were accepted as positive results (Bunce *et al.*, 1995).

2.5 Tumour-Associated Antigen Expression as Determined by Reverse Transcription PCR (RT-PCR)

2.5.1 Procedure for Total RNA Extraction

Plasticware used in the extraction of RNA was treated with a 0.1% diethyl pyrocarbonate (DEPC) overnight and then autoclaved prior to use, in order to inhibit RNases. Total RNA was isolated from cell lines by RNAzolTM B (Biogenesis Ltd., Poole, Dorset, UK) extraction according to the manufacturer's instructions. Basically, $2x10^6$ cells were resuspended in 400µl of RNAzolTM B and vortexed vigorously to lyse cells. After the addition of 40µl of chloroform, each sample was shaken. Samples were then left on ice for 5 minutes before centrifugation at 12000x g and 4°C for 15 minutes. The top aqueous layer was transferred to a clean eppendorf tube and an equal volume of isopropanol was added to it. Each sample was incubated at -20°C for 15 minutes prior to centrifugation at 12000x g and 4°C for 15 minutes. The top aqueous layer the RNA was washed in 70% ethanol and air dried before being resuspended in 11µl Sigma water (DNase and RNase free). Total RNA was analysed in a quartz cuvette (Starna, Heinauit, Essex, UK) using an Ultrospec 300 UV/visible spectrophotometer (Pharmacia Biotech, St Albans, Hertfordshire, UK). (OD)₂₆₀ was measured using Sigma water as a standard.
2.5.2 Reverse Transcription PCR (RT-PCR)

RT-PCR was performed on total RNA samples using Superscript II RNase H⁻ reverse transcriptase derived from the Murine Leukaemia Virus (Superscript[™] II, Gibco BRL), to synthesise first strand complementary DNA (cDNA). Briefly, 2.4µg of random primers (Gibco BRL) was added to 2.0µg of total RNA and the total volume in each reaction mix was adjusted to 12µl with Sigma water. The samples were heated to 70°C for 10 minutes and followed with a quick chill on ice for 2-3 minutes. To each reaction mix, 0.5mM of each dNTP, 0.1U of Superscript[™] II (Gibco BRL), 4µl of 5X First Strand Buffer [250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM MgCl₂] and 0.01M dithiothreitol (DTT) (both supplied with Superscript[™] II) were added. The total volume in each reaction tube was adjusted to 20µl with Sigma water. The contents of each tube were mixed and incubated for 1 hour at 37°C before being heated to 95°C for 5 minutes in order to denature the Superscript II enzyme. A negative control consisting of Sigma water taking the place of the RNA preparation was included.

2.5.3 Conversion of RNA to cDNA Control PCR

As a positive test for the conversion of RNA to cDNA, a PCR reaction designed to amplify human β -actin cDNA was performed. Primers for this reaction, as described by Nakajima-Iijima *et al.* (Nakajima-Iijima *et al*, 1985), were synthesised 'in house' at the Centre for Mechanisms of Human Toxicity, Leicester, UK, and were a gift from M Gould, Leicester, UK. Sense and anti-sense primer sequences were as follows:-

Sense primer sequence	5' TTCAACTCCATCATGAAGTGTGACGTG 3'
Anti-sense primer sequence	5' GATTCAGTATCAGGCGGATCTTCGTAA 3'

2.5.3.1 Reaction Components

1µl of cDNA was mixed with 5µl of 10X PCR buffer [200mM Tris-HCl (pH8.4), 500mM KCl], 2.5mM MgCl₂ (both supplied with Taq DNA polymerase, Gibco BRL), 1U Taq DNA polymerase (Gibco BRL), 0.2mM of each dNTP, 0.1µM of both β-actin sense and anti-sense primers and the volume in each tube was made to 50µl with Sigma water. Samples were overlayed with 60µl of mineral oil prior to thermal cycling in a PTC-100TM programmable Thermal Controller. Both positive (containing a known positive cDNA sample) and negative (containing Sigma water in the place of cDNA) control reactions were included. Everything was kept on ice throughout the procedure.

2.5.3.2 Thermal Cycling Conditions

The conditions for thermal cycling were as follows: 5 minutes at 95°C followed by 33 cycles of 30 seconds at 95°C, 30 seconds at 55°C, 1

minute at 72°C then a final extension of 4 minutes at 72°C.

2.5.3.3 Visualisation of PCR Products

PCR products were electrophorised at 70 volts for 70 minutes in a 1.5% agarose gel in 1X TBE buffer with loading buffer [0.25% bromophenol blue, 0.25% xylene cyanol, 15.0% (v/v) glycerol]. DNA was stained with 5μ g/ml ethidium bromide. Samples were viewed under a UV transilluminator and photographed with a Polaroid camera. Positive bands corresponding to 310bp were compared with a 1kb ladder (Gibco BRL) which was run alongside the samples. Contaminating genomic DNA produced a PCR product of 422bp and could thus be distinguished from cDNA.

2.5.4 PCR for Melanoma-Specific Gene Expression

2.5.4.1 Reaction Components

The primers used in the PCR reactions are shown in Table 2.2 and were a kind gift from S. Rogers, Sheffield, UK. Reagents and reaction tubes were kept on ice throughout the procedure. cDNA was diluted 1 in 5 in Sigma water before being used in each reaction. To each sample, 5µl of 10X PCR buffer (supplied with PrimeZyme, Biometra, Maidstone, Kent, UK) [100mM Tris-HCl (pH 8.8 at 25°C), 15.0mM MgCl₂, 500mM KCl, 1.0% (v/v) Triton X-100], 0.2mM of each dNTP, 0.4µM (or 0.8µM for MAGE PCRs) sense primer, 0.4µM (or 0.8µM for MAGE PCRs) anti-sense primer and 0.4U PrimeZyme (Biometra, Maidstone, Kent, UK) were added. The reaction volume was adjusted to 45µl total with Sigma water. 5.0µl of cDNA was added to each tube and the samples were mixed. A 50µl aliguot of mineral oil was then added to each reaction tube. The PCR block in a PTC-100TM programmable Thermal Controller was pre-heated to 94°C and the reaction tubes into the preheated block for thermal cycling. placed immediately were

Gene	Primer Orientation	Primer Sequence (from 5' to 3')	Reference
MAGE 1	sense	CGGCCGAAGGAACCTGACCCAG	Patard et al., 1995.
	anti-sense	GCTGGAACCCTCACTGGGTTGCC	
MAGE 2	sense	AAGTAGGACCCGAGGCACTG	Patard et al., 1995.
	anti-sense	GAAGAGGAAGAAGCGGTCTG	
MAGE 3	sense	TGGAGGACCAGAGGCCCCC	Patard et al., 1995.
	anti-sense	GGACGATTATCAGGAGGCCTGC	
Tyrosinase	sense	GGATAGCGGATGCCTCTCAAAG	Brasseur et al., 1995.
	anti-sense	CCCAAGGAGCCATGACCAGAT	
MelanA/MART1	sense	CTGACCCTACAAGATGCCAAGAG	Brasseur et al., 1995.
	anti-sense	ATCATGCATTGCAACATTTATTGATGGAG	
gp100	sense	GGAAGAACACAATGGATCTGG	Brasseur et al., 1995.
	anti-sense	CACAGCATCATATGAGAGTAC	

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Table 2.2. Sequences of primers used in the detection of MAGE and melanocyte differentiation gene expression.

MAGE 1	MAGE 2	MAGE 3	Tyrosinase	MelanA/MART1	gp100
94°C for 4 min	94°C for 4 min	94°C for 4 min	94°C for 4 min	94°C for 4 min	94°C for 4 min
94°C for 1 min	94°C for 1 min	94°C for 1 min	94°C for 1 min	94°C for 1 min	94°C for 1 min
72°C for 1 min	67°C for 2 min	72°C for 2 min	65°C for 2 min	60°C for 2 min	56°C for 2 min
72°C for 2 min*	72°C for 2 min*	72°C for 2 min*	72°C for 2 min*	72°C for 2 min*	72°C for 3 min*
30 cycles	34 cycles	33 cycles	25 cycles	24 cycles	28 cycles
72°C for 5 min	72°C for 5 min	72°C for 5 min	72°C for 5 min	72°C for 5 min	72°C for 5 min
	MAGE 1 94°C for 4 min 94°C for 1 min 72°C for 1 min 72°C for 2 min* 30 cycles 72°C for 5 min	MAGE 1MAGE 294°C for 4 min94°C for 4 min94°C for 1 min94°C for 1 min72°C for 1 min67°C for 2 min72°C for 2 min*72°C for 2 min*30 cycles34 cycles72°C for 5 min72°C for 5 min	MAGE 1MAGE 2MAGE 3 $94^{\circ}C$ for 4 min $94^{\circ}C$ for 4 min $94^{\circ}C$ for 4 min $94^{\circ}C$ for 1 min $94^{\circ}C$ for 1 min $94^{\circ}C$ for 1 min $72^{\circ}C$ for 1 min $67^{\circ}C$ for 2 min $72^{\circ}C$ for 2 min $72^{\circ}C$ for 2 min* $72^{\circ}C$ for 2 min* $72^{\circ}C$ for 2 min* 30 cycles 34 cycles 33 cycles $72^{\circ}C$ for 5 min $72^{\circ}C$ for 5 min $72^{\circ}C$ for 5 min	MAGE 1MAGE 2MAGE 3Tyrosinase94°C for 4 min94°C for 4 min94°C for 4 min94°C for 4 min94°C for 1 min72°C for 1 min67°C for 2 min72°C for 2 min65°C for 2 min72°C for 2 min*72°C for 2 min*72°C for 2 min*72°C for 2 min*30 cycles34 cycles33 cycles25 cycles72°C for 5 min72°C for 5 min72°C for 5 min72°C for 5 min	MAGE 1MAGE 2MAGE 3TyrosinaseMelanA/MART1 $94^{\circ}C$ for 4 min $94^{\circ}C$ for 1 min $94^{\circ}C$ for 2 min $72^{\circ}C$ for 2 min $65^{\circ}C$ for 2 min $60^{\circ}C$ for 2 min $72^{\circ}C$ for 2 min* 30 cycles 34 cycles 33 cycles 25 cycles 24 cycles $72^{\circ}C$ for 5 min $72^{\circ}C$ for 5 min $72^{\circ}C$ for 5 min $72^{\circ}C$ for 5 min

* 2 second extension per cycle

 Table 2.3. Thermal cycling conditions to detect MAGE and melanocyte differentiation gene expression.

In each PCR, a positive (containing a known positive cDNA sample) and negative (containing Sigma water in the place of cDNA) control were included.

2.5.4.2 Thermal Cycling Conditions

The conditions for thermal cycling are shown in Table 2.3.

2.5.4.3 Analysis of PCR Products

To each reaction tube, 45μ l of chloroform followed by 12.5μ l of sample buffer [0.25% (w/v) bromophenol blue, 50.0% (v/v) glycerol] were added. The samples were shaken by hand then spun for 4 minutes at 12000x g in a microcentifuge. 10µl of each sample was electrophoresed at 80 volts in 1.0% or 2.0% agarose gels in 1x Tris-acetate-EDTA buffer (TAE) [50X TAE buffer: 2M Tris, 50M EDTA, 1.14% (v/v) glacial acetic acid] for approximately 75 minutes. DNA was stained with ethidium bromide and visualised as described previously (section 2.4.6). Positive bands were compared with a 100bp (Gibco BRL) or 1kb ladder (Gibco BRL) which was run alongside the samples. PCR product sizes are shown in Table 2.4.

Gene	cDNA PCR Product Size (bp)	Genomic DNA Product Size (bp)
MAGE 1	421	no genomic band
MAGE 2	230	300
MAGE 3	725	805
Tyrosinase	383	no genomic band
MelanA/MART1	605	no genomic band
gp100	2039	no genomic band

Table 2.4. Products produced in the MAGE and melanocyte differentiation PCRs.

2.5.5 Analysis of Tumour Antigen Expression by Semi-Quantitative RT-PCR

Semi-quantitative RT-PCR was performed by S Rogers, Sheffield, UK, as previously described (Mulcahy *et al.*, 1996) in order to determine level of expression of MAGE 1, MAGE 2, MAGE 3, MelanA/MART1, gp100 and tyrosinase. The MAGE genes are expressed in a range of tumour types, including melanoma, and the testis.

MelanA/MART1, gp100 and tyrosinase genes are melanocyte differentiation antigens and are expressed in melanocytes and melanomas only.

2.6 EBV Genome Presence and Viral Protein Expression As Determined by PCR and Western Blot Analysis Respectively

2.6.1 PCR Analysis to Determine the Presence of the EBV Genome

2.6.1.1 Sample Preparation

Adherent cells were trypsinised, detached from the cell monolayer and washed twice in cold phosphate buffered saline (PBS) [80mM Na₂HPO₄, 20mM NaH₂PO₄.2H₂O, 100mM NaCl₂] and suspension cells were collected by centrifugation and also washed twice in cold PBS. Cells were counted using a haemocytometer and stored as cell pellets in aliquots of 5×10^6 cells at -20° C. On day of analysis, cell pellets were thawed on ice and resuspended in 200µl of lysis buffer [1ml of 10X PCR buffer (200mM (NH₄)₂SO₄, 750mM Tris-HCl (pH8.8 at 25°C), 0.1% (v/v) Tween 20) (supplied with Thermostable DNA polymerase Advanced Biotechnologies, Epsom, Surrey, UK), 20U Proteinase K (Roche Diagnostics Ltd., Lewes, East Sussex, UK), 0.5% (v/v) Tween 20, made up to 10ml with distilled water], and DNA was denatured overnight by incubating the samples at 37°C. To each sample, a further 300µl of lysis buffer was added. Samples were then inactivated by heating the samples to 94°C for 10 minutes. DNA quantification was not performed. Both negative (a cell sample known not to contain EBV, and a sample with Sigma water taking the place of DNA template) and positive controls (known to contain type 1 or type 2 EBV) were all included in each PCR.

2.6.1.2 PCR Amplification

To 5µl of each DNA sample, 15µl 10X PCR buffer (described in section 2.6.1.1), 0.8mM of each dNTP (Boehringer Mannheim, UK (Diagnostics and Biochemicals Ltd., Lewes, East Sussex, UK), 16.5µM sense primer, 16.5µM anti-sense primer, 1.25mM MgCl₂ and 2.5U Thermostable DNA polymerase (Advanced Biotechnologies) were added and the total volume of each reaction mixture made up to 60.5µl with Sigma water. PCR mixtures were then overlayed with 60µl mineral oil. PCR reagents and mixtures were kept on ice until thermal cycling in a PTC-100TM programmable Thermal Controller. The

primers (sequences shown below) used in this reaction were synthesised at Alterbioscience, The University of Birmingham, UK, and were a gift from D Croom-Carter, Birmingham.

Sense primer sequence5' AGAAGGGGAGCGTGTGTTGT 3'Anti-sense primer sequence5' GGCTCGTTTTTGACGTCGGC 3'

B95.8 (prototype EBV strain) co-ordinates: sense primer: 99939-99958 anti-sense primer: 100091-100072

2.6.1.3 Thermal Cycling Conditions

The conditions for thermal cycling were as follows:

5 minutes at 94°C followed by 40 cycles of 30 seconds at 94°C, 1 minute 30 seconds at 45°C and 2 minutes at 72°C then a final extension of 5 minutes at 72°C.

2.6.1.4 Visualisation of PCR Products

To each sample, 15μ l of loading dye [0.25% (w/v) xylene cyanol, 0.25% (w/v) bromophenol blue, 30% (v/v) glycerol] was added. Into the wells of a 2% agarose in 1X TBE buffer, 10µl of sample was added. A 1kb ladder was run alongside the samples in order to identify band sizes. Gels were electrophoresed at 180 volts for 2 hours and DNA was stained with ethidium bromide. Gels were visualised under UV with a transilluminator and a permanent record made by photographing the gel using a Polaroid camera. The primers used in the PCR amplified the EBV EBNA 6 gene. EBV type 1 produce a PCR product of 153bp and EBV type 2 produced a PCR product of 246bp.

2.6.2 Western Blot Analysis to Detect EBV Protein Expression 2.6.2.1 Sample Preparation and Protein Quantification

Adherent cells were removed from the cell monolayer by trypsinisation and washed twice in cold PBS. Suspension cells were also washed twice in cold PBS. Cells were then counted using a haemocytometer, pelleted at 4°C and stored until analysis in aliquots of $5x10^6$ cells at -20° C. On day of analysis, cell samples were thawed on ice and resuspended in 200µl urea buffer [9M urea, 50mM Tris (pH7.5)] and samples were sonicated using a Soniprep 150 (SANYO Gallenkamp PLC, Loughborough, Leicestershire, UK) to lyse cells.

Protein quantification was performed following a simplified protocol using the Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK) according to the manufacturer's instructions. This is colorimetric assay is based on the reaction of protein with a copper tartrate solution and a dilute Folin reagent. From each protein sample, 0.5µl was taken and added to 4.5µl distilled water in a flat bottomed 96 well plate. This was done in duplicate. A range of BSA protein standards were made by diluting BSA (supplied with Bio-Rad DC Protein Assay kit, Bio-Rad Laboratories Ltd.) from 2mg/ml to 0.1mg/ml in the same urea buffer as the cell samples (described above). 5µl of each standard was also added to the wells of the 96 flat-bottomed plate in duplicate. To each sample, 25µl of Bio-Rad Protein Assay Reagent A (an alkaline copper tartrate solution) was added and mixed gently. A 200µl aliquot of Bio-Rad Protein Assay Reagent B (a dilute Folin reagent) was then added and samples were incubated for 15 minutes at RT. After 15 minutes, the absorbance at 650nm was read using a MRX ELISA plate reader (Dynatech Labs, Billinghurst, West Sussex, UK). Protein samples were resuspended at 5mg/ml in sample buffer [62.5mM Tris (pH 6.8), 4% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue, 5M urea, 10% glycerol].

2.6.2.2 Protein Separation and Transfer

Standard 6% and 10% SDS resolving gels were prepared with a 3% stacking gel on top (Sambrook *et al.*, 1989). Samples were boiled for 2 minutes at 94°C before 100µg of protein from each sample was loaded into individual wells in the gel. High and low molecular weight markers (Sigma-Aldrich Company Ltd.) were loaded onto the gels in order to identify proteins of the correct size. SDS gels were electrophoresed for 20 minutes at 80mA followed by 21mA overnight in running buffer [25mM Tris HCl, 192mM glycine, 10% SDS]. Proteins were transferred onto Biotrace nitrocellulose membrane (Gelman Sciences) in transfer buffer [25mM Tris HCl, 192mM glycine, 20% methanol] by semi-dry electrophoresis for 4.5 hours at 85 volts. Protein transfer and equal loading was confirmed by irreversibly staining nitrocellulose membranes for 1 minute with Ponceau stain solution followed by a short rinse in distilled water to visualise protein bands. Ponceau stain was removed by washing the filters several times in distilled water.

2.6.2.3 Filter Staining and Probing

Each nitrocellulose membrane was washed briefly in 0.1% Tween 20 in PBS (PBS/Tween) followed by a 2 hour incubation in 5% marvel (Premier Beverages, Knighton Adbaston, Stafford, UK) in PBS/Tween on a platform shaker (Stuart Scientific Industries International (UK) Ltd., Loughborough, Leicestershire, UK) at RT. Enough primary Ab (described elsewhere, see Table 2.5) was added to the filters to ensure their entire surface areas were covered and they were then incubated overnight at 4°C with continuous agitation. The antibodies were gifts of D Croom-Carter, Birmingham who had optimized the Ab concentration and diluted the cell culture supernatants ready for use in Western blot analysis. The primary antibodies were then removed and the filters were washed in PBS/Tween for 1 hour. The appropriate secondary Ab directed against the primary Ab was diluted in 5% marvel in PBS/Tween and added to the filter, again, covering the entire surface area. The secondary antibodies, either goat anti-mouse Ig (diluted 1:1000) or rabbit anti-rat Ig (diluted 1:1000) or goat anti-human Ig (diluted 1:2000) (all purchased from Sigma-Aldrich Company Ltd.) depending on the primary Ab, were conjugated with horse radish peroxidase (HRP). Filters were again washed for 1 hour in several washes of PBS/Tween followed by a quick rinse in PBS, before being subjected to ECL™ chemiluminescence detection.

2.6.2.4 Protein Detection

ECL[™] Western blotting detection reagents (Amersham Life Science Ltd., Little Chalfont, Buckinghamshire, UK) were used to detect proteins immobilised on the nitrocellulose filter labelled indirectly with HRP-conjugated Ab. Equal volumes of reagent 1 and reagent 2 (both supplied with ECL[™] Western blotting detection kit) were mixed before being applied to the surface of the nitrocellulose filter for 1 minute. Filters were completely covered with SaranWrap[™] (trademark of The Dow Chemical Company. Genetic Research Instrumentation, Braintree, Essex, UK) and placed inside an X-ray film cassette (Genetic Research Instrumentation). In a dark room, autoradiography film (Hyperfilm ECL: Amersham Life Science Ltd.), was applied to the filter and the cassette closed for approximately 15 seconds. The X-ray film was developed using a Gevamatic 60 film developer (AGFA Gevaert Ltd., Brentford Middlesex, UK). From this film exposure it was possible to alter the length of time needed for the film to be exposed in order to detect the band of interest with minimal background staining.

Antibody	Antigen	Origins	Protein Size (kDa)	Reference
PE2	EBNA 2	Mouse mAb	66-97	Young et al., 1989.
1H4	EBNA 1	Rat mAb	66-97	Grasser et al., 1994.
CS1-4	LMP 1	Mouse mAb	45-66	Rowe et al., 1987a.
NZ1V0	EBNA 6 type 2	Human serum	110-200	Rowe et al., 1992.
E3cA10	EBNA 6 type1	Mouse mAb	110-200	Maunders et al., 1994.
BZ.1	BZLF 1	Mouse mAb	29-45	Young et al., 1991.

Table 2.5. Primary antibodies used to detect expression of EBV proteins. Protein sizes

 characteristically differ between individual EBV strains.

2.7 Western Blot Analysis in Order to Detect TAP and Lmp Protein Expression 2.7.1 Sample Preparation

Cells were collected and washed twice in cold PBS. Cells were resuspended at $1x10^7$ cells per ml of lysis buffer [1% Triton X-100, 150mM NaCl, 5mM EDTA, 50mM Tris (pH7.5), 5mM EGTA, 10mM sodium fluoride (NaF), 1mM sodium orthovandidate (Na₃VO₄.14H₂O), 0.5% NP-40, 1mM PMSF (added within 30 minutes of use)] and kept on ice for 10 minutes. Samples were then spun at 13000x g for 10 minutes at 4°C. The supernatant was removed and protein quantification performed as described in section 2.6.2.1. Aliquots of the protein were stored at -20°C. Protein aliquots were diluted to 1875mg/ml in sample buffer [62.8mM Tris, 15% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β -mecaptoethanol, 0.05% (w/v) bromophenol blue (pH6.8)] on day of use.

2.7.2 Protein Separation and Transfer

Standard 13% SDS resolving gels with 5% stacking gels on top (Sambrook *et al.*, 1989). Protein samples used to identify TAP expression were warmed to RT prior to loading, and those samples used to identify Lmp expression were heated at 94°C for 4 minutes. Into the lanes of the gels, 37.5µg of protein sample was loaded. In order to identify protein sizes, prestained, broad range SDS PAGE standards (Bio-Rad Laboratories Ltd.) and SDS PAGE low molecular weight standards (Pharmacia Biotech) were also loaded. The gel was electrophoresed in running buffer (described in section 2.6.2.2) for approximately 2 hours at 180 volts. Protein samples were transferred to Hybond-C super

nitrocellulose filter (Amersham Life Science Ltd.) in transfer buffer (described in section 2.6.2.2) at 78 volts for 45 minutes. Following transfer, proteins were visualised with Ponceau stain solution (described in section 2.6.2.2), rinsed in distilled water and given a final wash in PBS/Tween.

2.7.3 Protein Blocking, Washing and Probing Procedure

At RT, the nitrocellulose filters were submerged in 5% marvel in PBS/Tween on a platform shaker for 1 hour. The filter was rinsed quickly in PBS/Tween. Primary antibodies which were a kind gift of A Kelly, Cambridge, (described and used elsewhere, see Table 2.6) were diluted 1:1000 in 5% marvel in PBS/Tween then applied to the filter, covering the total surface area of the filter for 1 hour at RT on a platform shaker. Filters were then washed 4 times in PBS/Tween. Each wash lasted 15 minutes and filters were continuously agitated. The secondary Ab, a HRP conjugated swine anti-rabbit Ig (DAKO) was diluted 1:1000 in 1% BSA in PBS/Tween and applied to the filters. Filters were agitated for 1 hour at RT and followed with a repeat of the wash procedure. The filters were then washed in PBS alone before ECL^{TM} detection, as described previously (section 2.6.2.4).

Ab	Antigen	Origin	Protein Size	Dilution	Reference
AK14	Lmp 2	Rabbit polyclonal serum	28kDa	1:1000	Kelly et al., 1991.
BMF-1	Lmp 7	Rabbit polyclonal serum	23kDa	1:1000	Glynne et al., 1993.
AK13	TAP 1	Rabbit polyclonal serum	71kDa	1:1000	Kelly et al., 1992.
AK7	TAP 2a	Rabbit polyclonal serum	75kDa	1:1000	Powis et al., 1992.

Table 2.6. Primary antibodies used in the detection of TAP and Lmp proteins.

2.8 Flow Cytometric Analysis

2.8.1 Cell Surface Markers

Hybrid cells were analysed as clones and as 'bulk' populations, along with the relevant parent cell lines, for the expression of cell surface markers. Viable cells were stained by direct and indirect immunofluorescence with the relevant mouse anti-human monoclonal antibodies (mAb) including; W6\32 (Barnstable *et al.*, 1978) and L243 (Lampson and Levy, 1980) which recognise framework determinants on HLA class I and

class II molecules respectively, 142.2 (a gift of Dr J Bodmer, Oxford, UK), BB7.2 (Brodsky *et al.*, 1979), and GAP.A3 (Berger *et al.*, 1982) which recognise HLA -A1, -A2 and -A3 respectively, lymphocyte function-associated proteins CD11a (LFA-1) and CD58 (LFA-3), intracellular cell adhesion molecules CD50 (ICAM-3) and CD54 (ICAM-1) and costimulatory molecules CD40, CD80 (B7.1) and CD86 (B7.2). Also the cells were stained for appropriate cell type specific markers including the T-cell markers CD3, CD4, CD8, CD45RO and CD45RA and the B-cell marker CD19. mAbs were either directly conjugated with fluorescine isothiocyanate (FITC) or R-phycoerythrin (RPE), or indirectly labelled with FITC conjugated F(ab)'₂ rabbit anti-mouse immunoglobulin (Ig) (DAKO). Both negative (unlabelled) and positive (stained with conjugate only) controls were included in each indirect analysis. Table 2.7 lists the mAbs used for flow cytometry.

2.8.2 Immunofluorescence Procedure

Prior to cell staining, adherent cells were removed from the cell sheet by trypsin/EDTA treatment and suspension cells collected by aspiration. All cell samples were washed thoroughly in PBS. Typically, 2x10⁵ cells per sample were included and labelled with saturating amounts of mAb for 30 minutes on ice. Samples were washed twice with 0.5% bovine serum albumin (BSA) fraction V in PBS. If the mAb was not directly labelled, the sample was subsequently incubated with saturating amounts of FIT-C conjugated secondary Ab for 30 minutes on ice and in the dark and samples washed twice with 0.5% BSA in PBS. Samples were fixed in a 1% paraformaldehyde solution and then kept in the dark at 4°C for up to 2 days until analysis.

2.8.3 FACScan Analysis

Flow cytometry was performed on a Becton and Dickinson FACScan (Cowley, Oxford, UK) with LYSIS II and CellQuest software. Viable cells were gated on forward-scatter and side-scatter cell populations, above a threshold of 52.

mAb	Origin	Clone	Ig subclass	Label
MHC class I	tissue culture supernatant	W6\32	IgG _{2a}	indirect
MHC class II	tissue culture supernatant	L243	IgG _{2a}	indirect
HLA-A1	ascitic fluid	142.2	unknown	indirect
HLA-A2	tissue culture supernatant	BB7.2	IgG _{2b}	indirect
HLA-A3	tissue culture supernatant	GAP.A3	IgG _{2a}	indirect
CD3	DAKO	UCHT1	IgG ₁	FITC conjugated
CD4	DAKO	MT310	IgG ₁	FITC conjugated
CD8	DAKO	DK25	IgG ₁	FITC conjugated
CD11a	Serotec	B-B15	IgG ₁	indirect
CD19	DAKO	HD37	IgG ₁	FITC conjugated
CD40	Serotec	EA-5	IgG ₁	indirect
CD45R0	DAKO	UCHL1	IgG_{2a}	RPE conjugated
CD45RA	DAKO	4KB5	IgG ₁	RPE conjugated
CD50	Serotec	ICAM3.3	IgG ₁	indirect
CD54	Serotec	84H10	IgG ₁	indirect
CD58	Serotec	BRIC-5	IgG_{2a}	indirect
CD80	Serotec	BB- 1	IgM	indirect
CD86	Serotec	BU63	IgG ₁	indirect

Serotec Ltd, Kiddlington, Oxfordshire, UK

DAKO Ltd., High Wycombe, Buckinghamshire, UK

 Table 2.7. mAb used for flow cytometry.

2.9 Isolation and Analysis of Allogeneic Responder Cells For Use In Vitro 2.9.1 Responder Cell Donors

Responder cells for use in T cell stimulation experiments were obtained from healthy volunteer donors. The EBV status of the donors was determined by serological analysis (presence of IgM antibodies against the EBV capsid protein and/or IgG antibodies against the nuclear antigens) carried out by The Public Health Laboratories Services, Leicester Royal Infirmary, Leicester, UK.

The HLA -A, HLA-B and HLA -DR types of each donor was determined by sequence specific PCR as described (section 2.4). Genomic DNA was extracted from blood samples collected in potassium EDTA/2.7ml Monovette[®] tubes (Sarstedt, Numbrecht, Germany) using the NucleonTM II Genomic DNA Extraction Kit. Red blood cells were lysed by mixing blood samples with 4 times the volume of Reagent A [10mM Tris-HCl, 320mM sucrose, 5mM MgCl₂, 1% Triton X-100] for 4 minutes on a blood tube Rotator SB1 (Stuart Scientific Industries International (UK) Ltd.) at RT. The sample was centrifuged at 1300x g for 4 minutes and the supernatant was discarded. The cell pellet was then treated as described previously (section 2.4.1).

The HLA types and EBV serology of the donors used in the study are given in Table 2.8.

* <u></u> **. <u></u> **.	HLA-A	HLA-B	HLA-DR	EBV Status
Responder 1	A3	B7, B35	DR4, DR10	Sero-negative
Responder 2	A3, A9	B62	DR7, DR13	Sero-negative
Responder 3	A2, A24	B44, B5	DR4, DR15	Sero-positive
Responder 4	A1, A9	B15, B53	DR11, DR13	Sero-positive
Responder 5	A2, A3	B7, B35	DR1, DR2	Sero-positive
Responder 6	A2, A9	B7, B13	DR4, DR14	Sero-positive
Responder 7	A1, A2	B8, B27	DR1, DR3	Sero-positive

Table 2.8. EBV status and HLA genotypes of responders used in this study.

2.9.2 Isolation of Responder PBMC for Use In Vitro

Fresh heparinised blood was collected by venepuncture from healthy, allogeneic volunteer donors. PBMCs were obtained by carefully layering 20ml of blood diluted by the addition of an equal volume of serum free RPMI 1640, over 10ml of lymphocyte separation medium (LymphoprepTM: NYCOMED PHARMA AS Diagnostics, Majorstua, Oslo, Norway). PBMCs were separated into a buffy layer by centrifugation at 800x g (no brake) for 20 minutes at RT. The cells were then aspirated from the sample/medium interface and washed in RPMI 1640 growth medium prior to either further cell separation or immediate use *in vitro*.

2.9.3 Isolation of Responder T Cells for Use In Vitro by Magnetic Labelling

T cells were isolated from PBMCs by magnetic labelling cells and immunoseparation using the pan T cell microbead isolation kit and the Midi MACS cell separation system (Miltenyi Biotech, Bergisch, Gladbach, Germany) according to the manufacturer's instructions. T cells were negatively selected and remained untouched prior to use *in vitro*.

Briefly, monocytes were removed from PBMCs by allowing cells in RPMI growth medium to settle out and adhere to plastic tissue culture flasks. After one hour, non adherent cells were collected, washed in cold MiniMACS Buffer (MMB) [0.5% BSA and 5mM EDTA (pH8.0) dissolved in PBS, degassed and sterile filtered] and adjusted to 1×10^7 cells in 80µl of MMB. The cells were then incubated for 10 minutes at RT with 20µl of pan T cell isolation hapten-Ab cocktail per 1×10^7 cells, which contained monoclonal-hapten conjugated anti CD11b, CD16, CD19, CD36 and CD56 antibodies, all of which are directed against non-T cells. Cells were washed thoroughly in MMB to remove unbound Ab and then incubated for a further 15 minutes at RT with 20µl of magnetically labelled anti-hapten microbeads in 80µl of MMB per 1×10^7 cells. Cells were again washed with MMB, resuspended in 500µl of MMB (for up to 1×10^8 cells) and applied to a magnetic separation column.

For up to 10⁸ positively labelled cells, MidiMACS Separation Columns (type LS+) were used, and for up to 10⁷ positively labelled cells, MiniMACS Separation Columns (type MS+) were used. Prior to use, each MidiMACS Separation Column was 'primed' by passing 3ml of PBS followed by 3ml of 2% BSA in PBS and then 3ml of MMB just before cell separation. MiniMACS Separation Columns were 'primed' with only 1ml of each

solution. After applying the magnetically labelled cells to a MidiMACS Separation Column, each column then was washed at least 3 times with 3ml MMB (or 1ml for a MiniMACS Separation Column) and all flow through was collected and pooled. The magnetically labelled non-T cells were retained in the column and depleted from the cell population allowing the negatively selected CD3+ T cells to pass through the system untouched. Throughout the procedure MMB and cells were kept on ice, except were stated.

Cell purity of the separated cell populations was determined by direct immunofluorescent techniques and flow cytometric analysis using antibodies directed against various cell surface molecules, as described in section 2.8.

For several experiments (section 2.10.4), the column-bound cells, termed non-T cells were used. Cells attached to plastic were removed by trypsin/EDTA treatment and mixed with the cells retained in the column which were obtained using the plunger supplied with the Separation Columns. These cells are referred to in the study as non-T cells.

2.9.4 Separation of T Cells into T Cell Subsets

In certain experiments purified CD4+, CD8+, CD45RO+ and CD45RA+ T cells were required. In these instances, a fraction of the negatively selected CD3+ cells were labelled with anti-CD8, anti-CD4, anti-CD45RO or anti-CD45RA magnetic labelled microbeads according to the manufacturer's instructions (Miltenyi Biotech). Basically, 20 μ l of magnetically labelled microbeads per 1x10⁷ cells in 80 μ l of MMB was added. Cells were incubated for 30 minutes at 4°C then washed thoroughly in MMB, resuspended in 500 μ l MMB. The cells were then applied to a MiniMacs Separation Column following the procedure described in section 2.9.3. Anti-CD4 magnetic labelled beads leave the CD8+ T cells untouched and anti-CD8 magnetic labelled beads leave the CD45RA+ T cells untouched and anti-CD8 magnetic labelled beads leave the CD45RA+ T cells untouched and anti-CD8 magnetic labelled beads leave the CD45RA+ T cells untouched and anti-CD8 magnetic labelled beads leave the CD45RA+ T cells untouched.

2.10 In Vitro Assays Assessing Immunostimulatory Phenotype of Hybrid Cell Lines 2.10.1 Mixed Tumour Cell / Lymphocyte Cultures (MTLC)

MTLC were used to assess the immunostimulatory capacity of hybrid and parental cells *in vitro*. Stimulator cells were treated with 0.5ml of Mitomycin-C (either 50µg/ml for suspension cells or 100µg/ml for adherent cells) for 40 minutes then washed 3 times in RPMI 1640 growth medium. The cells were applied to a haemocytometer, counted and adjusted to 1×10^6 cells/ml. The cells were then triple diluted to 3.3×10^5 cells/ml and 1×10^5 cells/ml in RPMI 1640 growth medium, before 100µl aliquots of each dilution were dispensed into the appropriate wells of a 96 well U-bottomed plate.

To the stimulator cells, 100μ l of responder PBMCs in RPMI 1640 growth medium at $1x10^6$ cells/ml were added. Each cell combination was performed in triplicate. In addition, PBMCs were cultured alone and the stimulator cells were also cultured alone as controls. As a positive control, PBMCs were co-cultured against Mit-C treated HMy2 cells, which express high levels of MHC class I and MHC class II antigens and co-stimulatory and adhesion molecules. Cell co-cultures were incubated at 37° C in 5% CO₂. Each experiment was performed with a minimum of 2 different responders unless stated. Both EBV sero-negative and EBV sero-positive responders were included in the study.

After 6 days, cells were pulsed with 1µCi/well [methyl ³H]-thymidine (Amersham Radio Chemical Company, Amersham, UK) for 8 hours at 37°C in 5% CO2. Cells were then harvested onto glassfibre filter disks (Helis Bio Ltd., Newmarket, Suffolk, UK) using Semi-Automatic Cell Harvester (Skatron Instruments Ltd., Newmarket, Suffolk, UK). Each disk was then deposited into individual scintillation vials (Helis Bio Ltd.) containing 2ml scintillation fluid (Camberra Packard, Pangbourne, Berkshire, UK) and capped. Radioactivity was recorded as counts per minute (CPM) by a MINAXI 4000 series scintillation counter (United Technologies, Packard Instrument Company Inc., Downers Grove, Illinois, USA).

The degree of allogeneic stimulation caused by the parent or hybrid cell line was taken as the uptake of [methyl ³H]-thymidine in the MTLC - the background uptake of [methyl ³H]-thymidine by the stimulator cells alone.

2.10.2 T Cell Response to EBV B-LCL x Tumour Cell Hybrids and Relevant Parent Cell Lines in an In Vitro Proliferation Assay

In order to investigate the T cell response in an allogeneic proliferation assay, responder PBMC were separated into a CD3+ T cell fraction and CD4+ and CD8+ T cell subsets. The assay was performed essentially as described previously (section 2.10.1) except stimulator cells were dispensed at a single concentration of 3.3×10^4 cells per well. 1×10^5 CD3+, CD4+ and CD8+ T cells per well were cultured with the stimulator cells to give a total volume of 200µl per well. Triplicate wells consisting of either responder cells alone, or stimulator cells alone, were also included as controls. Each experiment was performed with both EBV sero-negative and EBV sero-positive responders. The experiment was also repeated separately using CD45RO+ and CD45RA+ T cell subsets as described above.

2.10.3 Blocking of Cell Surface Molecule Function

In order to determine the costimulatory molecules involved in the immune response directed against the tumour cells and the parent cell lines, MTLC assays were performed as previously described (section 2.10.1 and section 2.10.2), except for a single modification. Blocking antibodies directed against CD40 (mAb89: Coulter Electronics Ltd, Luton, Bedfordshire, UK) and CD80 and CD86 (CTLA-4 Ig fusion protein, Ancell, part of Alexis Corporation UK Ltd., Nottingham, UK) were included in the wells at saturating concentrations (0.5µg/ml) either singly, or in combination or with an isotype matched Ab control (mouse anti-human IgG₁, R & D Systems) at the same concentration. In a separate experiment, blocking antibodies against MHC class I (W6/32) and MHC class II (L243) were used as cell culture supernatants, and formed 10% of the final volume per well. A tissue culture supernatant at the same volume and the appropriate isotype matched Ab control (mouse anti-human IgG_{2a}, R & D Systems) at a saturating concentration (0.5µg/ml) was also included. Tissue culture supernatants were prepared by centrifugation of cell suspension at 800x g to remove cells and were frozen at -20° C until day of use. Mit-C treated stimulator cells were aliquoted at a concentration of 3.3x10⁴ cells per well and incubated with the appropriate Ab for 30 minutes at 37°C with 5% CO₂ prior to the addition of responder PBMC at a concentration of 1×10^5 cells per well to give a total volume of 200µl per well. Each Ab/cell mixture was performed in triplicate.

2.10.4 The Role of Non-T Cells in Stimulator-T Cell Co-Cultures

In order to investigate whether non-T cells can provide co-stimulatory signals to allow T cells to proliferate in response to stimulator cells which lack the expression of CD80 and CD86, assays were preformed essentially as described previously (section 2.10.2) except only CD3+ T cells were cultured with the stimulator cells, and $3.3x10^4$ or $1x10^5$ Mit-C treated responder non-T cells were also included in the co-cultures to give a final volume of 200µl per well. Triplicate wells consisting of stimulator cells and CD3+ T cells co-cultures and stimulator cells and Mit-C treated non-T cells co-cultures were also included. Control wells consisted of stimulator cells alone and each responder cell combination in the absence of stimulator cells.

In order to determine whether CTLA-4 Ig was able to block the T cell response to stimulator cells in the presence of non-T cells, antibody blocking assays were performed as described above except non-T cells were included in the stimulator-T cell co-cultures at a single concentration of 3.3×10^4 cell per well and CTLA-4 Ig was included as essentially as described previously (section 2.10.3).

2.10.5 Ability to Induce a Cell Specific CTL Response In Vitro

The ability of parent and hybrid cell lines to induce a CTL response *in vitro* was assessed in standard ⁵¹Chromium (⁵¹Cr) release assays, essentially as described elsewhere (Browning *et al.*, 1990). Basically, $1x10^7$ CD3+ T cells from healthy allogeneic donors were co-cultured with $2x10^6$ Mit-C treated stimulator cells (as described previously, 2.9.1) in 25cm² tissue culture flasks in a total volume of 10ml RPMI 1640 growth medium for 6 days.

On day 7 of the culture, viable cells were counted by trypan blue exclusion and use of a haemocytometer. $3x10^6$ stimulated CD3+ T cells were then incubated with 20U/ml IL-2 (a gift from Dr C Hewitt, Leicester, UK) and restimulated with $1x10^6$ appropriate Mit-C treated stimulator cells for a further 6 days in 10ml RPMI 1640 growth medium. T cells were distinguished from stimulator cells in the mixed cell population either by size or by flow cytometry. A sample of the cell population was stained with anti-CD3 mAb as described previously (section 2.8) in order to determine the percentage of CD3+ T cells in the population. Unstimulated T cells were also maintained over the 14 day period in 10ml of RPMI 1640 and also treated with 20U/ml IL-2 on day 7 of assay. On day 14 of assay, target cells were radiolabelled with 0.5mCi ⁵¹Cr sulphate (Amersham Radiochemical Company) for 1 hour, then washed thoroughly to remove excess radioactivity. $5x10^3$ labelled target cells in 50µl RPMI 1640 growth medium were added to duplicate wells of a 96 well U bottomed plate containing different dilutions of pre-stimulated T cells (effector T cells) or unstimulated T cells in 100µl growth medium. Effector cells were distinguished from stimulator cells as described previously prior to dispensing in wells. ⁵¹Cr release assays were routinely performed at effector:target (E:T) ratios of 30:1, 10:1, 3:1 and 1:1. As an assessment of NK activity, effector T cells were also incubated with K562 labelled target cells at the same E:T ratios. Effector T cells were also

After 4 hour incubation at 37°C and 5% CO2, 50µl of cell supernatant was collected from each sample, dispensed into a polystyrene test tube (Fisher Scientific UK), capped with a push on stopper (Sarstedt) and immediately counted in a universal gamma counter (1282 Compugamma CS: Perkin Elmer Life Science, Cambridge, UK). Each experiment was performed with a minimum of 2 different responders; both EBV sero-negative and EBV sero-positive responders were included.

Percentage specific lysis was determined as:

(release in test - spontaneous release) (maximal release - spontaneous release) x 100.

Maximal release was obtained by incubating 50 μ l of RPMI 1640 growth medium containing 5x10³ radiolabelled target cells in the presence of 100 μ l of 0.1M HCl and spontaneous release was the background release of ⁵¹Cr by target cells cultured alone in 150 μ l of RPMI 1640 growth medium.

2.10.6 Presentation of Tumour-Antigens to Antigen Specific, HLA Restricted CTL Clones

In order to determine whether or not hybrid cells retained the ability to present tumour associated antigens to CTL, ⁵¹Cr release assays were performed using HLA restricted CTL clones specific for tumour antigens MAGE 1, MAGE 3 and tyrosinase as described elsewhere (Herin *et al.*, 1987; Coulie *et al.*, 1994). These assays were performed

by Dr P Coulie at the Unite de Genetique Cellulaire, Université Catholique de Louvain, Brussels, Belgium.

2.11 Transfection of CD80 into Hybrid and Tumour Parent Cell Lines 2.11.1 Transformation of E. coli with pCR3 and pCR3.CD80

The plasmid pCR3 and the plasmid containing a cloned CD80 insert, termed pCR3.CD80 (as described by S. Thirdborough, 1998) were kindly provided by S. Thirdborough, Leicester, UK. The plasmids contain an enhancer-promoter sequence from the immediate early gene of human cytomegalovirus for high level gene transcription, a separate expression cassette for resistance to neomycin and a pBR322 vector sequence for replication and selection in *Escherichia coli* (E. coli). The plasmids were used to transform E.coli strain DH5 α bacteria by heat shock. A 100 μ l sample of competent bacteria (a gift from S Kingston, Leicester, UK), was cultured on ice for 30 minutes in the presence of 1µg of pCR3 or pCR3.CD80 prior to a heat shock at 42°C for 45 seconds. The bacteria were then placed on ice to cool for 2 minutes. To the sample, 1ml of Luria Bertoni (LB) medium [1% tryptone, 0.5% yeast extract, 0.5% NaCl] was added and the sample was incubated for 45 minutes at 37°C. 200µl of the bacteria culture was then spread evenly over LB agar plates [1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar] containing 50µg/ml ampicillin. The plates were inverted and cultured over night at 37°C. Following the over night incubation period, discrete bacterial colonies were picked and grown in 5ml selective LB broth containing 50µm/ml ampicillin over night at 37°C with continuous shaking.

2.11.2 Small Scale Plasmid Production and Analysis

To recover the plasmids, 1.5ml of bacteria from the selective overnight culture was taken. The sample was centrifuged at 12000x g for 5 minutes and the supernatant discarded. The pellet was then fully resuspended in 100µl Resuspension Buffer [50mM Tris-Cl (pH 8.0), 10mM EDTA, 100ug/ml RNase A] and left on ice for 5 minutes. 200µl of Lysis Buffer [200mM NaOH, 1%SDS] was then added and the solution inverted to mix the contents. The sample was then left on ice for 5 minutes. 150µl of Neutralization Buffer [3.0M potassium acetate (pH5.5)] was then added to the sample and incubated for a further 5 minutes. The sample was centrifuged for 15 minutes at 12000x g and the

supernatant was removed and put into a fresh eppendorf tube. 1ml of 100% ethanol was added to each tube to precipitate the DNA. The sample was washed in 70% ethanol, air dried and resuspended in 30µl sterile water. The Resuspension, Lysis and Neutralisation Buffers were all supplied with the QIAfilter Maxi Plasmid Extraction kit (QIAGEN Ltd., Crawley, West Sussex, UK).

2.11.3 Large Scale Plasmid Production

The bacteria were then grown over night at 37° C with continuous shaking on a larger scale in 100ml LB selective broth. The plasmids were extracted using the QIAfilter Maxi Plasmid Extraction Kit according to the manufacturer's instructions. Such a technique is based on a modified alkaline lysis procedure, followed by plasmid binding to QIAGEN Anion-Exchange Resin under low-salt and pH conditions. Plasmid DNA is eluted in a high salt buffer and precipitated with isopropanol. Plasmids pCR3 and pCR3.CD80 were washed in 70% ethanol, air dried and resuspended in sterile NANOpure water. The concentration of the plasmid DNA was determined by reading OD₂₆₀ as described previously (section 2.4.2). The plasmids pCR3 and pCR3.CD80 were then correctly identified by restriction analysis (Sambrook *et al.*, 1989).

2.11.4 Transformation of Adherent Cell Lines by Effectene Transfection

Cells were seeded into the wells of a six well plate and left to adhere overnight. Wells which were 60% confluent were transfected with either pCR3.CD80 or the empty pCR3 plasmid using Effectene Transfection Reagent (QIAGEN Ltd.) according to the manufacturer's instructions. Effectene transfection is based on a non-liposomal lipid. Basically, 0.4µg of plasmid DNA dissolved in NANOpure water was mixed with DNAcondensation buffer, buffer EC, (supplied with Effectene Transfection Reagent) to a total volume of 100µl. To the diluted DNA, 3.2µl of Enhancer (supplied with Effectene transfection reagent) was added, and the sample was vortexed for one second. The mixture was incubated for five minutes then pulsed by centrifugation. To the DNA-Enhancer mixture, 10µl of Effectene Transfection Reagent was added and mixed by vortexing for ten seconds. The sample was then incubated at RT for ten minutes to allow Effectene-DNA complexes to form. During Effectene-DNA complex formation, the cell monolayer was washed with PBS and 1600µl of DMEM growth medium was added to the well. To the Effectene-DNA complexes, 600 μ l of DMEM growth medium was added and the sample was mixed by pipetting up and down. The sample was added dropwise to the cells. The plates were then gently swirled and incubated at 37°C and 5% CO₂.

After 24 hours, the adherent cells were washed with PBS and DMEM growth medium containing 2mg/ml G-418 sulphate was added. This concentration G-418 sulphate killed non-transfected cells but allowed transfected cells to grow. After 15 days in selection, transfected cells were cloned at one cell per well (see section 2.3.4 for procedure) and cell numbers expanded. Expression of CD80 was determined by Ab labelling and flow cytometric analysis (as described in section 2.8).

Chapter 3

EBV B-LCL x Melanoma Hybrid Cell Generation and Phenotypic Characterisation

EBV B-LCL x Melanoma Hybrid Cell Generation and Phenotypic Characterisation

3.1 Introduction

This chapter provides an account of hybrid cell formation and selection, and describes in general EBV B-LCL. It also explains the necessity for characterising and cloning hybrid cells prior to their use in tumour immunotherapy.

3.1.1 Hybrid Cell Generation and Associated Problems

3.1.1.1 General Background

Hybrid cell formation has been studied extensively and used in many fields of biology, especially in biotechnology. Cell fusion has been used as a method for introducing exogenous genetic material into a cell and has also been used to map genes to chromosomes. Since chromosome loss in hybrid cells often occurs, especially when cells of different species are fused, it is possible to match retained cellular functions with retained chromosomes (Tsujimoto *et al.*, 1999). Kohler and Milstein in 1975 (Kohler and Milstein, 1975) were the first to introduce monoclonal Ab production by fusing Ab producing spleen cells from immunised mice with continuously dividing murine plasmacytoma cells. Hybridomas were formed that secreted antibodies with single epitope specificity, thus revolutionising the use of antibodies in cell biology. Hybridoma research is continually improving hybrid cell production, with monoclonal antibodies having many important applications, especially in biological research and in disease diagnosis and therapy.

This study was concerned with the generation of hybrid cells for potential use in tumour immunotherapy. At the start of the study the only published work on hybrid cells as potential agents in tumour immunotherapy was produced by Guo *et al.* in 1994 (Guo *et al.*, 1994). Guo and colleagues fused hepatocarcinoma cells with activated B cells using the rat as an experimental animal model and achieved promising results. Animals injected with hybrid cells were able to eradicate established tumours and prevent wild type tumour growth. Since then a number of reports on the use of hybrid cells using a variety of tumour cell lines and a range of APCs in tumour immunotherapy have been published, and are discussed elsewhere (section 1.7.5). Melanoma cells fused with EBV transformed B cells,

the focus of this study, have not been previously been generated or characterised with a view to using the hybrid cells as immunogens in cancer therapy.

3.1.1.2 Methods for Hybrid Cell Generation

Techniques used in the production of hybrid cells include virus mediated cell fusion (White *et al.*, 1981), various chemical fusogens, including PEG (Davidson *et al.*, 1976), and electrofusion (Zimmermann and Vienken, 1982; Bates *et al.*, 1987).

3.1.1.2.1 Virus Mediated Cellular Fusion

Virus treatment was the first method used for hybrid cell production. Some enveloped viruses have cell surface proteins that enable the virus envelope to fuse with the cell surface membrane. This can lead to cell fusion between adjacent cells. Inactivated Sendai and other viruses, including influenza, have been exploited for their fusogenic potential. The haemagglutinating virus of Japan (HVJ) is able to fuse cell membranes at near neutral pH for a variety of cell types. A glycoprotein in the viral envelope can efficiently agglutinate cells with HVJ receptors. However, many cells either do not express particular viral receptors or do so at low levels, rendering virally mediated cellular fusion impractical for many types of cells. The use of inactivated viruses as method for inducing cellular hybridisation is also labour intensive and so this method has been replaced with a simpler approach using fusogenic chemicals to fuse cell membranes.

3.1.1.2.2 PEG Mediated Cellular Fusion

Pontecarvo in 1975 first introduced PEG as a method for fusing mammalian cells (Pontecarvo, 1975). PEG is a fusogenic chemical and offers several advantages over other methods for producing hybrid cells in that it is inexpensive, relatively quick and simple to use and can also fuse a variety of cells which may differ in species (Hui *et al.*, 1985; Knutton and Pasternak, 1979; Power *et al.*, 1978). This technique was used in the production of EBV B-LCL x non B cell hybrids (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1991). For these reasons, PEG was chosen as the method for making EBV B-LCL x melanoma cell hybrids in this study.

PEG mediated cell fusion is a complex mechanism which is not very well understood (see Zimmerberg *et al.*, 1993). It involves cell aggregation and the disturbance of the lipid membrane leading to the formation of prefusion sites. The osmotic swelling of cells resulting from the sudden dilution of PEG, expands the prefusion sites allowing membrane fusion (Boni and Hui, 1987). Factors including the concentration and pH of the PEG solution (Sharon *et al.*, 1980), whether or not serum is present (Zola and Brooks, 1982) and length of time cells are exposed to the PEG solution (see Gefter *et al.*, 1977) have all been reported to affect hybridisation efficiency. In addition, cell size, the ratio of parent cells and cell origins have also been shown to affect fusion efficiency. These experimental parameters need to be co-ordinated in order to obtain the highest numbers of hybrid cells. PEG, however, is cytotoxic to certain mammalian cell lines (Engleman *et al.*, 1985) and can cause cell damage if the cells are exposed to it for too long. Fusion yields using this chemical are also low and in many cases the hybridisation procedure has to be repeated several times before any hybrid cells are obtained.

3.1.1.2.3 Electrofusion

More recently electrofusion as method for cell-cell fusion was introduced by Zimmermann in 1982 (Zimmermann, 1982). Using this technique cells are dielectrophoretically aligned by exposure to an alternating electric field. It is also possible to bring the cells into close contact before membrane fusion using alternative approaches which include growing the cells to confluent monolayers (Teissie *et al.*, 1982), pelleting cells by centrifugation (Abidor *et al.*, 1993) and using PEG (Li and Hui, 1994). The need to dielectrophoretically align cells with an alternating electric current is thus eliminated. A transient direct electric current is then passed through the cells which induces membrane permeabilisation and results in the joining of adjacent membranes.

Electrofusion has been reported as being more efficient than other methods for cell fusion (Krenn, 1995) and has several advantages. It is possible to alter conditions used in the hybridisation procedure by microscopically observing the cells align under the influence of the alternating current. A reduced number of cells are necessary for this technique to be successful, an important factor to be considered when limited numbers of cells are available from patient samples. This technique is also less labour intensive and has been used successfully for a variety of cell types. However, the electrofusion equipment required is expensive compared to the cost of PEG and low viability has been reported with some types of cells, especially when cells differing in sizes are fused (see Chang, 1989). As with PEG, optimal hybrid production depends on a variety of experimental parameters being co-ordinated for different cell types. For electrofusion, these factors include the strength and duration of the electric fields being applied to the cells.

3.1.1.3 Hybrid Stability

Inevitably, when two cells are fused together there is some chromosomal elimination. Factors including asynchronous DNA replication and disturbances in chromosomal metabolism are thought to effect chromosome loss. This in particular happens when two cells of differing species are brought together. When mouse-human hybrid cells are formed, human chromosomes are preferentially lost (Weiss and Green, 1967). It has however been reported that when cells of the same species are fused there is less chromosome loss (Eliceiri, 1972; Kuter and Rodgers, 1975). Although Olsson in 1983 (Olsson et al., 1983) reported a substantial loss of chromosomes in human-human hybrid cells during the first three to eight weeks after fusion, Ber et al. (Ber et al., 1978) reported human hybrid cells with near tetraploidy for most chromosomes in hybrid cells formed between two different human derived cell lines. Similarly, Kozbor and co-workers reported some variation in chromosome numbers for hybrids formed between an EBV B-LCL and an EBV B cell clone expressing antitetanus toxoid Ab, though hybrids were near tetraploid two months after isolation (Kozbor et al., 1982). Chromosomal content can be assessed by karyotpye analysis.

3.1.1.4 Hybrid Cell Selection Procedure

Since cellular hybridisation is essentially a random process, hybrid cells formed between the two different cells (heterologous hybrids) must be selected at the expense of the unfused parent cells or hybrids formed between cells of the same type (homologous hybrids). The selection process of hybrid cells is normally in the form of nutritional requirements or drug resistance differences between the two parental cell types. These markers can be introduced into the parent cells by plasmid transfection or by mutating the cells using UV or chemicals. Fusion of cells which are not able to grow *in vitro* with cells which can, requires only a single selection method and is directed only against the parent cells which are able to proliferate in culture.

In this study, the cell line termed HMy2 was used as the universal EBV B-LCL parent in the hybrid cells. Previous studies using HMy2 as a fusion parent have been described elsewhere (Edwards *et al.*, 1982; Kerr *et al.*, 1992). This cell line is a double mutant having a recessive HAT sensitive and dominant ouabain resistant phenotype, rendering hybrid cells resistant to double chemical selection with HAT and ouabain, whilst HMy2 cells are killed by HAT in the selection medium, and the other cell lines are killed by ouabain.

3.1.1.4.1 HAT Sensitivity

The cell line HMy2 lacks the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) after passaging parent cells in increasing concentrations of 8-azaguanine. 8-azaguanine is a cytotoxic guanine analogue which selects for mutants lacking HGPRT. Those cells which lack the enzyme HGPRT do not incorporate the toxic purine analogue into DNA and are able to survive in this medium whereas non-mutated cells incorporate this cytotoxic analogue into DNA and die. In medium containing HAT, HGPRT mutants are unable to grow as the cells are unable to follow the 'salvage pathway' in purine synthesis. The mutant cells are unable utilise hypoxanthine from the selective cell culture medium and since aminopterin blocks *de novo* purine synthesis, cells die from being unable to replicate DNA. Normal cells follow the 'salvage' pathway due to the presence of the enzyme HGPRT and are able to utilise hypoxanthine in nucleotide synthesis.

3.1.1.4.2 Ouabain Sensitivity

Ouabain is a steroid compound and a specific inhibitor of the Na⁺K⁺ activated ATPase of the plasma membrane (Glynn, 1964). This enzyme actively transports K⁺ into the cell and exports Na⁺ out. Permanent ouabain resistance is due to a mutation in one allele of the α -subunit of the Na⁺K⁺ ATPase. Normal cells are unable to grow in medium containing ouabain and differ in their sensitivities to ouabain. HMy2 cells are able to grow in ouabain, although how the ouabain resistant phenotype was generated could not be determined from published works. In a study by Contreras-Brodin *et al.*, an EBV B-LCL cell line (KR-4) was made ouabain resistant by γ radiation treatment (Contreras-Bodin *et al.*, 1991). Ouabain resistance can also be chemically induced (Lin and Kuo, 1990).

The concentrations required for selection must be determined separately for each cell type. The concentration of ouabain chosen for selection of hybrid cells in this study was approximately 30% higher than the minimal concentration required to kill 100% of the unfused tumour cells within 7–14 days, as determined in cell viability tests. Fusion of normal cells with mutated HAT sensitive, ouabain resistant cells produces hybrid cells only which are able to grow in the double chemical selective medium (containing HAT and ouabain). Unfused and cells fused between the same parental type (homokaryons) are unable to replicate due to their enzyme deficiency or chemical sensitivity.

3.1.2 Melanoma Cell Lines Used in this Study

The melanoma cell lines used as the tumour cell partner to HMy2 in this study were 518.A2, Gerl43 and DAUV. 518.A2, Gerl43 and DAUV have been used previously in the identification of tumour-associated antigens. Gerl43 is a human derived melanoma cell line from a tumour metastasis removed from patient MZ-2, from whose cells a panel of autologous anti-tumour CTL clones were obtained (Herin *et al.*, 1987) and the first melanoma antigen, MZ2-E, encoded by the MAGE 1 gene, was identified (van der Bruggen *et al.*, 1991). 518.A2 is also a human derived melanoma cell line which was shown to express MAGE 1, MAGE 3, tyrosinase, gp100 and MelanA/MART1 (Van Elsas *et al.*, 1997). Recently, IL-2 transfected 518.A2 cells induced a CTL response against a previously unidentified antigen termed CAMEL restricted by HLA-A2 (Aarnoudse *et al.*, 1999). In addition, IL-2 transfected 518.A2 cell have been used in a phase I-II study in patients with metastatic melanoma (Osanto *et al.*, 1993). DAUV, is a human derived melanoma cell line from a metastasis removed from patient LB33 (Lehmann *et al.*, 1995).

3.1.3 EBV and B Lymphocytes

3.1.3.1 General Background

EBV is a gamma-herpes virus. Most people carry the virus as a life long infection of the B cell pool in an asymptomatic state (Nilsson *et al.*, 1971; Gratama *et al.*, 1988). EBV has also been associated with several malignancies including endemic Burkitt's lymphoma (BL), undifferentiated nasopharygeal carcinoma (NPC) and immunoblastic lymphoma which can arise in immunocompromised individuals. By virtue of its receptor for the B cell lineage associated complement receptor CD21, EBV is able to bind and gain entry into the resting B cell by endocytosis. The viral receptor for CD21 is the major envelope glycoprotein gp350 (Tanner *et al.*, 1987). EBV infection is primarily latent in B cells although a lytic infection can occur (Miller, 1990). The only other type of cell permissive for viral replication is the mucosal epithelium, where the virus persists as a lytic infection (Sixbey *et al.*, 1984). How the virus penetrates epithelial cells is still largely unknown.

3.1.3.2 Transformation of B Cells with EBV

Transformation of B lymphocytes by EBV (EBV B-LCL) into permanently growing cell lines occurs readily *in vitro*, and results in the characteristic growth of cells in tight clumps due to the high level of expression of cell surface and adhesion molecules (CD11a, CD54 and CD58) (Thorley-Lawson *et al.*, 1985; Rowe *et al.*, 1985; Gregory *et al.*, 1988). EBV B-LCL display an 'activated' B cell phenotype, and have on the surface of the cell high levels of MHC class I and class II molecules and B cell activation markers (CD23, CD30 and CD31) which is comparable to the B cell phenotype upon stimulation with antigen or mitogen (Ehlin-Henrikkson and Klein, 1984; Rowe *et al.*, 1985). The expression of these activation markers by the virally infected cells also enhances EBV B-LCL immunogenicity and promotes CTL recognition and interaction. The CTL immune response plays an important role in controlling EBV B-LCL proliferation and helps to maintain the host in an asymptomatic state (reviewed in Rickinson and Moss, 1997).

3.1.3.3 EBV and Latent Viral Protein Expression

Latently infected cells carry numerous episomal copies of the EBV and express only a limited portion of the viral genome (Kieff, 1996). On the basis of the pattern of viral gene expression, EBV infected cells can be categorised into 3 different types of latent infection known as type I latency, type II latency and type III latency. The differences in the expression of the latent genes reflect the differences in the viral host cell phenotype.

3.1.3.3.1 Type III Latency

EBV B-LCLs constitutively display a limited set of viral proteins that are associated with type III latency. The viral latent proteins collectively are responsible for the growth and transformation of the B cell. The proteins include 6 nuclear antigens, EBNA 1, EBNA 2, EBNA 3, EBNA 4, EBNA 5 and EBNA 6. (EBNAs 3, 4, 5 and 6 are also referred to as EBNA 3a, 3b, LP and 3c) and 3 membrane proteins, LMP 1, LMP 2A and LMP 2B) (Henderson *et al*, 1993; Young *et al.*, 1989). The EBNA proteins are expressed from one of two promoters located in either BamHI C (Cp) (Bodescot *et al.*, 1986) or BamHI W (Wp) (Sample *et al.*, 1986; Speck *et al.*, 1986) whereas LMP protein expression is driven from 2 separate LMP promoters. A bidirectional LMP promoter region in BamHI N (Np) controls the expression of LMP 1 and LMP 2B whilst a separate EBV infection by targeting the latent antigens.

3.1.3.3.2 Type I Latency

In contrast to type III latency, type I latency is associated with the selective expression of EBNA 1 only and is seen in group 1 BL cells. These cells selectively down

regulate or do not express the B lymphocyte activation markers and also express low levels of surface activation antigens and adhesion molecules (Rowe *et al.*, 1987b; Gregory *et al.*, 1991). The expression of EBNA 1 only in these cells is driven from a separate promoter in the BamHI F region (Fp) where the BamHI W, C and N promoters are silent (Sample *et al.*, 1991). Group 1 BL cells can change the pattern of latent protein expression to that seen in type III EBV latency following a period of growth *in vitro*. This is associated with a change in viral promoter usage and expression of the EBV B-LCL cell surface phenotype (Rowe *et al.*, 1987b; Sample *et al.*, 1991).

3.1.3.3.3 Type II Latency

A third type of latency has more recently been identified and characterised and termed type II latency. This type of latency was first identified in NPC. EBNA 1 expression is driven from the Fp promoter and activation of one or more of the LMP promoters leads to the expression of LMP 1, LMP 2A and/or LMP 2B (Brooks *et al.*, 1992). Promoters Wp and Cp are silent in type II latency (Smith and Griffin, 1992) as seen in type I latency.

3.1.3.4 The Roles of the Viral Latent Proteins

The proteins associated with EBV latency are collectively responsible for B cell growth transformation and viral episome maintenance (reviewed in Kieff, 1996). EBNA 1, due to its ubiquitous expression in infected cells is required for the maintenance and replication of the viral episome (Yates *et al.*, 1985). EBNA 2, EBNA 3, EBNA 6 and LMP are essential for B cell growth transformation (Cohen *et al.*, 1989; Hammerschmidt and Sugden, 1989; Thomkinson *et al.*, 1993) whereas EBNA 5 enhances B cell transformation (Hammerschmidt and Sugden, 1988; Mannick *et al.*, 1991).

LMP 1 alone is able to transform rodent fibroblast cells (Kieff, 1996). LMP 1 is generally found in the plasma membrane, and acts like a constitutively activated growth factor, transmitting signals which would normally be received in response to CD40-CD40L interaction (reviewed by Farrell, 1998). LMP 1 can induce expression of various cell surface markers which include CD21, CD23, CD40 CD54, CD11a and CD58 (Wang *et al.*, 1988; Wang *et al.*, 1990a) and upregulate the expression of Bcl-2 to enhance cell survival (Henderson *et al.*, 1991). EBNA 2 has also been shown to trans-activate cellular genes, leading to the increased expression of various cell surface markers which include CD21 and CD23 (Wang *et al.*, 1990a), and EBNA 2 has also been shown to upregulate the proto-

onco gene, c-fgr (Knutson, 1990), and viral LMP 1 (Wang et al., 1990b) and LMP 2 expression (Zimber-Strobl et al., 1991). EBNA 6 can also upregulate CD21 expression (Wang et al., 1990a) and the expression of viral LMP 1 (Allday et al., 1993). EBNA 5 may complement EBNA 2, to induce G0 to G1 transition as marked by the induction of cyclin D2 (Sinclair et al., 1994). LMP 2 acts as a substrate for B cell src tyrosine kinases and blocks normal calcium immobilisation following the cross linking of surface IgM, CD19 or MHC class II, thus affecting transmembrane signal transduction and preventing the concomitant activation of the EBV lytic cycle (Miller et al., 1994). The effects of EBNA 3 or EBNA 4 on cellular gene expression have not yet been determined.

3.1.3.5 Immune Response Against EBV Latent Proteins

The host immune response, with MHC class I restricted CTLs playing a central role, is important in controlling both primary and latent EBV infections. The proteins associated with viral latency are the main antigenic targets for a CTL response against EBV. The immuno-dominant antigens are derived from EBNAs 3, 4 and 6 (Murray et al., 1992; Khanna et al., 1992; Gavioli et al., 1992) and memory CTL circulating in the T cell pool which are directed preferentially against these antigens and can be detected at high frequencies. A sub-dominant response which is detectable against LMP 2 and less frequently EBNA 2, EBNA 5 and LMP 1 has been shown, and very rarely has a response against EBNA 1 been demonstrated (Murray et al., 1992; Khanna, et al., 1992; Gavioli et al., 1992; Blake et al., 1997). The observed differences in immuno-dominance may in part be due to differences in the levels of expression of the epitope/MHC complexes on the cell surface, demonstrating the protein's accessibility to the MHC class I processing pathway (Rickinson and Moss, 1997). EBNA 1 contains an internal glycine-alanine repeat motif of over 200 residues in length which protects it from the MHC class I processing pathway (Levitskava et al., 1995). This however does not protect the protein from being degraded along the MHC class II processing pathway, as a CD4+ CTL clone reactivated from the CTL memory pool has shown specificity for an EBNA 1-derived peptide (Khanna et al., 1995). More recently, a HLA-DR restricted epitope derived from the EBNA 6 protein has been identified and CD4+ T cells recognising this epitope have been obtained from EBV carriers (Rajnavolgyi et al., 2000). Antigen specific CD4+ T cells may be important in maintaining EBV specific memory CTL.

Work to date on the immune response to EBV has focused mainly on EBV type I and Caucasian populations and needs to be viewed with caution when taken out of this context (see Rickinson and Moss, 1997).

3.1.3.6 Type 1 and Type 2 EBV

There are two types of EBV known as type 1 and type 2. Type 1 is more common in Caucasion and South-East Asian populations. EBV type 2 and type 1 are equally prevalent in some parts of Africa. The genomes for EBV type 1 and type 2 are very closely related, showing polymorphism for EBNA 2, EBNA 3, EBNA 4, and EBNA 6 genes. The predicted primary amino acid differences for the alleles EBNA 2, EBNA 3, EBNA 4 and EBNA 6 are 47%, 16%, 20% and 28% respectively (reviewed in Kieff, 1996).

3.1.3.7 Lytic (or Replicative) EBV

EBV normally establishes a latent infection in lymphocytes. Occasionally, B cells spontaneously undergo EBV lytic viral production, for reasons unknown, but usually it is the infected squamous epithelial cells that are permissive for lytic viral production. Permissive cells undergo cytopathic changes, including chromatin condensation soon after infection with EBV, so viral DNA replication can take place within the nucleus. During lytic infection, the Fp and LMP promoters are active so EBNA 1 and LMP 1 latent proteins are expressed. The virus also synthesises a collection of replicative (lytic) proteins (Sixbey *et al.*, 1984). CTL recognising immediate early and early EBV antigens are frequently detected in the blood of an infected individual. Kieff (Kieff, 1996) reviews the viral genes and proteins associated with a lytic EBV infection.

Lytic viral genes with homology to cellular genes have been described. BZLF 1, an immediate early gene, is similar to the jun/fos family of transcriptional activators (Packham *et al.*, 1990). BHRF 1, an early gene, has distant but functional and colinear homology to the cell survival gene Bcl-2 (Pearson *et al.*, 1987; Cleary *et al.*, 1986). It seem likely that BHRF 1 expression may prevent lymphocytes undergoing apoptosis during EBV lytic infection (Kieff, 1996). During late lytic infection, the virus produces a protein which is homologous to IL-10 (Moore *et al.*, 1990). BCRF 1 has nearly 90% amino acid sequence homology with IL-10 and possesses most of the features of human IL-10 *in vitro*. IL-10 is a negative regulator of NK and M\phi function and IL-10 also

inhibits the synthesis of several cytokines by Th1 cells (de Waal-Malefyt *et al.*, 1991). BCRF 1 most likely curtails initial NK and CTL responses to EBV infection. Viral IL-10 is also a potent B cell growth factor and induces the secretion of high levels of IgG, IgM and IgA (Rousset *et al.*, 1992).

3.1.4 EBV B-LCL x Tumour Cell Hybrids

Previous studies have demonstrated the potential to fuse EBV B-LCL with a range of human derived cells including both lymphoid and a diverse range of non-lymphoid cells, including erythroid, myeloid, fibroblast and epithelial cells (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1991; Kozbor *et al.*, 1982). The hybrid cells generated in these studies were not characterised with a view to using them as agents in tumour immunotherapy. Fusion of EBV B-LCL with somatic cells has been performed prior to this study to alter EBV latent gene expression (Klein *et al.*, 1976; Contreras-Salazer *et al.*, 1989; Contreras-Brodin *et al.*, 1991; Kerr *et al.*, 1992) and have shown cell phenotype dependence of EBV promoter usage. Careful phenotypic analysis of the hybrid cells at the clonal level, identifying the expression of various cell surface markers and studying EBV latent protein pattern of expression, revealed clonal variants from a single hybridisation (Kerr *et al.*, 1992).

EBV B-LCL fused with lymphoid derived cells consistently produced hybrid cells which retained EBV gene expression in the latency III state and displayed cell surface markers typical of the 'activated' B cell phenotype (Kerr et al., 1992; Contreras-Brodin, 1991). Hybrid cells formed between EBV B-LCL and non-lymphoid derived cells were often associated with a switch in the pattern of EBV gene expression from type III latency to either type I or type II latency depending on the EBV negative partner, which Contreras-Brodin et al. also associated with the extinguished expression of the activated B cell markers, MHC class II antigens and Ig gene expression (Contreras-Bodin et al., 1991; Kerr et al., 1992). In these studies, EBV B-LCL fused with HeLa (cervical carcinoma), SVAT5B1 (fibroblast) or K562 (erythroleukaemia) showed a type II pattern of EBV latent gene expression and did not have a dominant EBV B-LCL phenotype. Fusion of EBV B-LCL with K562 produced hybrids which had either the EBV B-LCL phenotype as the dominant cell phenotype or the non EBV B-LCL phenotype from a single hybridisation (Kerr et al., 1992). This was also true for hybrid cells formed between EBV B-LCL with HL60 (promyelocytic leukaemia). Again, EBV latent protein expression was dependent on the resulting dominant cell phenotype. EBV B-LCL dominant hybrid phenotypes retained EBV latency III pattern of gene expression and non EBV B-LCL dominant hybrid phenotypes expressed a latency I or II phenotype. These results showed that changes in cellular transcription factors are associated with hybrid cell formation. All hybrid cell lines produced between EBV B-LCL and EBV negative cells retained the viral genome and expressed at least EBNA-1 (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1991).

The hybrid cells generated by Kerr and colleagues and Contreras-Brodin and coworkers have highlighted the problems associated with random fusion and have shown that in addition to the existence of morphological heterogeneity within a hybrid cell population, changes in cellular transcription can result. Thus, there is a need for both cloning and characterising hybrid cells *in vitro* prior to using EBV B-LCL x tumour cell hybrids as agents in tumour immunotherapy.

3.1.5 Aims of this Work

The work presented in this chapter sought to demonstrate the feasibility of producing EBV B-LCL x melanoma cell hybrids using PEG as the chemical fusogen, and to confirm the hybrid nature of such cells. Confirmation of the hybrid nature of the cells was achieved in a number of ways. The first indicator of a cell's fused status was its ability to grow in double chemical selective media. The hybrid status was then confirmed by HLA genotyping, cell surface phenotyping, and the presence of the EBV viral genome by the hybrid cells.

Since there is scope for genetic loss during hybrid formation and as the cellular hybridisation is essentially a random process, hybrid cells were cloned and carefully characterised. EBV promoter switching can also occur when the differentiated cell phenotype is changed, for example when somatic cells hybrids are formed using EBV B-LCL as a fusion partner and the latent protein pattern of expression may be altered. EBV presence and EBV protein expression thus needed to be determined for all hybrid cells generated in the study. Lytic viral infection also needed to be investigated, as during lytic infection, EBV produces proteins which have homology to cellular proteins. The capacity for EBV B-LCL x melanoma hybrid cells to retain the expression of tumour-associated antigens is covered in chapter 5.

The presence of both parental HLA types in chemically selected and cloned hybrid cells was taken as further proof of their hybrid nature. Finally, hybrid cells should resemble APCs and possess both MHC class I and class II antigens and have the relevant co-stimulatory and adhesion molecules necessary to stimulate an effective T cell response
(Dunnion *et al.*, 1999). Hybrid cells must therefore be phenotypically characterised *in vitro* in order to identify those which may be of use in tumour immunotherapy.

3.2 Results

3.2.1 Hybrid Cell Generation

The cell line termed HMy2 was used as the universal EBV B-LCL parent in all hybridisations. This cell line has a HAT sensitive, ouabain resistant phenotype enabling it to be used as a parent cell line in somatic cell hybrids, and has been described previously (Edwards *et al.*, 1982). The human derived, EBV negative melanoma cell lines used as the tumour cell parents in a fusion were Gerl43, 518.A2 and DAUV. These cell lines, like normal cells, are sensitive to ouabain but are able to grow in HAT supplemented medium.

3.2.1.1 Viability Tests to Determine Hybrid Cell Selection Medium

Since cells to differ in their sensitivity to ouabain, viability tests with a range of ouabain concentrations were performed in order to predetermine the concentration of ouabain necessary for total killing of parent melanoma cells in the hybrid cell selection process. The concentration of ouabain used in the selective medium to kill the 518.A2 melanoma parent cells was 2.5x10⁻⁶M and that required to kill the Gerl43 and DAUV parent cells was 5x10⁻⁷M. The mutated EBV B-LCL cell line HMy2 was able to grow in growth media supplemented with ouabain at both of these concentrations of ouabain. HMy2 cells were reported to die in 2% HAT (Edwards, 1982). This was tested and confirmed. It was noticed that these cells took up to 10 days to die in growth medium containing 2% HAT. The melanoma cell lines were able to grow well in medium supplemented with 2% HAT.

3.2.1.2 Preliminary Experiments To Determine the Conditions Required for Cell Hybridisation

Preliminary experiments were performed on cell cultures in order to determine favourable conditions for somatic cell hybrid production (data not shown). The dilution of PEG is a crucial stage in the production of hybrid cells. The rate of PEG dilution was altered, as was length of time cells were exposed to the toxic effects of PEG and the effects of FBS on the dilution of PEG. Another parameter investigated was the pH of PEG and its effects on the yield of hybrids. In each instance, one parameter was altered to the protocol described in section 2.3. Three methods to assess hybrid cell formation were then employed. Firstly, a sample of recently PEG treated cells were observed microscopically and the number of hybrid cells formed were counted. Hybrid cells could be distinguished visually by their morphologic appearance (Li and Hui, 1994). Secondly, recently treated cells were plated at one cell per well in selective media in 96 well plates and the number of wells showing cell growth determined, so only viable hybrid cells were counted. Thirdly, two colour immunofluorescence was used as a measure of hybrid cell production. A marker expressed on the surface of each parent cell line only was chosen for mAb labelling cells and flow cytometric analysis. This would enable heterologous hybrid cells to be distinguished from homologous hybrid cells.

Each method utilised for determining fusion yield was hampered with various problems. It was possible microscopically to identify hybrid cells by their appearance due to the formation of a lumen bridge connecting two cells and due to the appearance of markedly enlarged cells. It was not possible to distinguish those hybrids formed between the same parent cell lines (homokaryons) and those formed between two different cell lines (heterokaryons). In addition, EBV B-LCL grow as tight clumps due to the high level of expression of cell adhesion molecules, and dividing cells also hindered the true assessment of the fusion yield. As fusion yield does not necessarily correlate with hybrid survival, many cells with fused membranes do not survive to the stage of nuclear division.

In order to overcome these problems with estimating fusion yield, cells were cloned immediately following PEG treatment. Low fusion yields were obtained following this method for determining hybrid cell survival. This may have been due to the stress on cells by seeding one cell per well in double chemical selective media. In hind sight, it may have been more advantageous to seed cells at increased numbers per well as described by Krenn *et al.* (Krenn *et al.*, 1995). Furthermore, two colour flow cytometry proved to be an unsuccessful way of monitoring hybrid cell generation in this study. This was due to the very low percentage of fused cells, meaning it was not possible to accurately quantify dual labelled cells.

To enhance cellular fusion, an alternative approach to hybrid cell generation was employed. A trial employing PEG combined with electrofusion was performed based on the work of Li and Hui (Li and Hui, 1994). This method aimed to combine the advantages of PEG with electrofusion, but was not successful and did not produce any fused cells able to grow in selective media. Tests using this approach however, were limited.

The preliminary experiments (data not shown) however, produced cells between HMy2 with 518.A2, Gerl43 and DAUV human derived melanoma cell lines which grew in

selective media following the protocol outlined in section 2.3. Until the cells had been confirmed as true hybrid cells by the presence of both parental HLA types, phenotypic characteristics inherited from both parents or the presence of the EBV genome, they were referred to as (chemically) 'selected cells', meaning the cells had survived the hybrid cell chemical selection process.

3.2.1.3 The Generation of Chemically Selected Cells Between HMy2 and Melanoma Cell Lines

Chemically selected cells were generated between HMy2 and 518.A2. The pH of the 50% w/v PEG solution in serum free medium was pH 8.2 and the protocol described in section 2.3 was used. It took 60 days before any cell growth was noticed in the selection medium. PEG solution at pH8.2 yielded cells able to grow in selective media for HMy2 x Gerl43 and also pH8.2 for the HMy2 x DAUV hybrid, although it took 120 days before cell growth in selection media was noticed in both cases.

After an initial growth in tissue culture flasks, cells were transferred to 48 well tissue culture plates and seeded at one cell per well in selective media. Growth in wells could be observed after at least two weeks. At this cell seeding concentration, plates showing less than 30% of wells showing growth was taken as representing clonal populations. These clones were phenotypically analysed, and at least one clone from each hybridisation experiment was chosen to be investigated in greater detail. HMy2 x DAUV was not cloned, and work carried out using these cells was done on an uncloned or 'bulk' culture, as preliminary analysis suggested that these cells were not true hybrids in spite of growing in selective medium.

3.2.2 Cell Morphology and Growth Pattern

HMy2 x 518.A2, HMy2 x Gerl43 and HMy2 x DAUV grew as adherent monolayers like the melanoma parent cells. Cellular morphology was similar for all selected clones as compared with the relevant tumour parent cell line (Figures 3.1, 3.2 and 3.3). The growth kinetics of HMy2 x 518.A2, HMy2 x Gerl43 and HMy2 x DAUV selected cells in double chemical selective media over an extended period of time *in vitro* were similar to the growth of the melanoma parent cells. Selected cells were grown routinely in selective media in order to preserve the cell phenotype. Like both parent cells, the selected cells recovered well from freezing in 10% DMSO in FBS and long term

storage in liquid nitrogen. Hybrid cells which grew like the HMy2 parent cell line in suspension (Figure 3.4) were not produced.

3.2.3 HLA Genotyping

3.2.3.1 Method Chosen

Hybrid cells and the parent cell lines were HLA genotyped by SSP-PCR using a method termed Phototyping (Bunce *et al.*, 1995). To achieve complete typing, many sets of primer mixes are required. The level of resolution of HLA specificities used in this study was lowered, but remained suitable for the purpose of allowing hybrid status to be genetically determined. This method, unlike serology, does not assess whether these molecules are being expressed on the cell surface. An example of an HLA-B genotype is shown in Figure 3.5.

3.2.3.2 HLA Genotypes of Parent and Hybrid Cells

Total DNA was extracted from parent (518.A2, Gerl43 and DAUV) and uncloned selected cells (HMy2 x 518.A2, HMy2 x Gerl43 and HMy2 x DAUV) for HLA genotype to be determined. All selected cells possessed the full complement of HLA genes from both parents, except for HMy2 x DAUV which possessed the HLA haplotype of the melanoma parent only (Table 3.1). HMy2 x 518.A2 and HMy2 x Gerl43 hybrid cells at the clonal level were also HLA typed and, like the uncloned bulk culture, possessed the haplotypes derived from both the melanoma and the EBV B-LCL parent cell lines. The cloned cell lines analysed included HMy2 x 518.A2 clone 1, clone 2, clone 3 and clone 4 and HMy2 x Gerl43 clone 3B5.

3.2.4 Cellular Phenotype as Determined by Immunofluorescence and Flow Cytometry

The cell surface phenotypes of selected cells formed between HMy2 and three melanoma cell lines were analysed by mAb labelling followed by flow cytometric analysis. To observe any variation in the expression of cell surface markers, cloned selected cells were analysed alongside the uncloned bulk population of selected cells. Individual HLA antigen expression was restricted to HLA-A1, HLA-A2 and HLA-A3 as these were the only informative HLA specific mAbs available for use in the laboratory.

Cell Line	HLA-A	HLA-B	HLA-DRB1
HMy2	A2, A3	B35, B62/B76	DR4, DR12
518.A2	A1, A2	B8	DR3, DR15
Gerl43	A1, A29	B37, B44	DR1, DR13
DAUV	A9, A28	B44, B13	DR15, DR13/DR11
HMy2 x 518.A2 bulk	A1, A2, A3	B35, B62/B76, B8	DR4, DR12, DR3, DR15
HMy2 x Gerl43 bulk	A1, A2, A3, A29	B35, B62/B76, B37, B44,	DR4, DR12, DR1, DR13
HMy2 x DAUV bulk	A9, A28	B44, B13	DR15, DR13/DR11

Table 3.1. *HLA genotypes as determined by SSP-PCR*. Sequence specific primers were used to determine the HLA genotypes of the selected cells and their relevant parent cell lines. HMy2 x 518.A2 and HMy2 x Gerl43 selected cells retained HLA haplotypes from both parent cell lines.

3.2.4.1 Phenotypic Analysis of HMy2, the Universal EBV B-LCL Parent.

The analysis of the cell surface phenotype of the EBV B-LCL cell line, HMy2, is shown in Figure 3.6. HMy2 expressed the phenotypic markers typical of an EBV B-LCL. Expression of all the markers tested (MHC class I, MHC class II, the co-stimulatory molecules [CD40, CD80 and CD86], adhesion markers [CD11a, CD50, CD54 and CD58] and the B cell lineage specific marker [CD19]) were observed. The cell line also expressed the HLA-A2 and HLA-A3 antigens but not HLA-A1 molecules by staining with the HLA specific mAbs. This was in accordance with the HLA genotype as detected by SSP-PCR. Expression of CD11a on this one occasion was low due to the age of the antibody. Other cell lines used in the study were labelled with new mAb which reproducibly stained HMy2 cells at levels three fold greater than that shown in Figure 3.6.

3.2.4.2 Phenotypic Analysis of 518.A2 and HMy2 x 518.A2 Hybrid Cells

The expression of various cell surface markers by 518.A2 cells is shown in Figure 3.7. The melanoma cell line expressed MHC class I antigens on the cell surface. More specifically, HLA-A1 and HLA-A2 antigens but not HLA-A3 antigens were expressed, as detected with antibodies specific for these HLA-A molecules, consistent with the HLA genotype as determined by SSP-PCR. However, the cells were devoid of MHC class II antigen, CD11a and CD50 expression and the B cell marker CD19. In addition the cells lacked the expression of the co-stimulatory molecules CD80 and CD86. The expression of CD40, CD54 and CD58 was detected.

Treatment of the cells in IFN- γ (data not shown) resulted in a three to four fold upregulation of MHC class I antigen expression. CD54 expression was also upregulated upon culturing the 518.A2 cells in IFN- γ . The expression of the other cell surface markers studied (MHC class II, CD11a, CD40, CD50, CD58, CD80 and CD86), were not affected by IFN- γ pretreatment.

The HMy2 x 518.A2 selected cells were analysed as a bulk culture and ten individual clones were subjected to a preliminary screening. Cells were screened for the expression of MHC class I, MHC class II, CD19, CD40, CD80 and CD86 only, since these molecules are important for providing signal 1 and signal 2 to activate a specific T cell response. This was in order to observe any phenotypic variation between the clones (data not shown).

The selected cells expressed MHC class I antigens and in all instances, individual HLA-A antigens (HLA-A1, HLA-A2 and HLA-A3) from both parental cell lines were detected on the cell surface. This was in accordance with the results from the SSP-PCR HLA genotyping, showing the presence of HLA haplotypes from both parental cell lines. The expression of the MHC class I molecules by the selected cells was never as great as the level detected on the surface of the EBV B-LCL parent cell line, but was as great as or up to two fold greater than the expression of MHC class I antigens by the melanoma parent cell line. All selected cell clones expressed CD40. What was unexpected was the lack of expression of MHC class II antigens by the selected cells, especially since the EBV B-LCL parental HLA-DR antigens had been detected by SSP-PCR, and the HLA-A antigens from both parents were co-dominantly expressed by the selected cells. In spite of being expressed by the EBV B-LCL cell line, the HLA-DR alleles derived from HMy2 were clearly not expressed on the cell surface of the cloned selected cells. Selected cells also consistently lacked the expression of CD19, CD80 and CD86 like the parent melanoma cell line. More detailed analysis of HMy2 x 518.A2 clone 2 is shown in Figure 3.7. HMy2 x 518.A2 clone 2 was chosen for more in depth analysis due to its enhanced stimulatory capacity compared with other cloned selected cell lines (chapter 4). Like the melanoma parent cell line, HMy2 x 518.A2 clone 2 expressed CD54 and CD58 but did not express CD11a and CD50. The cloned selected cells also behaved in a manner similar to the melanoma parent cells after IFN-y pretreatment (data not shown), upregulating the expression of MHC class I molecules three to four fold and also upregulating the expression of CD54. The expression of CD11a, CD40, CD50, CD58, CD80 and CD86 was not affected by IFN-y treatment.

The only observed phenotypic difference between 518.A2 and the hybrid cells was the range of HLA class I antigens expressed by the selected cells. The parental melanoma cell phenotype was dominant over the EBV B-LCL phenotype in ten individual HMy2 x 518.A2 selected cell clones and the uncloned bulk cell culture, with all showing similar cell surface marker expression. The eclipse of the B cell phenotype by hybrid cells formed between EBV B-LCL and non-haemopoetic cells has been reported previously (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1991).

3.2.4.3 Phenotypic Analysis of Gerl43 and HMy2 x Gerl43 Hybrid Cells

Phenotypic characterisation of the Gerl43 cell line is shown in Figure 3.8. The melanoma cell line expressed high levels of both MHC class I and MHC class II molecules. HLA-A1 antigen expression, but not HLA-A2 and HLA-A3 antigens, was detected on the cell surface as expected due the results of the SSP-PCR HLA analysis. The cell line lacked CD19, CD11a, CD50, CD40, CD80 and CD86 expression. The melanoma cells expressed CD54 and CD58. This melanoma cell line differed to the 518.A2 cell line due to its expression of MHC class II antigens and lack of CD40 expression. When cultured in IFN- γ (data not included), the cells upregulated the expression both MHC class I and MHC class II antigens two to three fold. The expression of the other cell surface markers (CD11a, CD40, CD50, CD54, CD58, CD80 and CD86) was not affected by IFN- γ pre-treatment.

Five cloned selected HMy2 x Gerl43 cell lines and a bulk cell culture were screened for the expression of MHC class I, MHC class II, CD40, CD80, CD86 and CD19 (data not shown). In each instance the clones were all found express MHC class I and MHC class II antigens at levels which were either similar to or slightly greater than the melanoma parent cells. The expression of MHC class II antigens was sometimes at a level similar to that seen for the parent EBV B-LCL, whereas the expression of the MHC class I molecules was not. The hybrid cells expressed HLA-A1 (originating from the melanoma parent) and HLA-A2 and HLA-A3 antigens (originating from the EBV B-LCL parent), as expected from the results of the SSP-PCR determining HLA genotype. Using this panel of mAbs, the expression of MHC class II antigens derived from either parent cell lines could not be determined. The selected cells lacked the expression of CD19, CD80 and CD86, like the melanoma parent cell line. The selected cells all expressed CD40 at low levels which was consistent and reproducible. The parent melanoma cell line did not express CD40 as determined by mAb labelling and flow cytometric analysis.

Figure 3.8 shows a more detailed phenotypic analysis of HMy2 x Gerl43 clone 3B5 which was chosen for more in depth analysis (chapter 4). CD54 and CD58 were expressed on the cell surface at levels similar to the melanoma parent. The selected cells did not express CD11a and CD50, like the melanoma parent cell line. Also, like the melanoma parent cell line, culturing the hybrid cells in IFN- γ resulted in a upregulation of MHC class I antigen expression. The expression of MHC class II antigens by this cell line was not affected, suggesting constitutive upregulation of MHC class II expression. There was no

induction of CD11a or CD50 expression by the selected cells following IFN- γ pretreatment, and the level of CD40, CD54 and CD58 expressed on the cell surface was also not affected. It would be interesting to look the expression of the different HLA-DR alleles by the selected cells using specific mAbs, and analyse cloned selected cells for MHC class II expression after IFN- γ pretreatment.

Analysis of HMy2 x Gerl43 selected cells showed the melanoma phenotype was dominant over the EBV B-LCL phenotype, although low levels of CD40 was seen in the hybrid cells. The level of expression of MHC class I and class II antigens and the range of HLA-A antigens expressed by the selected cells differed between the parent melanoma cell line and the selected cells. The dominance of the parent melanoma phenotype by the HMy2 x Gerl43 cells, as also seen in the HMy2 x 518.A2 cells, was in accordance with other results on hybrids formed between EBV B-LCL and non-haemopoetic cells (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1991).

3.2.4.4 Phenotypic Analysis of DAUV and HMy2 x DAUV Cells

The expression of various cell surface markers by the DAUV melanoma cell line is shown in Figure 3.9. The melanoma parent cells expressed MHC class I and MHC class II antigens. The cell line also expressed CD54 and CD58 but lacked the expression of CD40, CD50, CD80 and CD86 and did not express CD19. Culturing the cells in IFN- γ (data not shown), resulted in an upregulation of both MHC class I and MHC class II molecules by two fold. In addition, the cells also upregulated the expression of CD54 but there was no induction of CD40, CD50, CD58, CD80 and CD86 expression.

The HMy2 x DAUV selected cells were analysed only as an uncloned bulk cell culture for the expression of various cell surface markers (Figure 3.9). The selected cells expressed MHC class I antigens like the parent melanoma. MHC class II expression on the selected HMy2 x DAUV cells was down regulated as compared with the parent melanoma cell line. Like the melanoma parent cells, the selected cells expressed CD54 and CD58 but did not express CD40, CD50, CD80 and CD86. In agreement with the results of the SSP-PCR, the HMy2 x DAUV selected cells did not express HLA-A2 or HLA-A3 antigens (derived from the HMy2 parent), since the cells retained the HLA haplotype of the DAUV parent only. The selected cells cultured in IFN- γ behaved in a similar manner to the DAUV parent cells and upregulated the expression of MHC class I, MHC class II, and

CD54 (data not shown). The expression of CD11a, CD40, CD50, CD58, CD80 and CD86 was not affected by culturing the cells in IFN- γ .

Although the HMy2 x DAUV selected cells showed reduced expression of MHC class II antigens, it was upregulated to an extent similar to that seen for the parent melanoma cell line upon culturing the cells in IFN- γ . This suggests MHC class II expression may have been down regulated by culturing the selected cells *in vitro*, or else the selected cells were derived from MHC class II negative cells present in a heterogenous melanoma cell population. There was no indication by analysing the cell surface phenotype through mAb staining and flow cytometric analysis that the selected cells were true hybrids.

3.2.5 EBV Genome and EBV Protein Expression

Four clones (clone 1, clone 2, clone 3 and clone 4) and an uncloned bulk culture from the HMy2 x 518.A2 selected cells were chosen in order to determine the presence of the EBV viral genome and establish the pattern of EBV viral protein expression. From the HMy2 x Gerl43 selected cells, one clone (clone 3B5) was picked alongside the uncloned bulk cell population and an uncloned population of HMy2 x DAUV selected cells were also assessed for the presence and expression of the EBV viral genome. The relevant parental cell lines were also analysed for EBV viral genome and gene expression.

3.2.5.1 Presence of Viral Genome

Hybrid cells and the relevant parent cells were examined for the presence of the EBV viral genome by PCR analysis. As described in section 3.1.3.6, there are two types of EBV, type 1 and type 2 which differ in sequence similarity for EBNA 2, EBNA 3 EBNA 4 and EBNA 6 genes (reviewed in Kieff, 1996). The EBV B-LCL used in this study, HMy2, is known to be infected with type 2 EBV (Kerr *et al.*, 1992).

To distinguish between type 1 and type 2 EBV isolates, primers detecting the EBNA 6 viral gene were used in a PCR reaction. The EBNA 6 gene shows 28% sequence similarity between type 1 and type 2 EBV. Total DNA preparations from each cell line were analysed for the presence of the EBNA 6 gene. Type 2 isolates of EBV could be distinguished from type 1 isolates according to the sizes of the EBNA 6 PCR products amplified, with type 1 giving PCR product of 153bp and type 2 giving a product of 246bp. The control samples, B95.8 containing the type 1 virus (EBV B-LCL carrying the standard

type 1 EBV) and the sample IM12 (an EBV B-LCL derived from an infectious mononucleosis patient by spontaneous outgrowth from lymphocyte cultures without added EBV) containing the type 2 virus, show the difference in PCR product size between the two different EBV types (Figure 3.10). RAMOS (EBV negative BL cell line) was the EBV negative control cell line. RAMOS, B95.8 and IM12 cell samples were gifts from D Croom-Carter, Birmingham.

Figure 3.10 shows the results of the PCR to detect EBV genome. The results showed EBV type 2 was present in the EBV B-LCL, HMy2. The selected cells derived from the fusion of this cell with human derived, EBV negative melanoma cell lines 518.A2 and Gerl43 (but not DAUV) also showed type 2 EBV, giving further evidence of hybrid formation with the EBV B-LCL, HMy2. Two selected cell lines only did not yield EBNA 6 specific PCR products. These were HMy2 x 518.A2 clone 4 and HMy2 x DAUV. Other irrelevant bands in the lanes of these samples show that DNA was loaded, therefore PCR reaction failure, viral gene mutation or the absence of the virus from these cells may explain the negative result. The result itself is surprising since the presence of the virus has been detected in all previously described hybrid cell lines formed between EBV B-LCL and a range of lymphoid and non-lymphoid cells (Kerr et al., 1992; Contreras-Brodin et al., 1991) and therefore indicates these were unfused melanoma cells that had acquired resistance to the chemical selection. However, HMy2 x 518.A2 clone 4 retained both parent HLA genotypes as determined by SSP-PCR, and expressed HLA-A molecules from both parents as determined by flow cytometric analysis, suggesting these cells were EBV negative hybrids. Clearly, further work therefore remains to clarify this issue.

The PCR reaction was not quantitative. The band shown for the type 2 positive control (IM12) is faint which may have been due to a low copy number of EBV by the cell line, or due to the age and repeated freeze-thawing of the sample. Since the PCR reaction was not quantitative, it was also not possible to compare the EBV genome copy number between the individual cloned selected cells and the EBV B-LCL.

In order to confirm these findings, the PCR products were transferred onto nitrocellulose filter and probed with primers directed against the EBNA 6 found in type 1 isolates and with primers directed against EBNA 6 found in type 2 isolates in a Southern blot analysis kindly performed by D Croom-Carter, Birmingham (data not shown). In all instances, when the virus was present in the hybrid cells, EBV type 2 isolates were identified, confirming the initial finding from the PCR reaction. HMy2 x 518.A2 clone 4 and HMy2 x DAUV again were the only hybrid cells tested not to possess EBV using these

specific primers. The melanoma parent cell lines and the negative control cell line were negative for the presence of EBV as expected.

3.2.5.2 Viral Protein Expression

The presence of various viral gene products was assessed by Western blot analysis. The identification of EBNA 1, EBNA 6 and LMP 1 expression was undertaken in order for the type of viral latency to be determined. Latency I is associated with the expression of EBNA 1 only. Latency II is associated with EBNA 1 and LMP 1 and Latency III is associated with the expression of EBNA 1, EBNA 2, EBNA 3, EBNA 4, EBNA 5, EBNA 6, LMP 1 and LMP 2 expression. The expression of BZLF 1 was also assessed in order to determine whether or not the virus was lytic. BZLF 1 encodes for an immediate early protein which is an important transactivator of early viral lytic gene expression.

10% acrylamide SDS PAGE gels were run to look for the presence of LMP 1 and BZLF 1 proteins and 6% acrylamide SDS PAGE gels were run to look for the presence of EBNA 1, EBNA 2 and both type 1 and type 2 forms of EBNA 6. Equal protein concentrations were loaded into the gel wells and this was checked by reversibly staining the protein with Ponceau S. In all cases protein staining was even between the samples.

3.2.5.2.1 Antibody Specificity

All primary antibodies used in the Western blot analysis were monoclonal except that which recognised EBNA 6 type 2, and are listed in Table 2.5. The EBNA 6 type 2 specific Ab was a human serum which had strong reactivity against EBNA 6 type 2 viral antigen. The anti-LMP 1 Ab was a mixture of 4 murine mAbs recognising at least 3 separate determinants on the LMP molecule. Specificity of the antibodies used to determine the expression of EBNA 1 and EBNA 2, EBNA 6, LMP 1 and BZLF 1 was shown by running cell samples containing EBV type 1 and type 2 isolates along side test samples and by also including EBV negative samples. In this case, the type 1 EBV control was the cell line C2+Obaji and the type 2 control was C2+BL16 (both are cord blood cell lines transformed with BL derived EBV strains Obaji and BL16, see Rowe *et al.*, 1987b), both samples were a gift from D Croom-Carter. The melanoma cells which did not contain the EBV genome by PCR analysis and Southern blot detection (section 3.2.5.1) were included as negative controls, and were confirmed as negative for EBV protein expression.

3.2.5.2.2 EBNA 1 Expression

The protein EBNA 1 is ubiquitously expressed in all cells infected with EBV, being required for viral genome maintenance and viral replication. The results of the Western blot analysis showed those hybrid cells which retained the presence of the EBV genome also expressed the EBNA 1 gene (Figure 3.11). This is in accordance with other groups who showed EBNA 1 is consistently expressed in hybrid cells regardless of the dominant hybrid cell phenotype (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1991). EBNA 1, as expected, was also expressed in HMy2 and the positive controls. HMy2 x 518.A2 clone 4 and HMy2 x DAUV did not express EBNA 1. This consolidates the previous findings demonstrating the virus was not present in these cells (section 3.2.5.1).

The EBNA 1 specific bands for C2+Obaji, C2+BL16 and HMy2 show typical size variations as different strains of EBV express different EBNA 1 protein sizes. The EBNA proteins have repeat regions which differ in number between viral isolates, producing different sized proteins which can be visualised on immunoblots. Progeny from an EBV infected cell all have the same number of repeat elements. HMy2, HMy2 x 518.A2 and HMy2 x Gerl43 have equal sized EBNA 1 bands (although smaller breakdown products are also visible for HMy2 x Gerl43) indicating infection by an identical EBV strain.

Although not quantitative, comparing the band intensities visually gives an indication of the level of protein expression. EBNA 1 was highly expressed by the parent EBV B-LCL cell line HMy2, and at a level similar to the positive cell controls (C2+Obaji and C2+BL16). HMy2 x 518.A2 clone 1, clone 2, clone 3 and the uncloned bulk culture did not express EBNA 1 at levels as high as the parent EBV B-LCL, and even between these cloned cell lines and the bulk culture, there was variation in the level of expression of this protein. HMy2 x 518.A2 clone 2 produced a low intensity EBNA 1 specific band on the immunoblot whereas HMy2 x 518.A2 produced a strong EBNA 1 specific band. On the other hand, HMy2 x Gerl43 clone 3B5 and the uncloned cell culture expressed EBNA 1 at levels which were very similar to the parent EBV B-LCL. EBNA 1 expression by HMy2 x Gerl43 selected cells was detected as the full size protein plus smaller breakdown products. Kerr et al. also addressed the level of EBV protein expression by hybrid cells and compared them with the relevant parent cell lines. EBNA 1 expression was found to be lower in the non EBV B-LCL like hybrid cells compared with the parent EBV B-LCL for reasons unknown. This finding was similar to that of Contreras-Brodin (Contreras-Brodin et al., 1991). The discrepancies between the work of Kerr and co-workers (Kerr et al., 1992) and Contreras-Brodin and colleagues (Contreras-Brodin et al., 1991) with the

results for the level of EBNA 1 expression by the HMy2 x Gerl43 selected cells is not fully explicable.

3.2.5.2.3 EBNA 2, EBNA 6, LMP 1 and BZLF 1 Expression

Samples were probed with both EBNA 6 type 1 and type 2 specific antibodies. The expression of EBNA 6 was not detected in any of the selected cells but was visible in HMy2, being recognised by the type 2 Ab only and in the positive controls (data not shown). EBNA 2 expression was not detected in the selected cells, but again was detected in HMy2 and the positive control samples (data not shown). LMP 1 expression was detected in HMy2 and the positive control cell lines, but not in any of the selected cells (Figure 3.11). The faint marks on the immunoblot in the region of the LMP 1 protein for the selected cells were non-specific bands which were present in both EBV positive and EBV negative samples, and were not the same size as the LMP 1 protein found in HMy2. The presence of BZLF 1 was not identified by the BZ.1 Ab in the negative control (C2+BL16), HMy2 or the selected cells (data not shown), but was present in the positive control sample, C2+Obaji (data not shown).

The results of the EBV latent protein analysis have shown a latency III state of viral protein expression existed in the parent EBV B-LCL. The viral strain in this cell line was not lytic. Except for HMy2 x 518.A2 clone 4 and HMy2 x DAUV, all selected cells also showed EBV gene expression. Virus latent protein analysis showed the selected cells maintained a type I state of viral latency. The virus in these cells was not lytic.

3.3 Discussion

3.3.1 The Generation and Characterisation of Hybrid Cells for Potential Use in Tumour Therapy

Hybrid cells formed between APC and tumour cells have the potential for use as immunogens in cancer therapy, providing the fused cells retain the APC phenotype and process and present tumour-associated antigens effectively to T cells (reviewed by Stuhler *et al.*, 1998). Previous studies have demonstrated the need for careful phenotypic characterisation of hybrid cells due to chromosome loss and changes in the balance of cellular transcription factors (Lem *et al.*, 1988; Tripputi *et al.*, 1988; Bergman *et al.*, 1988). Other studies have shown the potential to derive cells with differing dominant phenotypes from a single hybridisation (Kerr *et al.*, 1992; Dunnion *et al.*, 1999). Based on these

findings, hybrid cells formed between EBV B-LCL and melanoma cells were generated and characterised *in vitro* for surface phenotype and EBV gene expression.

The potential to fuse the human derived, EBV negative melanoma cell lines, 518.A2 and Gerl43 with the EBV B-LCL cell, HMy2, to form stable hybrid cells, using PEG as the chemical fusogen, was shown. The fused cells were unequivocal hybrids due to a number of features of these cells (described later). Although the HMy2 x DAUV selected cells grew in double chemical selective media, they were not deemed true hybrids since the cells failed to fulfil the criteria (HLA genotypes from both parents and the presence of the EBV genome) to confirm hybrid cell status in this study.

3.3.2 HMy2 x DAUV Selected Cells

The HMy2 x DAUV selected cells were able to grow in double chemical selective media, which killed unfused parent cells and those cells fused between the same type. In spite of this, no other evidence showed the selected cells were true hybrids. The selected cells were morphologically and phenotypically similar to the melanoma parent cells. In addition, the selected cells did not contain the EBV genome. Studies by Kerr *et al.* and Contreras-Brodin *et al.* have shown all EBV B-LCL x somatic cell hybrids retain the viral genome (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1992; Contreras-Brodin *et al.*, 1991). Genetic analysis of the selected cells also showed the cells possessed the HLA genotype of the melanoma parent cell line only. The results imply either extensive genetic loss during hybrid cell formation or a subpopulation of unfused melanoma cells have survived the selection process. Karyotype analysis is used extensively in hybrid cell studies to confirm the fused cell status. It was not performed in this study due to the cost of having the samples sent away for testing. In addition, tumour cells are aneuploid which makes karyotype analysis difficult. In light of this evidence and the uncertain hybrid nature of the HMy2 x DAUV cells, further studies using these cells was limited.

3.3.3 HMy2 x 518.A2 and HMy2 x Gerl43 Hybrid Cell Confirmation

The first indicator of the hybrid nature of the HMy2 x 518.A2 and HMy2 x Gerl43 cells was their ability to grow in double chemical selective media which killed the unfused parent cell lines and homokaryons. Confirmation that the cells were hybrids was obtained at the genetic level as these cells were cloned and found to contain the HLA class I and HLA class II genotypes of both parent cell lines. Furthermore, the hybrid cells co-expressed the HLA-A antigens from both parent cells as detected by mAb labelling and

flow cytometric analysis. The EBV genome and EBV gene expression was also detected in all but one of the cloned hybrid cell lines and the uncloned bulk culture. The parent melanoma cell lines were negative for the presence of the EBV genome.

3.3.4 HMy2 x 518.A2 and HMy2 x Gerl43 Morphology and Phenotype

Like the parent melanoma cells, the hybrid cells grew as an adherent monolayer *in vitro*. Hybrid cells showing morphology and a growth pattern similar to the parent EBV B-LCL, which grew in tight clumps in suspension, were not produced. The morphology of other EBV B-LCL x adherent cells has been documented (Contreras-Brodin *et al.*, 1991; Kerr *et al.*, 1992). The adherent parent cells used in the production of these hybrids included fibroblast and cervical carcinoma cells, both of which formed hybrid cells with EBV B-LCL that had a dominant fibroblast appearance or a dominant epithelial appearance respectively. The data from this study are consistent with these findings.

In addition, hybrid cells with a cell surface marker phenotype similar to the EBV B-LCL parent were not produced in this study. The EBV B-LCL x melanoma hybrid cells did not express B cell specific surface markers. Like the hybrid cells produced in this study, the EBV B-LCL x fibroblast and EBV B-LCL x cervical carcinoma hybrid cells produced elsewhere were also associated with the extinction of the B cell markers (Kerr et al., 1992; Contreras-Brodin et al., 1991). It is of interest that MHC class II antigen expression was not induced in the EBV B-LCL x 518.A2 hybrid cells, especially since the cells co-dominantly expressed HLA-A antigens derived from both parent cell lines, and the MHC class II genes were expressed in the EBV B-LCL parent cells. EBV B-LCL x cervical carcinoma hybrid cells generated in a separate study also expressed MHC class I antigens but did not express MHC class II antigens (Contreras-Brodin et al. 1991). There is no documented evidence to show expression of MHC class II genes inherited from the tumour parent can be induced in hybrid cells following fusion of EBV B-LCL with somatic cells. MHC class II expression can be induced on a variety of cells including melanocytes following IFN-y treatment and has also been detected on the surface of melanomas, so the reason for this lack of expression of MHC class II antigens by the HMy2 x 518.A2 hybrid cells is not known. However, MHC class II regulation is complex and involves the interaction of a variety of cis and transacting regulatory factors which differ between cell types (reviewed by Rohn et al., 1996). CD80 and CD86 expression, which is normally restricted to APC only, was also not detected on either of the hybrid cells produced in this study. Normal immune function requires the appropriate constitutive and inducible expression of MHC class II antigens and CD80 and CD86 co-stimulatory molecules for the activation of naïve antigen specific helper T cells. It is likely that fusion of EBV B-LCL with melanoma cells has changed the balance of transcription factors and this has subsequently resulted in the transcriptional repression of various genes, including MHC class II, CD80 and CD86. Other studies have shown hybridisation of cells can result in the repression of tissue-specific genes (Lem *et al.*, 1988; Tripputi *et al.*, 1988; Beggs *et al.*, 1988). The expression of CD40 (albeit at low levels) was induced on the HMy2 x Gerl43 hybrid cells even when it was not expressed by the parent melanoma cell line, and was upregulated on the HMy2 x 518.A2 hybrid cells as compared with the parent melanoma. This molecule is expressed on a range of cell types, including professional APCs, eosinophils, endothelial cells, fibroblasts and carcinomas. CD40 interaction with CD40L is thought to play a more general role in immune regulation than CD80 / CD86 interaction with CD28 / CTLA (reviewed in van Kooten and Banchereau, 2000).

This study has therefore demonstrated the potential to alter gene expression through fusion of EBV B-LCL with melanoma cells. For EBV B-LCL x tumour cells hybrids to be of use in tumour immunotherapy, tumour-associated antigen expression must be maintained to drive a tumour specific immune response. This is addressed in chapter 5.

3.3.5 LCL-Like Phenotype Versus Non-LCL (Tumour)-Like Phenotype

Fusion of EBV B-LCL with EBV negative somatic cells produced hybrid cells which had either an LCL-like phenotype or a non-LCL-like phenotype, depending on the EBV negative somatic cell partner (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1991; Dunnion *et al.*, 1999). Dunnion *et al.* further addressed the differences in the stimulatory potential of LCL-like hybrids and non-LCL-like hybrids formed between HMy2 and a promyelocytic leukaemia, HL60. LCL-like hybrid cells which expressed high levels of MHC class I, MHC class II, CD40, CD80 and CD86 were highly stimulatory *in vitro* MTLC assays. In contrast, non-LCL-like hybrid cells did not express high levels of these cell surface markers and were poorly stimulatory in MTLC assays. Souberbielle have added to these finding and shown hybrid cells formed between murine derived M\u00f6 and a murine derived melanoma cell line had a melanoma-like phenotype and were poorly immunogenic in mice (Souberbielle *et al.*, 1998). The stimulatory potential of the hybrid cells formed between HMy2 with melanoma cell lines was therefore called into question,

since the hybrid cells also have a melanoma-like phenotype and lacked the expression of the co-stimulatory ligand molecules CD80 and CD86. The ability of the EBV B-LCL x melanoma hybrid cells to stimulate T cell responses *in vitro* is addressed in Chapter 4.

3.3.6 EBV Transformation of B Cells and Viral Protein Expression

EBV has the capacity to transform B cells into permanently growing cell lines. From a hybrid cell vaccine point of view, EBV B-LCL are good APC partners for fusion with tumour cells, since these cells are robust, grow well in culture and provide a continuous source of APC for use as fusion partners in immunotherapy. EBV gene expression is limited in B cells, and protein expression is associated with type III latency. Fusion of EBV B-LCL with non-lymphoid somatic cells can alter viral gene expression to type I or type II latency, depending on the somatic cell fusion partner and the dominant hybrid phenotype. EBV B-LCL fused with lymphoid somatic cells consistently produced hybrid cells with an LCL-like phenotype and type III EBV latent protein expression. Maintenance of type III EBV latency is likely to require B cell specific transcription regulatory factors to drive the expression of EBNA 1 and EBNA 2 to EBNA 6 from the BamHI W or BamHI C promoters (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1991). In this study, HMy2, as expected, expressed viral proteins associated with type III latency.

EBV genome was detected in the bulk hybrid cell culture and all but one of the cloned hybrid cells, a discrepancy between this study and the results of Kerr *et al.* and Contreras-Brodin *et al.* (Kerr *et al.*, 1992; Contreras-Brodin *et al.*, 1991). These groups both showed all hybrid cells formed between EBV B-LCL and a range of EBV negative somatic cells retained the EBV genome derived from the parent EBV B-LCL and expressed at least EBNA 1. At present it is not known why HMy2 x 518.A2 clone 4 cells did not contain the EBV genome. HMy2 x 518.A2 clone 4 was proved to be a true hybrid by HLA genotyping and the cells also co-dominantly expressed HLA-A antigens derived from both parent cell lines, therefore the data suggest HMY x 518.A2 clone 4 represents and EBV negative hybrid cell.

The B cell phenotype was not maintained by EBV B-LCL x melanoma hybrid cells, and the EBNA 1 protein was the only latent protein studied to be expressed by the hybrid cell infected with EBV, indicating type I EBV latency. This is in agreement with the findings of Kerr *et al.* and Contreras-Brodin *et al.* who also showed EBV B-LCL fused with non-B cells did not express the full spectrum of EBV latent proteins if the EBV B-LCL phenotype was not maintained.

Kerr et al. and Contreras-Brodin et al. also showed the level of EBNA 1 expression by the hybrid cells was consistently lower in the hybrid cells compared with the EBV B-LCL. In this study, although this finding was true for the HMy2 x 518.A2 hybrid cells, the HMy2 x Gerl43 hybrid cells expressed EBNA 1 at a level comparable to the parent EBV B-LCL. There was also variation in the level of EBNA 1 expression between the various clones HMy2 x 518.A2 cells. The reasons for the varying level of protein expression between the hybrid cell lines was not known. It may have been due to the number of viral episomes per cell. Quantitative PCR analysis to determine the presence of the viral genome would allow this to be determined. However, cell lines containing a single EBV genome can express EBNA 1 to levels seen for EBV B-LCL (Contreras-Brodin et al., 1991). BL cell lines often express EBNA 1 at lower levels compared with isogenic clones that have progressed to type III latency (Gregory et al., 1990). EBNA 1 expression only is typically associated with the BamHI F promoter, whereas expression of EBNA 1 alongside the other EBNA proteins is associated with BamHI C or BamHI W promoter usage. A hybrid formed between HMy2 and HL60 was found unusually to express both BamHI W and BamHI F transcripts (Kerr et al., 1992) demonstrating this promoter usage rule in hybrid cells may not always exist. Differences in viral promoter usage, gene regulatory factors and viral episomal copy number may all contribute to the varying levels of EBNA 1 expression by the hybrid cells. Promoter usage by the hybrid cells in this study could be assessed by analysing EBV latent gene transcripts using RT-PCR as described elsewhere (Kerr et al., 1992).

3.3.7 Problems Associated With EBV B-LCL x Melanoma Hybrid Cell Generation

This chapter has highlighted several problems associated with making EBV B-LCL x melanoma cell hybrids using PEG as the chemical fusogen. Fusion efficiency was low, and it took 4 months on two occasions before hybrid cell growth was observed. In spite of the attempts to optimise the protocol by changing various experimental parameters, the results were limited and the fusion efficiency was not substantially improved. An alternative approach for hybrid cell generation was employed combining electrofusion with PEG, yet again, no enhancement of fusion efficiency was observed.

This presents problems in a clinical setting using patient samples where only a small number of cells may be available for use. Technically, this problem could be overcome by employing electrofusion as the method for inducing cellular fusion. This approach has been used successfully by a number of groups (Stuhler and Walden, 1994.

Scott-Taylor *et al.*, 2000), and has been shown to result in higher fusion efficiencies under appropriate conditions. Equipment necessary for high efficiency electrofusion was not available for this study.

The hybrid cell chemical selection method used in this study may not have been entirely effective, as it seems a sub-population of unfused DAUV melanoma cells survived selection in spite of the preliminary experiments on ouabain killing concentrations. It may be that harsher selection conditions should have been employed, for example increasing the ouabain concentration up to 50% greater than that which killed the parent cells rather than the 30% increase used in this study, in order to prevent tumour cell outgrowth.

3.3.8 Hybrid Cells for Use in Tumour Immunotherapy

This chapter has shown some of the inherent difficulties in fusing EBV B-LCL with melanoma cells. Hybrid cells with the APC phenotype were not produced in this study. Since the procedure was performed with 2 melanoma cell lines, and as other work has also shown that EBV B-LCL fused with non haemopoetic cells can produce hybrid cells with a non B cell phenotype, this presents problems in the production of hybrid cells with an APC phenotype for use in tumour immunotherapy. Nevertheless, hybrid cells with an APC phenotype have been produced using haemopoetic cell lines (Contreras-Brodin *et al.*, 1991; Kerr *et al.*, 1992) which were highly stimulatory in *in vitro* studies (Dunnion *et al.*, 1999).

3.4 Future Work

Although this chapter has shown the feasibility for producing stable EBV B-LCL x melanoma hybrid cells, it has also highlighted some of the problems associated with producing hybrid cells using PEG as the chemical fusogen. This procedure should be optimised, especially if patient samples were to be used and only a small number of cells are available for use. Optimisation of this procedure could be done by labelling lipid membranes with fluorescent lipophilic dyes and microscopically determining the percentage of cells fused. Alternatively, electrofusion could be the method of choice, since it has been reported as more efficient than chemically induced cell fusion. Fusion efficiency was greater 90% when assessed by 2 colour flow cytometry and 30-40% when observed microscopically by Stuhler and Walden after electrofusing thymoma cells with B cell lymphoma cells (Stuhler and Walden, 1994), and fusion efficiency exceeded 30% by

combining a direct current with an electric pulse in a study by Scott-Taylor *et al.* (Scott-Taylor *et al.*, 2000).

This chapter has also shown that there are problems associated with fusing EBV B-LCL with melanoma cells in that an APC phenotype was not maintained. Souberbielle and colleagues have fused murine $M\phi$ with tumour cells and observed the same eclipse of the APC phenotype. In order to overcome this problem, melanoma cells have been fused with dendritic cells with promising results, but these cells were not characterised at the clonal level (reviewed by Hart and Colaco, 1997). Dendritic cells, the most potent APC, have the capacity to prime naïve T cells, but there are problems associated with using dendritic cells in that these cells are difficult to maintain in culture and they proliferate slowly, if at all. Purification of DCs is also hindered by the small number of cells available. Only 0.1-1% of the total PBMC population and 1% of the total spleen cells are DCs. In contrast, EBV B-LCL grow continuously and easily *in vitro*, producing large numbers of patient specific cells for use in therapy.

Unanswered Questions

Why were CD80, CD86 and MHC class II antigens not expressed on the cell surface by the HMy2 x 518.A2 hybrid cells and CD80 and CD86 not expressed by the HMy2 x Gerl43 hybrid cells?

Why did HMy2 x 518.A2 clone 4 not contain the EBV genome when all other EBV B-LCL x melanoma hybrid cells did?

Why was EBNA 1 expressed at a level similar to the EBV B-LCL in HMy2 x Gerl43 when in HMy2 x 518.A2 it was not?

Is it possible to change the phenotype of the hybrid cells to resemble that of the APC by simple *in vitro* manipulation?



Figure 3.1. Morphology of melanoma and EBV B-LCL x melanoma hybrid cells. A) 518.A2
magnification x300. B) 518.A2 magnification x1200. C) HMy2 x 518.A2 clone 2
magnification x300. D) HMy2 x 518.A2 clone 2 magnification x1200.



Figure 3.2. Morphology of melanoma and EBV B-LCL x melanoma hybrid cells. A) Gerl43 magnification x300. B) Gerl43 magnification x1200. C) HMy2 x Gerl43 clone 3B5 magnification x1200.



Figure 3.3. Morphology of melanoma and EBV B-LCL x melanoma selected cells. A) DAUV magnification x300. B) DAUV magnification x1200. C) HMy2 x DAUV (uncloned) magnification x300. D) HMy2 x DAUV (uncloned) magnification x1200.



Figure 3.4. *EBV B-LCL cellular morphology.* **A)** HMy2 magnification x300. **B)** HMy2 magnification x1200.





Figure 3.5. An example of HLA-B genotyping using the SSP-phototyping method. Samples were loaded into the wells of an agarose gel an electrophoresed in the direction from top to bottom. Bands were visualised with ethidium bromide and sizes were compared with a 1 kb ladder. Lanes 5, 27, 28, 30 and 31 were positive with band sizes of 606, 124, 421, 128 and 389 bp respectively. These bands show that the HLA genotype of this cell line (HMy2 x 518.A2 clone 2) was HLA-B8 (lane 5), HLA-B62/76 (lanes 27 and 28) and HLA-B35 (lanes 30 and 31). HLA-B8 was derived from the 518.A2 parent and HLA-B62/76 and HLA-B35 were derived from the HMy2 parent (see Table 3.1). The internal control gave rise to a 796bp fragment from the third intron of HLA-DRB1.



Figure 3.6. *Phenotypic characterisation of EBV B-LCL cells.* Cell surface expression of MHC class I and class II antigens, CD19 (B cell marker), CD11a, CD50, CD54 and CD58 (adhesion molecules) CD40, CD80 and CD86 (co-stimulatory ligand molecules) on HMy2 cells. Open areas are stained with specific mAbs. Solid areas are stained with secondary mAb only. The x-axis represents arbitrary fluorescence units and the y-axis shows relative cell number.



Figure 3.7. *Phenotypic characterisation of parent (518.A2) and selected cells (HMy2 x 518.A2 clone 2).* Cell surface expression of MHC class I and class II antigens, CD19 (B cell marker), CD11a, CD50, CD54 and CD58 (adhesion molecules) CD40, CD80 and CD86 (costimulatory ligand molecules) on 518.A2 and HMy2 x 518.A2 clone 2 cells. Open areas are stained with specific mAbs. Solid areas are stained with secondary mAb only. The x-axis represents arbitrary fluorescence units and the y-axis shows relative cell number. (Continued overleaf).

Figure 3.7 Cont.....





Anti-CD80

0

100

Counts

Sec. 2

Anti-CD86

0

100

Counts

60



Figure 3.8. Phenotypic characterisation of parent (Gerl43) and selected cells (HMy2 x Gerl43 clone 3B5). Cell surface expression of MHC class I and class II antigens, CD19 (B cell marker), CD11a, CD50, CD54 and CD58 (adhesion molecules) CD40, CD80 and CD86 (co-stimulatory ligand molecules) on Gerl43 and HMy2 x Gerl43 cells. Open areas are stained with specific mAbs. Solid areas are stained with secondary mAb only. The x-axis represents arbitrary fluorescence units and the y-axis shows relative cell number. (Continued overleaf).

Figure 3.8 Cont... Anti-CD86 Anti-CD50 Anti-CD40 Anti-CD11a Anti-CD80 Anti-CD58 Anti-CD54 Counts Counts Counts Counts Counts Counts Counts 10^e FL1-Height Gerl43 FL1-Height 10² FL1-Height 10⁻⁻ FL1-Height 10⁴ FL1-Height FL1-Height FL1-Height 10:5 Counts Counts Counts Counts Counts Counts Counts in hun han h HMy2 x Gerl43 clone 3B5 10-1 10⁴ FL1-Height 10⁴ FL1-Height 10² FL1-Height 10² FL1-Height 10² FL1-Height FL1-Height 10² FL1-Height







Chapter 4

A Comparison of the Immunostimulatory Capacity of Hybrid Cells with the Parent Cell Lines *In Vitro*, and the Role of CD80 Co-stimulation of T cell Responses

A Comparison of the Immunostimulatory Capacity of Hybrid Cells with the Parent Cell Lines *In Vitro*, and the Role of CD80 in Co-Stimulation of T Cell Responses

4.1 Introduction

Evaluating vaccination strategies *in vitro* or in animal models prior to establishment in clinical setting is of great importance. Since there is no direct animal equivalent of EBV B-LCL, *in vitro* functional studies were performed in order to determine the potential use of EBV B-LCL x melanoma hybrid cells as candidates in tumour immunotherapy. For use in tumour therapy, the hybrid cells must show enhanced immunostimulatory capabilities as compared with the parent tumour cells and induce a specific T cell response. In addition, the hybrid cells must retain the capacity to process and present antigen to antigen-specific, HLA-class I restricted T cells (described in chapter 5).

4.1.2 T Cells and T Cell Co-Stimulation in Tumour Immunotherapy

The majority of tumours express antigens which the immune system is capable of recognising as foreign (discussed in section 1.6). However, tumour growth and progression occurs in vivo, suggesting tumour cells fail to present antigens effectively. Naïve T cell activation requires signal 1, induced by the engagement of MHC/antigen complexes with antigen-specific TcR and co-stimulation (signal 2), involving the interaction of additional molecules on the surface of the T cells and APC (section 1.5.1). TcR engagement in the absence of co-stimulation induces T cell non-responsiveness, where the T cell is unable to respond to further stimulation (Jenkins and Schwartz, 1987). CD80/CD86 binding with CD28/CTLA-4 is one of the most important interactions in T cell co-stimulation, since ligation of CD28 prevents T cell anergy (June et al., 1994). Costimulatory signals do not necessarily have to be provided by the APC. Both in vitro and in vivo studies have shown the potential for third party cells (bystander) to provide costimulatory signals, enabling T cells to proliferate in response to antigen on a separate cell (discussed in section 1.5.2). In addition, the potential for antigen to be processed and represented by bystander cells has also been described in a process termed cross priming (discussed in section 1.4.2.9).

4.1.2 Fusion of EBV B-LCL with Tumour Cells to Enhance Tumour Immunogenicity

The relevance of T cells in the rejection of tumour cells has been shown in both animal and human studies (Greenberg *et al.*, 1991; Kawakami *et al.*, 1994; Clark, 1991) (section 1.2). One reason for the lack of tumour cells immunogenicity is due to the absence of T cell co-stimulatory ligand expression by the tumour cell. In order to overcome this deficit, much attention has been paid to manipulating tumour cells to express CD80. This strategy in animal models of cancer therapy has been met with both success and failure (section 1.7.4).

In order to overcome the poor immunogenicity of tumour cells, it was hypothesised that fusion of melanoma cells with EBV B-LCL might produce hybrid cells that have enhanced stimulatory capacities and are able to process and present tumour associated antigen to T cells in a manner which induces a specific tumour response (discussed in chapter 5). This hypothesis was based on the promising work of Guo et al. (Guo et al., 1994) in the rat model, which showed fusion of activated B cells with hepatocarcinoma cells produced hybrid cells which were capable of eradicating established unmodified tumours in the rat model. Since then, other work published on APC x tumour hybrid cells has shown the effectiveness of APC x tumour cell hybrids in vitro (Gong et al., 2000), in animal experiments (Stuhler and Walden, 1994; Gong et al., 1997; Celluzzi and Falo, 1998; Wang et al., 1998a; Cao et al., 1999) and in clinical trials (Trefzer et al., 1997; Kugler et al., 1998; Kugler et al., 2000). However, the indepth, in vitro analysis of cloned EBV B-LCL x tumour cell hybrids derived from a range of human tumour cells has been described in only one study to date (Dunnion et al., 1999). Dunnion et al. documented the expression of HLA class I and class II and a wide range of accessory molecule expression, including the T cell co-stimulatory molecules CD40, CD80 and CD86, generated between EBV B-LCL and marrow derived human cell lines, and demonstrated the importance of expression of CD40, CD80 and CD86 by the hybrid cells for optimal T cell stimulation. However, flow cytometric analysis showed the EBV B-LCL x melanoma cell hybrids generated in this study were phenotypically similar to the tumour cell parent, and lacked to expression of CD80 and CD86 (section 3.2.4).

4.1.3 Aims of this Work

The aims of this work were to look at the immunogenicity of the EBV B-LCL x melanoma hybrid cells generated in this study and compare them with the parental cell lines *in vitro*. The proliferative response of allogeneic PBMCs and separated T cells
(CD4+, CD8+, CD45RO+ and CD45RA+ subsets) to stimulation by the parent and hybrid cells was assessed in an *in vitro* model of an allogeneic immune response. The capacity of these cells to induce CTL activity was determined by standard ⁵¹Cr release assays after MTLC. In addition, the role of MHC class I and class II antigens and co-stimulatory ligand molecules (CD40, CD80 and CD86), as immunologically relevant cell surface markers, was determined by blocking the functions of these molecules with specific, mAbs in MTLC. Since the EBV B-LCL x melanoma hybrids lacked CD80/CD86 expression, the effects of CD80 expression by the hybrid cells were assessed by transfecting the cells with the gene for CD80.

4.2 Results

4.2.1 An In Vitro Model of an Allogeneic Immune Response

To evaluate the effect of cell hybridisation on the immunogenicity of human melanoma cells and EBV B-LCL cell lines, EBV B-LCL x melanoma hybrid cells and the parent cell lines were compared for their capacity to stimulate an allogeneic immune response *in vitro*. Hybrid cells and the relevant parent cell lines were co-cultured with PBMCs from normal, healthy, allogeneic individuals as described in section 2.10.1. The main objective of the *in vitro* stimulation assays was to identify the immunogenicity of the cells by stimulation of allogeneic responses and not the reactivation of memory CTLs directed against the EBV antigens which circulate in the blood of EBV sero-positive individuals (Rickinson and Moss, 1997). PBMC from EBV sero-negative donors were used in the majority of the assays to control against responses to EBV antigen expression in the context of both MHC class I and MHC class II molecules. However, due to the relative scarcity of EBV sero-negative donors, PBMCs from EBV sero-positive donors were also used in some experiments. Responder HLA type and EBV status are listed in Appendix 1. All assays were performed on several occasions (unless stated), and results representative of the assays are shown.

4.2.1.1 Response of PBMCs to Stimulation With Parent (HMy2 and 518.A2) and Hybrid Cells (HMy2 x 518.A2)

Figure 4.1 shows the response of PBMCs to stimulation with HMy2, 518.A2 and HMy2 x 518.A2 hybrid cells. HMy2 stimulated a strong PBMC proliferative response, which was used as a marker for the optimal allogeneic immune response in these assays. The melanoma cell line 518.A2 was consistently a poor stimulator of an allogeneic

immune response in the assays. The HMy2 x 518.A2 clones used in these experiments were chosen randomly from a selection which had been expanded for the analysis of cell surface marker expression (chapter 3). Despite having a dominant melanoma cell phenotype, all clones and the bulk culture were able to stimulate an allogeneic immune response which was greater than the parent melanoma cell line (Figure 4.1 A), although for clone 1, clone 3 and clone 4 it was not statistically significant (p > 0.05). These results were consistent in repeat experiments. The allogeneic response to HMy2 x 518.A2 clone 2 was significantly enhanced compared with the melanoma parent cell line. In some experiments this response was equivalent to that of the parent EBV B-LCL (see Figure 4.8 A). Maximum PBMC proliferation was seen when 3.3×10^4 hybrid cells were co-cultured with 1×10^5 responder PBMCs. The experiment was performed at least twice for Responder 1, Responder 4 and Responder 5 and a similar trend was continually seen. Based on these results, one clone was chosen for a more in-depth analysis. HMy2 x 518.A2 clone 2 (abbreviated to Cl. 2 in the Figures) was chosen for further investigation due to its enhanced stimulatory capacity compared with the melanoma parent cell line in the in vitro model of an allogeneic immune response (Figure 4.1 B).

Initially, both EBV sero-negative (Figure 4.1 A) and EBV sero-positive individuals (Figure 4.1 B) were used as donors of Responder PBMC and no qualitative difference was found between the two, ruling out a response to EBV antigen expression by the parent EBV B-LCL or the hybrid cells. This was in accordance with the findings of Dunnion *et al.* (Dunnion *et al.*, 1999), who also used EBV B-LCL x tumour cell hybrids in a similar system to investigate allogeneic immune responses *in vitro* to assess the immunogenicity of hybrid cells formed using marrow derived cell lines as tumour cell partners.

The *in vitro* PBMC proliferation assay was also performed following culture of the cells in IFN- γ (100U/ml) for three days before the assay. There was no significant difference in the proliferative response between the Responder PBMCs stimulated by 518.A2 and HMy2 x 518.A2 clone 2 cells and by those cells which had been treated with IFN- γ (data not shown) in spite of the phenotypic differences described in section 3.2.4. These include an upregulation of MHC class I antigens and CD58 expression upon IFN- γ treatment.

4.2.1.2 Response of PBMCs to Stimulation with Parent (Gerl43 and HMy2) and Hybrid Cells (HMy2 x Gerl43)

Figure 4.2 shows the allogeneic response of PBMCs to stimulation with cell lines HMy2, Gerl43 and hybrid cells, HMy2 x Gerl43. The unfused melanoma parent cells, Gerl43, were poor stimulators of an allogeneic PBMC response. Various clones of the hybrid cells were analysed alongside the uncloned hybrid cells and all were found to be significantly more immunostimulatory than the melanoma parent cells (Figure 4.2 A). The clones used in the assay were chosen randomly from those which had been expanded for use in cell phenotype analysis (chapter 3). HMy2 x Gerl43 clone 3B5 (termed Cl. 3B5 in all Figures) was chosen for further analysis due to its enhanced immunostimulatory capacity compared with the parent melanoma cell line (Figure 4.2 B) and was found to be consistently more immunostimulatory than the parent tumour cells. In some instances, the PBMC response directed towards the hybrid cells was as great as the response directed against the EBV B-LCL (see Figure 4.2 B).

The melanoma and the hybrid cells were examined for their immunostimulatory capacity after IFN- γ pretreatment. Gerl43 cells and HMy2 x Gerl43 clone 3B5 were cultured in IFN- γ (100U/ml) for three days prior to their use *in vitro*. There was no significant difference in the stimulatory potential between those cells which had been cultured in IFN- γ and those cells which had not (data not shown) in spite of phenotypic differences which are discussed in section 3.2.4. These include the upregulation of MHC class I and class II antigens and CD58 expression upon IFN- γ treatment.

4.2.1.3 Response of PBMCs to Stimulation with Parent (DAUV) and Selected Cells (HMy2 x DAUV)

The response of PBMCs from healthy individuals to stimulation by parent DAUV and HMy2 x DAUV selected cells is shown in Figure 4.3. The response of PBMCs from both EBV sero-negative and sero-positive individuals was similarly poor towards both parent tumour cell and the selected cells. It was decided not to investigate the stimulatory potential of the selected cells any further, since, for hybrid cells to be of use in tumour immunotherapy, they must be more immunostimulatory than the parent tumour cell line alone, and there was no evidence that HMy2 x DAUV selected cells were hybrids other than their double chemical resistant phenotype.

4.2.2 Induction of a CTL Response

For hybrid cells to be of use in tumour immunotherapy, it is generally accepted that the presentation of tumour associated antigen to both CD4+ and CD8+ T cells is required, to activate both antigen specific helper and cytotoxic T cell populations (Dunnion *et al.*, 1999). This may occur by the tumour cell either acting as the APC itself, presenting tumour associated antigen directly to T cells, or host APC taking up tumour antigen and processing and presenting it bound to MHC class I and class II molecules (cross priming) to T cells.

In order to determine whether or not the hybrid cells generated in this study were able to directly induce CTL activity against the parental tumour cell type, negatively selected T cells (ie depleted of bystander APC) were co-cultured with hybrid cells or the relevant parent cell lines (HMy2, 518.A2 or Gerl43). The T cells were restimulated after one week in the presence of IL-2 (20U/ml) and after two weeks the T cells were incubated with target cells labelled with ⁵¹Cr and cell lysis observed. In addition to the parent and hybrid cells, the cell line K562 (as an NK cell sensitive cell line) was also used as a target for cell lysis.

4.2.2.1 Induction of a CTL Response by HMy2, 518.A2 and HMy2 x 518.A2

The induction of a CTL response by HMy2, 518.A2 and HMy2 x 518.A2 clone 2 is shown in Figure 4.4. The parent cell line, HMy2, stimulated T cell activity against itself and the hybrid cell line HMy2 x 518.A2 only. The melanoma parent cell line, 518.A2, stimulated activity against itself, HMy2 and the hybrid cells.

The hybrid cell line stimulated CTL activity against itself, both parent cell lines and K562. These results were similar on two separate occasions with both an EBV seropositive and an EBV sero-negative donor. Induction of CTL activity against the tumour cell line 518.A2 by the hybrid cells was no better than the induction of the CTL response by the parent melanoma cells themselves. The relatively high levels of killing of K562, however, suggests significant NK cell-like activity was induced by the hybrid cells. Overall, these results suggest that fusion of 518.A2 with HMy2 does not improve the capacity of the tumour cells to induce a CTL response against the parent cell line.

4.2.2.2 Induction of a CTL Response by HMy2, Gerl43 and HMy2 x Gerl43

The induction of a CTL response by HMy2, Gerl43 and HMy2 x Gerl43 clone 3B5 is shown in Figure 4.5. HMy2 stimulated CTL activity against itself and also against

HMy2 x Gerl43. Gerl43 stimulated a low level of CTL activity against itself, HMy2 x Gerl43 and also stimulated lysis of K562, indicating NK-like cell activity. HMy2 x Gerl43 stimulated CTL activity against itself and to a lesser extent against the parent melanoma cell line, Gerl43, but it did not stimulate CTL activity against the parent EBV B-LCL cell line, HMy2. These results suggest that HMy2 x Gerl43 hybrid cells were no more effective at inducing a CTL response against the parent melanoma cells than the unfused parent melanoma cell line alone.

Collectively, the data from the ⁵¹Cr release assays show that the hybrid cells did not induce a specific CTL response against the parent tumour cells which was significantly greater than that induced by the unfused melanoma parent cells. These results indicate that hybridisation of the melanoma cells with EBV B-LCL did not produce fused cells with an enhanced ability directly to induce a specific CTL response. This may have been due to the lack of expression of CD80 and CD86 by the cells, but represents a difference between induction of CTL activity and T cell proliferation which was enhanced in MTLC (section 4.2.3). It is possible that CTL induction may occur when the hybrid cells are cultured with T cells in the presence of responder APC . This is something which remains to be investigated.

4.2.3 T Cell Stimulation Assays

In order to study further the cells responding in the proliferation assays (described in section 4.2.1), T cells were separated from PBMCs and used in the *in vitro* assays as described previously (section 2.10.2). Separated T cells were divided into CD4+ and CD8+ T cell subsets, or CD45RO+ (memory) and CD45RA+ (naïve) T cell subsets. All T cell populations were negatively separated by Ab labelling and magnetic cell sorting and remained 'untouched' prior to their use in the proliferation assays (section 2.9.3 and 2.9.4). Cell purity was determined by mAb labelling and immunofluorescence techniques followed by flow cytometric analysis. Stimulator cells were cultured at 3.3×10^4 cells per well.

4.2.3.1 Response of CD3+, CD4+ and CD8+ T Cells to Stimulation with HMy2, 518.A2 and HMy2 x 518.A2

The response of separated T cell populations to stimulation with HMy2, 518.A2 and HMy2 x 518.A2 hybrid cells is shown in Figure 4.6 A. HMy2, the parent EBV B-LCL, consistently stimulated a CD3+ T cell response of equivalent magnitude to the

PBMC proliferative response, indicating the responding cells in the MTLC were T cells. Both CD4+ T cells and CD8+ T cells responded directly to stimulation by EBV B-LCL, demonstrating the ability of these cells to directly stimulate CD8+ cells without the need for CD4+ T cell help. These results show EBV B-LCL were able to stimulate allogeneic T cell responses, since responder APC, which could have been present and acting as antigen processing and presenting cells in indirect allogeneic stimulation in PBMC cultures (Lechler *et al.*, 1990), had been depleted from the stimulator cell populations.

The parent melanoma cells stimulated a weak allogeneic PBMC proliferative response. However, responses of separated CD3+, CD4+, and CD8+ T cell populations were not significantly raised as compared with the unstimulated cell controls. The hybrid cells, despite stimulating an allogeneic PBMC response which was greater than the parent melanoma cells, were not able to stimulate significant CD3+ or CD4+ T cell responses. In contrast, CD8+ T cells, when cultured with the hybrid cells, showed a low, but significantly enhanced level of stimulation. This experiment was performed three times in total, including both EBV sero-negative (Figure 4.6 A) and EBV sero-positive Responders (data not shown), with consistent results. The low proliferative response of CD8+ T cells to stimulation by the hybrid cells was not significantly inhibited by blocking the interaction of CD80/CD86 with CD28/CTLA-4 with CTLA-4 Ig ($0.5\mug/ml$) (data not shown), whereas the response of PBMCs to stimulation by the hybrid cells was inhibited by CTLA4-Ig (Figure 4.9 A).

The reason for the low CD8+ T cell response to stimulation with HMy2 x 518.A2 clone 2 is likely to be due to the expression of allogeneic MHC class I by the hybrid cells. The level of MHC class I molecules or the enhanced range of allogeneic MHC expressed compared with the parent melanoma cells may have enhanced the allogeneic CD8+ T cell response. HMy2 x 518.A2 clone 2 did not express MHC class II antigens, preventing CD4+ T cells from responding. Since both the hybrid and the melanoma cells express MHC class I molecules on the cell surface and lack CD80/CD86 expression (as detected by mAb staining and immunofluorescence techniques), the reason for the proliferative response of CD8+ T cells to stimulation by the hybrid cells but not by the parent melanoma cells could not be ascertained from this experiment, but appears to be independent of CD80/CD86 interaction with CD28/CTLA-4. It was assumed that the lack of CD3+ T cell stimulation by the hybrids cells was due to the predominance of CD4+ T cells over CD8+ T cells in this population therefore fewer T cells were stimulated.

4.2.3.2 Response of CD3+, CD45RO+ and CD45RA+ T Cells to Stimulation with HMy2, 518.A2 and HMy2 x 518.A2

The response of separated CD45RO+ (memory) and CD45RA+ (naïve) T cells to stimulation with hybrid cells and parent cell lines was also assessed and the results are shown in Figure 4.6 B. HMy2 showed optimal CD3+ T cell stimulation and was able to stimulate both CD45RO+ and CD45RA+ T cells. CD45RO+ and CD45RA+ T cells were stimulated to a similar extent.

Again, the parent melanoma cell line, 518.A2, was not able to stimulate an allogeneic CD3+ T cell response, or to stimulate either CD45RO+ or CD45RA+ T cells. The hybrid cells also failed at stimulate significant CD3+, CD45RO+ or CD45RA+ T cell responses. The poor allogeneic T cell response stimulated by the hybrid and melanoma cells was consistent with previous findings (Figure 4.6 A).

4.2.3.3 Response of CD3+, CD4+ and CD8+ T Cells to Stimulation with HMy2, Gerl43 and HMy2 x Gerl43

The response of separated CD3+, CD4+ and CD8+ T cells to stimulation with HMy2, Gerl43 and HMy2 x Gerl43 hybrid cells is shown on Figure 4.7 A. The T cell response to stimulation with HMy2 cells has been discussed elsewhere (section 4.2.3.1). Gerl43 cells stimulated a PBMC response that was significantly lower than that of HMy2, and failed to stimulate a significant CD3+, CD4+ or CD8+ T cell response (as compared with the unstimulated T cell populations). The hybrid cells on the other hand stimulated a PBMC proliferative response which was sometimes as great as, or greater than, the response to stimulation with the EBV B-LCL. Separated CD3+, CD4+ and CD8+ T cells showed a lower level of stimulation by the hybrid cells than the PBMC response. However, the responses were significantly greater than those seen for the unfused melanoma parent cells. This result was consistent in two separate experiments, both performed with EBV sero-negative donors.

As with the HMy2 x 518.A2 hybrids, the responses of purified T cell populations to the HMy2 x Gerl43 were not inhibited by the addition of CTLA4-Ig ($0.5\mu g/ml$) (data not shown). Thus, both CD4+ and CD8+ T cells responded weakly to stimulation by the HMy2 x Gerl43 hybrid cells. This is most likely to be due to the expression of both MHC class I and MHC class II antigens on the cell surface, stimulating allogeneic responses in the absence of co-stimulation through CD80/CD86 interaction with CD28/CTLA4. The parent melanoma cells also expressed MHC class I and class II antigens and lacked CD80 and CD86 expression, but did not stimulate a T cell response. The reason for the lack of T cell response directed towards the parent tumour cell line could not be determined from this assay, but may have been due to the level of MHC class I and class II expression or the enhanced range of antigens expressed by the hybrid cells or to expression of alternative costimulatory molecules by the hybrid cells that were not expressed on the parent melanoma cells.

The results from this experiment are similar to those seen in the 518.A2 and HMy2 x 518.A2 T cell stimulation assays, except that only CD8+ T cells were stimulated by the MHC class I positive, class II negative HMy2 x 518.A2 hybrid cells, whereas both CD4+ and CD8+ T cells were stimulated by the MHC class I positive and MHC class II positive HMy2 x Gerl43 hybrid cells. These results suggest CD80/CD86 independent co-stimulation by the hybrid cells.

4.2.3.4 Response of CD3+, CD45RO+ and CD45RA+ T Cells to Stimulation with HMy2, Gerl43 and HMy2 x Gerl43

The response of CD3+, CD45RO+ (memory) and CD45RA+ (naïve) T cells to stimulation with HMy2, Gerl43 and HMy2 x Gerl43 clone 3B5 is shown in Figure 4.7 B. CD3+, CD45RO+ and CD45RA+ T cells showed a low level of stimulation by Gerl43 although this was not statistically different compared with the unstimulated control cells. In this experiment, however, the CD3+, CD45RO+ and CD45RA+ T cell responses directed against the hybrid cells were not significantly greater than the T cell responses directed against the parent melanoma cells, a result that contradicts earlier findings (Figure 4.7 A). This experiment was performed only once, and would need to be repeated in order to confirm the findings.

4.2.4 Antibody Blocking Assays

The observations described above raised questions as to why HMy2 x 518.A2 and HMy2 x Gerl43 hybrid cells were able to stimulate an allogeneic PBMC response which was significantly greater than the response directed against the melanoma parent cells, whereas separated T cells were not stimulated to a similar degree. To address these questions, mAb blocking assays were performed to inhibit the functions of MHC class I and MHC class II, and the T cell co-stimulatory ligand molecules CD40, CD80 and CD86.

4.2.4.1 Effects of Anti-MHC class I and class II Antibodies on PBMC Responses to HMy2 x 518.A2 and HMy2 x Gerl43 and the Relevant Parent Cell Lines (HMy2, 518.A2 and Gerl43

Antibodies W6/32 and L243 were used to block the function of MHC class I and MHC class II molecules respectively, and were used as cell culture supernatants at a final concentration of 15% (vol/vol) in the MTLC. An isotype matched, control Ab (mouse anti-human IgG_{2a} at a final concentration of 0.5µg/ml) and a cell culture supernatant from an EBV B-LCL at 15% (vol/vol) were used as controls.

Figure 4.8 shows the proliferative responses of PBMCs to stimulation with HMy2 x 518.A2 clone 2, HMy2 x Gerl43 clone 3B5 and the parent cell lines, and the effects of anti-MHC class I and anti-MHC class II mAbs on these responses. The proliferation of PBMC in response to stimulation by HMy2 was not significantly inhibited by the control antibodies or by the anti-MHC class I Ab. The anti-MHC class II Ab partially inhibited this response, but not significantly compared with the PBMC (no Ab) response.

The PBMC proliferative response directed towards the 518.A2 melanoma cell line was poor, and at a level comparable to the unstimulated PBMCs. The IgG_{2a} isotype control and the tissue culture supernatant controls both significantly enhanced cell proliferation. The anti-MHC class I mAb had no significant effect on PBMC proliferation in response to 518.A2, and the anti-MHC class II mAb significantly reduced PBMC proliferation to below background levels.

The IgG_{2a} isotype control had no significant effect on PBMC proliferation directed towards HMy2 x 518.A2 clone 2, whereas the tissue culture supernatant control significantly enhanced PBMC proliferation. Although the hybrid cells expressed MHC class I antigens, the PBMC response directed against the hybrid cells was not significantly blocked with anti-MHC class I mAb. Despite HMy2 x 518.A2 clone 2 lacking MHC class II expression, the anti-MHC class II mAb significantly reduced the magnitude of the PBMC response to stimulation with the hybrid cells, bringing the level of stimulation down to almost background levels.

The PBMC response to stimulation with Gerl43 was not significantly affected by the IgG_{2a} isotype control, the tissue culture supernatant or the anti-MHC class I Ab. The response was reduced with the anti-MHC class II Ab, but this was not significant compared with the PBMC (no Ab) control.

The PBMC response to stimulation with the HMy2 x Gerl43 clone 3B5 cells was not significantly affected by the isotype control, the tissue culture supernatant control or the anti-MHC class I Ab. PBMC proliferation in response to the hybrid cells was partially but significantly inhibited with the anti-MHC class II mAb.

The results from these experiments do not allow firm conclusions to be drawn. The PBMC response to stimulation with the cell lines was not inhibited by the anti-MHC class I antibody, in spite of the demonstration that CD8+ T cells were involved in these responses (Figure 4.6 and 4.7). Partial but inconsistently significant inhibition was seen with the anti-MHC class II antibody. This applied to cultures involving the MHC class II negative cell lines 518.A2 and HMy2 x 518.A2 as well as the MHC class II expressing HMy2, Gerl43 and HMy2 x Gerl43 cell cultures.

Unpurified cell culture supernatant antibodies were used in this experiment which is not ideal, as growth factors and other cellular by-products may have also been present. Also, the concentrations of the antibodies used in the assay were unknown. Although the isotype matched, non-specific IgG_{2a} and the tissue culture supernatant controls showed no inhibitory effects, in some instances they sometimes significantly enhanced the proliferative response. This made it difficult to determine the true extent of the inhibitory effects of the anti-MHC class I and MHC class II antibodies.

To demonstrate the effects of blocking MHC class I and MHC class II function, the experiment should be repeated using purified mAbs at saturating concentrations. In addition, the anti-class II mAb used in this assay (L243) recognises HLA-DR antigens only. This experiment could also be performed using an alternative anti-MHC class II blocking Ab which binds to all MHC class II antigens.

4.2.4.2 PBMC Responses to HMy2 x 518.A2, HMy2 x Gerl43 and the Parent Cell Lines (HMy2, 518.A2 and Gerl43) and the Effects of Anti-CD40 mAb and CTLA4-Ig

CD40, CD80 and CD86 are known to be important T cell co-stimulatory ligand molecules, present on the surface of APC. To investigate the importance of co-stimulation through CD40 and CD40L interaction, and CD80/CD86 interaction with CD28/CTLA-4 in the allogeneic PBMC response to stimulation by HMy2, tumour and hybrid cells, *in vitro* blocking assays were performed using anti-CD40, CTLA-4 Ig and an isotype matched control antibody, which were included in MTLC at a final concentration of 0.5µg/ml. The melanoma cells and hybrid cell lines did not express CD80/CD86 on the cell surface

(section 3.2.4). Therefore, inhibition of a proliferative response by blocking the interaction of CD80/CD86 with CD28/CTLA-4 would suggest that responder APC within the PBMC population were acting as bystander cells, providing the necessary co-stimulatory signals to enable the T cells to respond to allogeneic MHC class I and/or MHC class II molecules on the surface of the tumour and hybrid cells. This is a phenomenon known as trans (or remote or bystander) co-stimulation, and is discussed in section 1.5.2.

Figure 4.9 shows the proliferative response of PBMCs to stimulation with HMy2, HMy2 x 518.A2 clone 2, HMy2 x Gerl43 clone 3B5 and the parent tumour cell lines. The isotype matched, non-specific IgG₁ control had no significant effect on PBMC proliferation in any of the MTLC. The anti-CD40 mAb had no consistent blocking effect under any experimental conditions tested. These results suggest CD40-CD40L interaction did not play a significant role in the allogeneic PBMC response. Previous work with EBV B-LCL x tumour cell hybrids (Dunnion *et al.*, 1999) has also shown that blocking CD40-CD40L interaction has minor inhibitory effects on T cell proliferation, and that CD80/CD86 interaction with CD28/CTLA-4 was more important.

The addition of CTLA-4 Ig to the MTLC significantly inhibited the PBMC response towards HMy2, HMy2 x 518.A2 and HMy2 x Gerl43 hybrid cells, but not the parent 518.A2 or Gerl43 cells. Therefore, despite the hybrid cells not expressing CD80 or CD86, as determined by flow cytometric analysis, the enhanced stimulation by the hybrid cells was dependent, in part, on CD80/CD86 mediated T cell co-stimulation. HMy2 cells were able to stimulate T cells directly, so the results suggest CD80/CD86 molecules on the cell surface interact with the ligand receptors on the T cells and play a crucial role in direct T cell stimulation. Since the PBMC proliferative response towards the hybrid cells was partially inhibited by CTLA-4 Ig, these results indicate CD80/CD86 interaction with CD28/CTLA-4 played a role in T cell co-stimulation in MTLC involving the hybrid cells. As the hybrid cells were not able to stimulate separated T cells effectively, probably due to the lack of expression of CD80 and CD86, it is possible bystander cells in the PBMC culture provided the T cell co-stimulatory signals enabling the T cells to respond to stimulation by the hybrid cells through trans co-stimulation.

4.2.5 Mechanism for T Cell Co-Stimulation by EBV B-LCL x Melanoma Hybrid Cells 4.2.5.1 Addition of Responder Non-T cells to a T cell Culture Allows T Cells to Respond to 518.A2 and HMy2 x 518.A2 Clone 2 Cells.

Previous experiments supported a role for trans co-stimulation by responder APC within the PBMC population. In order to determine whether or not T cells showed enhanced stimulation by hybrid cells in the presence of responder APC, a MTLC was established with the following modifications. Stimulator cells were cultured at 3.3×10^4 cells per well with 1×10^5 CD3+ T cells. After the T cells had been subtracted from the PBMC population, the remaining cells (referred to in Figures 4.10 and 4.11 as non-T cells) were Mit-C treated to prevent them from replicating and added at concentrations of 3.3×10^4 cells per well and 1×10^5 cells per well to co-cultures containing stimulator and T cells. Each cell combination was performed in triplicate.

Figure 4.10 shows the results of the assay performed using a healthy, allogeneic EBV sero-negative donor. The T cell response to stimulation by the HMy2 x 518.A2 clone 2 hybrid cells was markedly enhanced when cultured in the presence of both 3.3×10^4 and 10^5 Mit-C treated non-T cells. The proliferative responses were also significantly enhanced compared with the autologous mixed lymphocyte reaction (MLR). A similar but less marked enhancement of proliferation was seen when T cells were cultured with the parent melanoma cell lines in the presence of responder non-T cells. The proliferative response was significantly increased when 1×10^5 Mit-C treated non-T cells were cultured with the autologous MLR but not with 3.3×10^4 Mit-C treated non-T cells compared with the autologous MLR.

The results from this assay show that T cell proliferation was significantly enhanced in response to stimulation by both the melanoma cells and the hybrid cells when responder non-T cells were present. When the numbers of responder non-T cells were increased, the level of stimulation also increased, further suggesting that T cells were costimulated indirectly from bystander cells in the PBMC population.

4.2.5.2 The Response of T Cells to Stimulation by Hybrid and Melanoma Cells Involves CD80/CD86 Interaction with CD28/CTLA-4

The assay described above (section 4.2.5.1) was repeated with CTLA-4 Ig included in the MTLCs, in order to determine if the T cell response involved CD80/CD86 interaction with CD28 and CTLA4. The isotype control (IgG₁) Ab, was also included in the assay. In each well, 3.3×10^4 stimulator cells (518.A2 or HMy2 x 518.A2 clone 2) were incubated with either 1×10^5 PBMCs, 1×10^5 T cells or 1×10^5 T cells with 3.3×10^4 Mit-C treated non-T cells. Each cell combination was performed in triplicate and carried out in the presence of CTLA4-Ig and IgG₁ isotype control.

Figure 4.11 shows the results of the assay using a healthy allogeneic EBV seronegative responder. Culturing cells with the IgG_1 isotype control had no significant effect on T cell proliferation. PBMCs alone were poorly stimulated by the unfused melanoma parent cells, and culturing the cells with CTLA-4 Ig did not significantly inhibit this effect. PBMCs showed an enhanced level of proliferation due to stimulation by the hybrid cells compared with the parent melanoma cells. This response could be significantly inhibited with CTLA-4 Ig, demonstrating the importance of CD80/CD86 interaction with CD28/CTLA-4 in the immune response.

Incubating separated CD3+ T cells with 518.A2 and HMy2 x 518.A2 showed the T cells were not effectively stimulated as described previously (Figure 4.6). In addition, Mit-C treated non-T cells alone did not respond to the stimulator cells. However, culturing CD3+ T cells with Mit-C treated non-T cells and 518.A2 or HMy2 x 518.A2 resulted in enhanced T cell proliferation, confirming previous findings (section 4.2.4.2).

The response to stimulation with 518.A2 of T cells plus Mit-C treated non-T cells was blocked to background levels by the addition of CTLA-4 Ig to the culture medium. The response of T cells plus Mit-C treated non-T cells to stimulation with the hybrid cells was partially inhibited with CTLA4-Ig, but this result was not statistically significant. This experiment was performed once and needs to be repeated to confirm these findings.

The results of this and the previous experiment have shown enhanced T cell responses to the melanoma cell line and the hybrid cells *in vitro* by co-culture in the presence of responder non-T cells. Proliferation was enhanced when the number of non-T cells were increased, suggesting either a bystander co-stimulatory role for these cells in the immune response, or that the responder APC were acting as antigen processing and presenting cells in indirect allogeneic antigen presentation. CTLA-4 Ig was, at least in part, able to inhibit cell proliferation, in spite of the hybrid cells and the parent melanoma cells lacking the expression of CD80 and CD86. This adds further evidence in support of CD80/CD86 trans co-stimulation being provided by responder APC within the non T cell population.

4.2.6 Rationale For Transfecting EBV B-LCL X Melanoma and Melanoma Cells With CD80

The work thus far has shown the hybrid cells were more immunostimulatory than the parent melanoma cells in spite of lacking CD80 and CD86 expression. The data suggests, however, that bystander cells in the responder cell cultures may have provided additional trans co-stimulatory signals which would allow T cells to respond to stimulation by the hybrid cells.

In order to investigate whether or not CD80 expression by the hybrid cells has an additive effect on the immune response, by allowing the direct co-stimulation of T cells, the gene for CD80 expression was transfected into the hybrid cells and parent cell lines. Transfection of CD80 into the parent melanoma cells would show whether CD80 expression by the melanoma cells was more effective as a strategy for melanoma therapy than cell hybridisation, at least for these cells, and also whether CD80 expression by the hybrid cells further enhanced their ability to stimulate T cell responses.

4.2.6.1 CD80 Transfection of EBV B-LCL x Gerl43 Hybrids and the Parent Melanoma Cell Line, Gerl43

518.A2, HMy2 x 518.A2, Gerl43 and HMy2 x Gerl43 clone 3B5 were transfected with a plasmid encoding for CD80 expression and neomycin resistance (pCR3.CD80) by Effectene transfection (section 2.11.4). Cells were selected in growth medium containing G418, then cloned at one cell per well into 48 well tissue culture dishes. Ten clones from each cell line were chosen randomly and expanded for further analysis. Mock transfections were performed using the empty plasmid expressing the neomycin resistance gene only (pCR3) and transfected cells were selected in the same way. Mock transfectants were used as an uncloned cell culture. These cells were termed 518.A2.pCR3, HMy2 x 518.A2.pCR3, Gerl43.pCR3 and HMy2 x Gerl43.pCR3. These cells did not express CD80 as determined by flow cytometric analysis (Figure 4.12).

The CD80 expressing cells were termed 518.A2.pCR3.CD80, HMy2 x 518.pCR3.CD80, Gerl43.pCR3.CD80 and HMy2 x Gerl43.pCR3.CD80. The level of CD80 expression by the CD80 transfected cells was determined by flow cytometric analysis. From each cell line, one clone was chosen for further analysis depending on the level of CD80 expression to achieve as close a match of CD80 expression as possible. 518.A2.pCR3.CD80 and HMy2 x 518.A2.pCR3.CD80 at similar levels (data not shown) which were comparable to Gerl43.pCR3.CD80. The level of CD80

expression by Gerl43.pCR3.CD80 and HMy2 x Gerl43.pCR3.CD80 which were chosen for further analysis is shown in Figure 4.12. It was not possible to match the level of expression of CD80 by Gerl43.pCR3.CD80 with HMy2 x Gerl43.pCR3.CD80 from the twenty clones isolated, as all HMy2 x Gerl43.pCR3.CD80 clones which expressed CD80, for unknown reasons expressed it at higher levels compared with the Gerl43.pCR3.CD80 clones. The level of CD80 expression by the two clones chosen for further analysis remained constant throughout the study.

4.2.6.2 The In Vitro Effects of CD80 Expression by Gerl43 and HMy2 x Gerl43 in PBMC and T Cell Proliferation Assays

To determine the effects of CD80 expression on the immunogenicity of melanoma and hybrid cells, MTLC assays were established as described previously (section 2.10.1). 518.A2.pCR3.CD80 and HMy2 x 518.A2.pCR3.CD80 did not stimulate significantly enhanced PBMC responses compared with the mock transfected cell controls (data not shown) which may have been due to the low level of CD80 expression, so were not included in future experiments. Figure 4.13 shows the PBMC response of a healthy EBV sero-negative donor to stimulation by CD80 expressing Gerl43 and HMy2 x Gerl43 cells and the mock transfectants. Gerl43.pCR3.CD80 cells did not show a significantly enhanced PBMC response compared with the Gerl43.pCR3 mock transfectants. This may have been due to the low level of expression of CD80. HMy2 x Gerl43.pCR3.CD80 cells were able to stimulate an allogeneic PBMC response which was always significantly greater than the HMy2 x Gerl43.pCR3 mock transfectants and greater than the response to HMy2. This may have been due to the increased level of CD80 expression compared with the parent melanoma cells.

As previous studies had shown PBMC but not T cell responses to direct stimulation with hybrid cells, separated T cells were cultured with the Gerl43 and HMy2 x Gerl43 hybrid cells transfected with CD80, in order to determine whether these cells could provide T cell co-stimulatory signals to stimulate T cells directly. The responses of separated T cells to stimulation by CD80 transfected and mock transfected cells is shown in Figure 4.14. Gerl43.pCR3 cells and HMy2 x Gerl43.pCR3 cells did not stimulate enhanced separated CD3+, CD4+ and CD8+ T cell responses, consistent with the parent tumour and EBV B-LCL x melanoma hybrid cells (Figure 4.7). However, Gerl43.pCR3.CD80 cells stimulated CD3+ and CD4+ separated T cell responses which were significantly enhanced compared with the mock transfectants. Similarly, separated CD3+ and CD4+ T cell

responses were detected against the HMy2 x Gerl43.pCR3.CD80 cells. These responses were significantly greater than the T cell responses directed against the melanoma cells expressing CD80.

CD8+ T cells were not stimulated by Gerl43.pCR3.CD80 or HMy2 x Gerl43.pCR3.CD80 despite both expressing MHC class I antigens. This may have been due to the low proliferative potential of the CD8+ T cells after separation from responder PBMC, since in this experiment the CD8+ T cells did not respond to stimulation by the parent EBV B-LCL either. It is, however, possible that CD8+ T cells were unable to respond to the melanoma cells expressing CD80 without CD4+ T cell help. This experiment was performed only once and would have to be repeated in order to confirm this.

The results of this assay show that separated T cells could be stimulated directly, without the need for responder APC, by transfecting both the melanoma (Gerl43) and the hybrid cells (HMy2 x Gerl43) with CD80. Both the PBMC response and the T cell response stimulated by the HMy2 x Gerl43 hybrid cells expressing CD80 were significantly greater than the allogeneic responses directed against the Gerl43 melanoma cells expressing CD80 and the hybrid cell mock transfectants. This enhanced level of T cell stimulation by the HMy2 x Gerl43.pCR3.CD80 cells compared with the Gerl43.pCR3.CD80 melanoma cells could have been due to one or a combination of reasons. It is also possible that the hybridisation of melanoma cells with EBV B-LCL in conjunction with CD80 transfection may have acted synergistically to enhance the melanoma cell immunogenicity. However, HMy2 x Gerl43 cells expressed CD80 at a higher level compared with the CD80 transfected Gerl43 cells and the level of expression of CD80 has been shown previously to alter the effectiveness of CD80 transfection on tumour cells, with cells expressing CD80 at higher levels being more effective (Wu et al., 1995). Both 518.A2.pCR3.CD80 and HMy2 x 518.A2.pCR3.CD80 expressed CD80 at levels similar to Gerl43.pCR3.CD80 and were also not significantly more effective at stimulating PBMC proliferation compared with the mock transfected cell controls, further suggesting the level of CD80 expression is an important factor for the use of CD80 transfected cells in tumour therapy.

4.3 Discussion

In order for EBV B-LCL x tumour hybrids to be of use in tumour immunotherapy, they must be more immunogenic than the unfused tumour parent. To assess the stimulatory potential of the hybrid cells generated in this study, a series of *in vitro* experiments were performed. The results from the *in vitro* experiments should reflect hybrid cell effectiveness in therapeutic cancer vaccination, although proof of the efficacy of the hybrid cells in cancer treatment will require *in vivo* studies.

4.3.1 DAUV and HMy2 x DAUV Selected Cells

The HMy2 x DAUV selected cells stimulated a poor allogeneic PBMC response *in vitro*, which was at a similar level to the response stimulated by the parental DAUV cells. This served to add further evidence to support the notion that these cells were not true hybrids, but were unfused tumour cells which had grown through the selection process. Since the selected cells were also poorly immunogenic, they were not characterised any further in this chapter.

4.3.2 Enhanced Stimulation of Allogeneic Responses by Hybrid Cells as Compared with the Melanoma Parent Cells

The EBV B-LCL x melanoma hybrid cells generated in chapter 3 were phenotypically similar to the melanoma parent cell line. In this chapter, the immunogenicity of the hybrid cells was compared with the relevant parental cell lines in allogeneic immune responses *in vitro*, and the functional roles of various immunological molecules was assessed.

The data in this study shows that the hybrid cells formed between EBV B-LCL and melanoma cells, 518.A2 and Gerl43, were consistently able to stimulate allogeneic PBMC responses that were greater than the relevant parental melanoma cell line. This was not due to EBV specific memory CTL, as there were no qualitative differences between the responses of EBV sero-negative and EBV sero-positive donors. HMy2 x 518.A2 cells expressed MHC class I antigens but not MHC class II antigens, and were able to stimulate directly separated CD8+ T cells. HM2 x Gerl43 expressed MHC class I and class II antigens and were able to stimulate directly separated CD8+ T cells. The hybrid cells had a dominant melanoma phenotype and lacked the expression of CD80 and CD86 T cell co-stimulatory ligand molecules. Furthermore, the T cell response was not blocked with CTLA-4 Ig, thus demonstrating the potential of hybrid cell to stimulate T

cells in a CD80/CD86 independent manner. Stimulation of separated T cells by the hybrid cells, however, was not optimal.

Separated T cells were able to respond more effectively to stimulation by the hybrid cells *in vitro* when responder non–T cells were also present in the co-culture. Several experiments performed in this study suggested that either responder APCs were providing bystander co-stimulatory signals, allowing T cells to respond more effectively to the hybrid cells, or, responder APC were processing and presenting antigen (ie indirect allogeneic stimulation) and stimulating T cells directly. Firstly, when Mit-C treated non-T cells were added to hybrid and melanoma cell cultures containing separated CD3+ T cells, T cell proliferation was enhanced, and was further elevated when the number of non-T cells was increased. Secondly, the T cell response was inhibited with CTLA-4 Ig, indicating that the interaction between CD80/CD86 and CD28/CTLA4 was involved in this effect.

These findings support the use of APC x tumour cell hybrids in tumour immunotherapy and further suggest maintenance of the APC phenotype by the hybrid cells is not essential for enhanced T cell stimulation. This is at variance to the work of others who, in similar experiments, showed hybrid cells which did not express CD40, CD80 or CD86 were non-stimulatory as compared with the parent tumour cell lines (Dunnion *et al.*, 1999).

4.3.2.1 CD80/CD86 Independent Co-Stimulation

Although CD80 and CD86 play an important role in T cell co-stimulation, it appeared that T cells were able to respond to the hybrid cells independently of CD80/CD86 co-stimulation. Besides CD80/CD86, other molecules can transduce T cell co-stimulatory signals. This study limited accessory molecule analysis (by flow cytometry) to CD11a, CD40, CD50, CD54 and CD58. There was no difference in the level of expression (or lack of expression) of these markers between the parent and hybrid cells, except for CD40 which was expressed by HMy2 x Gerl43 and not by Gerl43 (section 3.2.4). Ab blocking experiments performed in this study showed that CD40 was not important in lymphocyte co-stimulation. However, as EBV B-LCL cells were fused with melanoma cells, the potential for other activated B cell markers to by expressed by the hybrid cells exists. Other molecules known to be involved in T cell activation are listed in Table 1.1. In addition to ICAM and LFA interactions, another important interaction in T cell activation involves CD27 with its ligand, CD70 (Hintzen *et al.*, 1994). This interaction has been

shown to enhance alloantigen induced proliferation (Brown *et al.*, 1995). Expression of CD70 is restricted to activated B cells and scattered T cells in the tonsils, gut and skin, and CD27 expression has been shown on most T cells, B cells and NK cells. In addition, CD24 (heat stable antigen) (Liu *et al.*, 1992), SLAM (Cocks *et al.*, 1995) and CD43 (Sperling *et al.*, 1995) have also been shown to have T cell co-stimulatory functions independent of CD80/CD86 interaction with CD28/CTLA-4. More recently, new members of the B7 family have been discovered which co-stimulate T cell function but do not bind to CD28 or CTLA-4 (Dong *et al.*, 1999; Swallow *et al.*, 1999) (reviewed in Abbas and Sharpe, 1999). Further studies are required to identify the molecules involved in the CD80/CD86 interaction with CD28/CTLA-4 normally induces T cell anergy (Tan *et al.*, 1992). Whether or not the T cells stimulated directly by the hybrid cells are functional requires further study.

4.3.2.2 T Cell Co-Stimulation by the EBV B-LCL x Melanoma Hybrid Cells

The importance of CD80/CD86 mediated T cell co-stimulation was shown when the hybrid cells were cultured with responder non-T cells plus separated T cells. T cells showed enhanced stimulation which could be blocked with CTLA-4 Ig. Since the hybrid cells *per se* did not express CD80/CD86, it appears that responder non-T cells were providing the T cell co-stimulatory signals. The results can be explained in two ways. Firstly, CD80/CD86 mediated T cell co-stimulation may have been provided by responder non-T cells, allowing T cells to proliferate in response to MHC antigens on the stimulator cell surface. Several studies using cell lines and primary cells in a variety of *in vitro* models have shown that third party cells, expressing co-stimulatory signals can activate T cells stimulated by MHC antigen complex on a separate cell (trans co-stimulation) (Jenkins *et al.*, 1988; Ding and Shevach, 1994; Sun *et al.*, 1996; Smythe *et al.*, 1999). Bystander (or trans) co-stimulation has also been shown *in vivo* (Gurlo *et al.*, 1999).

Secondly, it is possible for responder APC to process and present allo-MHC (ie indirect allogeneic stimulation) and stimulate T cells directly. Presentation of allogeneic MHC class I and class II molecules as peptides by professional APC has been shown both *in vitro* (de Koster *et al.*, 1989; Essaket *et al.*, 1990; Chen *et al.*, 1990; Liu *et al.*, 1993) and *in vivo* (Benichou *et al.*, 1992; Fangmann *et al.*, 1992a; Dalchau *et al.*, 1992. Fangmann *et al.*, 1992b; Watschinger *et al.*, 1994; Gallon *et al.*, 1995; Benham *et al.*, 1995). The indirect presentation pathway was originally invoked to explain the host's ability to reject MHC-mismatched tissue grafts.

From this series of in vitro experiments, it was not clear why the hybrid cells were more immunostimulatory than the parent melanoma cell lines and the mechanism by which CD80/CD86 dependent T cell co-stimulation occurred could not be determined. It is possible that the enhanced levels and/or increased range of MHC class I and class II antigens on the cell surface may account, at least in part, for their increased stimulatory potential. A separate study fused an EBV B-LCL with a cervical carcinoma cell line (HeLa), resulting in hybrid cells which had a dominant tumour cell phenotype (Contreras-Brodin et al., 1991). These hybrid cells were able to stimulate enhanced PBMC proliferation compared with the parent tumour cell line, though were never as great at stimulating an allogeneic response compared with the EBV B-LCL parent cells, in MTLC in vitro (D. Dunnion, personal communication), similar to the findings with melanoma. It seems that fusion of EBV B-LCL with non-lymphoid cells may frequently produce hybrid cells with a dominant tumour cell phenotype which still have enhanced ability to stimulate T cell responses and therefore demonstrate potential as agents in tumour immunotherapy. Other experimental evidence has shown the potential for tumour antigens to be processed and re-presented by APC (in a similar way to allo-MHC) (a process known as cross priming) (Huang et al., 1994; Wu et al., 1995; Cayeux et al., 1997; Maass et al., 1995; Toes et al., 1996). These studies have shown the induction of strong CTL activity against tumour cells, even when the tumour cell failed to express CD80. Little is known about how the APC processes exogenous antigens for presentation in conjunction with MHC class I on the cell surface in vivo, although there has been some speculation (reviewed in Reimann and Schirmbeck, 1999). If cross priming occurs with EBV B-LCL x tumour hybrid cells, it would be interesting to determine whether tumour-antigens from hybrid cells are processed and presented more efficiently than tumour cells by APCs in in vivo experiments.

To further support the role of non-specific effector cells in tumour vaccination strategies, other studies have shown that tumour infiltrates containing various non-specific effector cells positively correlate with tumour regression. Golumbek *et al.* used an IL-4 transfected renal cell carcinoma vaccine in mice and found tumour regression was associated with activated M ϕ (Golumbek *et al.*, 1991), and Dranoff *et al.* engineered B16 melanoma cells to secrete GM-CSF and found tumour regression was a more powerful immunogen than tumour cells expressing IL-4 or IL-6 in mice (Dranoff *et al.*, 1993). Also, associated with tumour regression using the GM-CSF modified tumour cells, was an

extensive infiltrate containing monocytes, granulocytes and activated lymphocytes. Both CD4+ T cells and CD8+ T cells were required for the anti-tumour response, which is of interest since the transfected melanoma cells did not express MHC class II molecules.

If trans co-stimulation or cross priming does occur when hybrid cells are cultured with PBMCs *in vitro*, it is plausible that tumour therapy may be more effective *in vivo* if APC were drawn to the site of vaccination, either to increase T cell co-stimulatory ligand receptors in the local environment or to increase the number of APC in the environment for indirect antigen presentation. Hybrid cells genetically modified to secrete GM-CSF have proved to be more powerful immunostimulants (Cao *et al.*, 1999). Hybrid cells formed between DCs and a melanoma cell line and genetically modified to express GM-CSF showed enhanced anti-tumour effects compared with the unmodified hybrid cells. Non specific effector cells were thought to contribute to tumour rejection (Cao *et al.*, 1999).

4.3.2.3 Additional Reasons for Hybrid Cell Enhanced Immunogenicity Compared With the Parent Melanoma Cells

Additional differences in the ability to stimulate a T cell response between the hybrid and the parent melanoma cell lines may lie in their capacity to express, process and present antigen (discussed in chapter 5). The range of cytokines secreted by the EBV B-LCL x melanoma cells may also differ to those secreted by the parental melanoma cells. This may enhance hybrid cell immunogenicity and influence the nature of the immune response mounted in MTLC *in vitro*. EBV B-LCL are known to secrete a range of cytokines, including auto-stimulatory growth factors (eg IL-1) (Blazar *et al.*, 1986) and IL-12. It is also possible that cytokine secretion could enhance trans co-stimulation by upregulating T cell co-stimulatory ligand molecule expression on third party cells in the local environment, or by enhancing cross priming.

Melanoma cells have been reported to secrete factors which can suppress cellular immune responses including IL-10 (Chen *et al.*, 1994b; Lattime *et al.*, 1995) and TGF- β (Moretti *et al.*, 1997; Schmid *et al.*, 1995). This should be tested, as it is possible for the parent melanoma cells to secrete inhibitory factors when the hybrid cells do not, which would explain the enhanced T cell stimulatory capacity of the hybrid cell compared with the melanoma cells. It has also been reported for tumour cells to express FAS-L which upon interaction with FAS on the T cell cause T cell apoptosis (Hahn *et al.*, 1996). The highly aggressive murine melanoma cell line, B16, has been show to express substantial levels of FAS-L (Bohm *et al.*, 1998). Expression of FAS-L by the parent tumour and hybrid cells could be identified by flow cytometry for FAS-L expression or testing the capacity of T cells to proliferate in response to restimulation with mitogen after a period in culture with the melanoma or hybrid cells.

4.3.3 Induction of CTL Activity

In order for hybrid cells to be effective as agents in tumour immunotherapy, they should be able to induce a specific CTL response which is effective against the unfused parental cell line. The hybrid cells produced in this study did not show an enhanced capacity to induce specific CTL activity against the parent tumour cell line, as compared with the parent melanoma cells. However, the *in vitro* results may not reflect the *in vivo* status. In a clinical trial, patients with metastatic melanoma were treated with autologous *in vitro* expanded lymphocytes (Rosenberg *et al.*, 1988). The surprising result of study was that the lytic specificity of the lymphocytes in standard ⁵¹Cr release assays did not correlate with the clinical results. The analysis of a specific function (eg *in vitro* CTL activity) may not reflect *in vivo* response due to the complex cellular and cytokine interactions.

The inability of the EBV B-LCL x melanoma hybrid cells to induce specific CTL activity may be due to the absence of CD80/CD86 mediated T cell co-stimulation which would render the T cells anergic. Since this study has shown enhanced T cell stimulation occurs when non-T cells are also cultured with the hybrid cells, it would be interesting to perform the ⁵¹Cr release assays again, assessing specific CTL activity in the presence of responder APC.

4.3.4 Genetic Modification of Hybrid and Melanoma Cells

In agreement with the work of others (Guo *et al.*, 1994; Stuhler and Walden 1994; Dunnion *et al* 1999), the work presented in this chapter showed EBV B-LCL x melanoma hybrid cells were more immunostimulatory than the unfused parent tumour. In contrast, however, the hybrid cells did not retain the APC phenotype, and the presence of bystander cells to enhance the low level of CD80/CD86 independent T cell stimulation by the hybrid cells was required. In order to investigate CD80 mediated T cell co-stimulation directly by the EBV B-LCL x melanoma hybrid cells, tumour and hybrid cells were genetically modified to express CD80. It was difficult to directly compare the immunogenicity of the transfected Gerl43 and HMy2 x Gerl43 hybrid cells, as they did not express CD80 at similar levels as determined by mAb labelling and flow cytometric analysis. The CD80 transfected HMy2 x Gerl43 hybrid cells and Gerl43 melanoma cells were both significantly better at stimulating allogeneic T cell responses than the mock transfected cells. However, the proliferative response induced by the CD80 transfected hybrid cells was significantly better than the CD80 transfected melanoma cells. This may have been due to the increased level of CD80 expression by the hybrid cells compared with the parent melanoma cells. Wu *et al.* (Wu *et al.*, 1995) have shown the level of CD80 expression by transfected tumour cells affects the capacity to induce a tumour specific immune response. Tumour cells modified to express high levels of CD80 were rejected more effectively by mice than those expressing CD80 at lower levels.

Although Townsend and Allison (Townsend and Allison, 1993) showed CD80 transfection into tumour cells induced a T cell mediated immune response which was also able to protect against tumour rechallenge in mice, Wu *et al.* (Wu *et al.*, 1995) showed CD80 expressing tumour cells did not induce long term memory protection. This may have been due to differences in the inherent immunogenicities of the different tumour cell lines used (Chen *et al.*, 1992). The prolonged expression of CD80 by CD80 transfected melanoma cells has also been shown to activate NK cells (Yeh *et al.*, 1995; Geldhorf *et al.*, 1995), preventing the establishment of long-term memory (Chong *et al.*, 1996).

It would be of interest to compare the capacity of CD80 transfected hybrid and melanoma cells with unmodified hybrid and melanoma cells to induce CTL activity in standard ⁵¹Cr release assays. This assay would also show the effects of prolonged CD80 expression on LAK or NK cell development. It would also be of interest to determine whether or not T cells stimulated by the CD80 transfected hybrid cells and melanoma cell have been fully activated, and to compare the T cells to those stimulated by the non-transfected cells. Prolonged CD80 expression also down regulates T cell responses through CTLA-4 ligation (Walunas *et al.*, 1994), so CD80 transfection into the hybrid cells may not translate into an effective clinical vaccination strategy (Thirdborough, 1998). *In vivo* experiments would enable to effectiveness of this strategy to be determined.

4.4 Future Work

The work presented in this chapter has shown hybrid cells formed between EBV B-LCL and melanoma cells, despite having a dominant tumour cell phenotype, were more immunostimulatory as compared with the unfused parent melanoma cell line. This enhanced immune response was not due to a reactivation of EBV specific memory CTLs which circulate in the blood of EBV sero-positive individuals, as there was no qualitative difference between the responses of EBV sero-positive and EBV sero-negative donors.

Why the hybrid cells were more immunostimulatory compared with the parent melanoma cells needs to be determined. The EBV B-LCL x melanoma hybrid cells were able to stimulate allogeneic T cells in both a CD80/CD86 independent manner, and a CD80/CD86 dependent manner which required the presence of responder non-T cells. Future work requires establishing whether or not the T cells activated by the hybrid cells are functional or if the T cells are non-responsive. The range of cytokines secreted by the hybrid cells may differ to those of the melanoma cells and this could be tested by RT-PCR, flow cytometry or ELISA.

CD80 transfected HMy2 x Gerl43 hybrid cells were able to stimulate T cells directly, without needing responder non-T cells. Whether or not transfection of hybrid cells with CD80 is therapeutically more advantageous needs to be assessed. Of particular importance is whether the CD80 transfected cells are able to activate tumour specific T cells and whether the activated CTL are functional.

Unanswered Questions

Are the T cells stimulated by the EBV B-LCL x melanoma hybrid cells fully activated and functional?

Does bystander co-stimulation occur when hybrid cells are cultured with non-T cells and T cells, or do responder APC process and present antigen directly to T cells (ie indirect antigen processing and presentation [cross prime])?

Why do the hybrid cells stimulate an enhanced immune response compared with the parent melanoma cell line?

Do the hybrid cells secrete factors which activate T cells? Do the melanoma cells secrete immunosuppressive factors? Do the melanoma cells express FAS-L? Can CD80 transfected hybrid cells activate tumour-specific CTLs more effectively than CD80 transfected parent melanoma cells, and are hybrid cells better than CD80 transfected tumour cells?



Figure 4.1. Proliferative reponse of PBMCs to stimulation with parent (518.A2) and hybrid (HMy2 x 518.A2) cells. A) Proliferative responses of PBMCs from Responder 1, a normal, allogeneic, EBV sero-negative donor to stimulation with $3.3x10^4$ Mit-C-treated parent EBV B-LCL (HMy2) cells, parent tumour cells (518.A2) or EBV B-LCL x melanoma cell hybrid cells (HMy2 x 518.A2 clone 1, clone 2, clone 3, clone 4 and bulk culture) in MTLC *in vitro*. Cell proliferation is expressed in terms of ³[H] thymidine incorporation (mean +/- s.d.). B) Proliferative responses of PBMCs from Responder 4 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 5 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 5 (\blacksquare), a normal, allogeneic, sero-positive donor to stimulation with $3.3x10^4$ Mit-C treated HMy2 cells, 518.A2 cells or HMy2 x 518 clone 2 hybrid cells.

** highly significant enhanced stimulation compared with 518.A2 (p < 0.01).



Figure 4.2. Proliferative response of PBMCs to stimulation with parent (Gerl43) and hybrid (HMy2 x Gerl43) cells. A) Proliferative responses of PBMCs from Responder 1, a normal, allogeneic, EBV sero-negative donor to stimulation with $3.3x10^4$ Mit-C-treated parent EBV B-LCL (HMy2) cells, parent tumour cells (Gerl43) or EBV B-LCL x melanoma cell hybrid cells (HMy2 x Gerl43 clone 2B1, clone 3B5, clone 1A1 and bulk culture) in MTLC *in vitro*. Cell proliferation is expressed in terms of ³[H] thymidine incorporation (mean +/- s.d.). B) Proliferative responses of PBMCs from Responder 3 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 2 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 2 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 2 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 2 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 2 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 2 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 2 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 2 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 2 (\blacksquare), a normal, allogeneic, sero-positive donor and Responder 2 (\blacksquare), a normal, allogeneic, sero-negative donor, to stimulation with 3.3x10⁴ Mit-C treated HMy2 cells, Gerl43 cells or HMy2 x Gerl43 Clone 3B5 hybrid cells.

* significant enhanced stimulation compared with Gerl43 (p < 0.05).

** highly significant enhanced stimulation compared with Gerl43 (p < 0.01).



Figure 4.3. Proliferative repsonse of PBMCs to stimulation with parent (DAUV) and selected cells (HMy2 x DAUV). Proliferative responses of PBMCs from Responder 1, a normal, allogeneic, sero-negative donor and Responder \equiv (), a normal, allogeneic, sero-positive donor to stimulation with 3.3×10^4 Mit-C treated HMy2 cells, DAUV cells or HMy2 x DAUV selected cells in MTLC *in vitro*. Cell proliferation is expressed in terms of ³[H] thymidine incorporation (mean +/- s.d.).

Figure 6.4. Induction of CTL privity. Represent T with from Supports 1, a metric, ESV percentigative domon wave extincted in either with Mrs-C briefle provided and T with (C-), (Ddy2 (+W+), 518.A2 (+A+) or (Hdy2 x 518.A2 from 7.0 *G) collar rectification allow areas days in the presence of 30.2, (300/raf), and CTL activity determined against unput offic (Ddy2, 518.A2, HMy2 x 516.A2 close 2 and 2.552 on day feation by "Condesse assign Barky of separated CDS+ T offic was > 20%.



Figure 4.4. Induction of CTL activity. Separated T cells from Responder 1, a normal, EBV sero-negative donor were cultured *in vitro* with Mit-C treated unstimulated T cells (\checkmark), HMy2 ($-\bullet$), 518.A2 ($-\bullet$) or HMy2 x 518.A2 clone 2 ($-\circ$) cells, restimulated after seven days in the presence of IL2, (20U/ml), and CTL activity determined against target cells HMy2, 518.A2, HMy2 x 518.A2 clone 2 and K562 on day fourteen by ⁵¹Cr release assays. Purity of separated CD3+ T cells was > 98%.



Figure 4.5. Induction of CTL activity. Separated T cells from Responder 7, a normal, EBV sero-positive, EBV donor were cultured *in vitro* with Mit-C treated unstimulated T cells (\checkmark), HMy2 ($-\bullet$), Gerl43 ($-\bullet$) or HMy2 x Gerl43 ($-\circ$ -) cells, restimulated after seven days in the presence of IL2, (20U/ml), and CTL activity determined against target cells HMy2, 518.A2, HMy2 x 518.A2 and K562 on day fourteen by ⁵¹Cr release assays. K562 targets were not recognised by autologous T cells, HMy2 or HMy2 x Gerl43 stimulated T cells (data not shown). Purity of separated CD3+ T cells was > 94%.



Figure 4.6. Proliferative responses of separated T cell populations to stimulation with parent (518.A2) and hybrid cells (HMy2 x 518.A2). A) Proliferative responses of PBMCs, separated CD3+ T cells, separated CD4+ T cells and separated CD8+ T cells from Responder 1, a normal, allogeneic, EBV sero-negative donor to stimulation with $3.3x10^4$ Mit-C treated HMy2 cells, 518.A2 cells or HMy2 x 518.A2 clone 2, in MLTC *in vitro*. Cell proliferation is expressed in terms of ³[H] thymidine incorporation (mean +/- s.d.). Purity of separated T cell populations was > 98% for CD3+ T cells, > 92% for CD4+ T cells (with < 4% CD8 T cells) and > 86% for CD8+ T cells (with < 2% CD4+ cells). **B)** Proliferative responses of separated CD3+ T cells, separated separated CD45RO+ T cells and separated CD45RA+ T cells from Responder 2, a normal, allogeneic, EBV sero-negative donor to stimulation as above. Purity of separated T cell populations was > 97% for CD3+ T cells, > 94% for CD45RO+ T cells and > 91 % for CD45RA+ T cells. ****** highly significant enhanced stimulation compared with 518.A2 (p < 0.01).



Figure 4.7. Proliferative responses of separated T cell populations to stimulation with parent (Gerl43) and hybrid (HMy2 x Gerl43) cells. A) Proliferative responses of PBMCs, separated CD3+ T cells, separated CD4+ T cells and separated CD8+ T cells from Responder 1, a normal, allogeneic, EBV sero-negative donor to stimulation with $3.3x10^4$ Mit-C treated HMy2 cells, Gerl43 cells or HMy2 x Gerl43 clone 3B5, in MLTC *in vitro*. Cell proliferation is expressed in terms of ³[H] thymidine incorporation (mean +/- s.d.). Purity of separated T cell populations was > 98% for CD3+ T cells, > 92% for CD4+ T cells (with < 4% CD8+ T cells) and > 87% for CD8+ T cells (with < 2% CD4+ T cells). B) Proliferative responses of separated CD3+ T cells, separated separated CD45RO+ T cells and separated CD45RA+ T cells from Responder 2, a normal, allogeneic, EBV seronegative donor to stimulation as above. Purity of separated T cell populations was > 97% for CD3+ T cells, > 94% for CD45RO+ T cells and > 91% for CD45RA+ T cells.

** highly significant enhanced stimulation compared with Gerl43 (p < 0.01).



Figure 4.8. Blocking of MHC class I and MHC class II function in a primary, allogeneic immune response in vitro. A) Monoclonal antibody blocking of proliferative PBMC responses from Responder 1, a normal, allogeneic, EBV sero-negative donor to stimulation with $3.3x10^4$ Mit-C treated HMy2 cells, 518.A2 or HMy2 x 518.A2 clone 2 hybrid cells in MTLC *in vitro* showing inhibition of proliferation in the presence of anti-MHC class I mAb and anti-MHC class II mAb and responses to a non-specific, isotype matched mAb, IgG2a, or a cell culture supernatant. B) Antibody blocking of proliferative PBMC responses from Responder 1 to stimulation with $3.3x10^4$ Mit-C treated HMy2 x Gerl43 cells or HMy2 x Gerl43 clone 3B5 hybrid cells as described above.

(*)* (highly) significant inhibition compared with PBMC response.



Figure 4.9. Blocking of CD40, CD80 and CD86 function in a primary allogeneic immune response in vitro. A) Monoclonal antibody blocking of proliferative responses from Responder 2, a normal, allogeneic, EBV sero-negative donor to stimulation with $3.3x10^4$ Mit-C treated HMy2 cells, 518.A2 cells or HMy2 x 518.A2 clone 2 hybrid cells in MTLC *in vitro* showing inhibition in the presence of CTLA-4 Ig but not with anti-CD40 mAb or a non-specific, isotype matched mAb, IgG1. **B)** Antibody blocking of proliferative PBMC responses from Responder 3, a normal, allogeneic, EBV sero-positive donor to stimulation with $3.3x10^4$ Mit-C treated HMy2 cells, Gerl43 cells or HMy2 x Gerl43 clone 3B5 hybrid cells as described above.

* significant inhibition of PBMC response compared with PBMC response in the presence of IgG_1 (p < 0.05).

** highly significant inhibition of PBMC response compared with PBMC response in the presence of IgG_1 (p < 0.01).



Figure 4.10. T cells are stimulated by parent (518.A2) and hybrid cells (HMy2 x 518.A2) in the presence of Responder non-T cells. Response of $1x10^5$ PBMCs, $1x10^5$ separated CD3+ T cells or CD3+ T cells co-cultured with Mit-C treated non-T cells at $1x10^5$ cells per well or $3.3x10^4$ cells per well from Responder 1, a normal, allogeneic, EBV sero-negative donor, to stimulation with $3.3x10^4$ melanoma cells (518.A2) or hybrid cells (HMy2 x 518.A2) in MTLC *in vitro*. Cell purity was > 90% CD3+ T cells, and < 10% CD3+ T cells present in the non-T cell culture.

*1 significantly enhanced T cell response compared with 518.A2 as stimulator cells plus T cells, and the autologous MLR (p < 0.05).

*² significantly enhanced T cell response compared with 518.A2 as stimulator cells plus T cells (p < 0.05).

** highly significant enhanced T cell response compared with response to stimulation with 518.A2, stimulator cells plus T cells only, and the autologous MLR (p < 0.01).



Figure 4.11. T Cells respond to melanoma (518.A2) and hybrid (HMy2 x 518.A2) cells in the presence of Responder APC: effect of CTLA-4 Ig blocking. Response of PBMCs, T cells and T cells cultured with Mit-C treated non-T cells from Responder 1 to stimulation with melanoma cells (518.A2) or hybrid cells (HMy2 x 518.A2 clone 2) in MTLC *in vitro* in the presence of CTLA-4 Ig or isotype matched, non-specific IgG1 control. Cell purity was >95% CD3+ T cells and < 25% CD3+ T cells present in the non-T cell culture. * significant inhibition (p < 0.05). ** highly significant inhibition (p < 0.01).


Figure 4.12. Phenotypic characterisation of parent melanoma cells (Gerl43) and hybrid cells (HMy2 x Gerl43) transfected with CD80 by mAb labelling and immunofluoresence techniques followed by flow cytometric analysis. Gerl43 transfected with CD80 (Gerl43.pCR3.CD80) expressed CD80 at lower levels compared with hybrid cells transfected with CD80 (HMy2 x Gerl43.pCR3.CD80). Solid areas are mock transfected cell controls, and open areas are CD80 transfectants stained with anti-CD80 mAb.



Figure 4.13. Proliferative responses of PBMCs to stimulation with parent (Gerl43) and hybrid cells (HMy2 x Gerl43) either mock transfected or transfected with the gene for CD80. Proliferative responses of PBMCs from Responder 2, a healthy, allogeneic, EBV sero-negative donor, to stimulation with $1x10^4$ Mit-C treated EBV B-LCL (HMy2), mock transfected melanoma and EBV B-LCL x melanoma cells (Gerl43.pCR3 and HMy2 x Gerl43.pCR3.CD80 and HMy2 x Gerl43.pCR3.CD80 in MTLC in vitro.

* significantly enhanced PBMC response compared with mock transfected cell control (p < 0.05).



Figure 4.14. Proliferative responses of separated T cell populations to stimulation with parent (Gerl43) and hybrid cells (HMy2 xGerl43) mock transfected or transfected with the gene for CD80. Proliferative responses of PBMCs, separated CD3+ T cells, CD4+ T cells and CD8+ T cells from Responder 2, a normal, allogeneic, EBV sero-positive donor to stimulation with $3.3x10^4$ Mit-C treated parent EBV B-LCL (HMy2), mock transfected cells (Gerl43.pCR3 and HMy2 x Gerl43.pCR3) or cells transfected with CD80 (Gerl43.pCR3.CD80 and HMy2 x Gerl43.pCR3.CD80) in MTLC *in vitro*. Purity of the separated cells was > 97% for CD3+ T cells, > 95% for CD4+ T cells, and > 90% for CD8+ T cells (with < 1% CD4+ T cells). ** highly significant enhanced stimulation compared with mock transfected cell control (p < 0.01).

Chapter 5

Antigen Expression, Processing and Presentation

Antigen Expression, Processing and Presentation

5.1 Introduction

This chapter describes the antigen expression, and processing and presentation through the MHC class I pathway, of the EBV B-LCL x melanoma hybrids. The introduction describes the generation and use of tumour-specific CTLs in tumour immunology in general terms. Mechanisms by which tumour cells are able to avoid immune detection are described, with attention focused on TAP and Lmp defects. In addition, some viruses, including EBV, are known to employ methods which prevent the immune recognition of infected cells. This is also discussed in the context of EBV latency.

5.1.1 Melanoma Antigenicity and Immunogenicity

5.1.1.1 Antigen Identification

Tumour-specific CTLs circulate in low numbers in the blood from cancer patients and metastatic melanoma is often infiltrated with T lymphocytes (TILs). These cells can be cultured in vitro with the patients' tumour cells and expanded (thor Straten et al., 1996; Kirkin et al., 1995). The derived CTL clones exert lytic activity on autologous tumour cells and allogeneic tumours expressing the same tumour antigen and MHC matched restriction element (Kirkin et al., 1995). Peptides recognised by CTLs can be identified by transfecting CTL resistant tumour cell variants with DNA prepared from a cosmid library. Transfectants expressing the antigen can then be identified, ultimately leading to the identification of individual tumour antigens. This method is based upon the pioneering work of Boon et al. who used melanoma-specific CTL to identify the antigen, MAGE 1 (van der Bruggen et al., 1991). Since then, the approach has been further improved by transfecting the cDNA libraries into COS cells, leading to high levels of DNA expression (Brichard et al., 1993; Coulie et al., 1994; Wang et al., 1995). The draw back of these approaches is the requirement for determining the MHC restriction element prior to library screening. More recently, retroviral-based cDNA expression systems have been used to introduce cDNA libraries into autologous fibroblast or EBV B-LCL with high efficiencies to identify tumour antigens recognised by CD8+ T cells without knowledge of the restriction element (Wang et al., 1998b). Tumour specific CTL have been identified from a wide range of tumour types including melanoma (Herin et al., 1987; Knuth et al., 1989), renal cell (Finke et al., 1992), ovarian (Ioannides et al., 1991) and head and neck cancers (Yasumura *et al.*, 1993). Melanoma remains to date the best characterised tumour from an immunological point of view due to the relative ease of growing these cells *in vitro* compared with other tumours.

Several other methods have been employed to identify tumour antigens. One other method for identifying tumour involves peptide elution from tumour cells by acid treatment in vitro (Cox et al., 1994). Eluted peptides can be separated by high pressure liquid chromatography (HPLC), tested for their ability to stimulate cytokine secretion from CTL when pulsed onto MHC-matched APC, and sequenced by tandem mass spectrometry. Based on motifs and the identification of anchor residues for different HLA specificities, a computer programme has been designed to identify peptides for binding to other HLA molecules. Synthetic peptides generated from a known protein sequence can be tested for their ability to bind to particular MHC molecules (Celis et al., 1994). Some identified antigenic peptides have only been shown to induce cytotoxicity against peptide loaded cells, and it is not known whether or not these peptides are actually processed and presented naturally. More recently, cancer patients' Ab repertoires have been used successfully to serologically identify target antigens, some of which also represent targets for CTL (SEREX) (reviewed in Tureci et al., 1997). In order to demonstrate the antigen identified by SEREX is a T cell antigen, peptides derived from the putative antigen are used to generate CTLs in vitro and show that these T cells can also recognise tumour cells.

5.1.1.2 Melanoma Cell Antigenicity

A number of HLA class I binding antigenic epitopes from melanoma proteins have been characterised (Table 1.2). The melanoma-associated antigens which are recognised by CTLs belong to three main categories: cancer/testis-specific antigens (MAGE, BAGE, GAGE, PRAME and NY-ESO-1), melanoma specific antigens (tyrosinase, gp100, MelanA/MART1, TRP-1 and TRP-2) and mutated or aberrantly expressed antigens (MUM-1, CDK-4, β -catenin and N-acetylglucosaminyltransferase V) (section 1.6).

5.1.1.3 Immunogenicity of Melanoma-Associated Antigens

Although CTLs recognising tumour-associated antigen have been cultured artificially *in vitro*, in most cases the *in vivo* T cell response is insufficient, so tumour growth and progression often occurs. The MAGE proteins are thought to have low immunogenicity due to the long α helical stretches which stabilise the protein structure and

hinder its degradation by proteolytic cleavage (Kirkin *et al.*, 1998). Expression of the peptide/MHC complexes on the cell surface may be too low for recognition by specific CTL *in vivo*.

In vivo, tolerance normally exists for highly immunogenic 'self' antigens, but the immune system is not tolerant to low or intermediate immunogenic 'self' antigens. The melanocyte differentiation antigens are degraded peptide products from 'self' proteins which are expressed by normal melanocytes and melanomas. The immune system is not tolerant to these proteins, as T cell responses directed against the melanocyte differentiation antigens have been identified in vitro (Anichini et al., 1993). However, the in vitro response may not accurately reflect the in vivo immune status, as the majority of the characterised peptide epitopes have low or intermediate affinity for HLA class I antigens and can only activate CTL lysis at relatively high concentrations (Kawakami and Rosenberg, 1996; Wolfel et al., 1994). Expression of peptides derived from the melanocyte-differentiation antigens complexed with MHC molecules on the cell surface may be too low to induce a naïve T cell response in vivo. Tolerance to highly immunogenic peptides can be broken by presenting them in allogeneic MHC class I molecules at high concentrations (Sadonikova and Stauss, 1996). This is an important observation from an immunotherapeutic point of view.

Low immunogenicity against mutated gene products may be expected from the low probability that a high affinity binding peptide would be generated from a mutation in a protein. Low affinity binding peptides may not be expressed in conjunction with MHC molecules on the cell surface at high enough concentrations to prime a naïve T cell response.

5.1.1.4 Use of In Vitro Cultured CTL in Cancer Therapy

As described previously (section 1.2), it is generally accepted by Immunologists that amplification of tumour-specific CTLs *in vivo* can result in the eradication of tumours. Since priming of a naïve T cell response requires approximately 1000 fold higher concentration of antigenic peptides than required for lysis by an activated CTL, *in vitro* cultured tumour-specific CTL clones have been used in adoptive T cell therapy. After activation, antigen specific CTLs are capable of killing tumour cells expressing the peptide epitope at lower concentrations. Success using this approach has been shown *in vitro*, and in a number of animal studies using CTLs directed against melanoma-associated antigens (reviewed in Greenberg *et al.*, 1991). The adoptive transfer of EBV specific T cell clones

into patients with malignant EBV-associated B-cell lymphoproliferative disorders has shown the approach was safe and successful against virus infected cells (Papadopoulos *et al.*, 1994). The results from various other clinical trials using this strategy in cancer therapy have not been as encouraging. This may be due to the infusion of CTLs recognising only the strongest antigens into cancer patients, which does not take into account the great heterogeneity which exists in tumour masses *in vivo*. This approach may also select for antigen loss variants (Topalian *et al.*, 1990).

5.1.2 Tumour Escape From Immune Recognition

In spite of the presence of tumour-specific CTL in patients with cancer, and the promising results of animal studies and clinical trials, the host immune response is usually not adequate for complete tumour eradication. A variety of mechanisms, acting singularly or in combination, enable tumour cells to avoid immunological elimination. These mechanisms can occur in a range of different cancers. Such evasion tactics include loss or down regulation of MHC molecules preventing antigen presentation. This may be in the form of single, haplotype, locus or complete loss of HLA expression which may be due to the loss of expression of $\beta 2m$ or TAP down regulation (reviewed by Browning and Dunnion, 1997). Low expression of HLA/antigen complexes on the cell surface can induce T cell anergy. In addition, it has been reported for tumour cells to lack the expression of various co-stimulatory molecules, including CD40, CD80 and CD86, which play a vital role in T cell signalling. This would also render tumour-specific T cells anergic. The loss of expression of various adhesion molecules has also been documented, including VCAM-1 which prevents the invasion of T cells into the tumour site (Piali et al., 1995). Other mechanisms postulated to account for the escape of tumours from immune recognition include the expression of Fas ligand and the production of various immune suppressive cytokines (reviewed in thor Stratten et al., 1999).

5.1.2.1 Antigen Processing Deficiencies in Tumour Cells

Often associated with malignant transformation is the specific down-regulation of TAP and Lmp expression. These molecules play an important role in the processing and presentation of tumour-associated antigens to antigen specific CTLs.

In humans, the TAP and Lmp genes are located in a tight cluster in the class II region of the MHC complex situated on chromosome six (Beck et al., 1992). TAP 1 and

Lmp 2 are co-ordinately regulated by a common promoter (Wright *et al.*, 1995). The inducible expression of the TAP and Lmp genes with IFN- γ suggests these molecules are important in the host immune response. Cultured melanocytes constitutively express TAP 1, TAP 2 and to a lesser extent Lmp 7, but do not express Lmp 2 (D.J. Hicklin and S. Ferrone, unpublished data).

The TAP proteins are required for the efficient transport of antigenic peptides generated in the proteasome into the ER where they bind with empty MHC class I molecules. The MHC/antigen complex is then expressed on the cell surface. The TAP transporter is composed of two non-covalently associated subunits, TAP 1 and TAP 2. TAP 2 exists as two polymorphic forms in the human population, TAP 2a and TAP 2b, which are functionally identical. Cells with reduced expression or absence of TAP 1 and / or TAP 2 are unable to load peptides onto MHC molecules in the ER and are impaired in their ability to express MHC/antigen complexes on the cell surface.

In the proteasome, endogenous cellular proteins are degraded into peptides for presentation on HLA class I molecules. Lmp 2 and Lmp 7 proteins are β -subunits of the proteasome which alter its proteolytic activities, enhancing the production of peptides which bind preferentially with MHC class I molecules. Although defects in the expression of Lmp proteins do not affect MHC/antigen expression on the cells surface, Lmp negative human and mice cells are unable to present certain CTL epitopes.

The down-regulation of TAP and Lmp proteins has been shown in a range of tumours including small cell lung carcinoma (Restifo *et al.*, 1993a), cervical carcinoma (Keating *et al.*, 1994; Cromme *et al.*, 1994) and melanoma (Kageshita *et al.*, 1999). However, thor Straten *et al.* (thor Straten *et al.*, 1997) used cell lines and RT-PCR to demonstrate that the loss of TAP 1 and TAP 2 and Lmp 2 and Lmp 7 did not appear to be a common mechanism of immune escape in malignant melanoma.

To date, three more genes which are located in the class II region of the MHC, associated with antigen processing and IFN- γ inducible, have been identified. These are PA28 α and PA28 β , which are proteasome regulators, and tapasin. Mutants lacking tapasin show reduced levels of MHC class I molecules on the cell surface, similar to the defects of TAP negative mutants. Lmp 10 (MECL-1) is not MHC linked, but like Lmp 2 and Lmp 7, it replaces a homologous constitutive subunit of the proteasome after IFN- γ induction. Defects in the expression of these proteins by malignant cells have yet to be shown.

5.1.3 EBV Protein Affects Antigen Processing

In order to avoid immune detection, viruses have also been shown to specifically target antigen processing pathways.

EBV B-LCL display an activated B cell phenotype with high levels of MHC class I and class II molecules, co-stimulatory and adhesion molecules (Thorley-Lawson et al., 1985). EBV B-LCL are associated with the expression of the complete spectrum of viral latent proteins (latency III) and display viral antigens on the cell surface in the context of MHC class I molecules which are excellent targets for EBV specific CTL (reviewed in Kieff, 1996). In contrast, in BL cells, a malignancy associated with EBV, only one EBV protein, EBNA 1, which is resistant to antigen processing in the proteasome, is expressed (latency I) (Rowe et al., 1987b). Furthermore, BL cells are not recognised by HLA class I restricted CTLs (Rooney et al., 1985). Even when a known target antigen expressed by a vaccinia viral vector was transfected into BL cells, they were still poorly recognised by antigen specific CTL, indicating a defective MHC class I processing pathway (Rowe et al., 1995). This defect was found to be associated with a down-regulation of TAP 1, and TAP 2 to a lesser extent. BL cells also expressed a reduced level of MHC class I molecules on the cell surface which was associated with a reduced rate of MHC class I synthesis. The expression of the LMP 1 virally encoded protein in these cells was able to upregulate TAP and MHC class I expression and mimic the effects of IFN-y, therefore EBV has several mechanisms by which it can modulate antigen processing and presentation. In this study, the EBV B-LCL x melanoma hybrid cells, like BL cells, expressed EBNA 1 viral latent protein only.

5.1.4 Objectives of this Chapter

For APC x tumour cell hybrids to be of value in cancer therapy, they must retain the expression of tumour-specific antigens, and process and present these effectively to the host immune system. The ultimate aim using hybrid cells in cancer therapy is to use the patient's own tumour cells in the APC x tumour hybrids so the complete, unique spectrum of tumour-associated antigens is retained. In this study, the expression of shared, tumourspecific (cancer/testis-specific) (MAGE 1, MAGE 2 and MAGE 3) and melanoma-specific antigens (tyrosinase, gp100 and MelanA/MART1) by the EBV B-LCL x melanoma hybrids was determined by RT-PCR analysis. RT-PCR is used to determine the expression of genes at the mRNA level. These antigens were chosen as they have been extensively characterised and they are prevalent on melanoma cells. Approximately 40% of melanoma samples are positive for MAGE 1 and about 75% are positive for MAGE 2 or MAGE 3. Only a small proportion of melanoma samples do not express any of these three antigens. The level of expression of these antigens is variable between tumour cell lines. To observe the level of expression of the antigens by the hybrid cell, semi-quantitative RT-PCR was also performed. It is also known that expression of the gene does not always correlate with protein expression or presentation of antigenic peptides derived from the protein as some proteins, including the MAGE proteins, are not efficiently degraded in the proteasome, especially those which have structures which make them resistant to unfolding.

Hybrid cells must also be able to effectively present tumour-associated antigen in the context of MHC class I molecules in order to stimulate a specific CTL cell response. Hybrid cell susceptibility to lysis by antigen specific, HLA restricted CTL clones was assessed in ⁵¹Cr release assays. The CTL clones used in this study were generated by culturing irradiated melanoma cells with autologous lymphocytes *in vitro* and have been described previously (Herin *et al.*, 1987; Coulie *et al.*, 1994). The anti-MAGE 1 clone (82/30) recognises the antigenic peptide EADPTGHSY presented through HLA-A1 (Traversari *et al.*, 1992), the anti-MAGE 3 clone (297/22) recognises the peptide epitope FLWGPRALV presented through HLA-A2 (van der Bruggen *et al.*, 1994a) and the antityrosinase clones (IVSB and 210/9) recognise YMNGTMSQV and MLLAVLYCL presented through HLA-A2 (Wolfel *et al.*, 1994). These CTL clones do not lyse NK cell sensitive and autologous EBV transformed B lymphocyte targets.

In light of the effects EBV has on modulating antigen processing and presentation through one of its own gene products in infected cells, and considering the defects some tumours have in antigen processing and presentation, it was important to assess the expression of the various components associated with endogenous antigen processing by the hybrid cells. Since TAP and Lmp are important proteins in antigen processing, their expression by the hybrid cells was determined by Western blot analysis. The level of expression of these proteins by the hybrid cells was of further interest due to the inheritance of chromosome six (on which the MHC complex is situated) from both parent cell lines.

5.2 Results

5.2.1 Tumour Associated Antigen Expression by RT-PCR

To detect the expression of tumour-associated antigens by HMy2 x 518.A2 (uncloned bulk culture, clone 2, clone 3 and clone 4), HMy2 x Gerl43 (clone 3B5) hybrid cells, HMy2 x DAUV (uncloned bulk culture) selected cells and the relevant parent cell lines, a series of RT-PCR reactions were performed on total RNA extracts. The expression of the well characterised, cancer/testis-specific genes (MAGE 1, MAGE 2 and MAGE 3), and the melanocyte differentiation genes (MelanA/MART1, tyrosinase and gp100) was assessed using primers specific for each gene. Amplification products were size fractionated on 1-2% agarose gels, allowing genomic DNA contamination to be identified due to the differences in PCR product sizes compared with cDNA product sizes. For several PCR reactions, the primers were designed specifically across exons to avoid genomic DNA amplification.

5.2.1.1 Reaction Specificity

At each stage of the RT-PCR procedure, from the RNA extraction to the PCR reaction, a negative control (consisting of Sigma water with no DNA template) was included in order to test for possible contamination. A known positive control sample was also included at each appropriate stage. The human derived melanoma cell line, Gerl43 (passage 29), which is known to express MAGE 1, MAGE 2, MAGE 3 and tyrosinase, was used as the positive control for the expression of these genes, and a second human derived melanoma cell line, MEL-SK23, which is known to express gp100 and MelanA/MART1 genes, was used as the positive control for the expression of these genes (S. Rogers, personal communication). In all reactions, the negative control did not produce a PCR product and the positive control produced a PCR product of the correct size. Results of RT-PCRs are shown in Table 5.1.

5.2.1.2 Confirmation of cDNA Synthesis

The conversion of total RNA to cDNA was confirmed in a PCR reaction using primers detecting the β -actin gene to ensure the RNA was not degraded. The β -actin gene is a house-keeping gene and codes for a cyto-skeletal protein which is ubiquitously expressed. All cDNA samples; HMy2, 518.A2, HMy2 x 518.A2 (clone 2, clone 3, clone 4

Cell Line	MAGE 1	MAGE 2	MAGE 3	Tyrosinase	MelanA/	gp100
					MART1	
HMy2	-	-	+	· •	-	-
518.A2-less than 45 passages*	+	+	+	+	+	+
518.A2-passage 49 and 150	+	+	+	-	-	-
HMy2 x5 18.A2 bulk	+	+	+	-	-	-
HMy2 x 518.A2 clone 2	+	+	+	-	-	-
HMy2 x 518.A2 clone 3	+	+	+	-	-	-
HMy2 x 518.A2 clone 4	+	+	+	-	-	-
Gerl43-passage 33	+ .	+	+	+	-	-
Gerl43-passage 150	-	-	-	-	-	-
HMy2 x Gerl43 clone 3B5	+	+	+	+	-	-
DAUV	-	-	+	-	-	-
HMy2 x DAUV	-	-	+ .	-	-	-

Table 5.1. *Tumour-associated antigen expression is retained by the hybrid and selected cells.* RT-PCRs were performed using total RNA extracts from various clones and a bulk culture of HMy2 x 518.A2 hybrid cells, HMy2 x Gerl43 clone 3B5 hybrid cells, and a bulk culture of HMy2 x DAUV selected cells. All relevant parent cell lines were also assessed. A sample of the PCR product was electrophoresed in an agarose gel and visualised by ethidium bromide staining. Bands corresponding to the size of the specific cDNA PCR poducts were scored as positive samples. No bands or bands whose size corresponded to the amplified genomic DNA were scored as negative (-) samples.

* personal communication, Dr A. Murray, Sheffield.

and bulk culture), Gerl43, HMy2 x Gerl43 (clone 3B5), DAUV and HMy2 x DAUV, were included in the PCR. PCR products were size fractionated on an agarose gel and appropriate sized bands were visualised in all test samples. Figure 5.1 shows the data for HMy2, Gerl43, DAUV and HMy2 x DAUV. Similar results were seen for 518.A2 and HMy2 x 518.A2 (data not shown). Genomic DNA contamination could be identified in cDNA preparations due to size differences in the PCR products. Band intensities were equal for all cell lines, indicating the same amount of cDNA had been used in each PCR reaction.

5.2.1.3 Tumour-Associated Antigen Gene Expression in HMy2, 518.A2 and HMy2 x 518.A2 Bulk and Cloned Hybrid Cell Lines

Originally, the human derived melanoma cell line, 518.A2, tested positive by RT-PCR for the expression of MAGE 1, MAGE 2, MAGE 3, tyrosinase, MelanA/MART1 and gp100 (personal communication, Dr A Murray, Sheffield, UK). The melanoma cell line was received as a gift from Dr A. Murray, Sheffield, UK, at passage 45 and the cells were used at passage 49 as a parent cell line in EBV B-LCL x tumour cell hybrids. Total RNA was extracted from the melanoma cells at passage 49. RT-PCR analysis performed on this sample showed the melanoma cells expressed MAGE 1, MAGE 2 and MAGE 3, but did not express the melanocyte differentiation antigens (tyrosinase, MelanA/MART1 and gp100). The PCRs were repeated and confirmed by S Rogers, Sheffield, UK (section 5.2.2). The RT-PCR tests used in this study were performed using primers identical to those used by Dr A Murray, Sheffield, UK. The discrepancies between the two sets of results may have been due to differences in sensitivities of the assays in the two different laboratories or the cell line 518.A2 may have lost the expression of these genes following continuous growth in culture. Monitoring the expression of these three antigens up to passage 150 showed MAGE 1, MAGE 2 and MAGE 3 expression by the melanoma cell line was stable in vitro (Figure 5.2).

Total RNA was extracted from HMy2 x 518.A2 uncloned bulk culture, clone 2, clone 3 and clone 4. Of the MAGE genes assessed in this study, the hybrid cell clones and the uncloned hybrid cell culture expressed all three, demonstrating that HMy2 x 518.A2 hybrid cells retained tumour associated antigen expression, in spite of the melanoma cell being fused with an EBV B-LCL (Figure 5.2). Like the melanoma parent cell line, the hybrid cells did not express the melanocyte differentiation antigens (tyrosinase, gp100 and MelanA/MART1) as determined by RT-PCR (data not shown). To further enhance the

sensitivity of the tyrosinase PCR, the number of annealing cycles were increased from 25 to 40 cycles, but the samples remained negative for tyrosinase expression.

The results of the RT-PCR showed the RNA sample extracted from the EBV B-LCL cell line, HMy2, was positive for MAGE-3 expression. This was not genomic DNA contamination, as it would have produced a different sized PCR product (Figure 5.3). The unexpected finding was repeated and confirmed by Southern blot analysis, a method employed to detect very low levels of MAGE gene expression, kindly performed by Ms Sheila Rogers, Sheffield, UK (described in Mulcahy *et al.*, 1996). This result was surprising since the only normal tissues known to express MAGE genes are the testis and placenta, but consistent with the low level of CTL by a MAGE 3 specific CTL clone (section 5.2.3). It is possible EBV transformation of B cells may allow low levels of expression of the MAGE genes. However, the EBV B-LCL cell line did not express MAGE 1 or MAGE 2 and also did not express the melanocyte differentiation antigens, tyrosinase, gp100 and MelanA/MART1 as determined by RT-PCR.

5.2.1.4 Tumour-Associated Antigen Gene Expression in Gerl43 and HMy2 x Gerl43 Hybrid Cells

The melanoma cell line, Gerl43, was received at passage 29 as a gift of Dr P Coulie, Brussels, Belgium. At passage 33, the melanoma cells were used as fusion partners in the production of EBV B-LCL x melanoma hybrid cells. An RNA preparation was made from the Gerl43 cells at this stage and the cells were tested by RT-PCR for the expression of several melanoma-associated antigen genes. The expression of MAGE 1, MAGE 2, MAGE 3 and tyrosinase was detected, but the cells did not express gp100 or MelanA/MART1. This was in accordance with previous studies using this cell line (Coulie et al., 1996). The cell line was grown routinely in culture and at passage 150, a second RNA sample was made from the Gerl43 cell line. When this sample was tested by RT-PCR, the cells no longer expressed any of the genes tested in this study. The data indicated that, over a period of time in culture, the melanoma cells had lost the expression of MAGE 1, MAGE 2, MAGE 3 and tyrosinase. Furthermore, to detect the presence of the MAGE 2 and MAGE 3 genes in the Gerl43 cells total genomic DNA isolated from the melanoma cells at this later passage was used in the MAGE 2 and MAGE 3 PCR reactions, replacing cDNA. The MAGE 2 and MAGE 3 genes were detected in the genomic DNA sample, as different sized PCR products were produced when compared with the cDNA samples (data not shown). Table 2.4 lists the PCR product sizes produced in the RT-PCR reactions. This indicates the two MAGE genes had been down-regulated by the Gerl43 cells, after a period of growth in culture. This is also likely to be true for MAGE 1 since it is located on the same chromosome as MAGE 2 and MAGE 3. The MAGE 1 and tyrosinase specific primers do not amplify genomic DNA genes so were not used in PCR reactions performed with genomic DNA.

An RNA extract from HMy2 x Gerl43 clone 3B5 which had been grown in culture to passage 75 was made and RT-PCR reactions were performed. This cloned hybrid cell line retained the expression of MAGE 1, MAGE 2, MAGE 3 and tyrosinase but did not express gp100 or MelanA/MART1 (Figure 5.4). Surprisingly, the expression of these genes was identical to the Gerl43 parent cells at the start of the study indicating, the hybrid cells have stably retained tumour-associated antigen gene expression after a length of time in culture, even though the parent Gerl43 melanoma cells had not. However, the loss of antigen expression over time in culture by the parent melanoma cells indicates the potential for antigen loss and therefore periodic characterisation of antigen expression in the hybrid cells for use in cancer vaccination is necessary.

5.2.1.5 Tumour-Associated Antigen Gene Expression in DAUV and HMy2 x DAUV Selected Cells

The DAUV cell line expressed the tumour-associated antigen MAGE 3, but did not express MAGE 1 or MAGE 2 and also did not express the melanoma specific antigens tyrosinase, MelanA/MART1 or gp100. The HMy2 x DAUV selected cells demonstrated an identical pattern of expression of these tumour-associated antigens compared with the melanoma parent cells expressing MAGE 3, but not MAGE 1 or MAGE 2 and none of the melanocyte differentiation antigens (Figure 2.4).

5.2.2 Semi-Quantitative PCR Analysis To Detect the Level of Expression of Tumour-Associated Antigens by HMy2 x 518.A2 Hybrid Cells and the Relevant Parent Cell Lines

The RT-PCRs for MAGE 1, MAGE 2, MAGE 3, tyrosinase, gp100 and MelanA/MART1 were performed semi-quantitatively by S. Rogers, Sheffield, UK, as described elsewhere (Mulcahy *et al.*, 1996). Band intensities from the test samples were visually compared with the PCR products from a reference cell line known to express the antigen of choice. The positive control for MAGE 1, MAGE 2, MAGE 3 genes and tyrosinase expression was a cDNA sample from the cell line MZ-2 and the positive controls for MelanA/MART1 and gp100 gene expression was a cDNA sample from the

cell line MEL-SK23. From the control cell line, $2\mu g$, $0.2\mu g$ and $0.02\mu g$ of RNA was converted to cDNA and used in each PCR reaction to obtain a series of reference signals. The level of gene expression from the test samples was positively correlated with the band intensity, and compared with the reference signals. To detect very low levels of the MAGE gene expression (weak expression), Southern blot analysis was performed as described elsewhere (Mulcahy *et al.*, 1996).

The results from the semi-quantitative PCR analysis confirmed the results of the previous findings, with one exception, and further add to the results of the previous findings. The one difference between these results and the results of the previous findings was the weak expression of MAGE 1 by HMy2. MAGE 1 gene expression by HMy2 was not detected previously (section 5.2.1.3). Different HMy2 x 518.A2 clones and the bulk hybrid cell culture express the tumour-associated antigens at variable levels. All of the hybrid cell culture expressed the MAGE 1 gene at a level which was greater than the melanoma parent. The MAGE 2 gene was weakly expressed by 518.A2 parent cells and by the uncloned hybrid cell population, but expressed at a higher level by HMy2 x 518.A2 clone 2 and expressed at moderate levels by the 518.A2 melanoma cell line. However, the MAGE 3 gene was expressed at either weak or low levels by the uncloned bulk hybrid cell culture, HMy2 x 518.A2 clone 3 and clone 4. Table 5.2 shows the results of the semi-quantitative RT-PCR.

5.2.3 Ability of the Hybrid Cells To Present Tumour-Associated Antigens Through MHC Class I

By labelling cells with HLA-specific mAbs followed by flow cytometric analysis, it was shown previously HMy2 cells expressed the HLA class I molecules HLA-A2 and HLA-A3 (Figure 3.6) and the 518.A2 expressed HLA-A1 and HLA-A2 on the cell surface (Figure 3.7). HMy2 x 518.A2 clone 2 expressed HLA-A1, HLA-A2 and HLA-A3 on the cell surface (Figure 3.7). In addition, the melanoma cell line 518.A2 and HMy2 x 518.A2 hybrid cells expressed melanoma-associated antigens MAGE 1 and MAGE 3 at mRNA level. Unexpectedly, HMy2 also expressed MAGE 3 at the mRNA level as determined by RT-PCR (section 5.2.1.3). Tyrosinase gene expression was not detected by RT-PCR in any of the HMy2 x 518.A2 hybrid cells or the parent cell lines.

Cell Line	MAGE 1	MAGE 2	MAGE 3	Tyrosinase	MelanA / MART1	gp100
HMy2	W	-	++	-	-	-
518.A2	W	W	++	-	-	-
HMy2x518.A2 bulk	++	W	+	-	-	-
HMy2x518.A2 cl2	+++	+	+++	-	-	-
HMy2x518.A2 cl3	++	+	W	-	-	-
HMy2x518.A2 cl4	+++	+	W	-	-	-

Table 5.2 Enhanced expression of various tumour-associated antigens by HMy2 x 518.A2 hybrid cells compared with the parent melanoma cells. The level of gene expression was scored according to band intensity of PCR products stained with ethidium bromide, and compared with the control reference signals according to the following scale:

- = no gene expression.

W = weak gene expression (band intensity < 1 in 100 dilution of reference RNA and detectable by Southern blot).

+ = low level of gene expression (band intensity > 1 in 100 dilution of reference RNA but < 1 in 10 dilution).

++ = moderate level of gene expression (band intensity > 1 in 10 dilution of reference RNA but < undiluted).

+++ = strong level of gene expression (band intensity equal to or greater than the undiluted reference RNA).

The combination of HLA-class I and melanoma antigen expression allowed the capacity for hybrid cells to present melanoma-associated antigens to be tested in ⁵¹Cr release assays using antigen specific, HLA restricted CTL clones. The CTL clones used in this study recognised MAGE 1 presented by HLA-A1 and MAGE 3 and tyrosinase, both presented through HLA-A2, and have been described elsewhere (Herin *et al.*, 1987. Coulie *et al.*, 1994). The assays presented in the following experiments (Figuress 5.5, 5.6 and 5.7) were performed (as described in Coulie *et al.*, 1994) by Dr P Coulie, Brussels, Belgium, using previously described melanoma specific CTL clones (Herin *et al.*, 1987; Coulie *et al.*, 1994). Lysis of the ⁵¹Cr labelled target cells by the antigen specific, HLA restricted CTL clones was used as a measure of the cell's antigen presenting capability.

5.2.3.1 Presentation of the Tumour-Associated Antigen, MAGE 1, MAGE 3 and Tyrosinase by HMy2 x 518.A2 Clone 2 and the Parent Cell Lines

Figure 5.5 A shows the capacity of the melanoma cell line 518.A2 and cloned hybrid cell line, HMy2 x 518.A2 clone 2, to present MAGE 1 to antigen specific HLA-A1 restricted CTL clones. The parent melanoma cell line, 518.A2, was lysed by the MAGE 1 specific, HLA-A1 restricted CTL clone with relatively low efficiency. Various cloned hybrids derived from the fusion of 518.A2 with HMy2 were also tested for their ability present MAGE 1. HMy2 x 518.A2 clone 2 and the bulk uncloned cells were lysed with much greater efficacy than the parent cells. This may have been due to the enhanced expression of this gene compared with the melanoma parent cell line. HMy2 x 518.A2 clone 3 was lysed at a level which was similar to the melanoma parent cells (data not shown) whereas clone 4 was lysed with much greater efficacy than the parent cell line, which did not express HLA-A1, was not recognised by the antigen specific CTL clone.

The 518.A2 parent cells were pre-incubated in IFN- γ before being used as targets for recognition by the MAGE 1 specific, HLA-A1 restricted CTL clone in one experiment. Lysis of these cells by the MAGE 1 specific CTL clones was increased to levels similar to those by the clone 2 hybrid cells and to levels similar to Gerl43 cells from which this CTL clone was established (Figure 5.6). This may have been due to the partial down-regulation of the MHC class I processing pathway in the 518.A2 cell line which could be augmented by treatment with IFN- γ . Figure 5.5 B shows the capacity of the melanoma cell line and HMy2 x 518.A2 clone 2 and an uncloned hybrid cell culture to present MAGE 3 to antigen specific HLA restricted CTL clones. The parent melanoma cell line 518.A2, was lysed by the MAGE 3 specific, HLA-A2 restricted CTL clone with low efficiency. This was comparable to the level of lysis by the MAGE 1 specific, HLA-A1 restricted CTL clone, despite the MAGE 3 gene being expressed at higher levels as determined by semi-quantitative RT-PCR (Table 5.2). The uncloned hybrid cells, which expressed the MAGE 3 gene at a lower level than the 518.A2 parent cells, were lysed with much greater efficacy than the parent melanoma cell line, and at a level similar to that seen for HMy2 x 518.A2 clone 2 which strongly expressed the MAGE 3 gene. HMy2 x 518.A2 clone 3 and HMy2 x 518.A2 clone 4 were lysed with low efficiency (data not shown), in line with the weak expression of the MAGE 3 gene by these cells. The MAGE 3 specific CTL clone showed a low level of recognition of HMy2, supporting the expression of the MAGE 3 gene as determined by RT-PCR.

Two anti-tyrosinase, HLA-A2 restricted CTL clones (IVSB and 210/9) were used to assess the capacity of the parent and HMy2 x 518.A2 cell line to present tyrosinase epitopes through HLA-A2. 518.A2, HMy2 and HMy2 x 518.A2 clone 3 and clone 4 were not lysed by the two HLA-A2 restricted CTL clones specific for tyrosinase (specific lysis was less than 5% at each E:T ratio), supporting the results of the RT-PCR which showed the cell lines did not express this gene (data not shown). However, HMy2 x 518.A2 clone 2 was lysed by the IVSB tyrosinase specific CTL clone, and to a lesser extent by the second anti-tyrosinase, HLA-A2 restricted CTL clone (210/9) (Figure 5.5 C). This was surprising since the expression of the tyrosinase gene by the HMy2 x 518.A2 clone 2 was not detected by RT-PCR even when the sensitivity of the PCR was enhanced by extending the number of annealing cycles.

5.2.4 Expression of TAP and Lmp Proteins

Data from the experiments on CTL recognition of parent and hybrid cells suggested that there were differences in the MHC class I antigen processing pathway. Analysis of the MHC class I processing pathway was restricted to TAP and Lmp protein expression by Western blot in the hybrid and selected cells and the relevant parent cell lines. Protein lysates were made and each protein sample was quantified. Equal amounts of protein were loaded from each sample per lane of the SDS PAGE gel and then separated according to size by electrophoresis before being blotted to nitro-cellulose filter. Proteins were visualised with Ponceau S and sample loading was found to be identical for all lanes. It was decided to load known protein concentrations into the wells of the SDS PAGE gels since unequal protein loading was seen when gels were loaded according to cell numbers. The level of expression of the proteins was assessed by comparing band intensities visually.

5.2.4.1 Antibody Specificity

The production and use of the rabbit anti-human polyclonal serum raised against TAP 1, TAP 2a, Lmp 2 and Lmp 7 have been previously described (Table 2.5). The anti-TAP 1 Ab recognises a protein of 71 kDa, the anti-TAP 2a Ab recognises a protein of 75 kDa. An anti-TAP 2b Ab was not available for use in this study. The anti-Lmp 2 and Lmp 7 antibodies recognise proteins of 28 kDa and 29 kDa respectively. The specificity of the serum was demonstrated by the lack of reactivity in Western blots from T2 cells which contain a deletion mutation, meaning the cells are both TAP and Lmp deficient (DeMars, 1984), and by recognition of proteins of the appropriate size in the immunoblots of extracts from an EBV B-LCL. HMy2, being an EBV B-LCL, expressed the TAP 1, TAP 2a, Lmp 2 and Lmp 7 proteins when cultured both in and out of IFN- γ , and served as the positive control in all immunoblots.

5.2.4.2 TAP and Lmp Expression by HMy2, 518.A2 and HMy2 x 518.A2 Clone 2

HMy2 expressed TAP 1, TAP 2a, Lmp 2 and Lmp 7, as illustrated in Figure 5.7. TAP 1 and TAP 2a proteins were not detected in the 518.A2 and HMy2 x 518.A2 clone 2 hybrid cells unless the cells had been cultured in IFN- γ (Figure 5.7). In addition expression of Lmp 2 and Lmp 7 proteins was not detected by the melanoma and hybrid cells unless the cells were first pretreated with IFN- γ (Figure 5.7). HMy2 x 518.A2 clone 2 hybrid cells showed a reduced level of expression of Lmp 2 and Lmp 7 proteins and TAP 1 and TAP 2a proteins, compared with the 518.A2 parent cell line following treatment with IFN- γ . These results suggest that differences in CTL recognition described in section 5.2.3 were not due to differences in the levels of expression of TAP and Lmp proteins which are involved in the MHC class I processing pathway.

5.2.4.3 TAP and Lmp Protein Expression of Gerl43 and HMy2 x Gerl43 Clone 3B5

Western blots for TAP 1 expression by Gerl43 and the HMy2 x Gerl43 clone 3B5 showed this protein was expressed at low base levels, and the protein was upregulated to a

similar extent by both cell lines after culture in IFN- γ (Figure 5.8). The expression of TAP 2a was not detected in the Gerl43 cell line, even after the cells had been cultured in IFN- γ . However, HMy2 x Gerl43 clone 3B5 expressed low amounts of TAP 2a, but only after the cells had been pretreated with IFN- γ (Figure 5.8). TAP 2 is polymorphic and although TAP 2a is expressed in 80% of individuals (Powis *et al.*, 1992), these data would be compatible with TAP 2b being expressed by Gerl43. If Gerl43 did not express TAP 2a or TAP 2b, then a loss or down regulation of MHC class I expression on the cell surface would have been detected, which was not seen (Figure 3.8). The use of an Ab to detect the less common form of the protein (TAP 2b) would have shown if it was expressed by Gerl43 and the hybrid cells.

Gerl43 and HMy2 x Gerl43 clone 3B5 expressed low levels of Lmp 7 and lower levels of Lmp 2. Both proteins were upregulated to similar levels by the melanoma cells and the cloned hybrid cells after they had been cultured in IFN- γ (Figure 5.8). The level of expression of TAP 1, Lmp 2 and Lmp 7 proteins by Gerl43 was similar to the hybrid cells. It was not clear why Gerl43 and the hybrid cells expressed similar levels of TAP and Lmp proteins whereas 518.A2 and the hybrid cells did not.

5.2.4.4 TAP and Lmp Protein Expression of DAUV and HMy2 x DAUV Uncloned Selected Cells

The expression of TAP 1, TAP 2a, Lmp 2 and Lmp 7 was only detected after DAUV and HMy2 x DAUV had been pretreated with IFN- γ (Figure 5.9). The level of expression of the upregulated TAP and Lmp proteins by the HMy2 x DAUV selected cells compared with the melanoma cell line was similar, consistent with the previous results on these cell lines.

5.3 Discussion

5.3.1 Overview

For use in tumour immunotherapy, hybrid cells formed between EBV B-LCL and tumour cells must be more immunogenic than the unfused tumour cells. Chapter 3 confirmed that the hybrid cells formed between HMy2 and the melanoma cell lines 518.A2 and Gerl43 had a dominant tumour cell phenotype. The hybrid cells lacked the expression of the co-stimulatory molecules CD80 and CD86 which are important in T cell activation and proliferation. Nevertheless, the hybrid cells demonstrated the capacity to stimulate an

allogeneic immune response *in vitro* which was greater than the unfused melanoma parent cell line, for reasons at present unknown (chapter 4).

As potential immunogens in cancer therapy, EBV B-LCL x melanoma hybrid cells also need to fulfil other criteria. Hybrid cells must be able to retain the capacity to express tumour-associated antigen and present them to antigen-specific CTL, to generate an effective, tumour-specific immune response. In order to determine in some detail the expression of components of the endogenous antigen processing pathway, the presence and level of TAP and Lmp protein (as two subunits of the proteasome) expression was analysed. EBV B-LCL have a normal, functional MHC class I processing pathway and antigens expressed through MHC class I molecules on the cell surface are excellent potential targets for CTL recognition. Tumours have been shown to escape immune recognition through various mechanisms which include the down regulation of TAP and Lmp proteins. Being encoded within the MHC region, these proteins were of special interest, especially since chromosomes containing the MHC region derived from both parents were present in the hybrid cells, as shown by HLA-genotyping (section 3.2.3.2). EBV is also known to escape immune detection in BL cells by modulating antigen processing and presentation machinery (Rowe *et al.*, 1995).

This chapter has shown that cloned EBV B-LCL x melanoma hybrid cells stably retained the expression of well defined tumour-associated antigens, and expressed them at levels either similar to, or greater than, the melanoma parent cell line. The hybrid cells were also able to present the tumour-associated antigens effectively to antigen specific HLA restricted CTL clones with similar, or greater efficiency, compared with the melanoma parent cell line, although TAP and Lmp protein expression by the hybrid cells was not enhanced in the hybrid cells as compared with the tumour parent cell lines.

5.3.2 Hybrid Cells Retain the Expression of Tumour-Associated Antigen Expression Following Extended Culture

Tumours are antigenic, and evidence from *in vitro* and *in vivo* studies exists for tumour-specific T cell responses (reviewed in Boon and Van der Bruggen, 1996). Various strategies in tumour immunotherapy, including peptide-based and naked DNA vaccines, have required the identification of the tumour-associated antigens or peptides to stimulate a tumour-specific immune response. Antigen loss variants often arise when patients are vaccinated against one or several tumour antigens, and HLA-polymorphism in the human population has also hampered some approaches to therapy using tumour-specific peptides. Fusion of EBV B-LCL with tumour cells avoids these problems, as tumour antigen identification is not required and the patient is vaccinated against their own complete, unique, tumour-antigen spectrum. Prior to use in tumour therapy, an evaluation of tumour-associated antigen expression and antigen presentation by APC x tumour cell hybrids is required.

In this study, the capacity to express known tumour-associated antigens by the individual hybrid cell clones formed by fusion of 518.A2 melanoma cells with HMy2 as the EBV B-LCL, was maintained over a prolonged period of time in culture. HMy2 x Gerl43 clone 3B5 hybrid cells also retained the expression of the tumour-associated antigens after extended passage, even though the Gerl43 melanoma parent cell line did not. Two possible explanations have been ascribed to explain this result. Either fusion of the melanoma cell line with EBV B-LCL genetically stabilised tumour-associated antigen expression, or the parent Gerl43 cell in the hybrid may have been a sub-dominant cell which expressed the tumour-associated antigens in a heterogenous cell population.

Following prolonged passage, the antigenic phenotype of the Gerl43 cell line changed greatly relative to the original tumour. In addition, 518.A2 lost the expression of melanoma-associated antigen expression after 49 culture transfers. Tumour cells are notorious for their genetic instability (Albino and Fountain, 1993) and tumour masses are known to be composed of heterogenous cell populations (Fleuren *et al.*, 1995). Loss of antigen expression is therefore a common problem associated with growing tumour cultures *in vitro* and cells grown in culture for any length of time require careful monitoring. Tumour-antigen loss variants can arise both *in vivo* and *in vitro* when selective pressure is put upon the cells. Tumour escape variants can be selected for in immunocompetent hosts during the natural course of the disease or during immunotherapy (Garrido *et al.*, 1997;. Ferrone and Marincola, 1995).

Cloning the parent Gerl43 cell line from a cryopreserved sample frozen at the time of hybrid cell generation and testing the capacity of individual clones to maintain tumourassociated antigen expression is one way to determine whether the cell population is heterogenous at the start of the study, and whether or not antigen loss variants arise from these clones following extended culture transfer. The loss of antigen expression in culture in the parent cell line also has implications for the maintenance of vaccine cell lines (including hybrid cells) in culture, as antigen loss by a vaccine cell line would render it ineffective as an immunotherapeutic agent. These data emphasise the need for careful monitoring of vaccine cell lines maintained in culture over time. Only one HMy2 x Gerl43 hybrid cell clone was tested for tumour-associated antigen expression in the study due to limited resources available for the RT-PCR reactions. It would also be interesting to test the capacity of other HMy2 x Gerl43 cloned hybrid cells generated in this study to express tumour-associated antigens.

5.3.3 HMy2, the EBV B-LCL, Expressed MAGE 3 and MAGE 1

MAGE gene expression is detected in the testis and placenta only amongst normal tissues, and expression of the gene is also found in a wide range of tumours (excluding leukaemias and lymphomas). Consequently, MAGE proteins represent useful targets for specific anti-tumour immunotherapy and have been studied extensively. The MAGE genes are expressed in a higher proportion of metastatic melanomas than primary melanoma. It is thought that MAGE expression may either play a role in melanoma metastasis or, may be the result of aberrant gene expression due to a loss of genetic control in tumours (Brasseur *et al.*, 1995). Transcription factors regulating MAGE 1 expression are present in all tumour cell lines, whether they express the gene or not. De Smet *et al.* found the promoter for this gene was highly methylated in cells where the gene was silent and unmethylated in those expressing the gene, and concluded this was probably true for other MAGE-type genes (de Smet *et al.*, 1999).

A surprising result from this study was the expression of MAGE 3 at a moderate level by the parent EBV B-LCL, HMy2. Southern blot analysis and RT-PCR results consolidated this unusual finding. Furthermore, MAGE 3 specific, HLA-A2 restricted CTL clones recognised HMy2 target cells, although at low levels, supporting the expression of MAGE 3 by this cell line. Southern blot analysis also showed HMy2 weakly expressed MAGE 1, although expression of the gene was not detected by RT-PCR. In this study, two other EBV B-LCL cell lines were also tested for MAGE 3 expression. One EBV B-LCL cell line (AT) was negative for MAGE 3 expression. However, the second EBV B-LCL cell line, KR4, (described by Kozbor *et al.*, 1982) was positive for MAGE 3 expression. Like HMy2, this cell line also has a HAT sensitive phenotype and has been treated with γ -radiation to induce ouabain sensitivity. These results suggest MAGE 3 expression by the EBV B-LCL was not due to EBV, but during the generation of either the HAT sensitive or ouabain resistant phenotype the MAGE 3 promoter may have become demethylated, leading to the expression of the gene. A separate study showed MAGE 1 was expressed in human skin wound repair (Becker *et al.*, 1994), indicating the potential for this gene to be expressed in normal cells other than the testis and placenta. However, in a separate study the result could not be repeated (Brasseur *et al.*, 1995).

5.3.4 Different Levels of Expression of the Tumour-Associated Antigens by the Cloned HMy2 x 518.A2 Hybrid Cells

The cloned and an uncloned culture of HMy2 x 518.A2 hybrid cells and the parent melanoma cell line expressed MAGE 1, MAGE 2 and MAGE 3 genes at varying levels as determined by Southern blot analysis. Why the MAGE genes were expressed at different levels by the individual hybrid clones and the parent cell line is not known at present. The degree of demethylation of the MAGE promoters may determine the level of gene expression, although the exact mechanisms of MAGE gene regulation remain to be determined.

5.3.5 Recognition of the HMy2 x 518.A2 Hybrid Cells and Parent Cell Lines by CTL

In addition to expressing the tumour-associated genes, MAGE 1, MAGE 2 and MAGE 3 at differing levels, the HMy2 x 518.A2 cloned hybrid cells, a bulk cell culture and the melanoma parent cell line were recognised with varying levels of efficiency by MAGE 1 and MAGE 3 specific, HLA restricted CTL clones. HMy2 x 518.A2 bulk cells and clone 2 were recognised by both CTL clones with much greater efficiency than the parent melanoma cell line. The reasons for this are not clear but may relate to levels of antigen expression by the hybrid cells. HMy2 x 518.A2 bulk cells expressed MAGE 3 at low levels but were lysed almost as effectively by the MAGE 3 specific CTL clone as HMy2 x 518.A2 clone 2, which strongly expressed MAGE 3. In addition, 518.A2 expressed MAGE 3 at a moderate level but was poorly lysed by the MAGE 3 specific, HLA-A3 restricted CTL clone. However, MAGE expression by PCR may not correlate to protein expression. Other factors may play a role in determining cell susceptibility to lysis by CTL clones. Differences in the level of expression of HLA-A1 and HLA-A2 molecules on the cell surface may affect cell susceptibility to lysis by CTL clones (section 3.2.4). There was no qualitative difference in the level of expression of adhesion molecules (CD11a, CD50, CD54 and CD58) by the hybrid and melanoma parent cell lines. Expression of a protein does not necessarily correlate with antigen presentation on the cell surface. Kirkin et al. have proposed that the rigid secondary structure of various proteins, including MAGE proteins, protects them from proteolysis in the proteasome (Kirkin et al., 1998) and results in the low level of expression of these proteins on the cell surface. This

would make the expression of various components of the antigen processing machinery also an important factor in determining the level of expression of the cancer/testis-specific antigens on the cell surface.

It was unexpected to find HMy2 x 518.A2 clone 2 was recognised by two tyrosinase specific, HLA-A2 restricted CTL clones, despite the expression of this melanocyte specific gene not being detected by RT-PCR. It is possible that the RT-PCR reaction was not as sensitive as the ⁵¹Cr release assay, as a single MHC/peptide can serially engage and trigger up to approximately 200 TcRs (Valitutti et al., 1995) with T cells responding once 8000 TcRs have been triggered (with co-stimulation lowering the activation threshold to 1500) (Viola and Lanzavecchia, 1996). Although it is possible to detect a single copy of a gene by PCR analysis, the sensitivity of the RT-PCR used in this study is not known. Recently, a mAb specific for the tyrosinase protein has become commercially available. Detection of the tyrosinase protein with the mAb would further add to these findings.

5.3.6 Analysis of Cellular Antigen Processing Machinery

Efficient antigen presentation requires protein degradation in the proteasome and transport of degraded peptides into the lumen of the ER by the heterodimeric TAP transporter. It is well known for tumour cells to down regulate TAP expression as a means of escaping immunological recognition, as this leads the failure to transport peptides produced in the proteasome to the ER, and to express peptide/MHC class I complexes on the cell surface (Restifo et al., 1993a; Cromme et al., 1994). The specific down-regulation of Lmp proteins has also been associated with tumours (Restifo et al., 1993a; Singal et al., 1996). Lmp proteins are β -subunits of the proteasome and their absence prevents the generation of certain CTL epitopes (Driscoll et al., 1993; Gaczunska et al., 1993). In addition, the expression of the EBV latent protein EBNA 1 only, which is associated with type 1 EBV latency and seen in group I BL cells (Rowe et al., 1987b; Gregory et al., 1991), has been associated with defective endogenous antigen presenting capabilities (Rowe et al., 1995). BL cells down-regulate, though always express, TAP 1 proteins and TAP 2 proteins to a lesser extent and show a reduced rate of MHC class I synthesis whereas expression of Lmp 2 and Lmp 7 subunits of the proteasome was not affected (Rowe et al., 1995). Treatment of the cells with IFN-y or LMP 1 only restored antigenprocessing function (Rowe et al., 1995). EBV B-LCL on the other hand have a fully functional MHC class I processing pathway and express high levels of both TAP and Lmp proteins (Rowe *et al.*, 1995). Since the hybrid cells were formed between a melanoma cell line and an EBV B-LCL, and as the hybrid cells express EBV latency type 1, analysis of the antigen processing components was of interest.

In order to determine the level of expression of TAP and Lmp proteins by the hybrid and melanoma cell lines, Western blot analysis was performed. TAP and Lmp proteins were detected in HMy2 x 518.A2 and 518.A2 cells only after IFN- γ pre-treatment. However, HMy2 x 518.A2 clone 2 were effective targets for recognition by antigen-specific, HLA restricted CTL clones without needing IFN- γ treatment. These results suggest the TAP proteins, and possibly Lmp proteins, may have been expressed at levels below those required for detection by Western blot. By RT-PCR analysis, thor Straten *et al.* have shown loss of TAP 1, TAP 2, Lmp 2 and Lmp 7 is not frequent in recently established melanoma cells and suggest tumour escape variants are selected following immunotherapy (thor Straten *et al.*, 1997). RT-PCR analysis to detect low levels of TAP and Lmp proteins could be applied in this study.

The results of the Western blot analysis support the results of the ⁵¹Cr release assay for the melanoma cell line, 518.A2. The cell line was a much better target after treatment with IFN- γ by the MAGE 1 specific CTL clone, suggesting the MHC class I processing pathway was partially down regulated, but could be augmented by treatment with IFN- γ and Western blot analysis showed the expression of TAP and Lmp proteins was upregulated by culture of the cells in IFN- γ . The effect of IFN- γ treatment on CTL recognition of HMy2 x 518.A2 clone 2 was not tested.

HMy2 x 518.A2 clone 2 cells showed a lower level of expression of TAP and Lmp proteins compared with the melanoma parent cell line after culture in IFN- γ . Further analysis of these cells is required to identify why the hybrid cells expressed lower levels of the TAP and Lmp proteins, especially since the hybrid cells inherited the genes for these proteins from both parent cell lines. However, this did not appear to adversely affect the ability of the cells to process and present antigen to CTL clones.

Gerl43 and HMy2 x Gerl43 clone 3B5 displayed baseline levels of TAP 1, Lmp 2 and Lmp 7 which were upregulated after the cells had been cultured in IFN- γ . Unlike the HMy2 x 518.A2 hybrid cells, there was no marked difference in the level of expression of these proteins between the hybrid and melanoma parent cell lines. Like the HMy2 parent cell line, HMy2 x Gerl43 clone 3B5 expressed TAP 2a but only at a low level and after IFN- γ treatment. Since this gene had been inherited from the EBV B-LCL parent cell line which constitutively expressed both TAP and Lmp proteins, its expression only after IFN- γ treatment suggests a dominant down-regulatory factor in the Gerl43 parent. Expression of TAP 2b by the Gerl43 parent cell line was the most likely explanation for the lack of TAP 2a expression by these cells even after IFN- γ treatment as the melanoma cells expressed MHC class I molecules on the cell surface. In the absence of an Ab detecting this protein which is expressed in only 1 in 5 humans, this could not be confirmed.

5.3.7 DAUV and the HMy2 x DAUV Selected Cells

There was no indication from results in this chapter to suggest the HMy2 x DAUV selected cells were true hybrids, as the levels of expression of the TAP and Lmp proteins were similar to the DAUV cells and the range of tumour associated antigens expressed by both the parent melanoma and the selected cells was identical. These findings were consistent with all of the data on the HMy2 x DAUV selected cells.

5.4 Future Work

The work in this chapter has demonstrated the capacity for hybrid cells formed between EBV B-LCL and melanoma cells to retain the expression of tumour-associated and melanoma specific genes and express them at levels either similar to, or greater than, the parent melanoma cell line. Furthermore, the HMy2 x 518.A2 clone 2 and an uncloned bulk culture were able to present MAGE 1 and MAGE 3 antigens more effectively than the parent melanoma cell line. However, the parent melanoma cell lines expressed TAP and Lmp proteins at levels similar to, or greater than, the hybrid cells as detected by Western blot analysis, suggesting no intrinsic differences in the endogenous antigen processing pathway between parent melanoma and hybrid cells.

The increased expression and enhanced presentation of tumour-associated antigen by the EBV B-LCL x melanoma hybrid cells support the use of this strategy for the immunotherapy of cancer and have several important implications. Hybrid cells may have the potential to break natural tolerance which exists for 'self' peptides through enhanced antigen expression and presentation. Hybrid cells may also activate CTLs recognising subdominant antigenic epitopes due to the enhanced tumour-antigen expression and presentation. Increased antigen expression and presentation may also activate T cells which have been anergised through low antigen expression on the cell surface and hybrid cells may also have the potential to activate naïve T cells. To characterise hybrid cells completely at the pre-clinical level prior to their use *in vivo*, these factors require further investigation.

Unanswered Questions

Did the HMy2 x Gerl43 hybrid cells arise from a sub-dominant melanoma cell which expressed tumour-associated antigens, or lose antigen expression in culture after generation of hybrid cells that were stable for antigen expression?

What results in the different levels of expression of the tumour-specific genes by the EBV B-LCL x melanoma hybrid cells?

What factors determine the levels of CTL recognition of individual hybrid cells (eg antigen expression, MHC expression, adhesion molecules, antigen processing pathway)?

Why did the HMy2 x 518.A2 clone 2 hybrid cells express lower levels of the TAP and Lmp proteins compared with the melanoma parent cell line, yet present tumour-associated antigen more effectively?

Do Gerl43 cell express TAP 2b and do the HMy2 x Gerl43 clone 3B5 hybrid cells express both polymorphisms of the TAP 2 protein?

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Figure 5.1. Beta-actin PCR reaction to ensure cDNA has been synthesised from RNA. A positive result produced a band size of 310 bp and the cDNA was therefore used in subsequent PCR reactions. PCR products were loaded into the wells of an agarose gel and electrophoresed in the following order: Lane 1, 1kb ladder. Lane 2, negative control. Lane 3, HMy2. Lane 4, Gerl43 (passage 33). Lane 5, HMy2 x DAUV. Lane 6, DAUV. Lane 7, HMy2 x Gerl clone 3B5. Lane 8, Gerl43 (passage 150). Band intensities were equal for all cDNA samples used in the study.

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Figure 5.2. *Hybrid cells retain the expression of tumour associated antigens.* RNA extracts from HMy2 x 518 and the melanoma parent cells were converted to cDNA and tumour-associated antigen expression determined by RT-PCR using antigen specific primers. PCR products were electrophoresed in an agarose gel and visualised by ethidium bromide staining. **A)** Expression of MAGE-1. Samples were loaded as follows: Lane 1, 518.A2. Lane 2, HMy2 x 518.A2 clone 2. Lane 3, HMy2 x 518.A2 clone 3. Lane 4, HMy2 x 518.A2 clone 4. Lane 5, negative control. Lane 6, Gerl43 (passage 29) (positive control). Lane 7, 100 bp ladder. **B)** Expression of MAGE 2. Samples were loaded as follows: Lane 1, negative control. Lane 2, 518.A2 clone 4. Lane 5, HMy2 x 518.A2 clone 4. Lane 6, 100 bp ladder. **C)** Expression of MAGE 3. Samples were loaded as follows: Lane 1, Gerl43 (passage 29) (positive control). Lane 2, negative control. Lane 3, 518.A2. Lane 4, HMy2 x 518.A2 clone 2. Lane 5, HMy2 x 518.A2 clone 4. Lane 5, 100 bp ladder. **C)** Expression of MAGE 3. Samples were loaded as follows: Lane 1, Gerl43 (passage 29) (positive control). Lane 2, negative control. Lane 3, 518.A2. Lane 4, HMy2 x 518.A2 clone 2. Lane 5, HMy2 x 518.A2 clone 4. Lane 6, 100 bp ladder. **C)** Expression of MAGE 3. Samples were loaded as follows: Lane 1, Gerl43 (passage 29) (positive control). Lane 2, negative control. Lane 3, 518.A2. Lane 4, HMy2 x 518.A2 clone 2. Lane 5, HMy2 x 518.A2 clone 3. Lane 6, HMy2 x 518.A2 clone 4. Lane 7, 100 bp ladder.



Figure 5.3. *EBV B-LCL parent cells express tumour-associated antigen, MAGE 3.* A sample of cDNA from the cell line HMy2 was used in a PCR reaction with primers directed against the MAGE 3 gene. Amplified cDNA produced a 725 bp band and contaminating genomic DNA produced a band of 805 bp. MAGE-3 gene expression was unexpectedly positive. The expression of this gene was was not detected in an unrelated pro-myelocytic leukaemic sample, HL60. Samples from the PCR reaction were loaded into the wells of an agarose gel in the following order: Lane 1, HMy2 x 518.A2 clone 2. Lane 2, 518.A2. Lane 3, HMy2. Lane 4: HL60.

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Figure 5.4. *Hybrid cells retain the expression of tumour-associated antigens.* RNA extracts from HMy2 x Gerl43 hybrid cells, HMy2 x DAUV selected cells and the relevant parent cell lines were converted to cDNA and tumour-associated antigen expression was determined by RT-PCR using antigen specific primers. For each PCR reaction, both positve and negative controls were included. PCR products were loaded into the wells of an agarose gel and visualised by ethidium bromide staining. **A)** PCR reaction to detect MAGE 1 expression. Lane 1, HMy2 x Gerl43 3B5. Lane 2, Gerl43 (passage 150). Lane 3, HMy2 x DAUV. Lane 5, Negative. Lane 6, Blank. Lane 7, Blank. Lane 8, 100 bp ladder **B)** PCR reaction to detect MAGE 2. Lane 1, HMy2 x Gerl43 3B5. Lane 2, Gerl43 (passage 150). Lane 3, HMy2 x DAUV. Lane 4, DAUV. Lane 5, 100 bp ladder. **C)** PCR reaction to detect MAGE 3 expression. Lane 1, Gerl43 (passage 150). Lane 2, HMy2 x Gerl43 3B5. Lane 3, HMy2 x DAUV. Lane 4, DAUV. Lane 5, 100 bp ladder. **D)** PCR reaction to detect tyrosinase. Lane 1, HMy2 x Gerl43 3B5. Lane 2, Gerl43 (passage 150). Lane 3, HMy2 x DAUV. Lane 4, DAUV. Lane 5, 100 bp ladder. **D)** PCR reaction to detect tyrosinase. Lane 1, HMy2 x Gerl43 3B5. Lane 2, Gerl43 (passage 150). Lane 3, HMy2 x DAUV. Lane 4, DAUV. Lane 5, 100 bp ladder. **D)** PCR reaction to detect tyrosinase. Lane 1, HMy2 x Gerl43 3B5. Lane 2, Gerl43 (passage 150). Lane 3, HMy2 x DAUV. Lane 5, 100 bp ladder. **D)** PCR reaction to detect tyrosinase. Lane 1, HMy2 x Gerl43 3B5. Lane 2, Gerl43 (passage 150). Lane 3, HMy2 x DAUV. Lane 5, 100 bp ladder. **D)** PCR reaction to detect tyrosinase. Lane 1, HMy2 x Gerl43 3B5. Lane 2, Gerl43 (passage 150). Lane 3, HMy2 x DAUV. Lane 5, 100 bp ladder. **D)** PCR reaction to detect tyrosinase. Lane 1, HMy2 x Gerl43 3B5. Lane 2, Gerl43 (passage 150). Lane 3, HMy2 x DAUV. Lane 5, 100 bp ladder.



Figure 5.5. EBV B-LCL x melanoma hybrid cells process and present MAGE 1, MAGE 3 and tyrosinase to antigen-specific, HLA class I restricted CTL clones with greater efficiency than the parent melanoma cell line. A) Presentation of the melanoma antigen MAGE 1 by EBV B-LCL cells (HMy2) (--), melanoma cells (518.A2) (-), HMy2 x 518.A2 clone 2 (--) and HMy2 x 518.A2 bulk (--) to MAGE 1, HLA-A1 restricted CTL clone 82/30. B) Presentation of the melanoma antigen MAGE 3 by EBV B-LCL cells (HMy2) (--), melanoma cells (518.A2) (--), HMy2 x 518.A2 clone 2 (--) and HMy2 x 518.A2 bulk (--) to MAGE 3, HLA-A2 restricted CTL clone 297/22. C) Presentation of the melanoma antigen tyrosinase through HLA-A2 by HMy2 x 518.A2 clone 2 to tyrosinase, HLA-A2 restricted CTL clone 210/9 (--) and to tyrosinase, HLA-A2 restricted CTL clone IVSB (--). Data for parent cell lines HMy2 and 518.A2 was less than 1% lysis at all E:T for both tyrosinase specific CTL clones.



Figure 5.6. Melanoma parent cells present a melanoma antigen with greater efficiency after treatment with IFN-gamma to an antigen-specific, HLA class I restricted CTL clone. Presentation of the melanoma antigen MAGE 1 through HLA-A1 by melanoma cells (518.A2) (--), melanoma cells (518.A2) after IFN-gamma treatment (---), HMy2 x 518.A2 clone 2 (---) and melanoma cells (Gerl43) (---) to MAGE 1 restricted CTL clone clone 82/30.


Figure 5.7. TAP and Lmp proteins were detected by Western blot analysis only after IFN- γ treatment by HMy2 x 5 18.A2 clone 2 and 518.A2. Protein lysates were loaded into the wells of an SDS PAGE gel. A) Samples were probed with a polyclonal antibody directed against TAP 1. B) Samples were probed with a polyclonal antibody directed against TAP 2a. C) Samples were probed with a polyclonal antibody directed against Lmp 2. D) Samples were probed with an antibody directed against Lmp 7. Samples were loaded in the following order: Lane 1, markers. Lane 2, HMy2. Lane 3, HMy2 + IFN- γ . Lane 4, T2. Lane 5, T2 + IFN- γ . Lane 6, 518.A2. Lane 7, 518.A2 + IFN- γ . Lane 8, HMy2 x 518.A2 clone 2. Lane 9, HMy2 x 518.A2 clone 2 + IFN- γ .



Figure 5.8. TAP 1 and TAP 2a and Lmp proteins are upregulated by HMy2 x Gerl43 clone 3B5 hybrid cells after IFN- γ treatment. Gerl43 does not upregulate TAP 2a. Protein lysates were loaded into the wells of an SDS PAGE gel. Gels A), B), C) and D) were probed as described in Figure 5.8. Samples were loaded as follows for for A) and B): Lane 1, markers. Lane 2, HMy2. Lane 3, T2. Lane 4, T2 + IFN- γ . Lane 5, Gerl43. Lane 6, Gerl43 + IFN- γ . Lane 7, HMy2 x Gerl43. Lane 8, HMy2 x Gerl43 clone 3B5 + IFN- γ . Protein lysates were loaded as follows for C) and D): Lane 1, markers. Lane 2, HMy2. Lane 3, HMy2 + IFN- γ . Lane 4, T2. Lane 5, T2 + IFN- γ . Lane 6, Gerl43. Lane 7, Gerl43 + IFN- γ . Lane 8, HMy2 x Gerl43 clone 3B5. Lane 9, HMy2 x Gerl43 + IFN- γ .



Figure 5.9. TAP and LMP protein expression is similar for HMy2 x DAUV compared with the melanoma parent cell line. Protein lysates were loaded into the wells of an SDS PAGE Gels A), B), C) and D) were probed as described in Figure 5.8. and loaded as follows for A): Lane 1, markers. Lane 2, HMy2. Lane 3, T2. Lane 4, T2 + IFN- γ . Lane 5, DAUV. Lane 6, DAUV + IFN- γ . Lane 7, HMy2 x DAUV. Lane 8, HMy2 x DAUV + IFN- γ . Protein lysates were loaded as follows for B), C), and D): Lane 1, markers. Lane 2, HMy2. Lane 4, DAUV. Lane 5, DAUV + IFN- γ . Lane 4, DAUV. Lane 5, DAUV + IFN- γ . Lane 6, HMy2 x DAUV. Lane 7, HMy2 x DAUV.

Chapter 6

General Discussion

General Discussion

6.1 Introduction

Tumour-associated antigens, presented through HLA class I and class II molecules, which are recognised by T cells have been identified, mostly from melanoma, and a role for T cells in the host's immune response to melanoma *in vivo* has been ascribed. Clinical and experimental evidence supports this notion (section 1.2). However, T cells are generally unable to prevent tumour growth as disease progression occurs. Manipulating the immune system has become a promising means of treating cancer in humans, with animal studies and clinical trials supporting the potential benefits of this approach (section 1.7). The conventional ways of treating cancer have involved surgical removal of operable tumour and/or courses of radiation therapy or chemotherapy. Lack of specificity of radiation and chemotherapy leads to patients suffering from unwanted side effects. In contrast, immunotherapeutic approaches to cancer treatment aim to target tumour cells specifically, with minimal toxicity and long lasting effects.

Following the identification of tumour-associated antigens, a number of new peptide-based approaches for the treatment of cancer are being investigated. These include immunising with peptide alone (Marchand *et al.*, 1995), peptide administered with adjuvant (Weber *et al.*, 1999), peptide-loaded ex-vivo onto professional APC (Nestle *et al.*, 1998), and immunisation with naked DNA or plasmid encoding for antigen (Hirschowitz *et al.*, 1998). Several other approaches have aimed at enhancing the immunogenicity of tumours cells for use as genetically modified tumour cells. These include cytokine transfection (Belli *et al.*, 1997; Ellem *et al.*, 1997), MHC transfection (Nabel *et al.*, 1993; Wahl *et al.*, 1995), or transfection with T cell co-stimulatory molecules such as CD80 (Chen *et al.*, 1992; Townsend and Allison, 1993) into tumour cells. Many of these approaches are currently in use in a number of anti-cancer clinical trials for a variety of cancers.

The use of hybrid cells in cancer therapy has recently come under investigation in both animal models and in clinical trials as an alternative strategy for whole tumour vaccination (section 1.7.5).

This approach has several advantages over other approaches.

• There is no need to identify tumour-antigens, so hybrid cells can be used to treat a wide range of different tumour types.

- Each hybrid vaccine can be tailor made for the individual patient and should express the complete (unique) spectrum of antigen presented by the patient tumour cells, so both dominant and sub-dominant antigens are presented on the cell surface, reducing the risk of epitope loss variants arising.
- Any defects in antigen processing/T cell co-stimulation by the tumour cells may be corrected upon fusion with professional APC.
- There is no need for gene transfection, which is difficult in primary cell lines.
- Fusion of tumour cells with allogeneic APC may result in the expression of allo-MHC class I and allo-MHC class II molecules by the hybrid cells, which may serve as adjuvant and increase the frequencies of CTL and CD4+, T helper cell recognition.

In order for APC x tumour cell hybrids to be effective in cancer therapy, they must fulfill several criteria.

- The potential to fuse patient (primary) tumour cells with APC must be realised, although tumour cell lines expressing a variety of shared tumour-antigens which are expressed in a range of tumours (of same or different histological type) could potentially be used as fusion partners to APCs.
- Hybrid cells must retain the expression of, and capacity to, process and present tumourassociated antigens.
- Hybrid cells must be potent immunogens and stimulate a tumour-specific immune response which is greater than the unfused tumour cells alone.

The results of Guo and co-workers showed rats immunised with activated B cells fused with hepatocarcinoma cells mediated rejection of pre-established tumours and were protected against subsequent tumour cell challenge (Guo *et al.*, 1994). A number of other studies have since confirmed these findings using DCs as the APC fusion partner to a number of tumour types including melanoma (Cao *et al.*, 1999; Celluzzi and Falo, 1998), lymphoma (Wang *et al.*, 1998a), lung carcinoma (Celluzzi and Falo, 1998) and adenocarcinoma (Gong *et al.*, 1997). In addition, clinical trials in patients with both renal cell carcinoma (Kugler *et al.*, 1998; Kugler *et al.*, 2000) and melanoma (Trefzer *et al.*, 1997) have also shown the therapeutic effectiveness of this approach to the treatment of cancer (discussed in section 1.7.5).

The results presented in this thesis provide a detailed *in vitro* characterisation of human EBV B-LCL x melanoma hybrids for potential use as a cell based vaccine in cancer therapy. B cells can be readily transformed *in vitro* into EBV B-LCL which grow continuously and easily in culture. EBV B-LCL have an 'activated' B cell phenotype and express high levels of MHC class I and MHC class II antigens, and a wide range of surface accessory molecules including the T cell co-stimulatory ligand molecules CD80 and CD86 (Thorley-Lawson *et al.*, 1985; Rowe *et al.*, 1985; Gregory *et al.*, 1988). In addition, EBV B-LCL have been used previously as the professional APC in hybrids formed with a variety of human derived tumour cell lines (Dunnion *et al.*, 1999). Those hybrid cells which retained the professional APC phenotype were more immunogenic than the parent tumour cell alone *in vitro*, suggesting their potential use as immunotherapeutic agents. However, the use of EBV B-LCL as the APC partner for fusion with tumour cells has potential disadvantages, including the association of EBV with several malignancies (reviewed in Rickinson, 1996), and whether or not EBV B-LCL have the capacity to stimulate naïve T cells, or if this property is unique to DCs.

6.2 EBV B-LCL x Melanoma Cell Hybrids: Results Summary

This study has demonstrated the potential to fuse an EBV B-LCL with human derived melanoma cell lines to form heterologous, somatic cell hybrids. Clones of the hybrid cells were able to stimulate an enhanced T cell response in vitro compared with the relevant parent melanoma cell lines. CD8+ and CD4+ T cell subsets were stimulated in vitro in the absence of CD80/CD86 co-stimulation by the hybrid cells, but indicating the expression of MHC class I and MHC class II molecules, respectively, was required. T cell stimulation was enhanced when autologous non-T cell PBMC were added to the MTLC, suggesting the possibility for indirect presentation of allo-antigen by responder APC or Indirect allo-MHC antigen presentation or bystander cobystander co-stimulation. stimulation involved CD80/CD86 mediated co-stimulation, since the response could be blocked with CTLA-4 Ig. Transfection of CD80 into one of the cloned hybrid cell lines resulted in an increased level of direct T cell stimulation, demonstrating the importance of CD80/CD86 interaction with CD28/CTLA-4 in T cell stimulation. The precise mechanisms by which the EBV B-LCL x melanoma hybrid cells stimulated a T cell response is unclear, and it remains to be seen whether or not the stimulated T cells are tumour-specific and functional. In order to direct a tumour-specific T cell response, hybrid cells must retain tumour-associated antigen expression. The hybrid cells generated in this

study also fulfilled the role of retaining tumour-associated antigen expression, and processed and presented them efficiently to antigen specific, HLA restricted CTL clones. Overall this data suggests that EBV B-LCL x melanoma hybrids show enhanced T cell stimulation *in vitro* by a combination of B7 dependent (possibly bystander) co-stimulation and B7 independent co-stimulation. These results support the idea that APC x tumour cell hybrids represent a promising approach to cancer therapy.

6.3 EBV B-LCL as Parent APC in Hybrid Cells

EBV B-LCL were chosen as the APC for fusion with melanoma cells for several reasons. EBV B-LCL have an activated B cell phenotype and express high levels of MHC class I and class II antigens, a range of adhesion (CD11a, CD50, CD54 and CD58) and costimulatory ligand molecules (CD40, CD80 and CD86). In addition, B cells are easily transformed in vitro and are robust in culture. However, the hybrid cells generated in this study between EBV B-LCL and human derived melanoma cell lines had a phenotype which was similar to the tumour parent cell. The hybrid cells expressed only the EBNA 1 EBV latent protein (typical of BL cells) and not the complete spectrum of latent proteins seen in EBV B-LCL. The results described in this thesis show that, despite lacking what is considered to be a 'immunostimulatory phenotype' (Dunnion et al., 1999), the hybrid cells used in this study had a stimulatory capacity which was greater than the tumour parent cell line in vitro. This suggests other factors are important in T cell co-stimulation and for identifying those hybrid cells which may be of use in tumour therapy, and demonstrates the need for the careful in vitro characterisation of cloned hybrid cells prior to their use in cancer therapy. Wang et al. showed a 20% fusion efficiency rate between DCs and the murine derived B6 melanoma cell line produced enough hybrid cells for effective vaccination in mice, meaning selection for hybrid cells alone was not necessary (Wang et al., 1998a). However, the efficacy of the vaccine was enhanced when the hybrid cells were purified (Cao et al., 1999), and Dunnion et al. have shown the potential to derive hybrid cells with different phenotypes from a single hybridisation including both stimulatory and non-stimulatory subclones (Dunnion et al., 1999). In addition, Mø fused with a melanoma cell line produced hybrid cells which were phenotypically similar to the tumour parent and did not mediate anti-cancer immunity in vaccinated mice (Souberbielle et al., 1998). Collectively, these results suggest it may be advantageous to select EBV B- LCL x tumour hybrids with enhanced stimulatory capacities *in vitro*, prior to use in vaccination protocols.

The effects of various latent (and lytic) EBV proteins on normal cellular functions have been described, and are still under investigation (reviewed in Kieff, 1996). Apart from being involved in viral episome maintenance and viral DNA replication (Middleton et al., 1991), the expression of EBNA 1 only in BL cells is also known to down regulate antigen processing and presenting machinery, whereas the expression of LMP 1 is known to upregulate various cell surface markers including MHC class I and class II molecules, CD40, CD11a, CD54, CD58 (Wang et al., 1988; Wang et al., 1990a) and TAP protein expression (Rowe et al., 1995), suggesting the EBV latency state may influence hybrid cell immunostimulatory capacity. This idea is supported by the work of Dunnion et al. (Dunnion et al., 1999). Several EBV lytic proteins have homology to cellular proteins. Of particular relevance is the protein BCRF 1 which is homologous to IL10, a cytokine which has been shown to have immunosuppressive effects (Chen et al., 1994b; Lattime et al., 1995). Although B cells are rarely permissive to EBV replication, the hybrid cells were analysed for lytic infection. The EBV B-LCL x melanoma hybrid cells used in this study did not express BZLF 1, a viral protein which has been shown to disrupt viral latency in B cells (Petti et al., 1988), but expressed EBNA 1 only which is characteristic of the EBV latency I which is associated with BL cells. Despite the association of EBNA 1 expression with the down regulation of TAP and MHC expression in BL cells, the expression of EBNA 1 only by the EBV B-LCL x melanoma hybrid cells did not show any noticeable negative effects on antigen presentation. The results of this study have shown the hybrid cells were able to process and present antigen to antigen-specific, HLA class I restricted CTL clones, and were often more effective at presenting antigen than the melanoma parent cell line alone. The EBV B-LCL x melanoma hybrid cells also retained the expression of genes associated with tumour-antigens as detected by RT-PCR. Future work might show whether or not it is possible to transfect patient B cells with defined EBV proteins, for example LMP 1 to transform the B cell and upregulate the expression of various cell surface markers and antigen processing and presentation machinery for more effective T cell co-stimulation and antigen presentation.

The majority of people have been previously exposed to EBV and carry the virus in the latent state (Nilsson et al., 1971; Gratama et al., 1988). For cancer patients who have previously had an EBV infection and have circulating memory CTL, EBV B-LCL x tumour hybrid cells could be used in immunotherapy. The vaccine would also offer the

additional benefit of presenting antigenic EBV epitopes for presentation to memory CTL and helper T cells which may be of use in recruiting and activating tumour specific CTLs. However, EBV is associated with a range of malignant diseases, so clearly the use of EBV B-LCL in therapeutic cancer vaccines presents ethical problems. It is likely that the virus would have to be made replication deficient, for example by irradiating cells, in order for the vaccine to be safe.

Alternatives to EBV B-LCL as APCs in the generation of hybrid cells include DCs and $M\phi$. DCs have been identified as the most potent APCs so far and have been described as the optimal APC for stimulating naïve T cells (Steinman, 1991; Yewdell and Bennink, 1992). A number of immunotherapeutic cell-based vaccines using DCs pulsed in vitro with tumour peptides or proteins have led to the induction of anti-tumour immunity and disease regression in animal studies (Mayordomo et al., 1995; Porgador et al., 1995) and clinical trials (Bakker et al., 1995; Hsu et al., 1996). Over the past few years, the methods for DC isolation and culture in vitro have improved, so it is now possible to obtain large numbers of DCs from bone marrow and blood-derived precursors (Romani et al., 1994; Inaba et al., 1992). Studies using DCs as the professional APC parent cells for fusion with tumour cells generated hybrid cells, which, at variance to the EBV B-LCL x melanoma hybrid cells produced in this study and the 'non-stimulatory' EBV B-LCL x tumour cell hybrids produced by Dunnion et al. (Dunnion et al., 1999), expressed MHC class II antigens and co-stimulatory antigens (CD80 and CD86) derived from the parent APC (Gong et al., 1997; Wang et al., 1998a; Cao et al., 1999). In contrast to the hybrid cells produced in this study and the study by Dunnion et al, the DC x tumour cell hybrids were not cloned and analysed at the clonal level. In addition, the potential to fuse DCs with primary breast carcinoma cells has also been demonstrated. However, Dunnion et al. were unable to generate chemically selected hybrid cells using DCs as APC and a erythroleukaemia cell line (K562) and a T cell leukaemia cell line (CEM) which grew in culture. Other groups have since shown hybrid selection and growth in vitro is not necessary to induce potent anti-tumour responses in mice using DCs and lung carcinoma cells (Celluzzi and Falo, 1998), or melanoma and lymphoma cell lines (Wang et al., 1998a) and in human clinical trials using DCs fused with renal cell carcinoma cells (Kugler et al., 2000). In a clinical situation this would minimise the length of time taken between tumour excision and vaccine administration, and retain the original tumour-antigen phenotype (as antigen loss variants can arise after only a short period of growth in vitro).

M ϕ have also been used as the source of APC for fusion with melanoma cells (B16-F10) in a separate study in mice (Souberbielle *et al.*, 1998). The hybrid cells generated did not express MHC molecules or the co-stimulatory molecules (CD80 or CD86) and were not therapeutically effective in mice. The same murine melanoma model was used in a DC fusion producing hybrid cells which expressed both MHC class I and class II molecules and CD80 and primed lymph node T cells *in vitro* which when adoptively transferred into mice were effective against established tumours (Wang *et al.*, 1998), suggesting the choice of APC for fusion with tumour cells is a crucial factor.

6.4 Modifications to the Hybrid Cell Approach in Cancer Therapy

In a study by Celluzzi and Falo (Celluzzi and Falo, 1998), tumour cells and DCs were brought into close contact by culturing the cells together. The physical interaction of the two cell types was sufficient for these cells to be effective in immunisations against tumour cell challenge in mice. The mechanism by which these cells induced an antitumour immune response was attributed to either cross priming or bystander costimulation. In a similar manner, it is possible that the enhanced T cell response to the EBV B-LCL x melanoma hybrid cells generated in this study was also due to the close association of the hybrid cells with responder APCs, which may have provided CD80/CD86 mediated co-stimulatory signals to T cells, or it is possible responder APCs may have represented allo-MHC antigens in the context of a professional APC, in order to stimulate an allogeneic T cell response. The enhanced stimulatory capacity of the hybrid cells compared with the unfused melanoma cells may also have been due to more effective antigen presentation or increased levels of antigen expression by the hybrid cells compared with the parent melanoma cells so, culturing hybrid cells in vitro with APCs prior to immunisation may produce a more effective anti-cancer therapy. This may be of greater relevance to those hybrid cells which lack co-stimulatory molecule (CD80 and CD86) expression.

As an alternative approach to hybrid cell therapy, DCs modified to express GM-CSF have also been used as the fusion partners to a murine derived melanoma cell line (B16), producing more potent hybrid cells compared with unmodified DCs used in hybrid cell production (Cao *et al.*, 1999). Improvements in the therapeutic efficacy of this cell vaccination given to mice was presumably due to an increase in the numbers of DCs, CTLs, M ϕ and neutrophils recruited to the area of immunisation. These cells have been shown to play a role in T cell stimulation through both cross priming (discussed in section 1.4.2.9) and bystander co-stimulation (discussed in section 1.5.2). Genetically modifying APC x tumour cells to express GM-CSF may be an effective strategy for further enhancing hybrid cell vaccines especially if the hybrid cells lack co-stimulatory molecule expression.

As described in section 1.7.4, the genetic modification of tumour cells to express CD80 has been met with both success and disappointment. The work described in this study has shown it is possible to transfect EBV B-LCL x melanoma hybrid cells with CD80 and directly stimulate T cells *in vitro*. The unmodified hybrid cells did not stimulate an effective, direct T cell response. The work of others has shown CD80 co-stimulation is required only during the initiation of a T cell response, and that continued expression of CD80 increases NK/LAK cell mediated killing without generating a memory T cell response (Wu *et al.*, 1996). The beneficial effects of CD80 expression *in vivo* need to be assessed.

6.5 Immunodominance

Within a naturally processed 'non-self' protein, the CTL response to peptide epitopes is not normally equal, and the response is usually directed against a small number of immunodominant epitopes. Immunodominant peptides generally bind with high avidity to MHC molecules, and are presented at high levels on the cell surface. Subdominant epitopes can also cause T cell activation, although to a lesser extent than that caused by immunodominant epitopes (Adorini *et al.*, 1988; Brett *et al.*, 1988). The hierarchy of immunodominance can be seen in viral infections. CTL responses to EBV latent infection are preferentially directed towards immunodominant epitopes from the EBNAs 3, 4 and 6 with the precise epitope choice depending on the HLA type of the infected individual (Rickinson and Moss, 1997). MHC polymorphism in the population means the same peptide epitope will bind to different allelic MHC molecules with a range of affinities.

The majority of the well defined tumour-antigens described so far are the products of structurally unaltered genes ('self' proteins). Immunodominance applies to T cell responses to 'non-self' antigen and is also thought to apply to tolerance induction by 'self' proteins (Sercarz *et al.*, 1993). Immunodominant peptides may induce 'self' tolerance whereas subdominant or cryptic peptides fail to induce tolerance. If tolerance against epitopes of 'self' proteins exists, then the immunogenicity of that antigen is likely to be low. Although studies have shown tolerance to 'self' antigens can be broken by administering high doses of antigen ('beneficial autoimmunity'), it may also be important for T cells to engage MHC class I/subdominant antigen complexes on the tumour cells for which tolerance does not exist. Given the small number of well characterised tumourantigens identified so far and the uniqueness of individual tumours, it is likely that whole cell cancer vaccines will be the most favourable approach in cancer therapy. Hybrid cells formed between APC and tumour cells can be made individually for each patient. If tumour antigen expression is enhanced and more effectively presented on the cell surface when tumour cells are fused with APC, it may be possible to generate CTL responses directed against subdominant antigens, as were seen with CD80 transfected tumour cells (Johnston et al., 1996). The immunisation of mice with lymphoma cells genetically modified to express CD80 led to an increase in the numbers of tumour-antigens recognised by CTLs in vitro (Johnston et al., 1996). Silent, subdominant tumour-antigens did not stimulate detectable CTL responses using unmodified, wild type cells from the same tumour. Expansion of the T cell response to a greater number of tumour-antigens suggests this may be beneficial in a clinical situation by reducing the number of antigen loss variants which frequently arise in cancers. However, it remains to be determined whether an immune response against subdominant 'self' antigens can be raised in vivo without inducing autoimmune disease.

6.6 The Future

Clearly, the use of hybrid cells in tumour therapy has great scope for modification and, given the results presented in this thesis and the recent literature, hybrid cells have huge potential for use in a clinical setting. The areas of research which require further investigation include

- Determining the mechanism by which those hybrid cells which do not express the costimulatory molecules (CD80 and CD86) stimulate T cells (ie bystander co-stimulation or cross priming) and evaluate the therapeutic efficacy of EBV B-LCL x melanoma hybrid cells *in vivo*.
- Determining whether or not hybrid cells which do not express co-stimulatory molecules (CD80 and CD86) stimulate functional, anti-tumour T cells, and if not, do hybrid cells which are modified to express CD80 stimulate effective anti-tumour T cell responses?
- Determining whether or not genetic modification (eg transfection with GM-CSF) of hybrid cells further augments their capacity to stimulate an anti-tumour immune

response, especially if the hybrid cells do not express the 'immunostimulatory' phenotype.

- Determining which is the optimal professional APC partner for fusion with tumour cells (B cells, DCs or m\u03c6s).
- Determining what is the optimal immunisation regime with regard to dose (including number and frequency of doses) and the site of immunisation.
- Determining if hybrid cells induce autoimmunity and if so what is the significance (beneficial or deleterious).
- Determining if fully autologous APC x tumour hybrid cell vaccines are superior to semi autologous or completely allogeneic hybrid cell vaccines in *in vivo* vaccination protocols. Allogeneic APCs fused with autologous tumour cells may prove to be therapeutically more efficacious due to the expression relevant tumour-antigen presented with allo-MHC which may provide an adjuvant effect, providing the anti-tumour response is not masked.
- Determining if EBV B-LCL can be fused with *ex vivo* tumour cells to produce stable hybrids that grow in culture.

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