p53 EPITOPES AS POTENTIAL TUMOUR TARGETS FOR IMMUNOTHERAPY PROGRAMMES AGAINST CANCERS

A thesis submitted by

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Declaration

I hereby declare that no part of this thesis has previously been submitted in support of an application for any Degree or Qualifications of this, or any other University or Institute of learning.

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Summary

The tumour suppressor gene p53 is pivotal in the regulation of program cell death (apoptosis), and point mutations within the gene represent the most common genetic alterations in human cancers. This process can result in the overexpression and/ or accumulation of mutated and/ or wild-type p53 protein within the cell. Cytotoxic T lymphocytes (CTL) play a critical role in the immune defense by recognising peptide/MHC complexes on the surface of virally infected or tumour cells followed by lysis. Therefore, p53-derived peptides are potential candidates for immunisation strategies designed to induce anti-tumour CTL in patients.

Conformational changes in the p53 protein, generated as a result of point mutations, frequently expose the 240 epitope, RHSVV (amino acids 212-217). It is possible that peptides in this region could be processed and presented in a different manner to the wild type protein and therefore, have the potential to be recognised by tumour specific CTL.

In this study 15 peptides derived from the 240 epitope of the wild type p53 protein, amino acids 193-237, and peptides which are located nearby, and possess binding motifs for HLA-A2, were assayed for their ability to stabilise HLA-A2*0201 (HLA-A2) molecules in an MHC class I stabilisation assay. T2 cells processing pathway is defective and as a result produce mainly empty HLA-A2 molecules, which are unstable and quickly recycled. However when a peptide, possessing binding properties, is added exogenously it stabilises the complexes which can then be detected by immunofluorescence. Out of all the peptides tested seven (AA position 187-197, 188-197, 193-201, 196-204, 217-225, 263-272, 264-272) showed strong binding affinities for the HLA-A2 molecules (FR>1.5) at 26^{o}C with 5 peptides (187-197; 193-200; 217-224; 263-272 and 264-272) also stabilising the complexes at 37^{o}C. Three of these p53 peptides (188-197, 196-203 and 217-225) have not previously been identified. Since out of these three peptide 217-225 stabilised HLA-A2 molecules with the highest FR, it was chosen for the generation of CTL *in vitro*, using peptide 264-272 as a positive control.

Dendritic cells were generated from HLA-$A2^+$ donors and, after peptide-pulsing, were co-cultured with autologous $CD4^+$-depleted T cells *in vitro*. The two primary CTL thus generated (CTL-217, and CTL-264) were capable of specifically killing peptide pulsed T2 or JY but not either cells alone. These cells express wild-type p53 protein. In order to determine whether these peptides were endogenously processed, and test the hypothesis that different mutants with different p53 protein conformation would generate different peptides at the surface of the cell bearing them, a panel of target cells was generated. HLA-$A2^+$ SaOs -2 cells were transfected with p53 cDNA containing a point mutation at either position 175 (R →H) or 273 (R→H) (SaOs-2/175 and SaOs-2/273). Two HLA-A2 negative cell lines, A431 and SKBr3, naturally expressing p53 mutated at positions 273 and 175, respectively, were transfected with HLA-A2 cDNA. The results showed that both primary CTL were capable of killing SaOs-2/175 and SKBr3-A2 cells which bear the same mutation but not SaOs-2/273, A431-A2 or SKBr3 cells transfected with control vector. This suggests that these peptides were presented on the surface of SaOs-2/175 and SKBr3-A2 cells in a conformation dependent manner and are potentially useful for inclusion in cancer vaccines.

Key questions are whether patients whose tumours harbour a p53 mutation have demonstrable precursor CTL or whether they can be induced, and whether there is an association between the p53 gene mutation, HLA type and disease progression. In an attempt to answer some of these questions the immunological status of HNSCC patients with respect to p53 was studied by analysis of p53 serum antibody, and analysis of p53 protein expression by immunohistochemistry, when tumour samples were available. Results showed p53 accumulation in 6/10 of HNSCC and presence of anti-p53 antibodies in 2 out of 10 of these patients' sera, and 2 out of 14 of the totals of patients.

Cellular immune responses to p53 by CTL and proliferation although intended could not be done because of shortage of materials (protein and PBMC).

Therefore some of these questions remain unanswered and are open to further studies.

Abbreviations

A2Flu	A2 Peptide from the Influenza Virus Protein
AA	Amino Acid
AIM-V	
amp^R	Ampicilline Resistant Gene
APC	Antigen Presenting Cell
APES	
ATP	Adenosine Tri Phosphate
b3/a2 bcr/abl	Protein Fusion
BALB/c	Inbred Mouse Strain
β2-m	Beta 2 Microglobuline
β-Gal	β-Galactosidase
bcl-2	Gene Involved in the Inhibition of Programmed Cell Death
bcr	Break Point Cluster Region
BIP	Binding Inhibitor Protein
BSA	Bovine Serum Albumin
c-abl	Gene Encoding a Protein Tyrosine Kinase
CD3	Common T Cell Marker
CD4	T Helper Cell Surface Marker
CD8	CTL Surface Marker
CD14	Monocyte Lineage Marker
CD19	B-Cell Surface Marker
CD28	T Cell Receptor
CD40L/ CD40	Membrane Molecule Expressed by Stimulated T Cell/ Surface Receptor Expressed By Activated DC
CD54	ICAM-I Intercellular Adhesion Molecule
CD86 and CD80	Co-Stimulatory Molecules
CdK	Cyclin –Dependent Kinase
cDNA	Complementary DNA
CEM	T Lymphoblastoid Cell Line
CM1, CM2	Transfection Buffers
CML	Chronic Myelogenous Leukaemia
CMV	Cyto Megalo Virus
COS	Simian Kidney Cell Line
CTL	Cytotoxic T Lymphocyte(s)
CTLA-4	Cytolytic T Lymphocyte Associated Antigen-4
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribo Nucleic Acid
DOPA	Dihydroxyphenylalanins
E: T	Effector/ Target Ratio

E1A	Adenovirus Protein
E6 and E7	HPV Proteins
Eb	Epstein Bar Virus Protein
EBV	Epstein Barr Virus
Eco R*I*, Bam H*I*	Restriction Endonuclease
EDTA	EthyleneDiamineTetra-Acetic Acid (versine)
ELISA	Enzyme Linked Immunosorbent Assay
EMS	Ethyl Methane Sulphate
ER	Endoplasmic Reticulum
FACs	Fluorescence Activated Cell Sorter
Fas	Cell Surface Molecule Associated with Apoptosis
FCS	Foetal Calf Serum
FD	Fahrad
FITC	Fluoresceine Isothiacyanate
FR	Fluorescence Ratio
FSc	Forward Scatter
G418	Geneticin
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GTE	Glucose Tris EDTA
H-2b	Murine HLA-A2 equivalent
HER-2/neu	Growth Factor Receptor
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HNSCC	Head Neck Squamous Cell Carcinoma
HPLC	High Performance Liquid Chromatography
HPV	Human Papilloma Virus
HSP	Heat Shock Protein
ICE/Ced-3	Cell Death Proteases
IFN-γ	Interferon gamma
Ii	Invarian Chain
IL-	Interleukin
IP90	Calnexin
IR	Ionised Radiation
IU	International Unit
KDa = Kd	Kilo Daltons
KLH	Keyhole Limpet Hemocyanin
LB	Luria Bertani Medium
LCL	Lymphoblastoid Cell Line
LMP	Low Molecular Weight Protein
mcf	Median Channel Fluorescence
MDM2	Murine Double Minute 2

MEM	Modified Eagles Medium?
MHC	Major Histocompatability Complex
MLR	Mixed Lymphocytes Reaction
Modified-pBr322 , pcDNA3	Expression Plasmid
MT-1	Metallothionein Promoter
NK	Natural Killer
Nu/nu	Nude Mice
p.s.i	Pounds (pressure) per square inch
P1, P2, P3	Transfection Buffers
p15, N-acetylglucosaminytransferase V}	
P16, P18	Virus Associated Antigen from HPV
P1A	Murine Tumour Associated Antigen (found highly expressed in Tum$^-$ Variants)
p21	Ras Protein
p53	Tumour Suppressor Gene
P815A	Murine Mastocytoma Antigen
Pab	Polyclonal Antibody
PBL-T	Peripheral Blood Lymphocyte T
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
pCMV- β	Reporter Plasmid
Ph	Philadelphia
pRb	Retinoblastoma Protein
QBT, QC, QF,	Transfection Buffers
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SSc	Side Scatter
SV40	Simians Virus
T4	DNA Ligase
TAA	Tumour Associated Antigen
TAA	Tumour Associated Antigen
TAE	Tris/ Acetate/ EDTA Buffer
TAP	Transportor Associated Protein
TBS	Tris Buffered Saline
TCR	T Cell Receptor
TCR	T Cell Receptor
TE	Tris/ EDTA Buffer
Th1, Th2	T Helper Cell 1 or 2

TNF-α	Tumour Necrosis Factor alpha
TSA	Tumour Specific Antigen
Tum^-	Tumour Negative
WT	Wild Type
X-Gal	β-Galactosidase Substrate
XL1- blue	E. coli Plasmid Vector

CONTENTS

1.Introduction

MHC antigens are implicated in presenting target antigens for immune recognition and generating an immune response. Indeed epitope mapping of tumour antigens for MHC class-I and more recently class-II restricted binding motifs followed by subsequent immunisation with these peptides has induced immune responses in murine models as well as in humans against cancer expressing the antigen. This introductory chapter will present an account for tumour antigens presentation and their identification.

The task of the immune system is of two-fold. First it has to recognise any pathogen or other foreign material potentially harmful followed by an appropriate immune reaction destined to eliminate them. All cells of a given human being bear on their surface something in common that make them different from any other being, with the exception of twins. On their surface a group of proteins is present which are unique to each individual. These molecules are called HLA standing for Human Leukocyte Antigen in human and more generally referred to as the Major Histocompatibility Complex (MHC). These were first discovered when skin-graft rejections in mice were studied. These are of two classes, MHC class-I molecules and MHC class-II molecules. Class-I molecules are normally expressed on most nucleated cells (and on erythrocytes and platelets in some species) whereas class-II molecules are mostly restricted to antigen-presenting cells (e.g. dendritic cells, activated macrophages, B-cells and vascular endothelial cells). The level of MHC molecules is, however, modulable and their expression on cells can be changed by cytokines. Interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) are powerful inducer of MHC antigen expression on many cell types, which would otherwise express MHC molecules only weakly (Andalib, *et al.*, 1995).

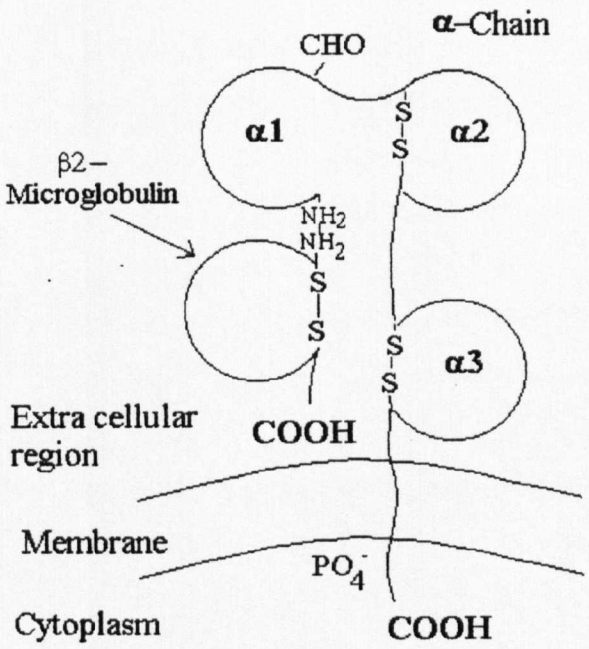
1.1 MHC Structure and Function

1.1.1 MHC Structure

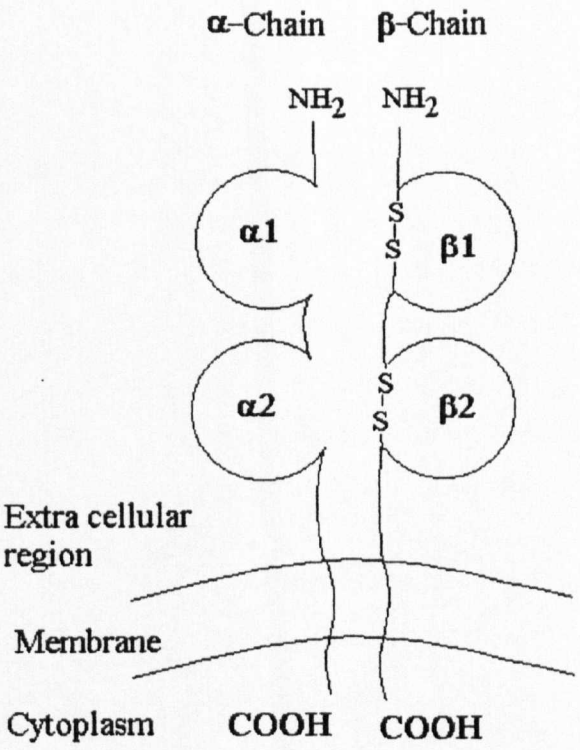
1.1.1.1 MHC Class-I Molecule

MHC class-I antigen structure consists of a 44KDa heavy chain, encoded by a number of genes on chromosome 6 (Trowsdale, et al., 1991) and composed of three extracellular domains (alpha-1, -2, -3), a transmembrane region and a cytoplasmic tail at the carboxyl terminus. Alpha-1 and -2 domains form a peptide-accommodating groove made-up of two antiparallel α-helices and overlaying a platform of antiparallel β-strands located on

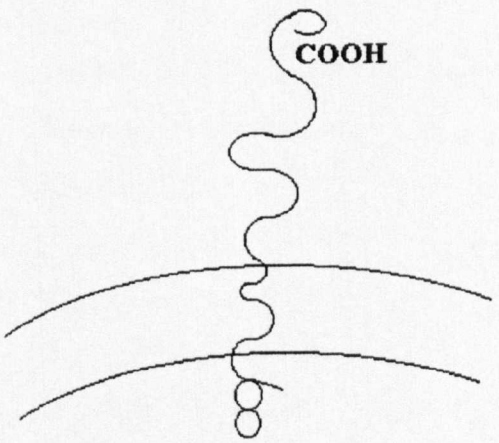
FIGURE: 1.1



1.a: MHC class I molecule



1.b: MHC class II molecule



1.c: The Invariant Chain

the top surface of the molecule. A non-polymorphic light chain of 12 KDa ß2microglobulin (ß2-m), encoded by chromosome 15 (Goodfellow, *et al.*, 1975) is non-covalently attached to the heavy chain (6) (Figure 1.a). Peptides are tightly bound in the groove and interact with both N and C termini and a subset of its amino-acid side chains, to MHC residues (Bjorkman[a], *et al.*, 1987). The complex formed is extremely stable with high affinity interaction. Two or three amino-acids position (anchor residues) are present, in the bound peptide, usually restricted to a vary narrow subset of amino-acid, leading to particular 'binding motifs' for each allele (See Section 3.1.2.1). Although, most MHC class-I peptides are of 8-11 amino-acids and derived from the endogenous processing of protein made inside the cell, some peptides arising from the degradation of exogenous protein are released from the APC phagosomes into the cytosol and are presented by MHC class-I molecules (Harding, *et al.*, 1994). The MHC class-I processing pathway process is explained in Section 1.1.2.1.

The human class-I region contains three major loci, called HLA-A, -B, -C, which direct the synthesis of class-I proteins. Three other genes (HLA-E, -F, -G) encode for 'minor' alleles, whose function are not fully determined yet. Each locus exists as allelic variants of MHC genes and is co-dominantly expressed. The frequencies of the principal alleles are as follows: HLA-A2 is present in 49.1% of Caucasians, HLA-A1 is present in 26.4% and HLA-A3 is present in 24.7%.

1.1.1.2 MHC Class-II Molecule

MHC class-II antigens, encoded by chromosome 6 (Trowsdale, *et al.*, 1991), are very similar to MHC class-I molecule. They are composed of heterodimeric membrane-integral glycoproteins, with tightly but non-covalently linked alpha (of about 34KDa) and beta (of about 28KDa) glycoprotein chains, each having a transmembrane domain and carboxyl terminal cytoplasmic tail (Figure 1.b) (Germain, *et al.*, 1994). The peptide-binding domain, forming open-ended pockets, shows greater degree of flexibility in the length of peptides that can accommodate within the groove. These vary from 11-25 amino acids and in contrast to MHC class-I peptide interaction specific residue interactions are formed throughout the extended peptide (Stern, *et al.*, 1994). The molecule is highly polymorphic and in man at least three isotypes (HLA-DR, -DP, -DQ) are co-expressed. In general, class-II MHC molecules bind peptides derived from

proteins that have been taken-up by antigen presenting cells (APC) through an endocytic process, but not always. This process is also discussed in Section 1.1.2.2.

1.1.2 MHC Function

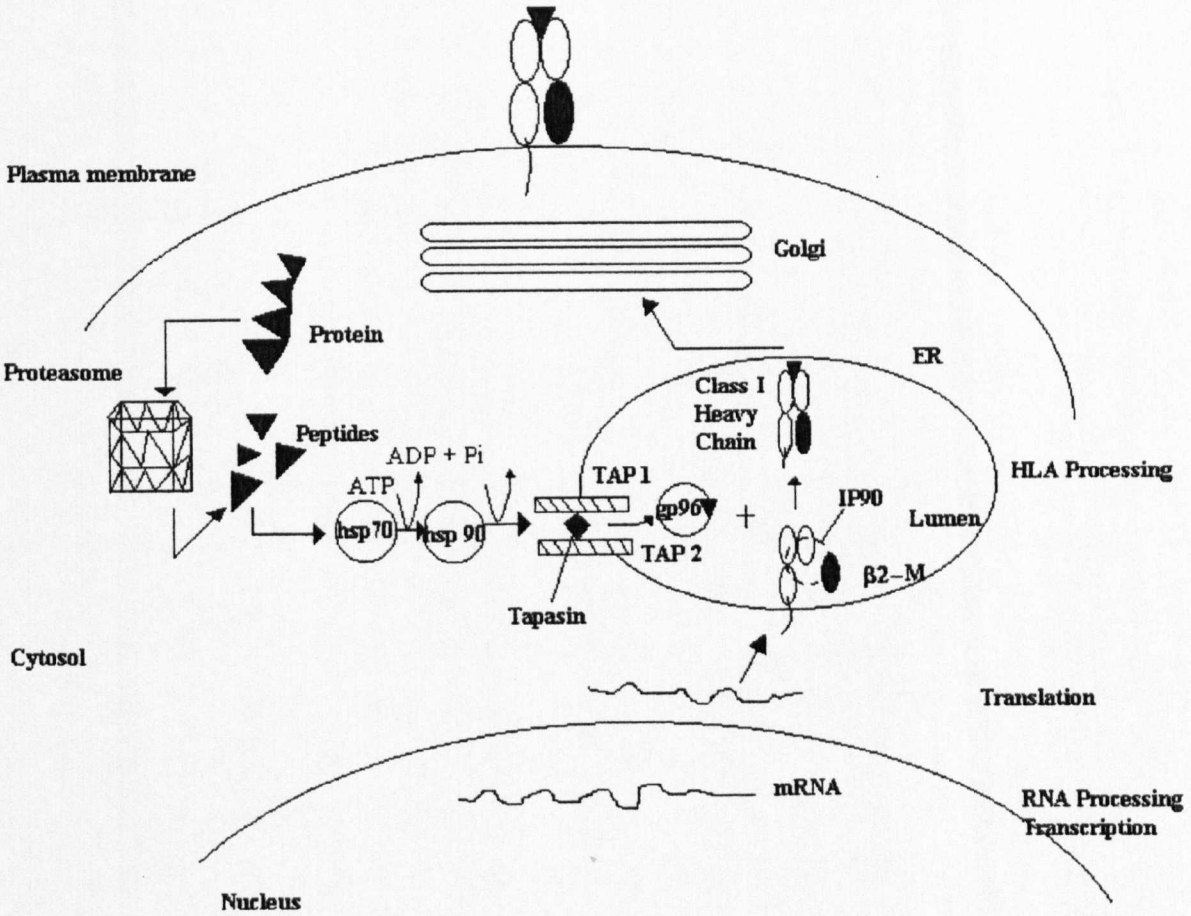
Apart from its role of aiding the immune system in distinguishing 'self' from 'non-self' MHC class-I and class-II molecules also function to collect peptides produced from endogenous cell protein, and transport them to the cell surface, where they can be surveyed by T-cells.

1.1.2.1 MHC Class-I Molecule

Most class-I binding peptides come from nuclear and cytolytic proteins (Benham, *et al.*, 1995). Proteins are processed in the cytosol by the major-cytosolic proteolytic complex, the proteasome, which exists as both a 20S and 26S form (Benham, *et al.*, 1995). Two of its subunits LMP2 and LMP7 (Low Molecular Mass Polypeptide) are encoded by the MHC gene complex (Glynne, *et al.*, 1991), which are responsible for the generation of small peptides. These peptides are transported via two heat shock proteins (HSP), of 70 and 90 KDa respectively, which needs ATP for each transfer (Srivastava, *et al.*, 1994). Peptides are then subsequently transported into the endoplasmic reticulum (ER) by TAP (transporter associated proteins), these consist of two subunits, TAP1 and TAP2. TAP proteins are structurally similar to the protein family of ATP driven membrane transporters, (a member of which is the P-glycoprotein involved in multidrug resistance of cancer cells (Spies, *et al.*, 1990). TAP proteins in turn transfer the peptides to another HSP of 96 KDa, called 'gp96', into the lumen of the ER (Deverson, *et al.*, 1990; Spies, *et al.*, 1990; Srivastava, *et al.*, 1993/1994). Finally peptides are delivered to empty complex MHC class-I/ß2-m, complex which occurred earlier (Ortmann, *et al.*, 1994). ß2-m, like the invariant chain for class-II molecules, plays an important role in the proper folding of the heavy chain since in the case of mutant cell line Daudi, that lacks ß2-m, virtually no class-I molecules were expressed on its cell surface. In this case the unproperly folded heavy chains never left the endoplasmic reticulum and were rapidly degraded (Restifo, *et al.*, 1996).

The dimerization of MHC class-I molecule and ß2-m, prior to peptide binding, appears to be mediates by a 90 KDa protein called calnexin (IP90) in human which then with the

Figure 1.2: MHC Class I Pathway

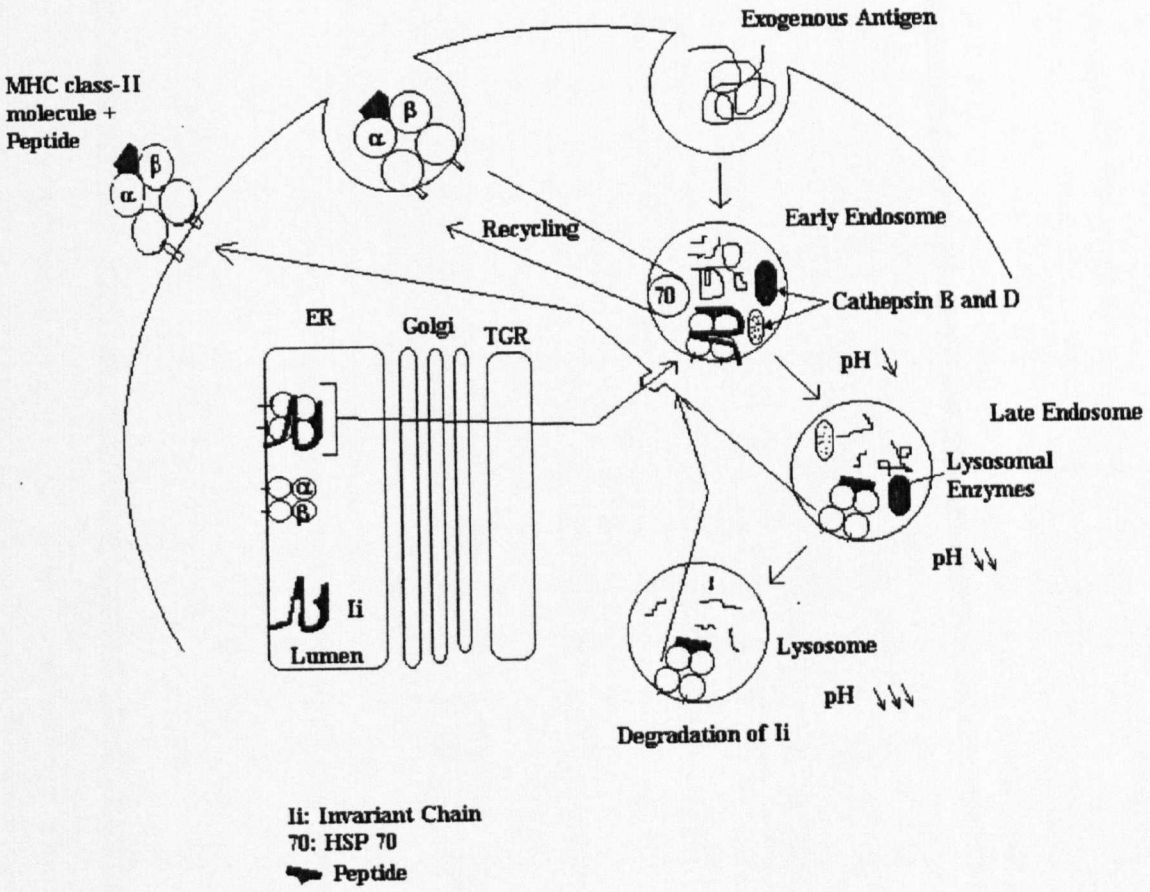


help of yet another transmembrane glycoprotein called tapasin facilitates their association with TAP molecules (Ortmann, *et al.*, 1994). This association allows dimmers to gain access to cytosol derived peptides before they get diluted or degraded in the lumen of the ER (Grandea III, *et al.*, 1995). In the human mutant T2 cell line, which has a homozygous deletion of the MHC class-II region located on chromosome 6 including the TAP genes, normal presentation of endogenously processed peptides does not take place even though cells produce high amounts of heavy chain class-I and ß2-m. Mostly, empty HLA-A2 isotypes are expressed, suggesting an important role of TAP molecules in antigen processing pathway (Towsend, *et al.*, 1989; Anderson, *et al.*, 1991). However when peptides are added exogenously, they bind to empty HLA molecule thereby stabilising the complex MHC / ß2 m. Yet in some cases other molecules apart from TAPs may be responsible for the antigen processing pathway. Followed by peptide binding, the complex is released and transported through the golgi apparatus to the cell surface (Figure 1.2). ER to golgi transport often requires correct tertiary or quaternary structure; inappropriately folded or unassembled proteins appear to be retained intracellularly either by inclusion in aggregates or by specific ER proteins such as calnexin or Binding protein (BIP). Calnexin and BIP, the latest being a member of the heat shock protein of 70 KDa (HSP 70), belong to group of molecules called molecular chaperones. Their predominant role, as stated, appears to be the prevention of intermolecular association between incorrectly unfolded polypeptide chains (Hartl, *et al.*, 1994). Its dissociation correlates with the completion of a distinct stage in folding. Thus calnexin seems to participate in regulating the intra-cellular transport of class-I molecules, probably by regulating assembly of intermediates since a close correlation between the rates of calnexin and the arrival of class-I molecule in the Golgi has been found (Degen, *et al.*, 1992).

1.1.2.2 MHC Class-II Molecule

MHC class-II molecules usually present exogenous antigens. These are internalised via the endocytic pathway and are subject to gradual degradation by a succession of enzymes active at different pH. Proteases cathepsin B and D, active in the early endosome (pH 6-6.5) (Guagliardi, *et al.*, 1990), are then joined by lysosomal enzymes in the late endosome (which are less active due to non-optimal pH of 5-5.5), and finally by

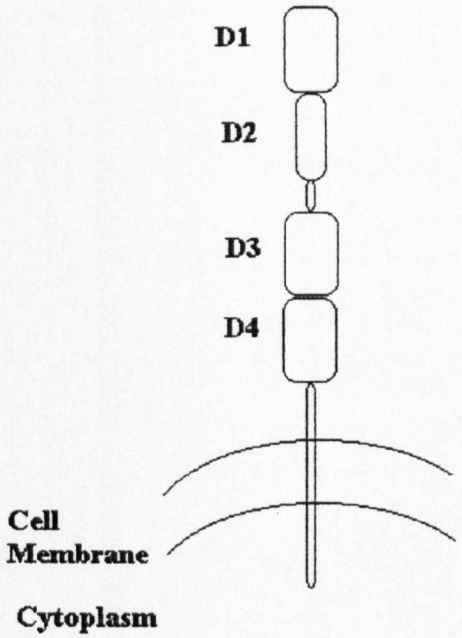
Figure 1.3: MHC Class II Pathway



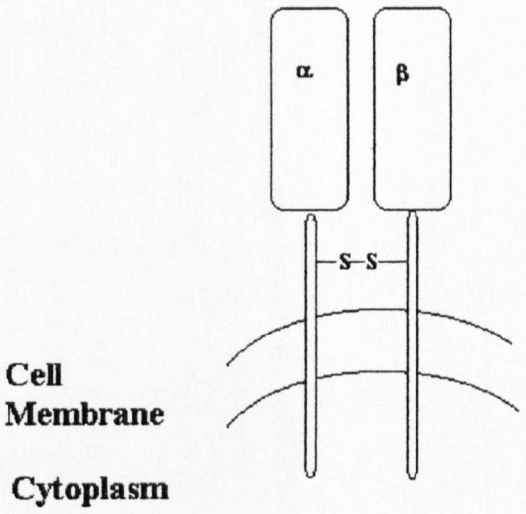
fully active lysosomal hydrolases in the lysosome (pH 4.5-5) (Ohkuma, *et al.*, 1978). Cathepsin B is necessary and may be sufficient for antigen processing alone (Takahashi, *et al.*, 1989), although the progressively decreasing pH of the endocytic pathway which activates most proteases plays an important part (McCoy, *et al.*, 1989). Newly synthesised class-II molecules with their associated invariant chain are delivered to early endosomes where they remain until the invariant chain is removed (Brodsky, *et al.*, 1991). The invariant chain is a glycoprotein, of 31-43 KDa, with an extra-cellular carboxyl terminus, a single transmembrane domain and an amino-terminal cytoplasmic tail and is encoded by chromosome 5 in human (Brodsky, *et al.*, 1991) (Figure 1.c). It has many functions, and among them is the protection of peptide-binding sites from the binding of peptides prior to the designated physiologic site (Hämmerling, *et al.*, 1990; Busch, *et al.*, 1996). This keep class-I and class-II pathways distinct and avoid the degradation of class-II molecule by activated proteases where the molecule is likely to bind peptides.

One other role of the invariant chain is to maintain the integrity of class-II molecule conformation. In its absence molecules folding differently are retained in the ER where they form aggregates in association with other molecular chaperons (Peterson, *et al.*, 1990). Analogous to its role in class-I assembly, discussed earlier, calnexin also retains alpha and beta chains of class-II molecule, enabling them to assemble and stabilise with the Ii in the ER (Williams, *et al.*, 1995). When the invariant chain is removed by proteolysis, class-II molecule becomes competent to bind peptides. Once complexed with peptides, the molecule is then released from either early or late endosome, transporting peptide to the cell surface. Internalisation and recycling of class-II molecules provides a second opportunity for empty class-II molecules to bind peptides. These processes therefore promote peptide exchange, with the possible involvement of a 70-Kd peptide-binding protein of the heat shock protein family (HSP) (Vanbuskirk, *et al.*, 1989) (Figure 1.3).

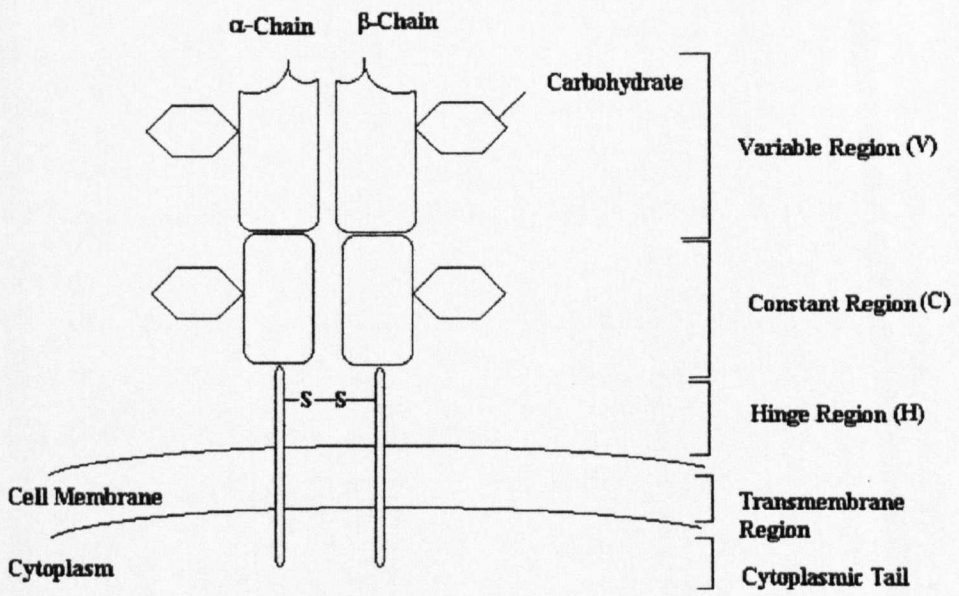
FIGURE: 1.4



1.4a: CD4 Marker



1.4b: CD8 Marker



1.4c: T-Cell Receptor

1.2 Tumour Antigens

As previously stated, the task of the immune system is two-fold. Once it has differentiated self from non-self and normal from abnormal cells, an 'immune' response then has to distinguish 'abnormal' from 'normal' cells. How this is achieved depends upon the type of molecular presentation by MHC molecules. MHC class-II molecules plus relevant peptides are recognised by immune cells bearing a CD4 marker (Figure 1.4a), also referred to as 'helper-T cells' because they enhance antibody production by activating B cells, promote other T-cell responses, and activate a variety of other immune effector cells (e.g., macrophages). MHC class-I molecules plus relevant peptides are recognised mostly by CD8 bearer T-cells (Figure 1.4.b) through their TCR (T-cell-receptor) (Figure 1.4.c), which requires to be of the same haplotype as the MHC molecule in question (MHC compatibility). This interaction, is necessary, but insufficient to lead to T cell activation (clonal expansion and differentiation) and destruction of the 'abnormal' cell by both the perforin / granzymes pathway (proteolyses) and the Fas / fas ligand-activated pathway (apoptosis) (Doherty, 1993). Indeed naïve T-cells require not only receptor binding, ensuring antigenic specificity but also a second signal delivered by co-stimulatory molecules such as CD80 (also called B7.1) and CD86 (also called B7.2) on the surface of APC via their interaction with CD28/CTLA-4 for activation. B-lymphocytes, macrophages or dendritic cells can deliver this second signal. T-cells reacting with self-protein have been eliminated prior to maturation by clonal deletion early in the development. T-cells are unable to respond to self-antigens since most cells cannot deliver this second co-stimulatory signal and instead induce tolerance where the T-cell in question is rendered anergic (Gimmi, *et al.*, 1993). $CD8^+$ T-cells are very effective cells which perform an immune surveillance function, effectively monitoring the contents of virtually all cells by detecting peptides that are presented in complex with MHC class-I molecules and displayed at the cell surfaces.

The evolution of a normal cell into a cancer cell is a complex multi-stage process that occurs at a molecular level leading eventually to the emergence of a clone of cells which no longer has the same growth control restraints that effect normal cells. Among all the molecular changes occurring in cancer cells are activation of oncogenes by mutation, rearrangements, transduction, or inactivation of tumour suppressor genes by mutation or deletion; and/or aberrantly glycosylated, aberrantly expressed or up-regulated self-molecules. All these changes may give rise to tumour associated antigen,

which are expressed by both normal and tumour cells but in higher quantity on tumour cells or to specific tumour antigen, which are only expressed by tumour cells.

1.2.1 Tumour Associated Antigens

Tumour associated antigens can be defined as antigens expressed by normal cells at particular stages of differentiation that are re-activated during the tumorigenic process or antigens express by normal cells that are overexpressed in tumour cells.

One mechanism by which oncogenes are activated in human cancers is gene amplification, which leads to increased expression of a normal self-protein that acts to drive cell proliferation. HER/neu proto-oncogene is amplified and overexpressed in 20-40% of invasive breast cancer. It encodes a receptor-like transmembrane glycoprotein with a relative molecular mass of 185Kda (p185) that is highly homologous to the to epidermal growth factor receptor (Bargman, *et al.*, 1986). Its function seems to be increasing activity of tyrosine-specific kinase (Coussens, *et al.*, 1985). CD4+ T-cells and antibodies have been shown to be present in the sera of patients with HER-2/neu-positive breast cancers (Disis, *et al.*, 1994). Tumour infiltrating lymphocytes (TIL) from patients HLA-$A2^{+}$ with ovarian cancer when expanded *in vitro* have demonstrated tumour-specific lysis in an HLA-A2 restricted manner (Yoshino, *et al.*, 1994).

Some mutations occurring in the genome, as said, are irrelevant to the tumoral transformation but can induce the activation of genes not normally active in normal cells. This may then leads to new peptides expressed and complexed with a MHC class-I molecule on the cell surface. These mutations may also generate mutated protein that once processed will generate binder peptides that did not bind before. That is exactly what happened when T. Boon (Boon, 1993), after exposing a culture of mouse tumour cells to a potent mutagen, transferred the clonal populations into tumour-free mice. Mice infected with the non-exposed tumour cells developed tumour almost always, while those infected with the mutagen-treated clones did not produce any malignancy. These cell clones were called Tum - for tumour negative. Yet, it was only in 1987 that the phenomenon was understood and three Tum- genes identified. Point mutation in two of the Tum- gene had rendered the affected peptide capable of binding to MHC molecules

and therefore recognisable by the host T-cells. The gene responsible for the tumour-rejection antigen (P815A) was then sequenced and called P1A. They also discovered that genes silent in normal cells can be activated by point mutations and this leads to exposure of new epitopes that can be targeted by host T-cells.

Since then a number of tumour associated antigens have been discovered (Boon, *et al.*, 1994) and at present three main tumour associated antigen groups can be described from the widely studied melanoma tumours.

The first group, testis-specific antigens, consists of developmental antigens that are not expressed by adult tissue, except testis, but are re-expressed in malignant cells, are all encoded by chromosome X, and belong to either of these families: MAGE, BAGE, GAGE, NY-ESO-1 and PRAME. MAGE-1, -2, -3 are expressed by melanomas (35, 58 and 60% respectively) (Brasseur, *et al.*, 1995) but also by other tumours such as breast cancers (Brasseur, *et al.*, 1992), lung cancers (Weynants, *et al.*, 1994), gastric carcinoma (Inoue, *et al.*, 1995), and bladder carcinoma (Patard, *et al.*, 1995). Their precise function is still unknown. Peptides encoded by MAGE-1 have been found to be presented by HLA-A1 or HLA-Cw(*)1601 and have been reported to induce specific cytolytic T-lymphocytes that recognise tumour cells expressing MAGE-1 gene (Salgaller, *et al.*, 1994; Van der Bruggen, *et al.*, 1994). Peptides encoded by MAGE-3, and presented by HLA-A2 or -A1, have also been found to generate CTL (Van der Bruggen, *et al.*, 1994; Gaugler, *et al.*, 1994).

The second group consists of differentiation antigens specific to the melanocyte lineage (tyrosinase, gp100/PmelA, and MART-1/MelanA). These antigens are expressed by tumour cells as well as by normal adult melanocytes but are in much higher level in melanoma cells. Tyrosinase is an enzyme that transforms tyrosine into dihydroxyphenylalanins (DOPA) an intermediate product in the synthesis of melanin. Tyrosinase has been shown to be recognised by HLA-A2 and -A24 restricted T-cell clones (Wölfel, *et al.*, 1994; Robbins, *et al.*, 1994) and by $CD4^+$ T-cells from TILS (Topalian, *et al.*, 1994). MART-1 gene, standing for melanoma antigen recognised by T-cells, encodes a protein that contain a hydrophobic region of 21 amino-acids long. This region may represent a transmembrane region (Kawakami[b], *et al.*, 1994/1996). Peptides from MART-1 have been reported to be specifically recognised by HLA-A2 restricted TILS (Kawakami[a], *et al.*, 1994). gp100 antigen is a type 1 transmembrane glycoprotein that may be involved in melanin synthesis. Peptides derived from the gp100 have been

shown to be recognised by HLA-A2 restricted anti-melanoma CTL line and TILS (Bakker, *et al.*, 1995).

The third group consists of the mutated or aberrantly expressed antigens including MUM-1, CDK4, beta-catenin, gp100-in4, p15 and N-acetylglucosaminyltransferase V (Kirkin, *et al.*, 1998). Other tumour-associated antigens include wild-type peptide epitopes of aberrantly expressed genes including cell cycle control genes such as cyclin D and E, or DNA repair genes such as p53. All of them may be very useful tools for future immunotherapy.

1.2.2 Specific Tumours Antigens

Among all oncogenes activated by point mutations, members of the ras gene family are the most frequently involved in human cancer and widely studied for their potential use for immunotherapy. The ras family consists of three related genes: ras H, ras K, and ras N, and encode inner membrane-bound proteins (p21 RAS). In human tumours, point mutations, i.e. replacement of one nucleotide by another one, are sufficient to convert normally functioning ras proto-oncogenes to potent oncogenes that efficiently induce neoplastic transformation. These mutations alter single amino acids (AA) at critical regulatory positions of the ras protein (p21) usually at residues 12 or 61 (Cooper, *et al.*, 1992). Thus a single AA substitution may create novel antigenic determinants which can be specifically recognised by host T-cells. Substitution of arginine for normal glycine at residue 12 has indeed shown to elicit specific MHC-class-II T-cells response (Peace, *et al.*, 1991) whereas substitution of glycine for aspartine at residue 13 did generate a class-I T-cell response being HLA restricted (Fossum, *et al.*, 1995).

Carcinogens are known to cause random mutations in the genome and methylcholonthrene is one of them. In methylcholonthrene-induced sarcomas, tumour cells express specific antigens that are unique to each tumour induced with the same component. Two tumour specific antigens have been isolated, one of 82 KDa and the other one of about 86 KDa, from the methylcholonthrene-induced sarcoma and both had tumour rejection activity specific for the tumour induced (DuBois, *et al.*, 1986).

Abnormal rearrangements of the genetic material like translocation of chromosomes, as said, can also activate oncogenes. In leukaemias and lymphomas additional translocations occur regularly, and in the case of chronic myelogenous leukaemia (CML) the 5' end of the c-abl proto-oncogene located on chromosome 9 is replaced by bcr gene

sequences on chromosome 22. The new chromosome is called Philadelphia chromosome (Ph) (Urban, *et al.*, 1992). The expression of which results in the production of an aberrant fusion protein, a tyrosine kinase with tumorigenic properties (McLaughin, *et al.*, 1987). Therefore the new protein may be immunogenic if it is appropriately processed intracellularly and if the resulting peptides possess the structural characteristics necessary to bind to both MHC molecule and T-cell receptor within that individual. T cells were shown to proliferate when incubated with dendritic cells pre-pulsed with 11-mer peptide from the b3/a2 bcr/abl fusion protein (Mannering, *et al.*, 1997).

Viral encoded gene products are strongly immunogenic and fall into a class of proteins that appear to facilitate cellular transformation. These potential tumour specific antigens represent obvious choices to evaluate for T-cell reactivity and against which to direct immune T-effector cells. Human papillomaviruses (HPV) are small DNA viruses that are associated with 90% of all cancers of the cervix. Approximately 70 different types of HPV have been identified but only 8 are capable of infecting the lower genital tract, transmitted during sexual intercourse. In pre- malignant lesions viral DNA appears to be episomal and replicates independently of cellular DNA, cells are able to produce mature viral proteins. Change to malignancy involves the integration, in a random fashion, of viral DNA into one several chromosome sites. It is associated with the loss of ability to produce viral particles. Studies have found that E6E7 portion of viral genome is constantly transcribed and these are known to carry information concerning immortalisation and transcription of a target cell. E6 and E7 are viral proteins expressed only by cancer cells, they constitute therefore specific tumour antigens potentially recognisable by host T-cells. Cells transfected with the E7 gene of HPV-16 were specifically rejected by mice immunised with MHC class-I, syngeneic fibroblasts expressing the same HPV gene and injection of antibodies specific for CD8+ cells prevented the rejection (Chen, *et al.*, 1991). Tumours expressing the E6 antigens were also specifically killed by immunised mice $CD8^+$ cells in a MHC restricted pattern (Chen, *et al.*, 1992).

The Epstein-Barr Virus (EBV) is directly implicated in the formation of immunoblastic B-cell lymphoma, Burkitt's lymphoma, and nasopharyngeal carcinoma (Masucci, *et al.*, 1993). EBV nuclear protein appear to be required for the maintenance of the transformed state and their expression is selectively retained in tumour cells as for many

viral proteins (Masucci, *et al.*, 1993). EBV peptides have been shown to elicit class-I-restricted $CD8^+$ T-cell immune response (Muuray, *et al.*, 1992).

Adenovirus products such as adenovirus 5 E1A gene products have also been shown to generate specific $CD8^+$ CTL immunity against an E1A transformed syngeneic cell line in H-2b mice and these *in vivo* primed $CD8^+$ T cells could be adoptively transferred and were capable of mediating the complete regression of E1A-induced tumours grown in nude (nu/nu) mice (Kast, *et al.*, 1989).

Breast, colon, and pancreatic adenocarcinomas all express aberrantly glycosylated mucin encoded by MUCI gene, thereby revealing previously cryptic mucin peptide epitopes. Mucin are large heavy glycosylated proteins expressed and secreted by ductal epithelial cells (Burchell, *et al.*, 1987). Their structure consists of a long polypeptide core, formed by a multiple tandem repeats of amino-acid sequences, surrounded by O-linked carbohydrate moieties (Roussel, *et al.*, 1988). CTL against normally cryptic epitopes present in the 20 amino-acids tandem repeats on both breast and pancreatic tumour targets have been generated in a MHC-unrestricted manner (Jerome, *et al.*, 1991).

The identification of tumour associated antigen has been possible by the progress made in molecular biology since the work of Thierry Boon and co-worker in 1991 where DNA from cancer cells were removed, cleaved and spliced into plasmids, carrying a drug resistance gene. Cosmids were then mixed with millions of cells known to have lost the antigen. Cells were then exposed to toxic drug and the survivors, containing the gene of interest, allowed to expand. Samples were in turn exposed to T-lymphocytes specific for the antigen. The DNA of the cells capable of inducing a CTL response were removed and sequenced. This technique has lead to the identification of a numbers of tumour associated antigens mentioned earlier. It is, however, important to stress out that these discoveries were only made possible because CTL were available to detect antigen in transfected cells.

Amongst all the tumour associated and specific antigens, p53 is recognised for its importance both as proto-oncogene or when mutated as an oncogene. In addition p53 has a potential role in immunotherapy against cancer, this is discussed below.

1.3 p53 Gene

1.3.1 p53 Function

p53 gene is a tumour suppressor gene located on chromosome 17 at band 1301 (Levine, *et al.*, 1991). It encodes a nuclear 53 KDa phosphoprotein whose half-life is very short in normal cells, 5-20 minutes. Normal p53 acts as a transcriptional factor (Fields, *et al.*, 1990) of different genes by forming tetramers which bind to specific DNA sequences. It is involved in apoptosis, in the control of cellular differentiation and appears to positively or negatively regulates the expression of particular genes in the cell.

The normal p53 serves to protect the genome and so has been called "the guardian of the genome" (Lane, *et al.*, 1992). As a consequence of DNA damage induced by Cisplatin, Mitomycin C, Etoposide regulator (Fritsche, *et al.*, 1993) or U.V irradiation (Hall, *et al.*, 1993) p53 levels increase, leading to p53 accumulation which in turn induces the transcription of the Waf1 gene localised on chromosome 6p21.2 (El-Deiry, *et al.*, 1993). The p21 ^{Waf1} increase leads to inhibition of the cyclin/Cdk mediated phosphorylation of pRb (Dulic, *et al.*, 1994) and also inactivates the DNA replication machinery by binding to proliferating cell nuclear antigen (PCNA) and DNA polymerase gamma (Waga, *et al.*, 1994), and as a consequence cells are arrested late in the G1 phase of replication (Kastan, *et al.*, 1991). p21 belongs to a family of cell cycle inhibitory proteins but none of the others however has-been found to be regulated by p53 (El Deiry, *et al.*, 1991). Tumour cells with mutated or absent p53 genes lose the ability to arrest in G1 following ionised radiation (IR) (Kastan, *et al.*, 1991) or G2 following DNA damage (Allday, *et al.*, 1995) and if normal p53 has some anti-proliferative properties its mutated counterpart however fails to block the progression of cell division and occasionally even promotes cellular proliferation (Martinez, *et al.*, 1991). p53 accumulation has been shown to be the result of an increased half-life of the protein rather than an increase in the protein synthesis since no mRNA augmentation was detectable after XRT treatment (Kastan, *et al.*, 1991). Another study showed that the overexpression of the temperature sensitive p53 Val 135 mutant transfected into non-tumorigenic murine myeloid precursor cell line, which is IL-3 and serum dependent for survival, accelerated apoptosis when cells were IL-3 deprived (Blandino, *et al.*, 1995). Similarly the transfection of p53 under control of a metallothionein MT-1 promoter in human colon tumour-derived cell line EB when stimulated by addition of zinc chloride resulted in a high level of p53 and induction

of apoptosis of cells grown in culture dishes as well as those grown in nude mice (Shaw, *et al.*, 1992). Overexpression of p53 in normal cells either arrests cell growth and/or induces apoptosis in response to certain stimuli. However p53 gene and its protein are not essential for cellular viability. Indeed a family of transgenic mice with both p53 alleles destroyed by an insertional mutation was still able to produce viable offspring, even though in two-thirds of them cancer developed over a three-to-six month period being mainly lymphomas (Donehover, *et al.*, 1992). On the other hand MDM2 (Murine Double Minute) null mice (which possess functional p53) are not viable, embryos dying before the day 5.5 of development (Montes de Oca Luca, *et al.*, 1995). In contrast mice with neither p53 nor MDM2 develop normally (Jones, *et al.*, 1995). MDM-2 is a murine gene that has originally been cloned from a spontaneous transformed BALB/c 3T3 cell line and that has been found to increase tumorigenic potential of cells when overexpressed (Cahilly-Snydler, *et al.*, 1987; Fakharzadeh, *et al.*, 1991). Wild-type p53 protein has shown to directly stimulate the MDM-2 product expression by binding to an element located on the first intron of the MDM-2 gene (Wu, *et al.*, 1993). MDM-2 product, a 90 KDa protein, then complexes with p53 protein (wild type and mutated one) and blocks further p53 function, which results in less MDM-2 (Wu, *et al.*, 1993) and other proteins being made. Factors that modify that balance by increasing MDM-2 levels (via gene amplification for e.g.) will then promote cell proliferation in the other hand inactivated MDM-2 would lead to growth arrest or apoptosis as it has been observed when MDM-2 gene was removed (Montes de Oca Luca, *et al.*, 1995).

Since p53 mRNA half-life is long (Dony, *et al.*, 1985) making transcriptional regulation rather ineffective, this is one way of controlling cellular p53 activity levels, avoiding accumulation of p53 that would lead to apoptosis after IR exposure for example. Inactivation of p53 may also be the result of other mechanisms such as protein-protein complexes with the transforming protein of tumour viruses such as SV40 and adenoviruses or Eb protein or as said complexes with MDM-2 protein. As a result p53 protein conformation is stabilised and its half-life increased, leading to accumulation within the cell. Different conformations of normal p53 depending on the state of the cell (quiescent or mitotic) have been reported (Milner, 1994). Two antibodies have shown to recognised specifically either the normal conformation (PAb 1620) or the mutated one (PAb240). P53 is mainly regulated post-transcriptionally and phosphorylation at serine

389 and in general increased phosphorylation has shown to be correlated with the suppressor form of p53 protein (Milner, 1994).

Apart from the WAF1 and MDM-2 genes other genes also positively regulated by p53 have been reported amongst them are Bax and bcl-2 (Miyashita, *et al.*, 1995), GADD45 (Kastan, *et al.*, 1992), and cyclin G (Zauberman, *et al.*, 1995).

1.3.2 p53 as Specific Tumour Antigen

p53 gene is present in all cells and mutation within the gene represent the single most common alteration identified so far in many types of human cancers, which is for some an early event. Point mutations, predominant mechanism of p53 protein inactivation are, for 95% of them located in four evolutionary conserved domains. Codons 175, 248, 273, and 281 represent the most frequently mutated codons and some are more commonly found in certain types of human cancers. p53 Peptides expressed at the surface of cancer cells with a single amino-acid substitution represent ideal candidates for tumour targeting immunisation since they would only be present at the surface of tumour cells. Some of these peptides have been identified and used to successfully immunise animals, which developed p53-specific CTL capable of specifically recognising mutated p53 peptide with a single amino-acid substitution but not its wild-type counterpart (Gabrilovich, *et al.*, 1995). More over these p53-derived peptides immunised animals were more resistant to tumour challenge (Noguchi, *et al.*, 1994). The main challenge remains however to generate a CTL response, which would recognise and destroy a tumour already established. This has partially been achieved in animals bearing palpable tumours after repeated immunisation with p53-peptide-pulsed dendritic cells (Gabrilovich, *et al.*, 1997). Tumour growth was much more efficiently inhibited when IL-12 was given along with the dendritic cells, only the addition of IL-12 resulted in CTL generation (Gabrilovich, *et al.*, 1997).

In addition significant CTL responses specific for mutant p53 have been observed in patients with breast cancer (Gabrilovich, *et al.*, 1995). These data strongly suggest that mutated p53 peptides are immunogenic and could potentially be used for *in vivo* vaccination. Although these 'mutant-derived' peptides would make highly specific tumour vaccines, with no toxicity for healthy cells, they are also patient-specific making this vaccine a difficult, expansive and time-consuming one. On the other hand a single peptide-based vaccine using p53 wild type epitopes would target a wide variety of

tumours expressing different mutated p53 proteins. p53 protein, as mentioned earlier, is expressed in all cells, which means that p53 peptides are to some extend expressed at the surface of all of them and are regularly surveyed by CTL. These would however not be activated since any self-reacting CTL would have been destroyed in the thymus or rendered tolerant in order to prevent autoimmunity. One might therefore think it wrong to produce a vaccine with self-peptides. However one has to remember that wild-type p53 protein expressed in normal cells has a very short half-life, around 20 minutes, and as a consequence, few peptides would have had the time to be produced. When however, p53 protein is stabilised as a result of mutation or as a result of complexes formed with other proteins, it accumulates within the cell which can and has been detected by immunohistochemistry. This would produce more peptides than should otherwise be and CTL, that do not normally react to self-peptide, would undergo clonal expansion and mediate cytolysis following recognition of high expression p53-peptides. Vierboom and co-worker have indeed demonstrated that when mutant p53 protein was overexpressed, processed and presented by MHC class-I molecules on the surface of murine tumour cells, tolerance was broken, and malignant cells recognised as 'abnormal' were killed by CTL generated against wild-type p53 peptide sequences (Vierboom, *et al.*, 1997).

Interestingly CTL precursors have been reported in normal individuals suggesting an ongoing immune response against cells expressing increased level of p53 protein (Röpke, *et al.*, 1995). Moreover antibodies against p53 protein have been found in a number of cancers bearing a mutation within the p53 gene. Such a humoral response requires antigen processing and presentation by APC as well as helper T-cell (Soussi, *et al.*, 1994) and all patients from whom a T-cell response was detected also had p53 antibodies, reinforcing the idea that tolerance can be broken as a result of p53 overexpression (Tilkin, *et al.*, 1995). Therefore wild-type p53 protein appears to be antigenic when express at high level and is the target for CTL after processing during the normal tumorigenesis process. There are different explanations for the non-destruction by the immune system of all tumours with a mutated p53. Cancer cells have indeed, been shown to evade the immune surveillance through different mechanisms. These include loss of MHC expression, generation of antigen-loss tumour variants, downregulation of antigen processing machinery and expression of local inhibitory molecules, such as tumour necrosis factor and Fas ligand. However, as far as p53 is concerned, the lack of

effective immune response towards cancer cells, *in vivo*, is at least not due to antigen loss since mutated p53 protein is expressed throughout the tumorigenisation process; from the smallest primary lesion to widespread metastatic disease (Sameshina, *et al.*, 1992). One possible reason could be that tumour cells require its continued presence in order to maintain their malignant phenotype. As for the other possible mechanisms of escape, β2-m mutations or defect in TAPs have been observed in some cancers but remains a relatively rare event in solid cancers (Chen, *et al.*, 1996 a and b). The decrease in MHC molecules has also been demonstrated in some cancers but is low and can be overcome by low doses of IFN-γ (Doyle, *et al.*, 1985). p53 'Wild-type' peptides-based vaccine therefore represents a very promising tool for immunotherapy against cancers.

Subtle mutations can affect the conformation of the entire protein, altering the structural integrity of domains far removed from the sites of mutation. As a result p53 is blocked in the promoter form leading to abnormal proliferation and cell transformation. Such conformational change may lead to exposure of normally cryptic epitopes or interfere with the normal processing pathway of the protein. Substitution of proline at codon 273 (Arg → Pro) appears to have such an effect on the protein (Milner, 1994).

More generally p53 gene hotspot mutations have been classified into two categories. (i) Those which induce a conformational change in p53 protein to a mutant conformation, for example the point mutation at amino-acid position 175; and (ii) those which retain the wild type conformation, for example the point mutation at position 273 (Milner, *et al.*, 1990/1995). Conformational changes in the p53 protein are frequently recognised by the monoclonal antibody PA240, due to unfolding of the protein and exposure of the 240 epitope, RHSVV (residues RHSVVV 212-217). It is therefore possible that peptides in this region are preferentially processed from conformational mutants rather than wild-type conformation protein and if they are naturally processed and presented at the cell surface of tumour cells in the context of MHC class-I, they could be useful as targets for CTL tumour specific immunotherapy for a wide range of cancers.

CTL only respond when antigen presenting cells (APC) express the same HLA class-I allele as the host from which the T-cells are derived, and it is necessary to determine whether specific epitopes from p53 protein bind to specific HLA haplotypes.

In a previous study thirty-seven overlapping peptides (34 nonamers, 2 ten amino-acids in length and one eleven AA in length) from the 240 epitope region were synthesised

(residues 193-237), and investigated using a flow cytometry based MHC stabilisation assay for their ability to bind HLA-A2 and HLA-A3 molecules. In this study further p53 peptides were tested for their capacity to bind to MHC class-I antigens using the T2 cell line expressing HLA-A*0201 (HLA-A2) molecules. Peptides found to bind were then assessed for their ability to generate anti-p53 CTL using first whole PBMC as APC then dendritic cells. Tumour cells HLA-A2 positive or transfected with the HLA-A2 gene and expressing either conformational mutant p53 proteins or wild-type conformation p53 proteins, either 'naturally' or transfected with mutated p53 gene, were used as targets.

This study assessed the potential of p53 epitopes as a target for CTL and their use in immunotherapy programms against human cancers.

CHAPTER 2: MATERIALS AND METHODS **Pages:**

2.1 MATERIALS

2.1.1 General laboratory reagents and equipment

a) Glassware

Pyrex glassware was washed in RBS detergent, rinsed several times in tap water, and finally in de-ionised water, dried in a hot air oven at 80°C, before sterilisation at 160°C for 2 hours.

b) Plastic and disposable equipment

Item	Supplier
'Universal' tubes	Bibby Striline
1.5 Cryovials	Nalge
10ml / 30mls Plastic pipettes	Costar
15ml centrifuge tubes	Bibby Sterilin
15ml, 50ml Polypropylene tubes	Costar
25cm^2, 75 cm^2 Tissue culture Flasks	Costar
5ml Bijoux	L.I.P
6-, 12-, 24-, 48-, and 96-well plates	Costar
90mm Petri dishes	Phillips Harris Scientific
C10 tips for Gilson p2/p10 pipettes	Gilson Medical Electronics
Cuvettes for electroporation	Equibio Ltd
Disposable scalpels	Swann-Morton
Ependorf tubes	Sartedt
FACS Tubes	Elkay
Glass coverslips	Chance-Proper; BDH
Latex examination gloves	Ansell Medical
Lumaplate^{TM}-96	Canberra Packard
Paper Tissue	Jamont
Plastic and glass Pasteur pipettes	Scientific Laboratory
Slides (For cytospin and immunostaining)	BDH Merck
Sterile disposable scalpels	Swann- Morton

Sterile syringes and needles	Becton Dickinson Supplies
Topseal™-S microplate heat sealing film	Canberra Packard
White tips for automated pipette	Sarstedt
Yellow and blue pipette tips	Sarstedt

c) Equipment

Item	**Supplier**
96 well Plate Harvester- Filtermate 196	Canberra Packard
Centrifuges	Beckman GPR
Cytospin Centrifuge	Shandon Cytospin 2
DynaBead Separation Unit	DYNA
Electrophoresis Gel Tanks	Bio Rad; BRL
FACSort	Becton Dickinson
Gamma Counter – Topcount ™	Canberra Packard
Incubators	Sanyo Gallenkamp
Microscopes	Leitz Dialux light microscope
Plate Sealer – Micromate 496	Canberra Packard
Shaker Incubator	Sanyo Gallenkamp
U/V/VIS Spectrometer	Perkin Elmer

d) Chemicals Reagents

Unless otherwise stated all chemicals used in the preparation of solutions and buffers were of high and mostly of Analar grade (BDH Merck Ltd).

Item	**Supplier**
2-mercaptoethanol	Gibco Life Technologies
Bromophenol Blue	BDH Merck
Calcium Chloride	BDH Merck

Chloroform	BDH Merck
DePex Mounting Medium	BDH Merck
Ethanol	BDH Merck
Glacial Acetic Acid	BDH Merck
Glucose	BDH Merck
Glycerol	BDH Merck
Isoamyl Alcohol	Sigma
Liquid Nitrogen	British Oxygen Company
Manganese Chloride	Sigma
Methanol	BDH Merck
Paraformaldehyde	BDH Merck
Phenol	Fisons
Potassium Acetate	BDH Merck; Sigma
Potassium Bicarbonate	Sigma
Presept	Johnson and Johnson
RBS	Chemical Concentrated
SeaKem Agarose	FMC Bioproducts
Sodium Acetate	BDH Merck
Sodium Chloride	BDH Merck
Sodium Dodecyl Sulfate	Fisons
Sodium Hydroxide	BDH Merck
Tris-Chloride	BDH Merck
Tryptone	Difco
Tween 20	Promega
Yeast Extract	Unipath

e) Water

All water used in this study was of ultrapure quality (>18 Ohms) unless otherwise stated and was obtained from an installed Permulab deionising water system.

2.1.2 Materials and Solutions for Tissue Culture

a) Cell lines

Cell line name	Type	Source	Culture media used	Adherent or suspension line
JY	Lymphoblastoid	CRC Paterson Institute, Manchester	RPMI + 10% FCS	Suspension
K562	Erythro-leukaemic	ICRF, London	RPMI + 10% FCS	Suspension
T2	Lymphoblastoid	Gift from Paterson Institute	RPMI + 10% FCS	Suspension
Rosi	Lymphoblastoid	Gift from Ludwig Institute	Iscove's + 10% FCS	Suspension
Jesthom	Lymphoblastoid	Gift from BTS	RPMI + 10% FCS	Suspension
SaOs-2	Osteosarcoma	Department of Human metabolism clinical Biochemistry, Sheffield	Dulbecco's + 10% FCS	Adherent
A431	Head and Neck	ECACC	MEM + insulin + 10% FCS	Adherent
SKBr3	Breast	ATCC	McCoy's 5A	Adherent
MCF-7	Breast carcinoma	ICRF, London	Dulbecco's + 10% FCS	Adherent

b) Growth Media

- AIM-V Medium

was purchase from Life Technologies Ltd and contained glutamine, streptamycin sulphate (50 mcg/ml) and gentamin sulphate (10mcg).

- Dulbecco's medium

Foetal calf serum (FCS): This was purchased from Life Technologies Ltd and stored at 80°C.

- ISCOVES and McCoy's 5A Media

Were purchased from Life Technologies

- MEM Medium

This was purchased from Sigma and supplemented with 20mM L-Glutamin.

- RPMI Medium

Was purchased from Imperial Laboratories

c) Mycoplasma Testing

All cell lines were periodically tested by Mrs. Janet White (Institute for Cancer Studies, Sheffield) for mycoplasma contamination, with a DNA hybridisation assay using a Gen-probe rapid detection kit (Gen-Probe Incorporated).

d) Trypsin

Trypsin (Gibco Life Technologies Ltd.) was prepared as a sterile 2.5% v/v solution in saline, diluted to 0.25% v/v solution with PBS, and stored at -20°C prior to use and thereafter at 4°C.

e) Versene

Ethylenediaminetera-acetic acid (EDTA) (BDH Merck Ltd) was prepared as a 0.02% w/v solution in PBS, autoclaved at 10 p.s.i for 10 minutes, and stored at 4°C.

f) Bovine Serum Albumin (BSA)

This was purchased as a 30% solution from Advanced Protein Products, subsequently diluted to a 10% solution by adding 100ml BSA to 200 ml sterile PBS, and then kept at 4°C.

g) Phosphate Buffered Saline (PBS)

Oxoid Phosphate Buffered Saline tablets (Unipath Ltd) were dissolved in distilled water as per manufacturer instructions (1 tablet per 100ml water), and sterilised by autoclaving at 15 p.s.i for 15 minutes.

h) Trypan Blue

Trypan blue powder was prepared as a 0.1% w/v solution in PBS. Residual, undissolved powder was removed by filtration prior to autoclaving at 15 p.s.i for 15 minutes.

2.1.3 Materials for MHC Stabilisation Assay

With the exception of the Flu peptide, all peptides were synthesised and dissolved in 10% DMSO, 90% PBS + BSA 0.1% to a peptide concentration of approximately 20mM and stored at -20°C in 10µl aliquots.

2.1.4 Materials for Flow Cytometry

a) 1% Paraformaldehyde solution

This was prepared by adding 1g of paraformaldehyde powder to 100ml PBS, with the pH adjusted by adding few drops of 0.4M NaOH solution. This solution was placed in a water-bath at 37°C for 1-2hrs for the paraformaldehyde to dissolve; and then kept in glass bottle at 4°C.

b) Antibodies

Antibody	Specificity	Amount used	Obtained from
HB-82	HLA-A2 and HLA-B27	1st Batch: 25µl of 1/10 dilution 2nd Batch: 75µl Neat	The supernatant derived from a hybridoma cell line This was a kind gift from Dr. J. Bartholemew. Paterson Labs., Manchester.
FITC	Goat anti-mouse F(ab')2 IgG conjugated to the fluorescent molecule Fluorescein isothiocyanate used as secondary antibody	100µl of 1/100 dilution	Bradsure Biologicals
DO-7 IgG2b	Wild-Type and Mutant p53 protein	30µl of 1/10 dilution 1/100 dilution for immunostaining	Dako
Class-I IgG2a Conjugated	HLA-A, -B, and –C	10µl	Dako
Class-II IgG2a Conjugated	HLA-DR, -DP, and –DQ	5µl	Dako
CD54 IgG1 Conjugated	ICAM-1	10µl	Dako
CD86 IgG1 Conjugated	B7.2	5µl	Dako
CD80 IgG1 Conjugated	B7.1	5µl	Dako
CD14 IgG2a	CD14	5µl	Dako
CD1a IgG1	CD1a	5µl	Dako

All antibodies were non-conjugated mouse antibodies, unless otherwise stated, and dilutions were made in PBS/BSA 0.1%. Isotype controls for each antibody were used at the same concentration.

2.1.5 Materials for Transfections

a) Liposomes

All of them were purchased from Life Technologies.

b) Buffers

- CM1 buffer was prepared as follows:

  10 mM Sodium Acetate (pH5.6)

  50 mM Manganese Chloride

  5 mM Sodium Chloride

- CM2 buffer was prepared as follows:

  10 mM Sodium Acetate (pH5.6)

  5 mM Manganese Chloride

  5% Glycerol

- Qiagen Maxi-Prep Buffer Composition

Buffer	**Composition**	**Storage**
Buffer P1 (Re-suspension buffer)	50mM Tris-HCl (pH 8.0) 10mM EDTA 100µg/ml Rnase A.	4°C
Buffer P2 (Lysis buffer)	200mM NaOH 1% SDS.	RT
Buffer P3 (Neutralisation Buffer)	3.0M potassium acetate (pH5.5).	RT
Buffer QBT (Equilibration Buffer)	750mM NaCl 50 mM MOPS (pH 7.0) 15% ethanol 0.15% Triton X-100	RT

Buffer QC (Wash Buffer)	1.0 M NaCl; 50 mM MOPS (pH 7.0) 15% ethanol.	RT
Buffer QF (Elution Buffer)	1.25 M NaCl 50 mM Tris-HCl (pH 8.5) 1 mM EDTA.	RT

c) Selection Antibiotics

Geneticin (G418) purchased from Gibco Life Technologies Ltd. was added to the medium of transfected cells at a pre-determined optimum concentration.

Ampicillin

d) GTE solution

50mM Glucose

10mMEDTA

25mM Tris-HCl, pH 8.0

The solutions were prepared in batches of 100ml, autoclave and stored at 4°C.

e) NaOH/SDS solution

0.2N NaOH

1% SDS

Water

This solution was prepared immediately before use

f) 3M Sodium Acetate solution

5M solution of Na acetate	60 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

The solution was stored at 4°C.

g) DNA Loading buffer

The solution was prepared by dissolving 0.25g of bromophenol blue and 40g of sucrose in 100 ml of distilled water. The solution was mixed overnight, filtered and the pH adjusted to 7.0.

h) Tris/EDTA (TE) and Tris/Acetate/EDTA (TAE)

The solution was prepared as follows

10 mM TrisHCl (pH 8.0)

1 mM EDTA

TAE buffer was prepared as above with addition of Glacial Acetic Acid.

Both solutions were stored at RT.

i) E. *coli* XL1 Blue

Was purchased from Invitrogen and stored at -20°C prior to use.

j) Ethidium Bromide

Ethidium bromide solution was prepared by dissolving ethidium bromide in distilled water to give a final concentration of 0.2mg/ml.

k) Ligase

Ligation of DNA fragments was performed with a T4 DNA ligase purchased from Boehringer Mannheim (U.K). This ligase was stored at -20°C prior to use.

l) Luria Bertani (LB) medium

LB medium was prepared as follow:

Distilled water	1 litre
Tryptone	10g
Yeast Extract	5g
Sodium Chloride	5g
Sodium Hydroxide	1 ml

The pH was adjusted to 7.5 with sodium hydroxide. The solution obtained was autoclaved at 15 p.s.i for 15 minutes and stored a RT. For the preparation of agar plate 1.5 % bacto-agar (Difco Laboratories) was added to the above recipe and after cooling, ampicillin selecting antibiotic (Sigma Aldrich Chemical Company Ltd.) was added.

m) Freezing Mixture

The solution was prepared as follow:

LB	66%
Glycerol	40%
Sterile $Mg2^{+}$	$1/25^{th}$ volume

kept at 4°C.

n) Molecular Size Marker

A 1Kb DNA ladder suitable for sizing linear double stranded DNA fragments from 500 bp to 12 Kb was purchased from Gibco Life Technologies Ltd. In 10 mM Tris HCl (pH7.5), 50 mM sodium chloride and 0.1 mM EDTA, and stored at -20°C prior to use.

o) Phenol Chloroform

Was prepared as follows:

Phenol	25 parts
Chloroform	24 parts
Isoamyl alcohol	1 part.

p) Plasmid vectors

- Bluescript SK was kindly supplied by the Immunex Corporation.
- P53 cDNA, mutated at either amino-acid position 175 (Arg->His) or 273 (Arg->His), subcloned into a pBR322 derived plasmid and under the control of the human CMV promoter were a generous gift from Prof. T. Soussi (Marie-Curie, France).

- The HLA-A2 cDNA subcloned pcDNA3 (Invitrogene) was a generous gift from Dr. Robbins, NIH, USA.

- Reporter Plasmid: pCMV-β was purchased from Pharmacia.

q) Rnase

Rnase, Dnase free, was purchased from Boehringer Manheim (U.K) Ltd. and stored at -20°C prior to use.

r) Restriction Enzymes

Both enzymes and buffer were purchased from Boehringer Manheim (U.K) Ltd. and stored at -20°C prior to use.

s) Staining Reagents

In situ β-Galactosidase staining of transfected cells:

Stock solutions	**Storage conditions**
20mg/ml Xgal in dimethylformamide	-20°C in propylene tube, in the dark
50mM potassium ferricyanide	4°C
50mM potassium ferrocyanide	4°C

Working solutions

Fixative solution 4°C

PBS containing 2% formaldehyde

0.05% glutaraldehyde

Stain solution 4°C

PBS containing 5mM potassium ferricyanide

5mM potassium ferrocyanide

2mM $MgCl_2$

Substrate/Stain solution Used immediately

1mg/ml Xgal in stain solution.

2.1.6 Materials for Blood Separation

a) Heparin

Was purchased from c.p Pharmaceuticals

b) J-Prep

Was purchased from VH-Bio

2.1.7 Materials for LAK Assay

a) PBMC

Were obtained after from blood separation from healthy donors.

b) Interleukin-2 (IL-2)

Was a generous gift from Glaxo Pharmaceuticals

2.1.8 Materials for CTL Generation and Chromium Release Assay

a) Mitomycin C

Mitomycin C was obtained from Sigma Aldrich Chemical Company Ltd under the form of powder (2mg) and was dissolved in 5ml of PBS and stored in aliquots of 1ml wrapped in aluminium at -20°C.

b) Recombinant Cytokines

All interleukins were diluted in PBS supplemented with 0.1% BSA to the desired concentration and stored at -70°C prior to use.

- **Interleukin-2 (IL-2)**

  Was a generous gift from Glaxo Pharmaceuticals

- **Interleukin-4 (IL-4)**

  Was purchased from Peprotech

- **Interleukin-7 (IL-7)**

  Was purchased from Peprotech

- **Interleukin-12 (IL-12)**

  Was purchased from Hoffman, La Roche

- Interferon Gamma (IFN-γ)

Was a kind gift from NCI Biogen

- Tumour Necrosis Alpha (TNF-α)

Was a kind gift from Strangeways Laboratories

- (GM-CSF)

Was purchased from La Roche

c) Antibody

Monoclonal antibody MA2.1 specific for HLA-A2 and HLA-B17 molecules was obtained as supernatant from a hybridoma cell line Purchased from the ATCC.

d) Radio-isoptope

^{51}Chromium in the form of sodium chromate (185 Megabecquerels in 5 ml) were purchase from Amersham International Ltd.

2.1.9 Materials for Immunohistochemistry

a) APES Slide Coating Solution

A 1% solution of APES in acetone was used to coat glass slides. This solution was prepared in a fume cupboard, by adding 3ml of 3-aminopropyltriethoxysilane (APES) solution (Purchased from Sigma) to 300ml acetone in a glass trough.

b) Tumour Specimens and Cryostat Sections

Head and Neck squamous cell carcinoma (HNSCC) tumour tissue was obtained from consented patients undergoing surgery at the Royal Hallamshire Hospital, Glossop Rd, Sheffield. Tumour tissue, after collection from the Department of Pathology, was snap frozen in liquid nitrogen, prior storage at –70°C.

Tumour material was positioned within aluminium foil cylinders placed on cork mats, and embedded in OCT compound, using isopentane, liquid nitrogen, and Cryospray 22 (chlorodifuoromethane) coolant (Bright Instruments Co. Ltd.). Sections were cut using a Bright Instrument Crystat (Model OTF/AS), and a 228x38x6mm tungsten tipped knife (Bright Instruments).

c) VECTASTAIN ABC Kit

Was purchased from Vector Laboratories.

d) PAPpen

Was purchased from Binding Site Limited

e) Scott's Tap Water

Was prepared as follows:

Sodium Bicarbonate	(BDH)	8.75g
Magnesium Sulphate	(BDH)	50g
Tap Water		2.5 litres

f) Haematoxylin

Was purchased from BDH

g) Picolyte Resin

Was purchased from Phase Separations

2.1.10 Materials for Immunofluorescence

a) TBS

The solution, for one litre, was prepared as follows

Tris	6.055g
NaCl	8.766g

pH was adjusted to 7.5 with HCl

b) 2% Blocking Reagent

The solution was prepared as follows

Tween 20	0.1g
TBS	100ml
1M NaOH	1ml

The mixture was microwaved for 50 seconds before addition of 2g Boehringer Blocking Agent. The solution was stirred until dissolved. The pH was adjusted to 7.5 with 1M NaOH once the solution has cooled down to RT.

c) DAPI

4', 6-diamidino-2-phenylindole (DAPI) was purchased from Sigma and diluted to 1: 1000 in TBS from stock solution 0.5mg/ml. The solution was stored at 4°C.

d) Fluorescent Mounting Medium

Was purchased from Dako

2.1.11 Materials for Mixed Lymphocyte Reaction

Radio-Isotope

^{3}H-thymidine was purchased from Amersham International Ltd as 1ml solution with a radioactive concentration of 37 Mbq/ml, and stored at 4°C.

2.2 Methods

2.2.1 MHC Stabilisation Assay

2.2.1.2 Principle

Cell line 174.CEM T2 (T2) is a human lymphoblastoid cell line (174.CEM T2) defective in processing. These cells express 'empty' HLA-A2 molecules, due to homozygous deletion of the MHC class-II region located on chromosome 6 including the TAP1 and TAP2 genes, which encode the peptide transporter proteins. Although these cells express high levels of cytoplasmic MHC class-I heavy chains and β2m no complexes can be detected at the surface of the cell. This is due to the fact that no peptide reached the endolasmic reticulum and empty class-I/β2m complexes expressed at the cell surface are very unstable and are rapidly degraded and recycled. However, if a peptide, added exogenously, possesses some binding properties, it will stabilise the complex, which can then be detected by antibody staining.

2.2.1.2 Method

Confluent T2 cells were washed twice in serum free RPMI-1640 culture medium and counted with Trypan blue to exclude non viable cells. Cells were then resuspended at a concentration of 4.10^6 cells/ml of medium. 40 µl (160 000 cells) of T2 cells were put into a U-bottom 96 wells tissue plate culture. Then 10µl of the appropriate peptide concentration (100µM, 10µM or 10µM) were added to wells. The Flu peptide (GILGFVFTC) was used as a positive control, at the same concentration and the fluorescence of the cells with DMSO (treated as a peptide) was taken as a negative control. The plate was then incubated overnight (minimum of 18 hours) at 26°C in 5% CO_2. T2 cells were then harvested from each well into separate tubes by adding 200µl of cold PBS/BSA (0.1%) mixing thoroughly and transferred to the appropriate FACS tubes. This was repeated and the cells washed with cold PBS/BSA to block any unspecific sites.

2.2.2 Flow Cytometry

2.2.2.1 Principle

Flow cytometry can broadly be described as the cytological analysis and measurement of a cell suspension as particules flow in a liquid stream passing through a beam light. The

cells are channelled into a fine core stream and then passed through a laser beam, one cell at a time to be analysed.

The light emitted can be refracted or simply deviated. The angle at which the emergent light is bent and how long the cell takes to pass through the laser beam allow the size of the cell to be determined. This is measured by a forward scatter detector in the direct path of a laser beam and is thus termed Forward Scatter (FSc). As the cell passes through the laser beam, some of this light is reflected back from the cell in multiple directions because a cell may have a high level of intracellular organelles. The rougher or more irregular or granular a particle is the more it will scatter the illuminating beam to the side. This Side Scatter (SSc) light is measured be a detector placed behind the stream, at right angle to the laser beam.

As well as measuring the size and granularity of a cell, flow cytometry is used to detect fluorochromes conjugated to antibodies against a protein of interest. Here fluorescein isothiacyanate (FITC) is used either as secondary antibody or conjugated to the first antibody. When the cells are passed through the correct wave length laser beam and excite the dye, longer wavelengths of light are emitted by the fluorescein molecules which are then detected. The amount of light measured is proportional to the number of fluorescein molecules bound to the cell.

2.2.2.1 Method

a) Preparation of Cells for intra-cytoplasmic Antigen Detection

Cells to be analysed were washed in PBS/BSA (0.1%), counted using a Trypan blue exclusion assay (See Section 2.2.2.2) and resuspended at $1x10^6$ cells per FACS tubes. Excess PBS/BSA (0.1%) was added to each tube and centrifuged at 400g for 5 minutes. The supernatant was removed and cells were fixed by addition of 200µl of 1% paraformaldehyde for 10 to 15 minutes at RT. Excess PBS/BSA (0.1%) was added to tubes and centrifuged at 400 g for 5 minutes. Cells were then permeabilised by addition of 200µl of 70% methanol for 5 minutes at RT. Excess PBS/BSA (0.1%) was added to tubes and centrifuged at 400 g for 5 minutes. Antibody staining and antigen detection were performed as described below.

b) Immunofluorescence Staining

The quantitative determinations of the presence of either stable cell surface peptide/MHC complexes following T2 peptide binding assays or surface molecules of any cells were performed by indirect immunofluorescence and by direct immunofluoresce when primary antibody was already conjugated to FITC.

Cells to be analysed were washed in PBS/BSA (0.1%), counted using a trypan blue exclusion assay (See Section 2.2.2.2) and resuspended at 0.5 to $1x10^6$ cells per FACS tubes. Excess PBS/BSA (0.1%) was added to each tube and centrifuged at 400g for 5 minutes. The supernatant was removed, cells were resuspended and primary monoclonal antibody was added to each FACS tube. Optimal concentrations for each primary antibody are listed in Section 2.1.4b. Tubes were then incubated at 4°C for 30minutes. All cells were then washed twice in cold PBS/BSA (0.1%). Afterwards 100µl of a 1:100 dilution of second antibody FITC-labelled F $(ab')_2$ fragments of goat anti-mouse IgG were added to all tubes when primary antibody was not already conjugated and tubes were then put at 4°C for another 30 minutes. Cells were then washed twice in cold PBS/BSA. After the last centrifugation at 400g for 5 minutes the pellet was resuspended in 300µl of PBS/BSA or serum free medium. Cells were fixed by addition of 300µl of 1% paraformaldehyde if FACS analysis was not done the same day and left at 4°C until analysis.

Following excitation by the light source of the Flow Cytometer, the fluorescence emitted by the FITC was then detected and measured for a large number of cells of the test, and converted into digital form. Data was only collected for the live cells of the sample, which were first gated.

The Fluorescence Ratio, for the T2 peptide-binding assay, was calculated from the median channel fluorescence (mcf) data for each peptide as follows:

$$\text{Fluorescence Ratio} = \frac{\text{(Median Channel of Fluorescence (mcf) test peptide)}}{\text{(Median Channel of Fluorescence (mcf) DMSO}}$$

Where DMSO was the 'True' negative control for the assay.

FR > 1 were considered as positive.

2.2.3 CTL Generation Assays

2.2.3.1 Blood Separation

60-120mls peripheral blood was obtained from healthy volunteers and placed immediately into a bottle containing heparin at 10IU/ml. This was diluted 1:2 in sterile PBS. 30mls aliquots were layered onto 15mls J-Prep in a 50mls tube for separation. Tubes were then centrifuged at 400g with brakes off. The leukocyte interface was carefully collected into fresh pre-chilled tubes. The tubes were filled with chilled PBS and spun down at 600g with acceleration and brakes on for 15 minutes at 4°C. The supernatant was discarded and the cells were resuspended in chilled PBS and then transferred into one tube. This tube was then filled with chilled PBS and spun down at 400g at 4°C for 6 minutes under the same conditions. The supernatant was discarded and the cells were resuspended in 1 to 3mls of AIM-V before counting with Trypan blue.

2.2.3.2 Trypan Blue Exclusion Assay

For PBMC, cells were counted by adding 100µl of the cell suspension to 0.6% glacial acetic acid. This was pipetted thoroughly and 100µl added to 900µl Trypan blue solution. Cell line cultures were diluted in Trypan blue alone before counting. Cells were counted using a haemocytometer, with the cell count being number of cells counted in four squares divided by four, times the dilution factor. This gives the number of cell $x10^4$ per ml.

2.2.3.3 Induction of CTL using PBMC as APC

On Day 0: 120mls of blood were taken from healthy HLA-$A2^+$ donors and PBMC were separated using J-prep as described in Section 2.2.3.1. $5x10^6$ PBMC (in 200µl) were then put into a 24-well-plate with 50µg of peptide. The plate was put at 37°C for 1hr. Then 1.8ml of AIM-V medium, containing IL-7 at 25ng/ml (final concentration in the wells) and KLH at 5µg/ml (final concentration in the wells) were added to each well and the plate was put back at 37°C.

On Day 3: 10U/ml of IL-2 with or without 16.5 U/ml (final concentration) of IL-12 were added to each well.

On Day 7: $CD4^+$ cells were removed from wells depending on method followed as described below:

Cells from 2 or 3 wells were pooled, washed and resuspended in 1ml of PBS. Anti-$CD4^+$ antibody, which were coupled with magnetic-beads, were added to the cells at a ratio of 7:1 (7 beads for 1 $CD4^+$ cells, normal PBMC contain about 40% $CD4^+$ cells). The tube was then put on a carrousel at 4°C for 30 minutes. $CD4^+$ cells were separated, after addition of 8mls of cold PBS + 2% FCS, by putting the tube against a magnetic source. The supernatant containing all $CD4^-$ cells was transferred into a fresh tube and spun down. Cells were resuspended in 1ml of AIM-V and counted. These cells were then transferred into a new well of a 24-well plate. On the same day cells were also restimulated. Autologous PBMC and allogeneic PBMC from $HLA\text{-}A2^+$ donors were thawed out, washed with PBS and resuspended in 1ml of AIM-V with 50µg/ml of peptide (all PBMC were kept separated until the end). Cells were incubated for 1hr at 26°C. 4mls of AIM-V along with 250µl of FCS, 8µl of β2-m (200µlg/ml) and 100µl of Mitomycin C (400µg/ml) were then added to the cells. These were incubated for 2 hours at 37°C. Cells were then washed twice with PBS, resuspended in 1ml of AIM-V and counted with Trypan blue. The cell concentration was adjusted to $1x10^6$ cells/ml and 1ml placed in each well. 10 U/ml of Il-2 (final concentration) +/- 16.5 U/ml (final concentration) IL-12 were added to the cells and 1ml of it was added to each well.

On Day 14: Cells were semi-cloned and restimulated. Cells from each well were washed in PBS, resuspended in 1ml of AIM-V and counted. 100µl of a concentration of $5x10^4$ cells/ml was seeded in each well of a 96 well-plate. The remaining cells were used as bulk cultures. Stimulator cells consisted of autologous PBMC and PBMC from two $HLA\text{-}A2^+$ donors. These were thawed out, washed in PBS and resuspended in 1ml of AIM-V. 50µg/ml of relevant peptide were added to the cells and the tubes were put at 26°C for 1 hour. 4mls of AIM-V medium was then added to each tube as well as 250µl of FCS, 8µl of β2-m and 100µl of Mitomycin C. Tubes were then put at 37°C for 2 hours. All cells were then washed twice with PBS, resuspended in 1ml of AIM-V and counted. Rosi cells were Mitomycin C treated following the same protocol as for the other cells and used as feeder cells. The cells were then mixed together to a final concentration of $1x10^6$ cells/ml consisting of at least 20% autologous cells, 10-20% Rosi

cells and 30% of each HLA matched PBMC from 2 Donors. 10 U/ml IL-2 in the well +/- 16.5U/ml final concentration of IL-12 was added to the mixture. 1ml of the restimulating mixture was added to each of the 24-well-plates and 100µl were added to each well of the 96-well-plates (According to relevant peptide).

On Day 21: Cells were restimulated as described above.

On Day 28: A cyto-assay was performed to assess the cytolytic activity of the bulk cultures as well as the cytolytic activity of each semi-clone.

2.2.3.3 Generation of CTL using Dendritic Cells as APC

Day 0: 120 ml of blood was separated, plasma was kept/ heat inactivated (56°C 30 minutes) and stored in the fridge until needed.

Half of the PBMC were frozen at $1x10^7$ cells/ml and called PBMC (1) to be used later as effector cells. The rest were plated at $2\text{-}3x10^6$ cells/ml in a T175 flask in RPMI + 10% FCS. The flask was put at 37°C for 2 hrs. The non-adherent cells were removed, spun down, resuspended in 1ml of AIM-V and counted. Cells were frozen at $1x10^7$ cells/ml and called PBMC (2). 2mls of RPMI + 10% FCS were added to the adherent cells and the plate was put back at 37°C.

Day 1: Cells were removed from the flask, washed in PBS, resuspended in 1ml of medium and counted. $5x10^6$ cells/ml were plated in a 6-well plate (1ml) and the plate was incubated at 37°C for 45 minutes. Following the incubation, the non-adherent cells were discarded, wells were washed gently twice with PBS and filled up with 2mls of medium + 1000 U/ml GM-CSF + 500 U/ml IL-4 for the generation of dendritic cells. The plate was put at 37°C.

Day 2: Cells were pooled together, spun down, resuspended in 1ml of medium and counted. Cells were diluted to $1x10^6$ cells/ml/well in a 24-well plate in 1ml of medium containing the same cytokines.

Day 7: Cells were pooled together, spun down, resuspended in 1ml of medium and counted. Some cells were used for FACS analysis the rest were diluted to $0.5x10^6$ cell/ml of medium containing GM-CSF and TNF-α (100 U/ml) and 1ml was seeded into each well of a 24-well plate in 1ml.

Day 10: Cells frozen on day 0 (PBMC (2)) were thawed out and depleted of $CD4^+$ cells as described in Section 2.2.2.3 (Day 7). $2x10^6$ PBMC ($CD4^-$) were added to $0.2x10^6$

dendritic cells in 2mls of medium consisting of RPMI + 17% AIM-V, 5% autologous serum (heated inactivated: 56°C for 30 minutes) from day 0, 10 U/ml IL-2 and 5ng/ml IL-7 (all final concentration).

Day 17: Autologous PBMC were thawed out, peptide pulsed + β2-m for 1hr at 26°C and Mitomycin C treated for 2hrs at 37°C. After two washes with PBS the concentration of these antigen presenting cells was adjusted to $0.1x10^6$ cells/ml and were added to $1x10^6$ responder cells in a 24-well plate in 2mls of medium containing: RPMI, 17% AIM-V, 5% human serum from day 0 and 10 U/ml IL-2.

Day 24: Cells were restimulated as follows: To $1x10^6$ responder cells were added $0.1x10^6$ antigen presenting cells (consisting of autologous PBMC, LCL and Rosi cells, as feeder cells, all peptide-pulsed, except Rosi cells and Mitomycin C treated) in 2mls of medium containing RPMI, 17% AIM-V, 5% autologous serum from day 0 and 10 U/ml IL-2.

Day 31: Cells were restimulated as described above.

Day 38: A cyto-assay was performed using appropriate target cells

2.2.4 Chromium Release Assay

2.2.4.1 Target Cells

Target cells were spun down and resuspended in 1ml of growth medium + 10% FCS and counted with Trypan blue. $1x10^6$ cells per target were put in a 15ml tube. 3.7Mbq of ^{51}Cr were added to each target +/- anti-HLA-A2 antibody: MA2.1 (1:2 dilution) tubes were then put at 37°C for 1hr. Cells were then washed twice with growth medium + 10% FCS and resuspended in 1ml of the same medium + /- 10μg of peptide. Tubes were put back at 37°C for another hour. After the incubation cells were washed once more in medium, resuspended in 1ml and counted with Trypan blue. For the assay cells were resuspended in $2x10^4$ cells/ml and 50μl were added to each relevant well of the 96-well-plate.

2.2.4.2 Effector Cells

Bulk cultures: Each set of cells from each well was resuspended, transferred into a universal and washed with PBS before counting. A range of E: T (Effector/ Target) ratios were set up ranging from 100 or 50:1 with dilutions down to 2.5:1. Each E: T

ratio was assayed in duplicate in a non-sterile 96-well-plate. 40x excess of cold K562 (NK sensitive cells) was added to each well and the cells were incubated 1hr at 37°C prior addition of target cells.

Clones: Cells from each clone were resuspended and 2x 50µl were transferred into 2 wells of a non-sterile 96-well-plate. 50µl of K562 at $80x10^4$cells/ml were added to each well. Cells left from each clone were restimulated as for day 21.

4 wells were used to determine maximum release (containing 50µl of 1% SDS in AIM-V +/- 50µl k562) and 4 others to determine spontaneous release (containing 50µl of AIM-V +/- 50µl k562). After addition of the target cells, plates were incubated at 37°C for 4 hours. 50µl of supernatant were then removed from each well and put into a lumaplate (containing solid scintillant). Plates were left overnight in the oven to dry and counted.

The percentage of cytotoxicity was calculated for each well as follows:

[(Experimental release - spontaneous release) / (Maximum release - spontaneous release)] X 100

2.2.5 Preparation of DNA Constructs

2.2.5.1 Preparation of Bactoagar Plates

LB medium containing 1.5% of Bactoagar was prepared according to the recipe described in Section 2.1.5l. Once cooled the selecting antibiotic ampicillin was added and the mixture poured into 9cm petri dishes and left to set at RT. Once set, the plates were placed up side down in an oven to dry at 37°C for 2 hrs.

2.2.5.2 Preparation of E. *coli* XL1 Blue Frozen Stock

A cell suspension of E.coli XL1 was plated on a LB-agar (1.5%) plate and incubated o/n at 37°C. The next day, several colonies of 1-2 mm in diameter were picked and used to inoculate 10ml of LB medium. The culture was shaken (225rpm) at 37°C The following morning, the overnight cultures were diluted with 1 volume of freezing mixture (Section 2.1.5m), dispensed into 0.5ml aliquots and stored at -70°C until required.

2.2.5.3 Preparation of Competent XL1-Blue

An XL1 stock tube (Section 2.2.4.2) was taken and kept in liquid nitrogen to prevent thawing of the cell. The surface of the frozen cell suspension was scratched using a

sterile pipette tip to obtain a clump of cells, which was streaked for a single colonies on an agar plate. The plate was then incubated at 37°C o/n. and several colonies were subsequently picked and transferred into 10 ml of LB and shaken (225 rpm) o/n at 37°C. The following morning 200 ml of LB was inoculated with 2 ml of one of the o/n cultures and shaken at 37°C until the optical density reached 0.375 at 590 nm wave length. Cells were then put into 4x 50ml tubes and spun at 3000rpm/1600g for 10 minute at 4°C. The supernatant was discarded, making sure that all drops were removed, and each pellet was resuspended in 10ml of ice-cold CM1 (Section 2.1.5b). After 20 minutes incubation on ice cells were spun at 1100g for 5 minutes and resuspended in 1 ml of ice-cold CM2 (Section 2.1.5b). Aliquots of 0.2 ml were either frozen at -70°C or used immediately.

2.2.5.4 Transformation of XL1-Blue

Aliquots of competent cells (Section 2.2.4.3) were thawed out and kept on ice. To each 0.2 ml of cells 3.4 µl of 10% (v:v) 2-mercaptoethanol were added and mixed by gently swirling. Cells were left on ice for 10 minute, mixing by hand occasionally. For each transformation, 100 µl of the above mixture was placed into a pre-chilled falcon tube, and 1 to 5 µl of DNA was then added to each tube and mixed gently. Tubes were placed on ice for 30 minutes before immersion and gently shaking in a 42°C water bath for 45 seconds and then returned to incubate on ice for a further 2 minutes. Subsequently 800µl of LB were then added to each tube and the tubes were shaken at 37°C for 1 hour. A 50µl aliquot of the transformed cells were plated on an LB-agar plate (containing 100µl of ampicillin) and incubated o/n at 37°C o/n. As a control for the efficiency of transformations, a control transformation was performed with 1ng of circularised plasmid pTZ18R (Section 2.1.5p). A negative control in which no DNA was added was also included to ensure that cells did not already contain an amp^R plasmid, and that the solutions were not contaminated with an amp^R plasmid.

2.2.5.5 Mini-Prep DNA Isolation from Transformed XL1-Blue

Colonies were picked from transformation plates (Section 2.2.4.4) using a sterile pipette tip, inoculated into 10ml of LB supplemented with 100 µg/ml ampicillin and incubated at 37°C o/n with shaking (225 rpm). The next day 0.5ml of sterile freezing mixture was mixed with 0.5 ml of o/n culture and stored immediately at -70°C for use as long term

stocks of transformants. The remaining cells of the o/n culture were pelleted by centrifugation at 3000g for 10 minutes at RT. The supernatant was removed completely and the cells were resuspended in 200µl of GTE solution (Section 2.1.5d). Cells were then transferred to a 1.5ml ependorf tube, ensuring that all cells were resuspended, and the tube was left to stand for 5 minutes at RT. Subsequently 400 µl of NaOH/SDS solution (Section 2.1.5e) was added to each ependorf and tubes were inverted several times to ensure sufficient lysis of the cells. Tubes were then placed on ice for 5 minutes. Finally 300 µl of ice-cold acetate solution was added to each tube to cause re-annealing of the plasmid DNA, and after a further incubation on ice for 5 minutes the tubes were centrifuged at 13,000g for 5 minutes. Approximately two thirds of the supernatant was transferred into a fresh tube, and *an equal volume of phenol-chloroform was added. Gently, each tube was inverted several times and then spun at 13,000 rpm in a microcentrifuge for 1 minute. Two thirds of the aqueous top layer, containing the DNA, was transferred into a fresh tube and an equal volume of isopropanol was added to the plasmid solution, and mixed by inverting the tubes several times. Tubes were left for 2 minutes at RT to precipitate the DNA and then spun at 13,000 rpm for 5 minutes. The supernatant was removed and the pellet was washed in 1 ml of 75% ethanol and spun at 7000 rpm for 5 minutes. The supernatant was removed and any traces of ethanol allowed to evaporate by leaving tubes with the lid open at RT for 5-10 minutes. The pellet was then dissolved in 50 µl of TE buffer and stored at -20°C*.

2.2.5.6 Digestion of Plasmid DNA with Restriction Enzymes

RNA contained in plasmid mini-preps (Section 2.2.4.5) was degraded by adding ~0.5-1µl RNase (500µg/ml) into the restriction digest. The appropriate restriction enzyme and buffers were then added as per manufacturers instructions, and the reactions incubated at 37°C water bath for 1-2 hrs and then analysed by gel electrophoresis.

2.2.5.7 Agarose Gel Electrophoresis

A 0.8% electrophoretic gel was prepared with SeaKem agarose and TAE buffer (Section 2.1.5h) with 0.5µl ethidium bromide per 100 ml gel and allowed to set in an appropriate sized gel tray with gel comb inserted. Electrophoresis buffer was supplemented with 0.5µl ethidium bromide per 100 ml buffer. Electrophoresis buffer was added to the

electrophoresis tank until the gel was just covered. Samples were diluted with ¼ volume bromophenol blue buffer and loaded into the wells using a p20 Gilson pipette. The gel was then run for 2-3hrs at 80 mVolts and photographed on a U.V transilluminator.

2.2.5.8 Purification of DNA Bands from Agarose Gels

DNA bands were excised from agarose gels (Section 2.2.4.7) using a scalpel and transferred into eppendorf tubes. DNA purification from the agarose gel slices was performed using the QIAEX II kit (QIAGEN), following the manufacturer's protocol. Briefly gel slices were weighed in eppendorfs and solubilised by adding 3 volumes of buffer QX1 (Section 2.1.5b) and 2 volumes of water to 1 volume of gel. 30µl of QIAII (Section 2.1.5b) was then added and samples incubated at 50°C for 10 minutes. Samples were then centrifuged for 30 seconds and the pellets were washed with 500µl of buffer QX1. Pellets were then washed twice with 500µl of buffer TE (Section 2.1.5h) and air-dried for 10-15 minutes. The DNA was eluted from the bead pellet in 20µl of 10mM Tris-HCl, pH 8.5. The purity as well as the quantity of DNA obtained was checked by electrophoresis against different dilutions of a mass ladder.

2.2.5.9 Ligation of DNA Constructs

The appropriate mammalian expression vector was linearised using the appropriate restriction enzyme as described in Section 2.2.4.6, to enable insertion of the required cDNA. An agarose minigel was run to check that the vector was completely linearised. In order to avoid the vector religating with itself, the 5' end phosphate was removed as follows. The equivalent of 1µg of DNA was removed from the linearised vector solution to serve as a control for the ligation assay see below. To the remaining DNA solution (20µl) was added 5µl of 10x SAP buffer (Section 2.1.5r), 1µl of SAP and the final volume adjusted to 50µl with water. The tube was then briefly centrifuged and incubated for 30minutes at 37°C. The DNA was extracted with phenol-chloroform, precipitated with Ethanol and dissolved in 20µl of TE buffer as described in Section 2.2.5.5 from* to *). In an ependorf tube 60ng of the purified cDNA insert (Section 2.2.4.8) was mixed with 100ng of SAP-treated vector (molar ratio between insert: vector being 3:1), 1 unit of T4 DNA ligase and 4µl T4 ligase buffer and adjusted to 20µl with water. To assess the effectiveness of SAP in preventing religation of the vector to itself, two controls

were also prepared with T4 DNA ligase, buffer and water but with either SAP-treated vector alone or untreated vector alone (saved above) in the absence of cDNA insert. All the tubes were incubated at 14°C overnight. The ligated DNAs were then transformed into XL1 as described in Section 2.2.5.4.

2.2.5.10 Maxi-Prep DNA Isolation from Transformed XL1-Blue

Agar plated single colonies obtained from frozen stocks of transformed cells (Section 2.2.5.4) were grown in a 5 ml culture at 37°C at 300 rpm for 8 hours to obtain mid-log phase cells. This culture was diluted 1 in 500 into 100-500 ml LB-amp and cultures o/n. The plasmid DNA was then purified using the QIAGEN Maxiprep Kit. Briefly, the bacterial cell pellet obtained from the culture was re-suspended into 10ml of solution P1 (Section 2.1.5b), making sure that no cell clumps were left. To this cell suspension 10ml of buffer P2 (Section 2.1.5b) was added and the mixture was incubated for 5 minutes at RT. After this incubation period 10mls of chilled buffer P3 (Section 2.1.5b) was added and mixed by inverting the tube a 4-5 times and subsequently transferred into a QIAfilter cartridge.

The mixture was left undisturbed for 10 minutes at RT. In the meantime a QIAGEN TIP500 column was equilibrated with 10mls of buffer QBC (Section 2.1.5b). The lysate was then filtered through the QIAfilter into the TIP500 and allowed to enter the column by gravity. The column was washed with 2x 30 ml of buffer QC (Section 2.1.5b) and DNA bound to the column was eluted with 15 ml of buffer QF (Section 2.1.5b). The DNA was precipitated with 10.5mls of isopropanol and spun at 15000g for 30 minutes at 4°C. The supernatant was then discarded and the pellet washed with 5ml of 75 % ethanol. After spinning tube at 15000g for 10 minutes at 4°C the supernatant was again discarded and the pellet left to air dry for 5 minutes. The DNA was then dissolved in 400-500µl of TE (Section 2.1.5h). A small amount of DNA was digested with the appropriate restriction enzyme and run on an agarose gel to check the identity of the construct.

2.2.5.11 Sterilisation of Plasmid DNA for Transfection

To sterilise the DNA obtained from a Maxiprep (Section 2.2.5.10) 1/10 volume of 3M sodium-acetate and two volumes 100% ethanol were added to precipitate the DNA. The precipitate was spun at 13 000 rpm in a microcentrifuge for 5 minutes and washed in 75% ethanol. Tube was then spun at 7000 rpm for 5 minutes and the supernatant removed in a sterile flow cabinet. The pellet was left to air dry for 1 minute in the sterile cabinet and dissolved in sterile TE buffer (Section 2.1.5h). The concentration of the DNA was estimated using a spectrophotometer.

2.2.6 Transfection of Mammalian Cells

2.2.6.1 Geneticin Selection

The concentration of Geneticin required to select for transfected cells was evaluated by growing the parent cell lines in various concentrations of Geneticin for a minimum of two weeks. The lowest concentration amount of Geneticin that killed all the cells after two weeks of culture was chosen as the concentration to be used to select for transfected cells. This experiment was performed in 24-well-plates with 2 ml of medium per well. The cells were seeded into the wells such that they could undergo 2-3 doublings before reaching confluence. Cells were left to adhere o/n before addition of Geneticin. Concentrations of Geneticin were 0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 μg/ml final concentration.

2.2.6.2 Transfection of SaOs-2 cells

2.2.6.2.1 Determination of Most Effective Liposome Preparation

In order to determine the most effective liposome reagent for transfecting SaOs-2 cells they were transiently transfected with pCMV-β, a reporter gene which express the E. coli β-galactosidase gene under the control of the human CMV promoter. Cells were grown in a 24-well plate until 70-80% confluence was reached. To prepare the DNA: Liposome mixtures, 300μl OPTIMEM was added to wells A1, B1, C1 and D1 of a 24-well plate and the following was added to each well:

- A1: 20μl of LIPOFECTIN,
- B1: 10μl of LIPOFECTAMINE,
- C1: 15μl of CELLFECTIN,

- D1: 10µl of DMRIE-C

The plate was left at RT for 30minutes during which the culture medium from the cells was removed and replaced by 1ml of serum free OPTIMEM. Then 4.32µl of DNA at 3.7µg/µl was transferred into an ependorf tube containing 1.5 ml of OPTIMEM to obtain a final concentration of 10.66 ng/µl. and then 300µl of this mixture was then added to each well containing the liposomes. The plate was then left at RT for a further 15 after which wells A1-D1 were double diluted across the plate in OTIMEM to obtain serial dilutions of each liposome: DNA mix. The medium from the cells was then removed and replaced with the contents of the corresponding well in the liposome: DNA plate. The cells were then incubated at 37°C for 5hrs after which the liposome: DNA mixtures were removed and replaced by cell culture medium and the cells cultured for 24 hours. Cells were stained for β-galactosidase expression (Section 2.2.6.3).

2.2.6.2.2 Determination of the Optimum Concentration of DNA/Lipofectamine

Cells at 80% confluence in a 24-well plate were used for this experiment. Eppendorfs containing 0.68, 1.35, 2.71 and 5.41 µg of plasmid DNA stock pCMVβ were diluted to a final volume of 136.5 µl in OPTIMEM. In separate eppendorfs 1.87, 3.75, 7.59, 11.24, 14.99 and 20.60 µl Lipofectamin were diluted to a final volume of 94 µl in OPTIMEM. Then 21 µl of each DNA solution was mixed with 21 µl of each Lipofectamin solution so that a total of 24 different DNA: Lipofectamin combinations were generated.

These mixtures were then left at room temperature for 45 minutes. During this time incubation, the medium from the cells was replaced with serum free DMEM. Subsequently 166µl of OPTIMEM was added to the DNA: Lipofectamin mixtures and the medium in each well was replaced one by one with the appropriate DNA: Lipofectamin mixture. The plate was then incubated for 5 hrs at 37°C, after which wells were emptied and 1ml of pre-warmed medium was added and the plate was cultured at 37°C for 24 hours. The cells were then stained for β-gal expression (Section 2.2.6.3).

2.2.6.3 Assessment of β-galactosidase Reporter Gene Expression

Cells transfected with pCMVβ in a 24-well plate were washed once with PBS and fixed with 0.5 ml of fixative solution (Section 2.1.5s) for 5 minutes at room temperature. The cells were then washed twice with PBS and then 0.5 ml of substrate/stain solution (Section 2.1.5s) was added and the plate incubated for 2 hrs at room temperature. Wells were observed under a light microscope and the proportion of blue (β-gal-positive) cells was evaluated.

2.2.6.4 Transfection of SKBr3 and A431 Cells

Cells were harvested and resuspended in 1ml of growth medium and counted with Trypan blue to assess viability and cell density. The concentration was adjusted to 5 $x10^6$/ml and 0.4 ml was dispensed into electroporation cuvettes in addition to 10μg of linearised DNA in 10μl. Cells were electroporated in a Gene Pulser (Biorad) at various voltages with the capacitance set at 960 μFD. The cells were then transferred into 10mls of fresh culture medium in a T25 flask and incubated at RT for 20 minutes to allow cells to recover before culturing them at 37°C for 24-48 hours.

The efficiency of the electroporation was assessed by antibody staining followed by FACs analysis.

2.2.7 Immunofluorescence Staining of SaOs-2 Cells Transfected with Mutated p53 or Vector Alone

Cells were grown in chamber slides until 60% confluency was reached. Cells were then washed with PBS and fixed for 10 minutes at RT with 1% paraformaldehyde. Cells were washed again with PBS and then permeabilised by incubation with 70% of methanol for 10 minutes. After washing the cells three times with PBS, 2% of blocking agent in TBS/Tween (Section 2.1.10b) was added and the cells were incubated for 30 minutes at RT in a moist box. P53 antibody was diluted in 1:100 in 1% of blocking reagent (Solution at 2% diluted with TBS) and added to the cells after removal of the blocking reagent solution, cells were incubated at 37°C for 1hr. Cells were then washed three times with TBS (Section 2.1.10a) and the secondary antibody, FICT-Conjugated anti-mouse antibody was added to the cells after dilution 1:100 in 1% blocking reagent. The chambers were left at 37°C for 30 minutes in a moist box. Slides were washed 3x 5

minutes in TBS and then DAPI (Section 2.1.10c) or ethidium bromide were added to the cells for 2 minutes. Then cells were washed 3x 1 minute in TBS. Slides were drained and mounted carefully under an ethanol-washed coverslip using the DAKO fluorescent mounting medium. Slides were observed under a fluorescent microscope using the middle (UV) and green filters.

2.2.8 Determination of p53 Expression in HNSCC Sections by Immunohistochemistry

Sections of 8μ thick were cut from a frozen block and layered on APES coated slides. These were left to air dry over-night. The following day sections were fixed in acetone for 10 minutes and then stored at -20°C until needed. The staining was done using the mouse ABC kit and the avidin-biotin amplification system. Briefly sections were re-hydrated in PBS for 5 minutes before addition of blocking serum, normal rabbit serum, for 30 minutes. The excess was then removed and either the first antibody DO7, or the control isotope antibody, IgG2b, were then added (50μl of 1/100 dilution) for 2 hrs. All sections were washed three times with PBS and the second antibody (goat anti-mouse) supplied with the kit, was then added to all sections for 30 minutes. The sections were again washed three times with PBS. The avidin-biotin complex was then added to the sections and left for 30 minutes. Sections were washed again, then the substrate DAB, added and left for 2 to 8 minutes. The sections were rinsed with double distilled water and the nuclei stained with Haematoxilin (30 seconds). Sections were washed with tap water first and then in Scott water (turning the Haematoxilin into a bluish colour).
Using increased alcohol bath concentrations (70%, 95% then absolute ethanol twice) sections were dehydrated and were finally put into a xylene bath and mounted in Picolyte. Sections were left to dry at 56°C before cleaning with a quick bath in the xylene to remove the excess of Picolyte.

2.2.9 LAK Assay

PBMC were freshly isolated from healthy donors as written in method Section 2.2.3.1. $4x10^6$ PBMC/ml were incubated in AIM-V with 500U/ml IL-2 in a T25 flask for four days at 37°C. Another flask was set up with the same amount of PMBC but without any

IL-2. Cytotoxic activities of IL-2 activated PBMC were assessed in a standard 4hrs chromium release assay as written in Section 2.2.4.

Briefly all transfected cells were versene/trypsinised, counted and labelled with 3.7Mbq of ^{51}Cr for 1hr at 37°C. Cells were then washed twice in RPMI+10% FCS and resuspended in 1ml of medium and incubated for another hour at 37°C. Cells were washed again, resuspended in 1ml of medium and counted. Each transfected cell line was added to IL-2 activated PBMC or untreated PBMC at different Effectors: Targets ratios, all were tested in duplicate.

2.2.10 RNA Extraction from Frozen Tumours

A small piece of loose tumour was placed into a homogenizer along with 1ml of RNAzol. The tumour was broken down in the homogenizer and solution was transferred into an ependorf. Small pieces were further broken by sucking and plunging the solution through a syringe 10-15 times. 100µl of chloroform was then added to all eppendorfs, which were then vigorously shaken. Tubes were incubated on ice for 5 minutes. *Eppendorfs were then centrifuged for 15 minutes at 4°C, maximum speed. The top layer was removed and transferred into fresh eppendorfs to which was added an equal amount of isopropanol. These were then put at -70°C for a least 30 minutes and spun down for 15 minutes at maximum speed. The supernatant was discarded and the pellet washed in 1ml of 80% ice-cold ethanol*. Pellets were dissolved in a mixture containing DEPC water, NaCl, and ethanol. Tubes were put back at -70°C for 2 hours. Thereafter the procedure * was repeated. After removal of all ethanol RNA was dissolved in a small amount of DEPC water and 1µl was removed and added to 99µl of DEPC water for spectrometry analysis. For long term storage at -70°C, RNA was re-precipitated by addition of more DEPC water, NaCl, and ethanol.

2.2.11 Mixed Lymphocyte Reaction

200µl of $2x10^5$/ml dendritic cells, obtained from healthy HLA-$A2^+$ donor after 10 days in culture with cytokines, see Section 2.2.3.3, were put into each well of the first column of a U/bottom 96-well plate. These were then diluted to $5x10^2$ dendritic cells/ml in RPMI + 10% FCS, final volume 100µl/well. Then 100µl of $2x10^6$/ml of either autologous or

allogeneic non-adherent PBMC, obtained as described in Section 2.2.3.3, were added to each of the well containing dendritic cells.

Cultures of dendritic cells mixed with non-adherent PBMC were assayed in triplicate. Dendritic cells and non-adherent PBMC were also cultured on their own at the highest of the concentrations as controls for proliferation in RPMI + 10% FCS.

The plate was then incubated for four days at 37°C + 5% CO_2 in humidified incubator. Then 1μCi (0.037Mbq) of ^{3}H-thymidine was added to all the wells for 18hrs. The plate was then harvested, the cellular content of each well was recovered onto a plate containing filter paper and original wells were washed. The plate with filter paper was then left o/n to air dry. A Gamma Counter detected the amount of ^{3}H-thymidine incorporated by cells after addition of 25μl liquid Scintillin and after the plate was sealed.

2.3 Names and Addressed of Suppliers

BDH Merck Limited

Fourways, Carlton Rd Industrial Estate, Atherstone, Warwickshire, CV9 1JH, UK.

Bibby Sterilin Limited

Tilling Drive, Stone, Stafforshire, ST15 05A, UK.

Bio Rad Laboratories Limited

20 Alfred Nobel Drive, Hercules, California, 94547, USA.

BRL- Bethesda Research Laboratories

Life Technologies Incorporated, Gaitherssburg, Maryland, 20877, USA.

Canberra Packard

Brook House, 14 Station Rd, Pangbourne, Berkshire, RG8 7DT, UK.

Becton Dickinson Limited

Between Towns Rd, Cowley, Oxford, OX4 3LY, UK.

Costar Limited

10 the Valley Centre, Gordon Rd, High Wycombe, Buckinghamshire, HP13 6EQ, UK.

DAKO Limited

16 Manor Courtyard, Hughenden Avenue, High Wycombe, Buckinghamshire, HP13 5RE, UK.

European Collection of Animal Cell Cultures (ECACC)

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

Gibco Life Technologies Limited

P.O. Box, Washington Rd, Abbotsinch Industrial Estate, Paisley, PA3 4EP, UK.

Glaxo Institute for Molecular Biology S.A.

46 Route des Acacias, 1211 Geneva 24, Switzerland.

Jamont Limited

Oughtibridge Mill, Oughtibridge, Sheffield, South Yorkshire, S30 3DN, UK.

Pepro-Tech E.C Limited

23 St. James's Square, London, SW17 4JH, UK.

Phillip Harris Scientific

Sainsbury Way, Hessle, North Humberside, HU13 9NX, UK.

Promega Limited

Delta House, Chiworth Research Centre, Southampton, SO16 7NS, U.K.

Qiagen Limited

Unit 1, Tillingbourne Court, Dorking Business Park, Dorking, Surrey, RH4 1HJ, UK.

Sarstedt Limited

68 Boston Rd, Beaumont Leys, Leicester, LE4 1AW, UK.

Scientific Laboratories Limited

Wilford Indutrial Estate, Nottingham, NG11 7EP, UK.

Sigma Aldrich Chemical Company Limited

Fancy Rd, Poole, Dorset, BH17 7NH, UK.

Nalge Company

Rochester, New York, 14602-0365, USA.

Swann-Morton Limited

Owlerton Green, Sheffield, South Yorkshire, S6 2BJ, UK.

L.I.P (Equipment and Services) Limited

Dockfield, Shipley, West Yorkshire, BD17 7AS, UK.

Ansell Medical

Ansell House, 119 Ewell Rd, Surbiton Surrey, KT6 6AY, UK.

Unipath Limited

Wade Rd, Basingstoke, Hantd, RG24 0PW, UK.

Amersham International plc.

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

British Oxygen Company (BOC) Limited

10 Priesley Rd, Guildford, Surrey, UK.

Fisons Scientific Equipment

Bishop Meadow Rd, Loghborough, LE11 0RG, UK.

Johnson and Johnson Medical Limited

Coronation Rd, Ascot, Bershire, UK.

Chemical Concentrated Limited

Unit 9, Chapel Park, Church Rd Business Centre, Sittingbourne, Kent, ME10 3RW, UK.

FMC Bioproducts

191 Thomaston St, Rockland, ME 04841, USA.

Difco Laboratories

Detroit, Michigan, USA.

Unipath Limited

Wade Rd, Basingstoke, Hants, RG24 0PW, UK.

Invitrogen BV

De Schelp 12, 9351 NV Leek, The Netherlands

Boehringer Mannheim

House, Bell Lane, Lewes, East Sussex, BN7 1Lg, UK.

Gen-Probe Incorporated

Unit 5, Kingsway Business Park, Olfield Rd, Hampton, Middlesex, TW12 2HD, UK.

Gilson Medical Laboratories S.A.

BP45, F95400 Villiers-le-Bel, France.

Pharmacia Biotech

23 Grosvenor Rd, St. Albans, Hertfordshire, AL1 AW, UK.

Immunex Research and Development Corporation

52 University St, Seattle, Washington, 98101, USA.

ICRF

London

Ludwig Institute

Brussels, Belgium.

Dr. Robbins

NIH, USA.

Dr J. Bartholomew

Paterson Laboratories, Manchester.

Prof. T. Soussi

Institut Marie-Curie, Paris, France.

CHAPTER 3: IDENTIFICATION OF P53 PEPTIDES THAT STABILISE MHC CLASS-I MOLECULES

3.1 Introduction

The immune system has evolved to respond to and eliminate dangerous foreign organisms such as bacteria that invade the body or virally infected cells, without in normal circumstances, damaging any healthy cells. This is achieved by immune defence mechanisms. Bacteria and toxins are normally eliminated by antibodies, whereas infected cells are killed by cytotoxic T cells (CTL). These cells are able to recognise small processed fragments of viral proteins, called peptides, in association with MHC class-I molecules on the surface of infected cells. It has long been hoped by immunologists that tumour cells would also display 'antigenic' fragments, differents from those expressed by normal cells, which would mount an immune response that would result in the eradication of abnormal cells. It is only recently, however, with the work of Thierry Boon and co-workers that definite evidence of the existence of such tumour associated antigens has been demonstrated. In addition, recent advances in understanding antigen processing and presentation has provided insight into the importance of the amino-acid sequences of peptides for binding to particular MHC class-I molecules and subsequent T cell recognition.

These new developments have led to renew interests in the possibilities of using the immune system to fight cancers. A major pre-requisite for antigen specific immunotherapies are the identification of tumour associated antigens (TAA). Several different types of TAA have been identified and classified into different groups such as unique tumour antigen (E.g. Melanoma antigens), shared tumour-specific antigens (E.g. MAGE gene family products), tissue-specific differentiation antigen (E.g. MART-1/Melan-A, gp100 and tyrosinase), oncogenes and tumour suppressor gene products (E.g. Ras, p53, Her-2/neu) and virus-associated antigens (p16 and p18 from HPV). These are discussed in Section 1.2. Several different techniques have been developed to identify TAA. These include genetic, biochemical and reverse immunology, approaches detailed below.

3.1.1 Identification of Tumour Antigens

3.1.1.1 Genetic Approach

The identification of tumour antigens had for many years involved the fractionation of cell extracts or membrane preparations. These fractions were then tested for their ability

to confer a protective immunity in murine models. Most of these techniques failed and so were replaced by a molecular approach involving expression cloning of cDNA libraries. This work was pioneered by Thierry Boon and co-workers (Boon, 1993), who discovered that clones generated from tumour cells exposed to a potent mutagen when re-injected into mice did not produce any tumour whereas the parent cells did. These tumour negative clones (called tum$^-$ variants) were found to be eliminated by the immune system. However when the parent tumour cells were re-injected in animals previously treated with the tum$^-$ no tumour growth develop. They had acquired resistance to the original tumour cells but not to unrelated cancer cells. DNA from tum$^-$ variants was removed, cleaved and inserted into plasmids (rings of bacterial DNA) bearing a gene which imparts resistance to a toxic drug. On exposing the cells to the toxic drug only those that incorporated the plasmid survived. These cells were then allowed to multiply and each clone was exposed to T lymphocytes that specifically recognise the tum$^-$ cells or antigen of interest. Any cell that induced a lymphocyte response (such as proliferation or cytokine production) was assumed to produce the antigen, and thereby the corresponding gene (Boon, 1993). Surprisingly, it was found that the gene identified (P1A gene) was also present in the original tumour cells, but somehow, expressed only weak antigens that are not able to induce a powerful immune response (van den Eynde, *et al.*, 1991). Therefore, it was postulated that some human tumours similar to murine tumours can be antigenic (express antigens that can be used to generated an immune response) without being immunogenic (incapable of generating an immune response) and could be rendered immunogenic. Using this technique tum$^-$ variants (antigen loss variants) were made from a cell line derived from a mastocytoma, p815 and a cosmid library was prepared using the genetic material of the newly obtained p815 tum$^-$. These were, in turn, transfected back into the parental cell line, p815, which do not express the gene. These were then tested for their ability to stimulate T cells (generated by immunizing mice with killed tum$^-$ variants) to multiply. The gene responsible for the stimulation of the T cells was identified and found to be expressed not only by the tum$^-$ variants but also by the parental cell line and in normal mouse tissue. However, in the tum$^-$ variants a point mutation had occurred within the gene giving rise to proteins with a single amino-acid substitution. These proteins were chopped into small peptides with different abilities to bind to MHC class-I molecules and therefore be presented at the surface of the cell to be surveyed by T-cells. The point mutation within the tum$^-$ variant

protein had rendered a non-binding peptide to a binding one. From a different tum[-] variant clone a different gene was identified and shown to be present but was not expressed in normal cells.

Using this genetic approach, with the availability of tumour specific CTL, the first human tumour associated antigen was identified. The MAGE (melanoma antigen) gene family was cloned from a melanoma cell line derived from a patient with melanoma, MZ-2 (van der Bruggen, *et al.*, 1991), BAGE (van der Bruggen, *et al.*, 1991), and GAGE (van den Eynde, *et al.*, 1995).

The technique was then modified and simplified to search for additional melanoma antigens. cDNA libraries were made from tumour cell lines and transfected into COS cells (chimpanzee cells) where the vectors expressing the cDNA multiplied episomally (DNA do not integrate into the host genome) to reach a very high copy number. Selected clones were then co-transfected with the appropriate HLA gene. These were then tested for their ability to stimulate TNF-release by tumour specific cytotoxic T lymphocytes. Using this method the tumour associated antigens tyrosinase (Brichard, *et al.*, 1993), melan-A (Coulie, *et al.*, 1994), gp-100 (Kawakami[b], *et al.*, 1994) were discovered. This approach has also led to the identification of several tumour associated antigens in some cases unique antigens (E.g. CDK-4, Wölfel, *et al.*, 1995; casp-8, Mandruzzato, *et al.*, 1997).

These gene cloning methods rely however on having a good supply of cytolytic T cells specific for the tumour studied.

Another approach to identify TAA relies on protein biochemical methods, whereby MHC bound peptides are acid eluted, purified and sequenced.

3.1.1.2 Elution Technique

The HLA-A2 molecules on the surface of cells can be purified by papain digestion of plasma the membrane. Indeed, papain cleaves the heavy chain at residue 271, 13 residues from the transmembrane region, resulting in a molecule composed of $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 2$-m (Springer, *et al.*, 1976). Subsequently, purified HLA-A2 molecules can be crystallised to learn more information of the groove structure where peptides bind their amino-acid sequence (Bjorkman[b], *et al.*, 1987). Peptides can then be eluted by acid treatment and fractions run through HPLC and mass spectrometry. This can provide information on peptide length and amino-acids which might be important for binding. Many tumour-

associated peptides have been extracted either from immunoaffinity purified or directly from the tumour cell surface by acid elution. These bulk peptides have then been resolved by reverse phase high performance liquid chromatography (RP-HPLC) (Storkus, *et al.*, 1993; Slingluff, *et al.*, 1993; Franksson, *et al.*, 1993). HPLC fractionated tumour peptides were then pulsed onto irrelevant target cells bearing the appropriate HLA allele, and were used as targets to tumour-specific T-cells. This technique allowed the identification of a number of potential T-cell epitopes perceived by the immune system in situ without any information of the identity of the original tumour protein involved in generating the immunogenic peptides (E.g. melanoma antigens).

Peptides bound to MHC class-I molecules have been found to be mainly of nine amino-acids in length with predominant amino-acid at position 2 and 9 (Leucine or Isoleucine at position 2 and a valine at position 9 for HLA-A2 molecules) for each MHC class-I allele (Bjorkman[b], *et al.*, 1987; Falk, *et al.*, 1991; Hunt, *et al.*, 1992; Drijfhout, *et al.*, 1995). These residues are called 'anchor residues', and can be described as either 'preferred' or 'tolerated' which indicates whether the amino acid at a particular position associates with a specific HLA molecule with respectively either a strong bond or a weak bond. Examples of anchor residues for common HLA alleles are shown in Table (1). Peptides of 10 and 11 amino-acid in length have also been identified, as well as residues at non-anchor positions playing an important role in the binding affinity of the peptide bearing them (Drijfhout, *et al.*, 1995). Identification of 'anchor residues' make it possible to screen any protein sequences for the identification of potentially immunogenic peptides that could be used for the production of a cancer vaccine.

3.1.1.3 Protein Sequence Screening Approach

T cell epitopes are nowadays mainly discovered by screening the potential tumour antigen sequence for nine amino-acids long peptides bearing 'preferred' or 'tolerated' anchor residues for any particular HLA-molecules. Once the peptides are identified and synthesised it is necessary to know the interaction between peptide and MHC. This determines an important intermediary step in antigen presentation. Immunogenic peptides form indeed relatively stable MHC-peptide complexes with a low dissociation rate in comparison with non-immunogenic peptides (van der Burg, *et al.*, 1996).

3.1.2 Peptide Binding Assays

A number of different methods have been used to determine peptide binding to MHC molecules, including the use of purified MHC class-I molecules and techniques based on stabilisation of empty MHC class-I molecules at the cell surface.

3.1.2.1 Using Purified MHC class-I Molecules

Cells (usually T2 cells) were lysed and MHC class-I molecules were purified using immunoaffinity columns of protein A-purified monoclonal antibodies and then eluted from these columns. Either T2 cells lysat alone (Connan, *et al.*, 1994) or purified MHC class-I molecules were incubated with different concentrations of cold or radio-labelled-peptides (Olsen, *et al.*, 1994; Celis, *et al.*, 1994) along with β2-m for a set time. The amount of bound peptide was then determined either by gamma scintillation counter, ELISA or FACS analysis depending on the method followed. The ability of test peptides to compete with a radiolabelled standard peptide for binding to a class-I molecule was measured in these assays. Alternatively E. coli (Parker, *et al.*, 1992) or drosophila cells (del Guercio, *et al.*, 1995) can be used to produce MHC class-I molecules. Using this method more details have been found about peptide binding motifs (Ruppert, *et al.*, 1993). (The purification of MHC molecules combined with the elution technique has allowed the discovery of naturally occurring peptides from the MHC class-I pathway (Rötzchke, *et al.*, 1991). Secondary pockets within the HLA-A2 molecule have indeed been discovered. Amino-acid at position 1, 3 and 7 were found capable of strengthening (case of Y, F, W, S, T, or C) or reducing (case of D, E, P, R, K, H) the binding of peptides nine amino-acid in length still bearing the proper anchor residues at positions 2 and 9.

Table 1: HLA-Specific Binding Motifs.

HLA Allele	Amino Acid Position			References
	2	3	9/10	
A1	**TMS**	**DE**	**Y**	Kubo, *et al.*, 1994; *Di Brino, *et al.*, 1994
		AS		
A2	**LM**		**LIV**	Rammensee, et al., 1993; Ruppert, *et al.*, 1993; Kast, *et al.*, 1994; Kubo, *et al.*, 1994.
	IVAT		AM	
A3	**LMIVSATF**		**KRY**	Di Brino, *et al.*, 1993; Kubo, *et al.*, 1994.
	CGD		A	
A11	**MLVSATGN**		**K**	Kubo, *et al.*, 1994.
	CDF		R	
A24	**YF**		**FLIW**	Kubo, *et al.*, 1994.
	M			

Bold type indicates '**preferred**' amino acids

Normal type indicates '**Tolerated**' amino acids

3.1.2.2 Using RMA-S Cells or T2 Cells

The mutant mouse cell line RMA-S was derived from the Rauscher virus-induced H-2b lymphoma RBL-5 by exposure to the mutagen ethyl methane sulphate (EMS) and repeated rounds of treatment with antisera against class-I molelcules and complement (Ljunggren, *et al.*, 1985; Karre, *et al.*, 1986). These cells synthesise both class-I heavy chains and β2-m, but most of the heavy chains do not associate with β2-m and remain intracellular (Ljunggren, *et al.*, 1989). The human cell line (T2) was derived between the T1 cell line (a fusion between the human cell line .174 which contains a homozygous deletion of the class-II region of the MHC) and the T lymphoblastoid cell line (CEM) but without the two intact copies of chromosome 6 derived from the CEM cells (Salter, *et al.*, 1985/1986; Cerundolo, *et al.*, 1990). T2 cells lack the TAP1 and TAP2 genes, which encode the transporter proteins, but still produce high amounts of the MHC heavy chain and β2-m, but are unable to present endogenously synthesised peptides. The amount of HLA-A2 molecules expressed on the surface of these mutants is very low due to the degradation of unstable empty complexes (MHC class-I molecules/β2-m). However, if a peptide, with high affinity for the HLA-A2 molecules, is added exogenously it will bind to the molecules and thereby stabilise the complexes. This in turn can be detected by indirect staining of the HLA-A2 or H-2b molecules on the surface of the T2 cells or RMA-S cells respectively using a FACS machine, see Section 2.2.2.

These mutant cell lines with defects in antigen processing have great potential and have been extensively used for the determination of the binding affinity and detailed motifs of selected peptides (Nijman, *et al.*, 1993; Drijfhout, *et al.*, 1995). Different conditions for the assay have been set up including one, using the T2 cells, which is used in our lab (established by McIntyre, *et al.*, 1995).

In addition to methods using biological assays, computer programs are now available to find out all potential binder of 8, 9 or 10 amino-acid in length as well as their half-life (HLA binding motifs program).

3.1.3 p53 peptides as CTL epitopes

P53 protein is the most commonly mutated gene in human cancers. Mutations within the gene induce an accumulation of the p53 protein due to its increased half-life. This therefore allows more peptides to be synthesised and thus presented at the cell surface in

association with MHC class-I molecules. Self-peptide do not, normally, elicit an immune response but an overexpression of it might. Indeed, CTL have been shown to be able to discriminate between the amount of p53 peptide present on normal cells and that present on tumour cells (Vierboom, *et al.*, 1997). p53 represents therefore an ideal candidate for immunotherapy against cancer. A number of studies have evaluated p53-encoded peptides for binding to MHC molecules and their ability to induce CTL activity. The protein sequence has been screened for any 8 or 9 amino-acids long peptides with the two anchor residues at position 2 and 9. A summary of published work is shown in Table (2). Peptides binding to MHC molecules was assessed by one of the method mentioned earlier and most results were comparable. Peptides 24-32, 25-33, 65-73, 65-74, 66-74, 69-76, 69-78, 78-86, 122-130, 129-137, 187-197, 210-218, 263-272, 264-272, 322-330 were found to bind with strong affinity to HLA-A2 molecules. However only peptides 25-35, 65-73, 65-74, 129-137, 149-157, 187-197, 263-272 and 264-272 were capable of generating CTL *in vitro* either in human or in transgenic mice bearing the α1 and α2 domains of the human HLA-A2 molecules. These CTL killed peptide pulsed target. Interestingly peptide 149-157 does not belong to the high binder group. Peptides 264-272 and 149-157 have so far been the only ones generating CTL capable of killing tumour targets as well as peptide-pulsed targets indicating that these are naturally endogenously processed. Peptide 187-197 has been found by Theobald, *et al.*,(1997)to be tolerated by HLA-A2 restricted CTL in transgenic mice.

The aim of this work was to identify new p53 peptides capable of binding with strong affinities to HLA-A2 molecules on the surface of T2 cells. These will then be used to generate CTL response *in vitro*.

TABLE 2: SUMMARY OF WORK DONE SO FAR USING P53 PEPTIDES IN HUMAN OR TRANSGENIC MICE

Authors	Title	Peptides studied	Resutlts
Nijman H. W., et al (1993)	Characterisation of cytotoxic T lymphocyte epitopes of a self-protein, p53, and a non-self-protein, influenza matrix: Relationship between major histocompatibility complex peptide binding affinity and immune responsiveness to peptides	25-33 (1.77) 65-73 (2.59) 187-197 (2.63) 264-272 (2.69)	Bind strongly. Only 264-272 peptide induces CTL
Houbiers J. G. A., et al (1993)	In vitro induction of human cytotoic T lymphocyte responses against peptides of mutant and wild type p53	21-31 (1.2) 24-32 (1.7) 25-35 (2.8) 65-74 (1.4) 65-73 (2.2)..... 113-122 (1.3) 129-138 (1.4) 129-137 (1.3) 132-145 (1.3) 168-176 (1.2)..... 187-197 (2.6) 193-203 (1.4) 256-265 (1.3) 263-272 (1.6).....	 No killing 20% killing Between 20 and 65% killing

		264-274 (1.2) 264-272 (1.9) 339-407 (1.8)	
Stuber G., et al (1994)	Identification of wild-type and mutant peptides binding to HLA-A2 assessed by a peptide loading-deficient cell line assay and a novel major histocompatibility complex class I peptide binding assay	24-32* 42-50* 43-52* 65-73** 92-100* 110-118* 129-137** 132-140 136-144* 159-167* 186-196* 187-195** 193-201* 245-253 250-258 256-265* 263-272** 264-272** 322-330** 331-339	1<*<1.5 **>1.5, considered as high binders.
Zeh III H. J., et al (1994)	Flow cytometric determination of peptide-class I complex formation. Identification of p53 peptides that bind	24-32 (low) 42-60 (does not reconstitute HLA-A2) 66-74 (high)	

	to HLA-A2	129-137 (high) 186-196 (low) 193-201 (low) 256-264 (low) 264-272 (high) 322-330 (high)	
Nijman H. W., et al (1994)	P53, a potential target for tumour directed T-cells	24-32 (1.6) 25-35 (2.4)* 65-73 (1.9) 65-74 (2.1)* 122-130 (1.7) 139-147 (1.5) 149-157 (1.5) 187-197 (2.2)* 193-203 (1.2) 256-265 (1.2) 264-272 (1.7)*	* Generate CTL
Gnjatic S., et al (1995)	Mapping and ranking of potential cytotoxic epitopes in the p53 protein: effect of mutations and polymorphism on peptide biding to purified and refolded HLA molecules	25-35 65-73 129-137 187-197 263-272 264-272 187-195 256-264	Bind strongly Bind moderately

Theobald M., et al (1995)	Targeting p53 as a general tumour antigen (Transgenic mice)	* 25-33 ↑↑↑ ** 25-35 ↑↑↑ * 31-39 ↑↑ 31-40 ↑↑ 42-50 ↑ * 42-52 ↑↑ *** 65-73 ↑↑↑ 69-76 ↑↑↑ 69-78 ↑↑↑ 69-79 ↑ 73-81 ↑ 78-86 ↑↑↑ Δ 110-119 ↑ Δ 117-125 ↑ Δ 121-129 ↑	Δ 122-130 ↑ 129-137 ↑↑↑ Δ 146-155 ↑ *** 149-157 ↑↑ Δ 161-169 ↑ * 187-195 ↑↑↑ Δ 187-197 ↑ * 210-218 ↑↑↑ Δ 229-237 ↑ * 255-264 ↑↑ Δ 255-265 ↑↑ * 263-272 ↑↑↑ *** 264-272 ↑↑↑ 322-330 ↑↑ Δ 339-347 ↑	↑ Low binder ↑↑ Moderate binder ↑↑↑ High binder Lytic activity: 0<*<7 10<**<50 50<***<100 Δ Not determined 149-157 and 264-272 kill SW480 and MCF-7 when pre-treated with IFN-γ and TNF-α
Röpke M., et al (1995)	T cell-mediated cytotoxicity against p53-protein derived peptides in bulk cultures and limited dilution cultures of healthy donors	24-32 (1.8)..... *129-137 (1.6)..... *264-272 (1.7).....		Bulk results: * generate CTL on day 21 Precursor frequencies: <1:300 000 < 1:300 000 1: 54 000 1: 27 000 1:110 000 1:110 000

		249-257 (1.2)	1: 33 000 <1: 330 000
Röpke M., et al (1996)	Spontaneous human squamous cell carcinoma are killed by a human cytotoxic clone recognizing a wild-type p53-derived peptide	264	CTL kill T2 cells pulsed with peptide and squamous cell carcinoma
Theobald M., et al (1997)	Tolerance to p53 by A2.1 restricted cytotoxic T lymphocytes (transgenic mice)	65-73..... 129-137..... 149-157..... 187-195..... 187-197..... 210-218..... 255-264..... 264-272.....	Kill T2 + peptide No killing Kill T2t + peptide No killing Kill T2 + peptide but not when CTL come from mice $p53^{+/+}$ υTolerant No killing No killing Kill T2 + peptide
Gnjatic S., et al (1998)	Accumulation of p53 protein allows recognition by human CTL of a wild-type p53 epitope presented by breast carcinoma and melanomas	264	CTL kill T2 + peptide M8 +/- peptide MDA-MB-231 +/- peptide MCF-7 (+/- mutation in p53 gene)+/- peptide

Theobald M, et al., 1998	The Sequence Alteration with a Mutational Hotspot in p53 Protects Cells from Lysis by Cytotoxic T Lymphocytes Specific for a Flanking Peptide Epitope	149-157 264-272	CTL generated with peptide 264-272 did not kill target cells expressing a mutational hotspot at residue 273. CTL generated with peptide 149 could. SaOs-2/175 were killed by both CTL lines
Chikamatsu K, et al., 1999	Generation of Anti-p53 Cytotoxic T Lymphocytes from Human Peripheral Blood Using Autologous Dendritic cells	149-157 264-272	Bulk Cultures only killed peptide-pulsed Target. Clones killed: - SaOs-2/148 - SCC-9 - PCI-13

3.2 Results

3.2.1 Selection of P53 Peptides

The protein sequence screening approach has been extensively used on the p53 protein. Many peptides of 9 to 11 amino-acid in length, bearing the 2 preferred anchor residues, have been identified and tested for their ability to bind or stabilise MHC class-I molecules using one of the methods mentioned earlier. All these studies are summarised in Table (2). Looking at the p53 protein sequence one region has never really been studied. This region (residues 193-237) corresponds to the epitope RHSVV (residue 213-217) known as the 240 epitope. In normal cells or in non-conformational mutants (mutants that retain the wild-type conformation of the protein) this epitope is not exposed but rather buried in a high hydrophilic mass. On the other hand, conformational mutants (175-like) loose the native conformation of the protein and as a result of the mutation expose that region which can be detected by the monoclonal antibody 240. In this study 9, 10 and 11-mers peptides from the 193-237 region and slightly extended region, bearing either 2, 1 or none of the preferred or tolerated anchor residues, were identified and synthesised, to be assessed for their binding properties to MHC class-I molecules. Peptides 187, 263 and 264 have been chosen as positive control for their well documented binding capability. These peptides are listed in Table (3).

Indeed, wild-type and mutant phenotypes represent alternative conformations of the p53 protein with the wild-type phenotype associated with the suppressor function of the protein, and mutant phenotype associated with cell proliferation. This change of conformation induced by cell growth is critical for p53 function but transient (Milner, 1990).

Table 3: DETAILS OF p53 ENCODED PEPTIDES ASSESSED FOR MHC BINDING

Peptides position	Sequence	Anchor Residue at position:		
		2	9/10	11
186-194	DGLAPPQHL	-	P	
*187-197	GLAPPQHLIRV	P	-	P
◊188-197	LAPPQHLIRV	T	P	
193-221	HLIRVEGNL	P	P	
194-222	LIRVEGNLR	T	-	
196-224	RVEGNLRVE	T	-	
202-210	RVEYLDDRN	T	-	
210-218	NTFRHSVVV	T	P	
211-219	TFRHSVVVP	-	-	
217-225	VVPYEPPEV	T	P	
224-232	EVGSDCTTI	T	P	
225-233	VGSDCTTIH	-	-	
229-237	CTTIHYNYM	T	T	
◊263-272	NLLGRNSFEV	P	P	
264-272	LLGRNSFEV	P	P	

T: Tolerated anchor residue *11-mer

P: Preferred anchor residue ◊10-mer

As can be seen four peptides have the 2 preferred anchor residues at position 2 and 9 or 11; peptides 193-221, 187-197, 263-272, and 264-272. One peptide has only one preferred anchor residue at position 9, peptide 186-194. Two peptides do not have any anchor residues, peptides 211-219 and 225-233. Three peptides have only one tolerated anchor residue at position 2, peptides 194-222, 196-224, and 202-210. Four peptides have two anchor residues, a tolerated one at position 2 and a preferred one at position 9, peptides 188-197, 210-218, 217-225, and 224-232. One peptide has only two tolerated anchor residues at position 2 and 9, peptide 229-237.

3.2.2 MHC Stabilisation Assay: Titration of HLA-A2 Antibody

In order to determine the optimum concentration of the primary antibody required to bind all the available surface peptide/MHC complexes in the T2 peptide binding assay every new batch of antibody were titrated in the same way.

Isolated HLA-typed human PBMC (see Section 2.2.3.1), stored in liquid nitrogen, were quickly thawed out, washed in PBS, and counted by Trypan Blue exclusion and used for the antibody titration. Flow cytometric analysis, as described earlier, was used to determine the optimum concentration of antibody to use. A typical result is shown in Table (4).

Table 4: Staining of HLA-A2-typed PBMC with Various Volumes of Monoclonal Antibody HB-82.

Volume of Antibody added to the cells (µl)	Median Channel of Fluorescence
0	24.0
25	338.0
50	359.5
75	358
100	351

As can be seen from these data and figure (1) there was a steady rise in the median channel fluorescence with increasing volume of HB-82 up to 50µl which then started to decrease by 75µl and 100µl. In order to be sure to saturate all of the binding sites 75µl of antibody was chosen for use in the experiments.

3.2.3 MHC Stabilisation Assay: Evaluation of Method.

The MHC Stabilisation assay was carried out as described in the method chapter Section2.2.1.2. Briefly, empty HLA-A2 molecules expressed on the surface of the T2 cells can be stabilised by addition of exogenous peptide, if peptide possesses any binding affinity for the HLA-A2 molecules. The number of stabilised molecules, which is indirectly proportional to the amount of peptide bound on the surface of the cells can be

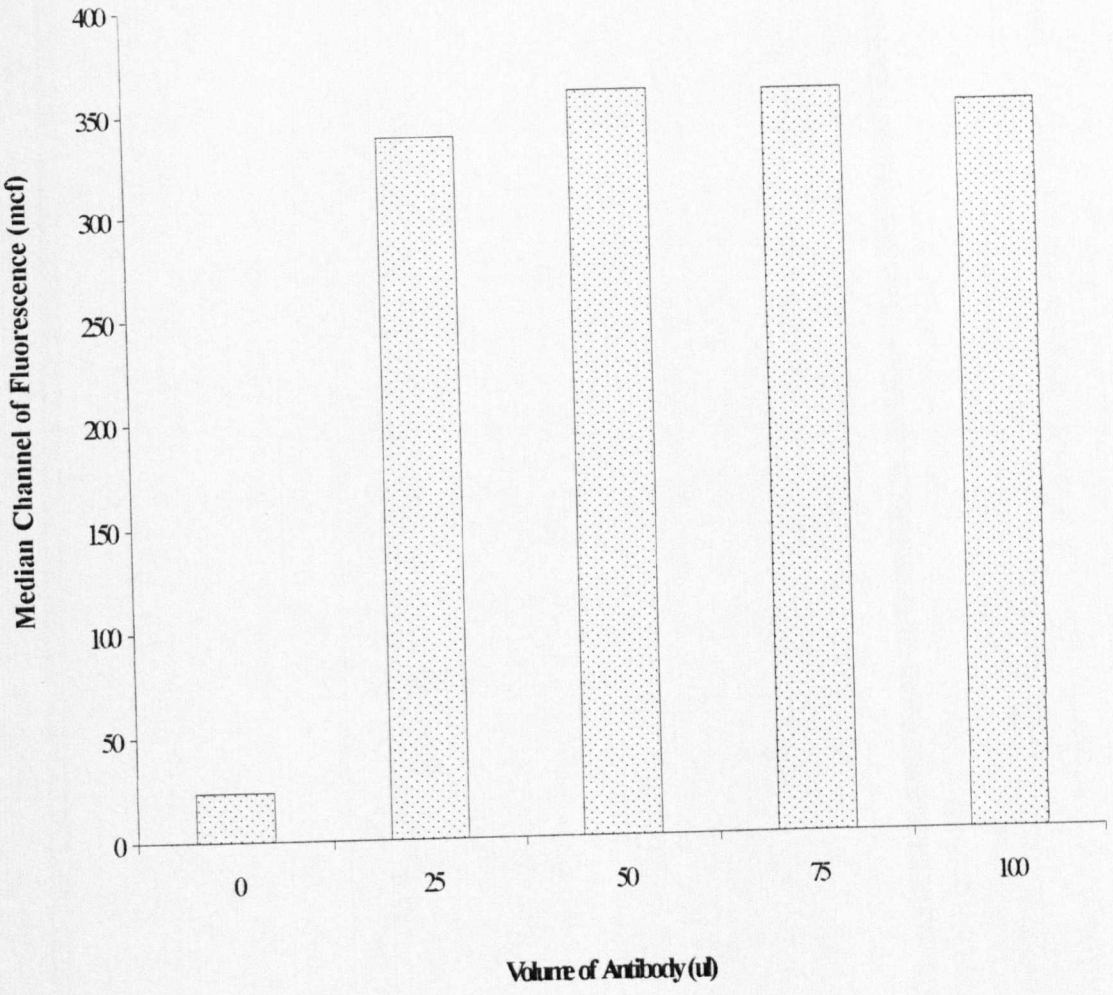


Figure 1: Titration of a New Batch of Primary Monoclonal Antibody HB-82 on HLA-A2-Typed Human PBMC

detected by indirect staining using an HLA-A2 antibody (HB-82) as first antibody and a goat anti-mouse F(ab)'2 as secondary antibody. The fluorescence, emitted per cell, is measured by a FACS-sorted machine. An fluorescence ratio (FR) is then calculated as follows:

$$FR = \frac{\text{Mean channel fluorescence of tested peptide}}{\text{Mean channel fluorescence of DMSO}}$$

Any peptide with an FR> 1.5 was considered as a high binder, with an 1<FR<1.5 peptide was considered as moderate binder and with an FR<1 peptide was considered as non-binder. Before testing the ability of any of the p53 peptides chosen to stabilise the HLA-A2 molecules at the surface of the T2 cells, it was necessary to first establish the efficiency of the positive (HLA-A2 Flu peptide, Bednarek, *et al.*, 1991) and negative (DMSO) controls.

Table 5: Positive And Negative Controls For Peptide Binding To HLA-A2 At 26°C.

Controls		Mean Fluorescence Ratio			
		0 (μM)	**1** (μM)	**10** (μM)	**100** (μM)
	Cells alone	1.06	-	-	-
'True' Negative Control	DMSO	1.00	-	-	-
Positive Peptide Control					
Influenza virus Matrix AA 58-66: GILGFVFT	A2Flu	-	1.14	1.67	2.61

It can be seen from these results and figure (2) that the Flu peptide showed a high affinity for the HLA-A2 molecules since it reached a mean FR of 2.61 at 100μM and was therefore suitable for use in the T2 binding assay as a positive control. DMSO was chosen as a 'true' negative control since all the peptides were dissolved in it. The cells

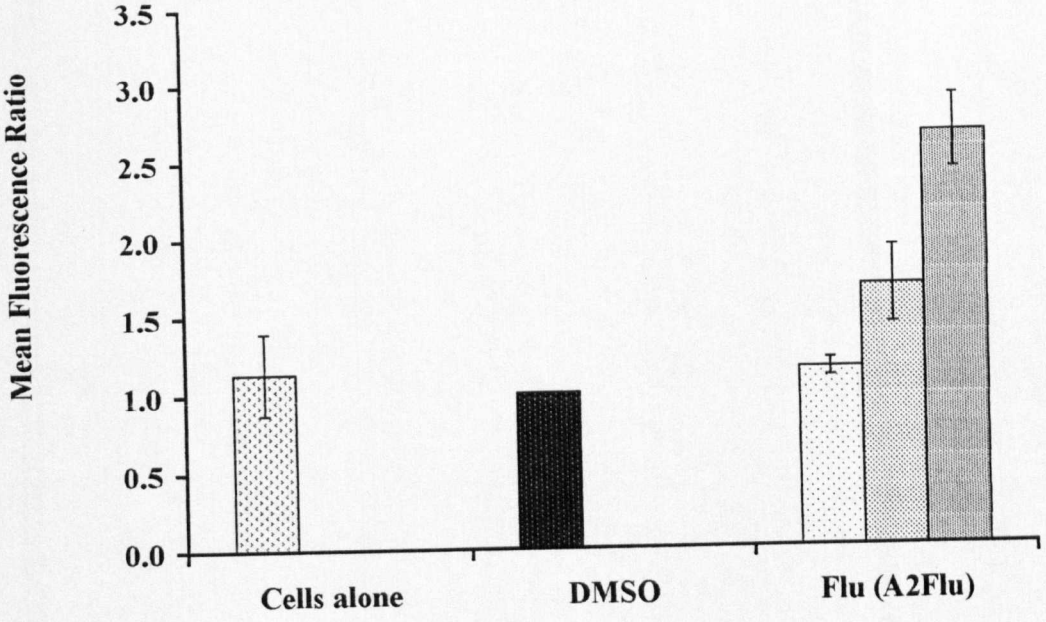


Figure 2: Positive and Negative Controls for MHC Stabilisation Assay Performed at 26°C.

alone showed no greater fluorescence than the DMSO and therefore no stable HLA-A2 molecules on their surface.

3.2.4 MHC Stabilisation Assay: Results of p53 Encoded Peptides in the Assay Performed at 26°C.

T2 cells were incubated with 1μM, 10μM or 100μM of each p53 peptides, or A2Flu or DMSO for a minimum of 18 hrs at 26°C. After antibody staining, the level of fluorescence, corresponding indirectly to the amount of peptide bound, was detected by flow cytometry. The fluorescence ratio was obtained by dividing the mean channel fluorescence of each peptide by the mean channel fluorescence of the DMSO.

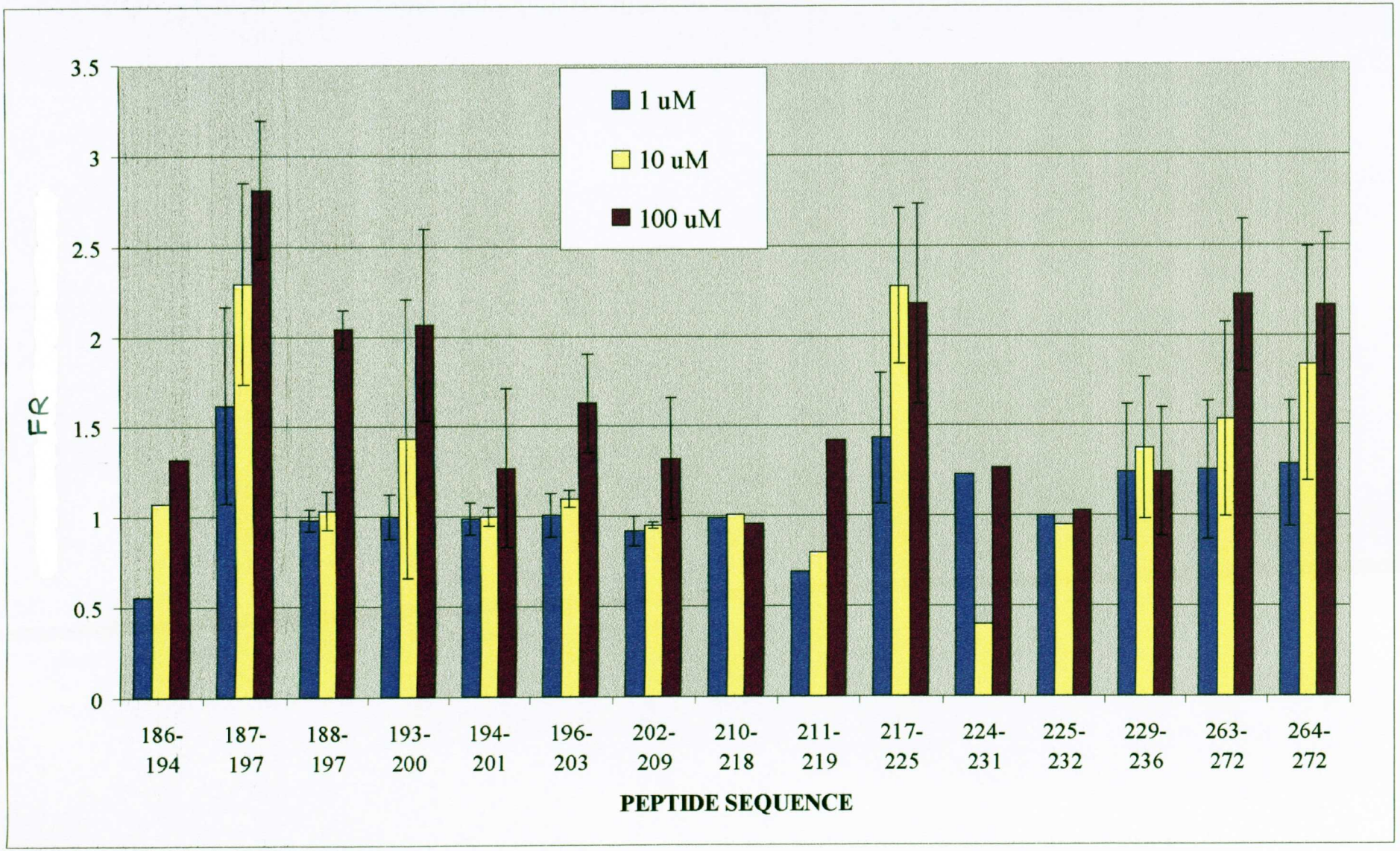
All peptides were tested 3 times in 3 independent assays. Peptides with a mean fluorescence ratio > 1.5 were considered as 'high' binders and are shown in bold. Peptides with a mean fluorescence ratio < 1.1 were considered as non-binders, peptides with an FR comprised between 1.1 and 1.5 were considered as intermediate binders. Results are summarised in table 6.

Table 6: T2 Binding Assay of p53 Peptides at 26°C

PEPTIDES	*SEQUENCE*	*MEAN FR AT 1μM*	*MEAN FR AT 10μM*	*MEAN FR AT 100μM*
186-194	DGLAPPQHL	0.56	1.07	1.32
187-197	GLAPPQHLIRV	1.62	**2.30**	**2.82**
188-197	LAPPQHLIRV	0.98	1.03	**2.04**
193-201	HLIRVEGNL	1.00	1.43	**2.07**
194-202	LIRVEGNLR	0.99	1.00	1.27
196-204	RVEGNLRVE	1.01	1.10	**1.63**
202-210	RVEYLDDRN	0.92	0.95	1.32
210-218	NTFRHSVVV	0.99	1.01	0.96
211-219	TFRHSVVVP	0.69	0.80	1.42
217-225	VVPYEPPEV	1.43	**2.28**	**2.18**
224-232	EVGSDCTTI	1.23	0.40	1.27
225-233	VGSDCTTIH	1.00	0.95	1.03
229-237	CTTIHYNYM	1.24	1.38	1.24
263-272	NLLGrNSFEV	1.25	1.53	**2.22**
264-272	LLGRNSFEV	1.28	**1.84**	**2.17**

As can be seen from table (6) and figure (3) 7 peptides (in bold) stabilised HLA-A2 molecules with high affinity (FR> 1.5) and were considered as high binders. Of which peptide 196 gave a range FR of <1 and >1.5. 6 other peptides stabilised the MHC molecules with moderate affinity (1< FR< 1.5) and were considered as moderate binders. Two peptides did not show any binding affinity (FR<1) and were considered as non-binder.

Figure 3: Results of MHC Stabilisation Assay Performed at 26°C



3.2.5 MHC Stabilisation Assay Performed at 37°C

The MHC stabilisation assay was also performed at 37°C a more physiological temperature. Initial experiments validated the assay by using positive and negative controls (as described previously) at 37°C and these results are listed below in table (7).

Table 7: Positive and Negative Controls for Peptides Binding to HLA-A2 at 37°C

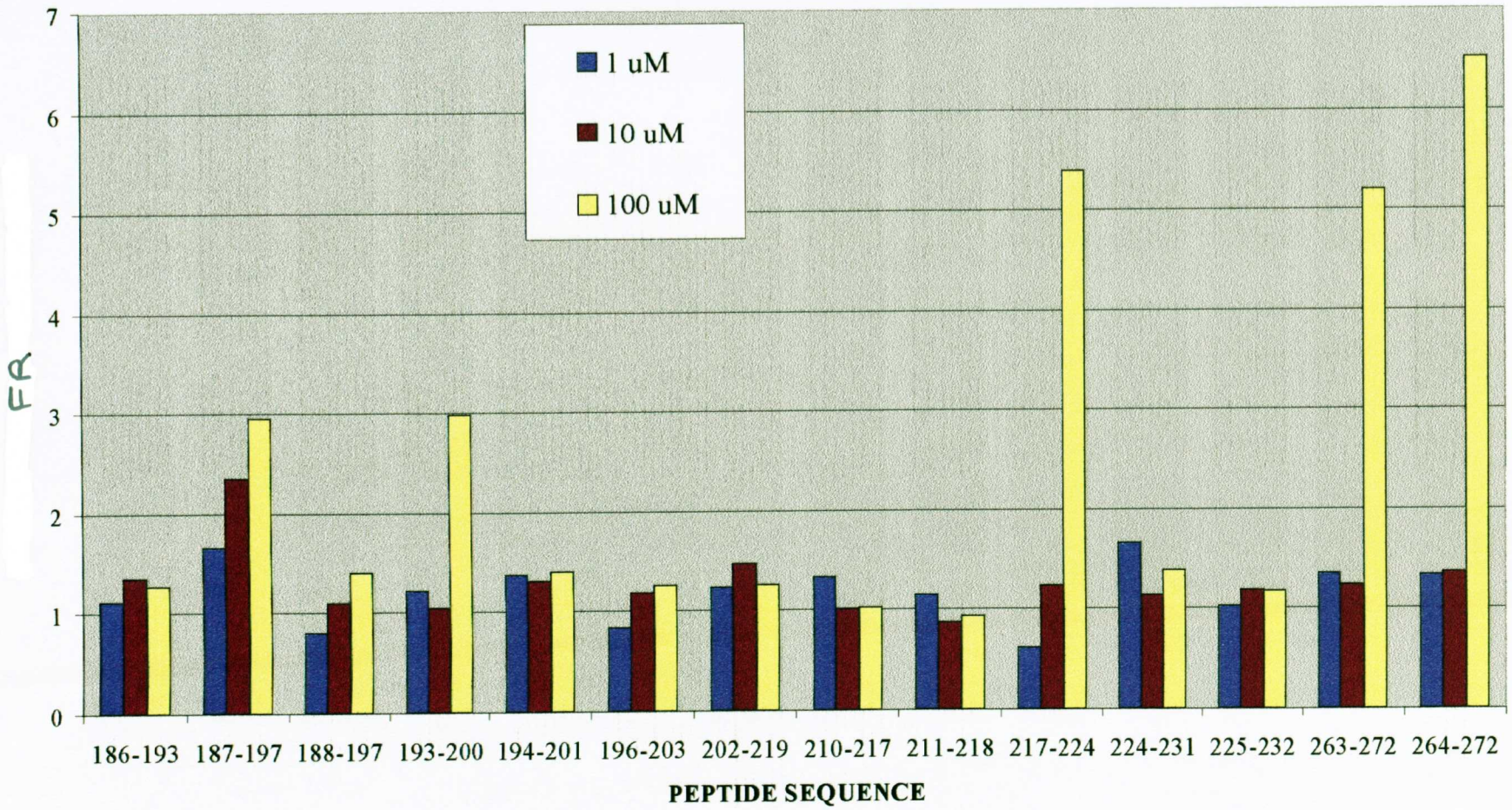
Controls		Mean Fluorescence Ratio			
		0 (μM)	1 (μM)	10 (μM)	100 (μM)
	Cells alone	1.2	-	-	-
'True' Negative Control	DMSO	1.00	-	-	-
Positive Peptide Control	A2Flu*	-	2.36	2.72	2.90

*A2Flu peptide used as a positive control gave consistantly high FR at all 3 concentration used in the assay. The p53 encoded peptides were assessed in the T2 binding assay at 37°C and the results are shown in Table (8) and Figure (4).

Table 8: T2 Binding Assay of p53 Peptides at 37°C

PEPTIDES	*SEQUENCE*	*MEAN FR AT 1μM*	*MEAN FR AT 10μM*	*MEAN FR AT 100μM*
186-194	DGLAPPQHL	1.12	1.36	1.28
187-197	GLAPPQHLIRV	1.66	2.36	**2.95**
188-197	LAPPQHLIRV	0.81	1.11	1.40
193-201	HLIRVEGNL	1.23	1.05	**2.99**
194-202	LIRVEGNLR	1.38	1.31	1.40
196-204	RVEGNLRVE	0.84	1.19	1.27
202-210	RVEYLDDRN	1.24	1.47	1.27
210-218	NTFRHSVVV	1.34	1.02	1.04
211-219	TFRHSVVVP	1.16	0.89	0.94
217-225	VVPYEPPEV	0.63	1.25	**5.39**
224-232	EVGSDCTTI	1.66	1.14	1.39
225-233	VGSDCTTIH	1.03	1.20	1.18
263-272	NLLGNSFEV	1.35	1.25	**5.20**
264-272	LLGRNSFEV	1.34	1.36	**6.51**

Figure 4: Results of MHC Stabilisation Assay Performed at 37°C



From Table (8) and Figure (4) it can be seen that most of the peptides which showed a strong binding affinity for HLA-A2 molecules, with an FR > 1.5, at 26°C also showed a strong binding affinity for HLA-A2 molecules at 37°C. Only two peptides did not. Peptide 196, however, was found to be border line with an FR between the two side of 1.5 when the assay was performed at 26°C, at 37°C the same trend seemed to happen.

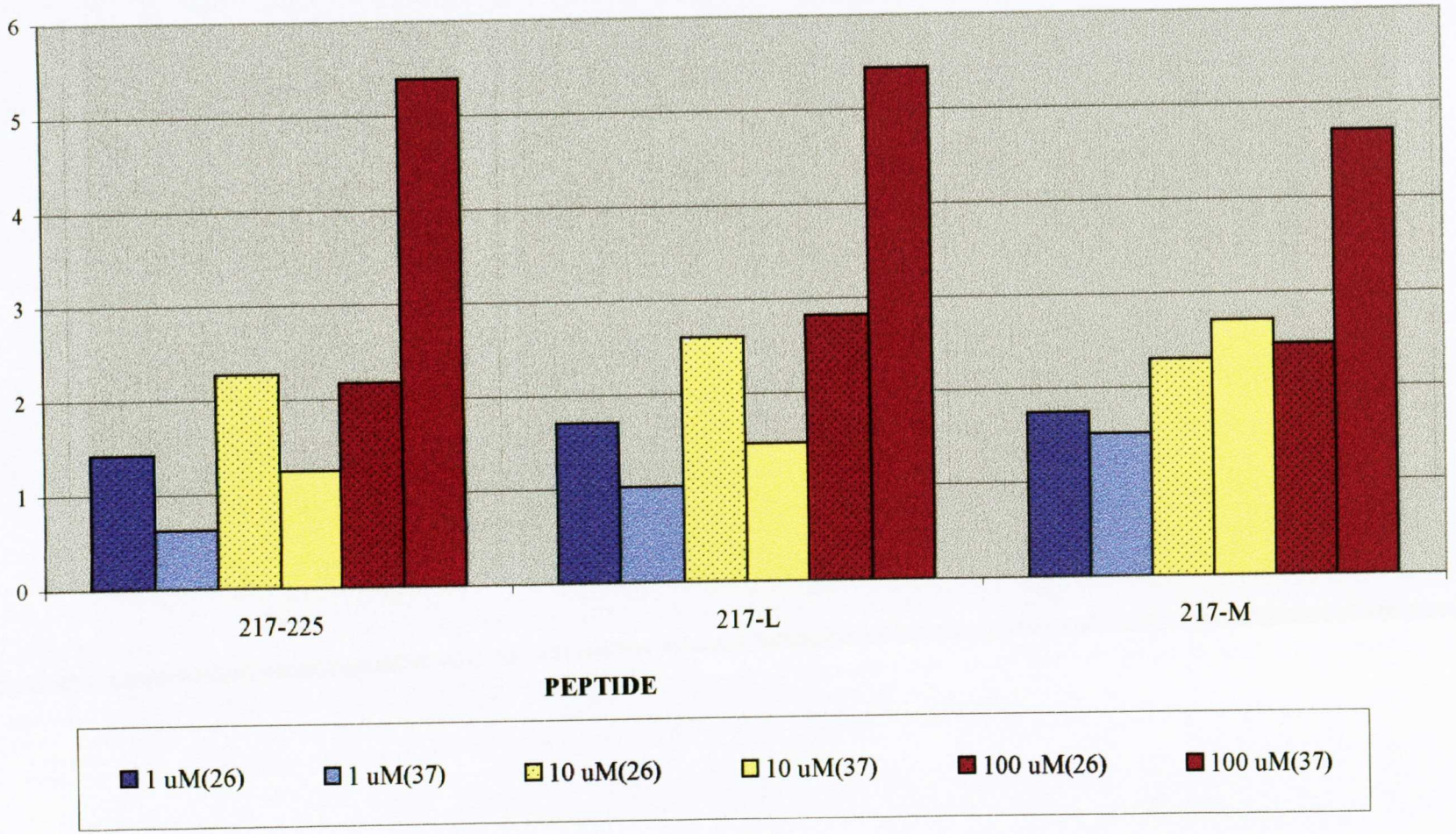
3.2.6 Effect of Amino Acid Replacement on MHC Stabilisation Assay of the 217 Peptide.

Peptide 217 is one of the peptides that have not been, so far, studied for its ability to bind to HLA-A2 molecule. In these experiments it was found to have, both at 26°C and 37°C, an FR > 1.5 even though it contains only a tolerated anchor residue at position 2 and a preferred one at position nine. Changing a 'non-preferred' anchor residue for a preferred one has proven in the past to improve the binding affinity of the peptide for HLA-A2 molecule (Lipford, *et al.*, 1995). In order to assess whether amino-acid substitution had an effect on binding of peptide 217, the second amino-acid was changed for a preferred one. These have the same amino-acid sequence than the original 217-peptide except for the position two where a substitution has been made. Peptide 217-L contains a Leucine instead of the Valine and peptide 217-M a Methionine instead of the Valine. These peptides were tested for their ability to stabilise MHC class-I molecules at the surface of the T2 binding assay along with the original peptide 217. As can be seen, from table (9) and figure (5), the 217-L peptide had the highest FR of the three, however the improvement was not really significant.

Table 9: Effect of A Single Amino-Acid Substitution on Peptide Binding Efficiency of Peptide 217 in a T2 Binding Assay.

PEPTIDES	SEQUENCE	MEAN FR AT 1μM		MEAN FR AT 10μM		MEAN FR AT 100μM	
		26°C	37°C	**26°C**	37°C	**26°C**	37°C
217-225	VVPYEPPEV	**1.43**	0.63	**2.28**	1.25	**2.18**	5.39
217-L	VLPYEPPEV	**1.71**	1.03	**2.61**	1.47	**2.83**	5.44
217-M	VMPYEPPEV	**1.75**	1.52	**2.31**	2.71	**2.46**	4.72

Figure 5: Results of MHC Stabilisation Assay Performed at 26°C and 37°C. Effect of Amino-Acid Replacement at Position 2 on Peptide 217-225



FR

95

3.3 Conclusions

Results obtained with peptides 187-197, 229-237, 263-272, 264-272, were in accordance with published work. Peptides 193-201 and 210-218 however gave opposite results to published work.

Nine new peptides have been tested for their ability to bind and stabilise the MHC complexes on the surface of the T2 cells in a T2 binding assay. Two peptides, peptide 188 and 217, have been found to bind strongly to the HLA-A2 molecules of the T2 cells and were considered as high binders. These have never been, so far, considered as potential immunogenic peptides. The 217 peptide was chosen for its particular interest since it corresponds to the p53 protein region exposed in 175-like mutant, epitope 240 and has not previously been used to generate CTL.

3.4 Discussion

3.4.1 Assay

The T2 binding assay is a very easy, quick and non-hazardous (no raodio-labelled isotope involved) assay for testing the ability of a given peptide for its ability to bind to HLA-A2 molecules. The results obtained with this assay using well-known peptides where similar in FR and conclusions. The assay was first carried out at 26°C because it as been previously reported (Cerundolo, *et al.*, 1991; Stuber, *et al.*, 1994) that by incubating the peptides with the T2 cells at 26°C, rather than 37°C, it was possible to increase the sensitivity of the assay. However because of its non-physiological use in prospective of patient immunotherapy all the peptides were also tested at 37°C. Binding affinity properties of most peptides were found similar at 26 and 37°C. Assays performed at 26°C however detected more peptides binding (total of 7) than assays performed at 37°C (total of 5).

3.4.2 Anchors Residues and Binding Affinity

- Presence of anchor residues is necessary for peptides binding....

Peptides containing no anchor residue (peptides 211 and 225) were not found to stabilise the HLA-A2 molecules at the surface of the T2 cells whereas peptides containing two preferred anchor residues (peptides 187, 193, 263 and 264) showed strong binding properties (FR >1.5). Peptides with only one tolerated anchor residue did not show high

binding capability. However, peptides with one preferred anchor residue at position 9 and either one or none tolerated anchor residue at position 2 did show strong affinity for HLA-A2 molecules (FR > 1.5) with the exception of peptides 210 and 224. This illustrates the need of two anchor residues for peptide binding even though peptide can bind with only one anchor residue.

- .....but not sufficient.

Factors other than size and anchor residues are also critical in determining whether or not a peptide will bind HLA-A2 molecules. Indeed the presence of other residues at non-anchor positions can either increase or abolish completely the binding affinity of peptide bearing them. So for peptide of nine amino-acid in length the presence of charged amino-acid (Acid: P, E, or D) at position 1 or position 6 (Basic: H, R or K) are of most detrimental effects on the peptide binding affinity (Ruppert, *et al.*, 1993; Drijfhout, *et al.*, 1995). On the other hand aromatic residues were associated with high affinity binding at position 3 and 7. Residues with OH- or SH- containing side chains such as S, T, or C were favoured at position 4 while A was favoured at position 7 and P was favoured at position 8. In addition positive contribution as been found for aromatic and positively charged residues at N-terminus especially Y and K as well as T (Drijfhout, *et al.*, 1995). The trend was found to be similar for peptides of 10 amino-acid in length with non-anchor position shifted to the right and an exception for position 3 where aliphatic rather than aromatic residues were associated with high binding affinity (Ruppert, *et al.*, 1993; Drijfhout, *et al.*, 1995).

Not only do peptides have to bind to MHC molecule with sufficient binding affinity but they have to remain attached. Computer programs are now available to determine the half-life of a given peptide.

3.4.3 Peptide Affinity and Immunogenicity

Despite the presence, in a given antigen, of peptides with both anchor residues only a few will generate CTL *in vitro*. One important parameter is the determination of the efficiency of peptide to bind to MHC molecules. Indeed a strong correlation between immunogenicity and the stability of MHC-peptide complex has been reported (van der Burg, *et al.*, 1996). A peptide with a poor ability to bind to HLA-A2 molecules was also found to be non-immunogenic. By improving its binding ability it was possible to

enhance its immunogenicity (Lipford, *et al.*, 1995). This however does not automatically imply that peptides with moderate binding affinity are immunologically non-important. This was the case of the WT p53 149 peptide found to have a moderate binding affinity (Nijman, *et al.*, 1994) and yet capable of eliciting CTL *in vitro* capable of killing peptide-pulsed target as well as tumour targets (Theobald, *et al.*, 1995/1997). Another important point is that a peptide naturally processed and possessing a strong binding affinity for MHC class-I molecules would have been presented to naïve T-cells in the thymus and any reactive T-cells would have therefore been eliminated. This has been reported for peptide 187-197 where T-cells recognised and killed targets when they were elicited in mice with no p53. Whereas T-cells from mice with a normal p53 did not kill the same target but were able to kill T2 (transfected with a mouse equivalent for HLA-A2: H-2b) cells pulsed with the peptide (Theobald, *et al.*, 1997).

When looking at a protein sequence if one selects peptides only based on their amino-acid sequence and length one would miss potentially immunogenic peptides. To test every single peptides for binding and then for their ability to generate CTL *in vitro* would however be time and money consuming. Kern, *et al.*, 1998, came up with an attractive alternative. Peptides of 15 amino-acids in length were used to stimulate PBMC which were then analysed by a four colours flow cytometry after 6h of incubation. Intracellular IFN staining serving as an indicator of T-cell activation. Time is indeed gained by using PBMC instead of CTL clones, by using large number of peptides at the same time and assessing PBMC after only 6 hours. Using this method Kern, *et al.*, discovered two new peptides capable of eliciting an IFN response by PBMC stimulated with them while these did bot bear any anchor residues in their sequence.

CHAPTER 4: GENERATION AND CHARACTERISATION OF TRANSFECTED CELLS TO BE USED AS TARGETS FOR CTL

4.1 Introduction

There are many reasons for introducing genes into cells. Indeed, after cloning an unknown gene the only way to analyse its characteristics is by re-introducing it into various cell types. Genetic elements responsible for its regulation of expression, the effect of its overexpression in a given cell line or the consequence of its introduction into a cell that does not normally express it can all then be determined following gene transfer. Gene transfer has also been used for therapeutic purposes such as in the treatment of many different classes of disorders including inherited genetic diseases (e.g. Cystic Fibrosis), viral infections (e.g. HIV) and cancers (Moelling, *et al.*, 1997). Many methods of gene transfer are now available, all with different advantages and disadvantages.

4.1.1 Transfection Techniques

Transfection of cells can either be transient or stable. In a transient transfection, the transfected cells are normally used 1-7 days after introduction of the DNA. However, to avoid numerous transfections it is sometimes preferable to perform stable transfection by permanently maintaining the transfected DNA in the cell using a selectable maker. Gene transfer techniques are normally divided into viral, using adenoviruses or retroviruses, both very useful for *ex-vivo* applications but limited use for *in vivo*; and non-viral physicochemical methods such as 'gene gun', in situ injection of naked DNA, use of polycations and Liposomes, or electroporation. Most non-viral techniques can be used for transient or stable transfection.

4.1.1.1 Viral Vectors

DNA can be introduced into eukaryotic cells by using viral vectors. Certain viral gene transfer systems will result in the overexpression of chosen proteins (e.g. vaccinia systems) or will allow single-copy stable integrants of expressed genes (e.g. retroviral systems).

DNA viruses such as adenoviruses can infect both dividing and non-dividing cells. Adenoviruses however, do not integrate into chromosomal DNA but replicate as episomal (extrachromosomal) elements in the nucleus of the host cell and their expression is therefore only transient.

In vivo one of the major disadvantages of using adenoviruses for gene transfer is that a potent anti-adenovirus immune reaction is elicited involving both cellular response and humoral mechanisms.

Retroviruses are generally used for stable gene transduction into a cell line or into cells in an animal. Because retroviral DNA integrates into the genome of target cells it replicates during mitosis providing each daughter cell with a copy of the transgene. However, one of the major disadvantages of retroviral gene transfer is that retrovirus can only transduce proliferating cells, limiting their application *in vivo* (Günzburg, *et al.*, 1995).

Viral systems can be much more efficient than non-viral transfection systems since their efficiencies of gene transfer are far superior. However, the construction of a recombinant virus requires considerable effort, and like non-viral systems they are inefficient for tissue-targeted delivery. In addition, some modification of their genetic material is necessary to reduce immune reaction in the host.

4.1.1.2 Non-Viral Vectors

The two most commonly used non-viral gene transfer techniques for *in vitro* transfection, apart from calcium phosphate precipitation, are liposome-mediated and electroporation-mediated gene transfer.

In liposome-mediated transfection, the mechanism by which liposomes mediate the transfection into cells is not well understood. Lipids are mixed and left to incubate at room temperature with pure DNA before adding the mixture to the cells which are then incubated at 37°C for 2-6 hours, depending on the optimal condition determined for the specific cell line. It is thought that negatively charged phosphate groups on DNA bind to the positively charged liposomes in a ratio that results in a net positive charge for the complex. The positively charged liposome-DNA complex then mediates binding to negatively charged sialic acid residues on the cell surface. It has been postulated that the liposome-DNA complex either fuses with the cell surface or is taken up by endocytosis ultimately resulting in the release of the DNA into the cytoplasm and subsequent transport to the nucleus (Gao, *et al.*, 1995).

Electroporation uses an electric field to create transient pores in the cell membrane. The DNA is then thought to diffuse into the cell through the pores (Weaver, *et al.*, 1993). Several parameters involved in the electroporation procedure play an important role in the success of the gene transfer into sensitive cells. These include ionic strength and volume of electroporation, medium, voltage, capacity, DNA concentration, temperature and pulse decay time (Kalinski, *et al.*, 1997). The most critical parameter is the voltage of the electric pulse which needs to be fine-tuned for each cell line in order to optimise the transfection efficiency.

4.1.2 Potential Applications of Transfection in Anti-Tumour Therapy

4.1.2.1 To Eradicate Tumour Cells Directly

Mutations in the tumour suppressor gene p53 represent the most common genetic alterations observed in human cancers (Hollstein, *et al.*, 1991) and make it an attractive target for gene-based therapy. Many tumours bearing an inactive p53 gene become resistant to chemotherapy and radiotherapy (Righetti, *et al.*, 1996). *In vitro* transfection of target cells with recombinant adenoviruses expressing the wild-type p53 cDNA resulted in the expression of wild-type p53 protein for 1-2 weeks and restored their sensitivity to chemotherapy and radiotherapy (Gallardo, *et al.*, 1996; Geng, *et al.*, 1998).

Recently another way to specifically infect and kill tumour cells using the p53 cDNA has been established. The E1B region of the human adenovirus encodes a protein of 55kD that binds and inactivates p53 (Yew, *et al.*, 1992). This represents a vital step for virus replication. Bischoff and co-workers engineered an E1B-defective adenovirus unable to produce the 55kD protein. Without this gene the virus cannot inactivate p53 and is therefore unable to replicate in normal cells bearing functional p53 gene (Bischoff, *et al.*, 1996). However this virus when introduced into tumour cells bearing inactive p53, can replicate and thereby lyse p53-deficient human tumour cells. When used in p53-deficient human cervical carcinomas grown in nude mice, complete regression was observed in 60% of the tumours (Bischoff, *et al.*, 1996).

4.1.2.2 Induction of an Immune Reaction against Tumour Cells

Although there is ample evidence for the presence of tumour-associated antigens on a variety of tumours, an adequate anti-tumour immune response rarely occurs (Boon, *et*

al., 1994). Following the development of new gene transfer techniques the two main approaches in immuno-gene-therapy against cancers have been the genetic manipulation of tumour cells or of antigen presenting cells.

There are many reasons why effective immune responses against tumour neo-antigens do not occur. These include loss or down regulation of the antigen by the tumour cell (Uyttenhove, *et al.*, 1983), down regulation or loss of MHC molecules (Ferrone, *et al.*, 1995) as well as the expression of local inhibitory molecules such as Fas ligand (Rabinowich, *et al.*, 1998). However in order to obtain an effective immune response effector T-cells need to be fully activated. This requires at least two signals. The first signal is delivered by the T cell receptor (TCR) following recognition of a specific antigenic peptide bound in the cleft of an appropriate MHC molecule. The second signal is provided by costimulatory molecules on the surface of professional antigen presenting cells (APC). These APC become activated by cytokines released during for example an inflammation, which are absent during tumour transformation. So even when tumour cells present on their surface sufficient level of antigen complexed with appropriate MHC molecules thereby providing signal one to T-cells if they do not receive the other correct signal no immune response will occur. There are therefore two major explanations to account for the unresponsiveness of T-cells, either they do not recognise the tumour cell (because of lack of antigen or MHC molecules) or become anergic as a result of the absence of the second signal (Staveley-O' Carroll, *et al.*, 1998). For these reasons many tumours are poorly immunogenic, but with recent genetic advances the immunogenicity of tumour cells can be enhanced by genetically engineering tumour cells to produce cytokines which will recruit and activate APC at the site of the tumour or express costimulatory molecules such as B7.1 rendering them capable of directly presenting antigen to T-cells (Iezzi, *et al.*, 1996; Hoshino, *et al.*, 1998).

Alternatively professional antigen presenting cells can be engineered to express either cytokines or tumour antigen (Westermann, *et al.*, 1998; Alijagic, *et al.*, 1995; Reeves, *et al.*, 1996). Interleukin-7 has been shown to provide a potent costimulatory signal to induce the proliferation of T cells and the generation of cytotoxic T cells (Grabstein, *et al.*, 1990; Jicha, *et al.*, 1991). The transfection of dendritic cells (professional APC) with the IL-7 gene significantly increased their capacity to induce T-cell proliferation (Westermann, *et al.*, 1998). Therefore dendritic cells (DC) that have been simultaneously

loaded with tumour antigen peptides and gene-modified to secrete specific cytokine(s) may amplify the response of tumour-specific T-cells. This can further be improved upon if DC are genetically modified to express tumour-associated antigens (TAA), since (i) presentation of tumour antigen peptides by MHC molecules persists for longer periods and (ii) other unidentified tumour peptides may be presented by the host cell MHC molecules. Indeed, genetically modified dendritic cells are able to raise a potent specific anti-tumour immune response *in vitro* (Alijagic, *et al.*, 1995; Reeves, *et al.*, 1996).

4.1.3 Selection of Target Cells and DNA

Once a peptide has been shown to bind to an appropriate MHC molecule (using a T2 binding assay), and is recognised by CTL (causing lysis of the peptide-pulsed T2 cells), it is necessary to demonstrate that it is naturally processed from endogenous p53 protein and presented at the cell surface of cancer cells in association with MHC class-I molecules. Only peptides, which are naturally processed may be relevant targets for cancer therapy. In order to assess whether peptides are naturally processed, the relevant genes can be transfected into cells and the resulting transfectants assessed for recognition by peptide-specific CTL.

p53 protein overexpression, present in more than 60% of cancers, is often a consequence of protein stability acquired by point mutation in the gene. These mutant proteins can be classified into two groups depending on the effect of the gene mutation on the protein conformation. Mutants may conserve the 'wild type' conformation for example p53 mutant 273 (e.g. R->H mutation at amino-acid 273) or acquire a new conformation for example p53 mutant 175 (e.g. R->H mutation at amino-acid 175) (Milner, *et al.*, 1995). Different protein conformations lead to different proteolytic products when compared with wild type p53 degradation (Bargonetti, *et al.*, 1993).

273-like mutants exhibit similar proteolytic fragments to wild type protein while 175-like mutant fragments are quite differents. Therefore it is possible that different MHC-binding-peptides are generated from these two classes of mutants. This is in accordance with the

Figure 4.1a:

pCMVneoBam is an expression vector containing the cytomegalovirus constitutive promoter and the neomycine resistance gene under the control of the simian virus 40 promoter. pBR322WTp53 is derived from pCMVneoBam by insertion of a wild-type p53 cDNA.

pBR322-273: is derived from pBR322WTp53 by insertion of a single point mutation at codon 273 Arg->His.

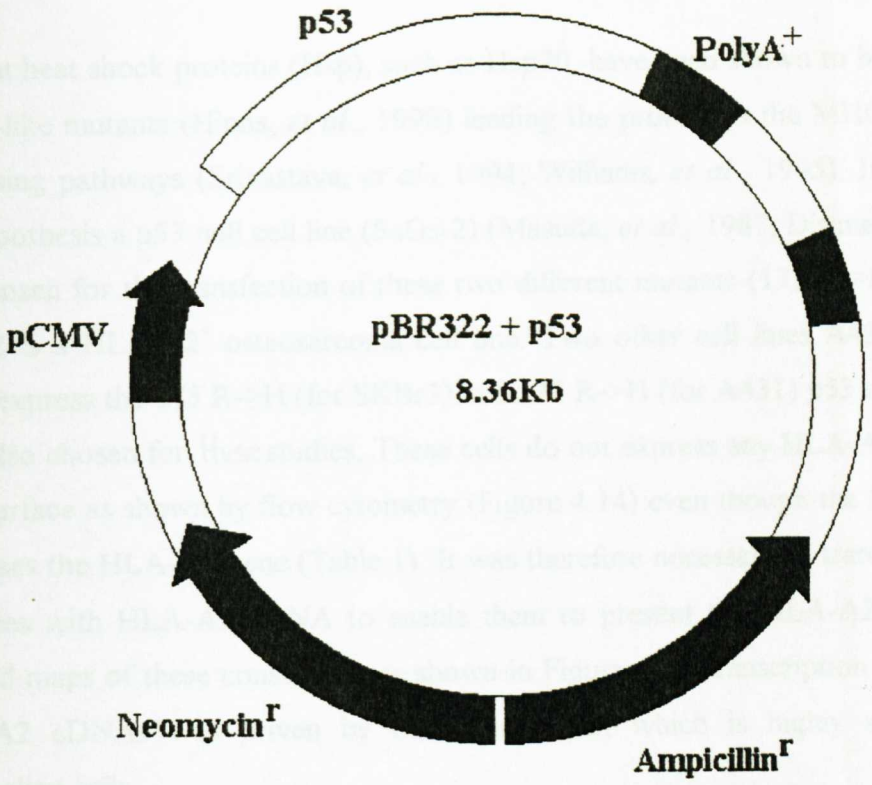
pBR322-175: is derived from pCMVneoBam by insertion of a mutant p53 cDNA.

Insertion of cDNAs was done using Bam *H* I restriction site while all other restriction sites were destroyed.

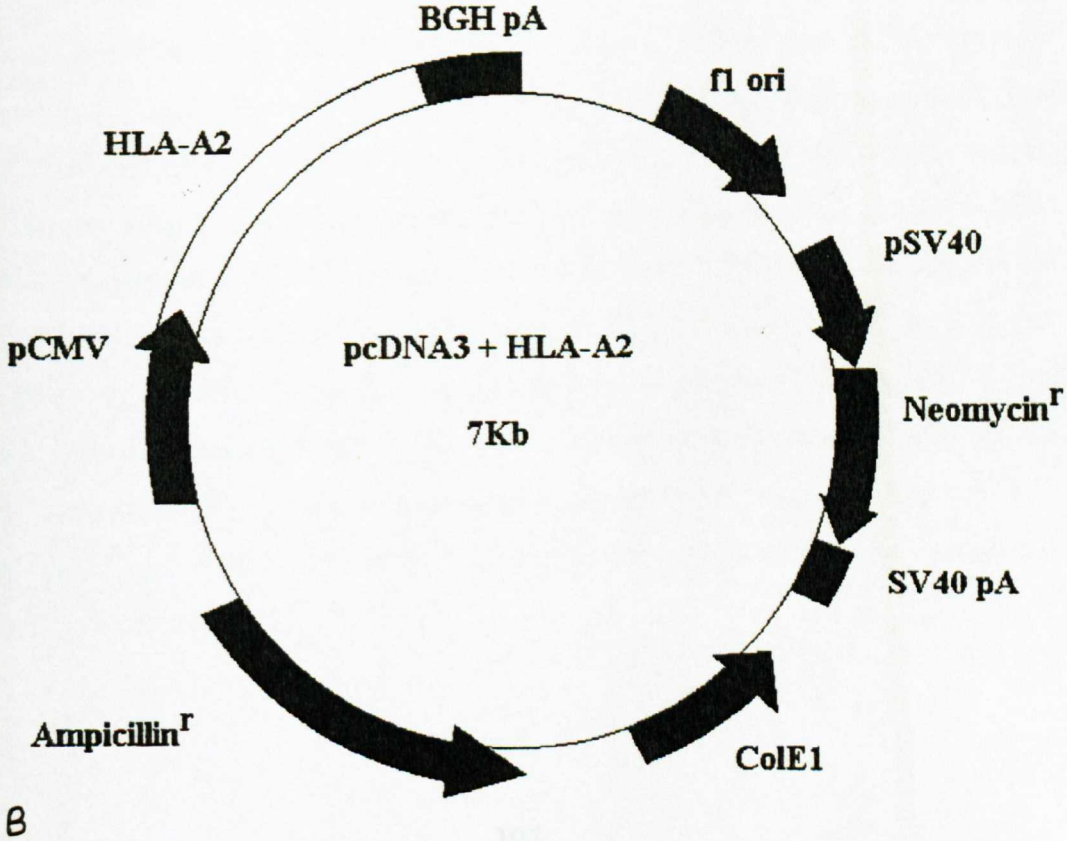
For pBR322-175 only one Bam *H* I restriction site remained.

Figure 4.1b:

pcDNA3 is an expressing vector containing the cytomegalovirus constitutive promoter and the neomycin resistance gene under the control of the simian virus 40 promoter. HLA-A2 gene was inserted.


p53
PolyA+
pBR322 ori
pCMV
pBR322 + p53
8.36Kb
Neomycinr
Ampicillinr

A


BGH pA
f1 ori
HLA-A2
pSV40
pCMV
pcDNA3 + HLA-A2
7Kb
Neomycinr
SV40 pA
Ampicillinr
ColE1

B

Figure 4.1:

fact that heat shock proteins (Hsp), such as Hsp70, have been shown to bind specifically to 175-like mutants (Hinds, *et al.*, 1990) leading the protein to the MHC class-I or -II processing pathways (Srivastava, *et al.*, 1994; Williams, *et al.*, 1995). In order to test this hypothesis a p53 null cell line (SaOs-2) (Masuda, *et al.*, 1987; Dittmer, *et al.*, 1993) was chosen for the transfection of these two different mutants (175 R->H; 273 R->H). SaOs-2 is a HLA-A2$^-$ osteosarcoma cell line. Two other cell lines A431 and SKBr3, which express the 175 R->H (for SKBr3) and 273 R->H (for A431) p53 mutant proteins were also chosen for these studies. These cells do not express any HLA-A2 molecules at their surface as shown by flow cytometry (Figure 4.14) even though the SKBr3 cell line possesses the HLA-A2 gene (Table 1). It was therefore necessary to transfect these two cell lines with HLA-A2 cDNA to enable them to present the HLA-A2 p53 peptides. Plasmid maps of these constructs are shown in Figure 4.1. Transcription of the p53 and HLA-A2 cDNAs was driven by CMV promoter, which is highly active in many mammalian cells.

4.2 Results

4.2.1 Preparation of Transfection Grade DNA Constructs

4.2.2.1 Preparation of pBR322-273 and pBR322-175

Competent E. coli strain XLI-Blue were transformed with plasmid pBR322+273 as described in Section 2.2.5.4. A number of transformed colonies were then cultured o/n in selective liquid medium and the amplified transforming plasmid DNA was purified from each mini-culture as described in Section 2.2.5.5. To confirm the identity of the constructs, purified DNA was digested with restriction enzyme Bam *H*I and analysed by agarose gel electrophoresis as described in Section 2.2.5.7. As seen in Figure 4.2, digestion of the purified plasmids with Bam *H*I gave two fragments of 6.5 and 1.8 Kb, corresponding to the expected fragment sizes of pBR322 and p53 cDNA. Frozen stock cells of XL1/pBR322-273 clone I were used to inoculate a large scale culture (500ml) and DNA was isolated as described in Section 2.2.5.10 to generate highly pure DNA suitable for mammalian transfection. The above procedure was also used to obtain a sufficient amount of highly pure DNA of plasmid pBR322-175. Following digestion with Bam *H*I a single linear fragment of ~8Kb was obtained, consistent with the size of pBR322-175 (Figure 4.3).

4.2.1.2 Preparation of Transfection Grade Empty pBR322 DNA

In order to obtain the empty pBR322 plasmid (i.e. plasmid without p53 cDNA insert) for use as a control for p53 plasmid transfections, 10μg of pBR322-273 were cut using Bam *H*I restriction digestion enzyme and run in an agarose gel (Figure 4.4.a). The 6.5 Kb bands corresponding to the empty plasmid were cut from the gel, pooled and the DNA was purified using the QIAEX II kit (as described in Section 2.2.5.8). To determine the concentration of the purified DNA, for the purposes of DNA ligation, 2, 1 and 0.5 μl of the solution was electrophoresced on an agarose gel and compared with serial dilutions of a high mass ladder (see Figure 4.4b). The concentration of the purified DNA was estimated to be 0.9 μg/μl. The linearised empty plasmid DNA was then circularised via intramolecular ligation as described in Section 2.2.5.6 and introduced into competent XLI-Blue cells (see Section 2.2.5.4).

Transfection grade empty plasmid DNA was then generated by amplification of a selected colony and purification of the DNA using the maxiprep method (Section 2.2.5.10).

Pure empty plasmid was cut using Bam *H*I restriction enzyme and electrophoresced on an agarose gel in order to confirm the absence of p53 cDNA. The only band present on the gel after digestion with Bam *H*I was a 6Kb fragment corresponding to the empty plasmid.

Figure 4.2: Gel electrophoresis of clone 1 pBR322-273

Purified DNA from each clone was digested with restriction enzyme Bam *H* I or left unchanged and analysed by agarose gel electrophoresis.

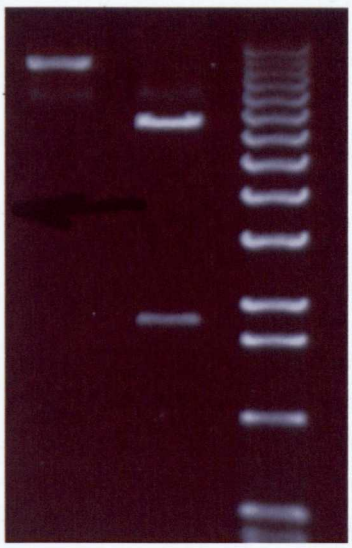
This is representative example of all the DNA preparations from XL I-Blue/pBR322-273.

The two bands appearing on the gel, after digestion (cut), corresponded to pBR322 empty vector (6Kb) and p53 cDNA (1.8Kb) respectively.

The two faint bands appearing a t around 8.5-9Kb were denatured plasmid DNA which are resistant to restriction digestion.

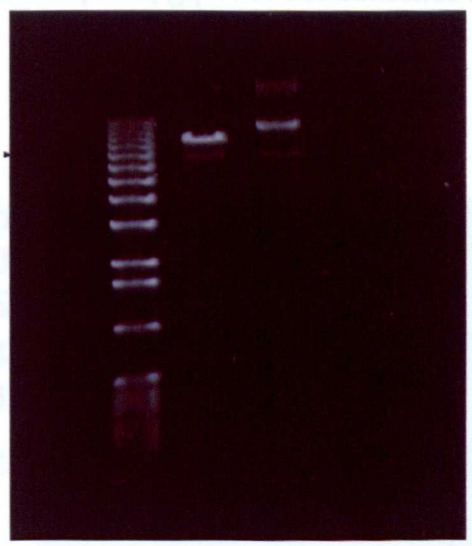
Figure 4.3: Gel electrophoresis of clone 1 pBR322-175

This is a representative of a number of colony DNAs. This is from XLIpBR322-175 clone 1. The two faint bands appearing at around 11 Kb were denatured plasmid DNA which are resistant to restriction digestion.



Uncut (pBR322-273) Cut 1Kb Ladder

Figure 4.2: Midi-prep Plasmids



1Kb Ladder Cut Uncut (pBR322-175)

Figure 4.3: Midi-prep Plasmids

Figure 4.4a: Separation of empty pBR322 vector from p53 cDNA

Pure DNA was cut using Bam *H*I restriction enzyme and run in an agarose gel. The 6Kb bands correspond to the empty plasmid.

Figure 4.4b: Determination of the quality and quantity of pBR322 empty removed from the gel (Bands figure 4.5a) and purified.

4μl of high mass ladder contained 200ng of 10Kb fragment, 120ng of 6Kb fragments, 80ng of 4Kb fragment and 60ng of 2Kb fragments. 2 μl (lane 1), 1μl (lane 2) and 0.5μl (lane 3) of solution was electrophoresced on an agarose gel and compared with serial dilutions of a high mass ladder. Purified empty fragment band on lane 1 compared with 6Kb band intensity of high mass ladder. The brightness of the band compared with 90ng of high mass ladder giving a concentration of 0.9μg/μl of purified empty pBR322.

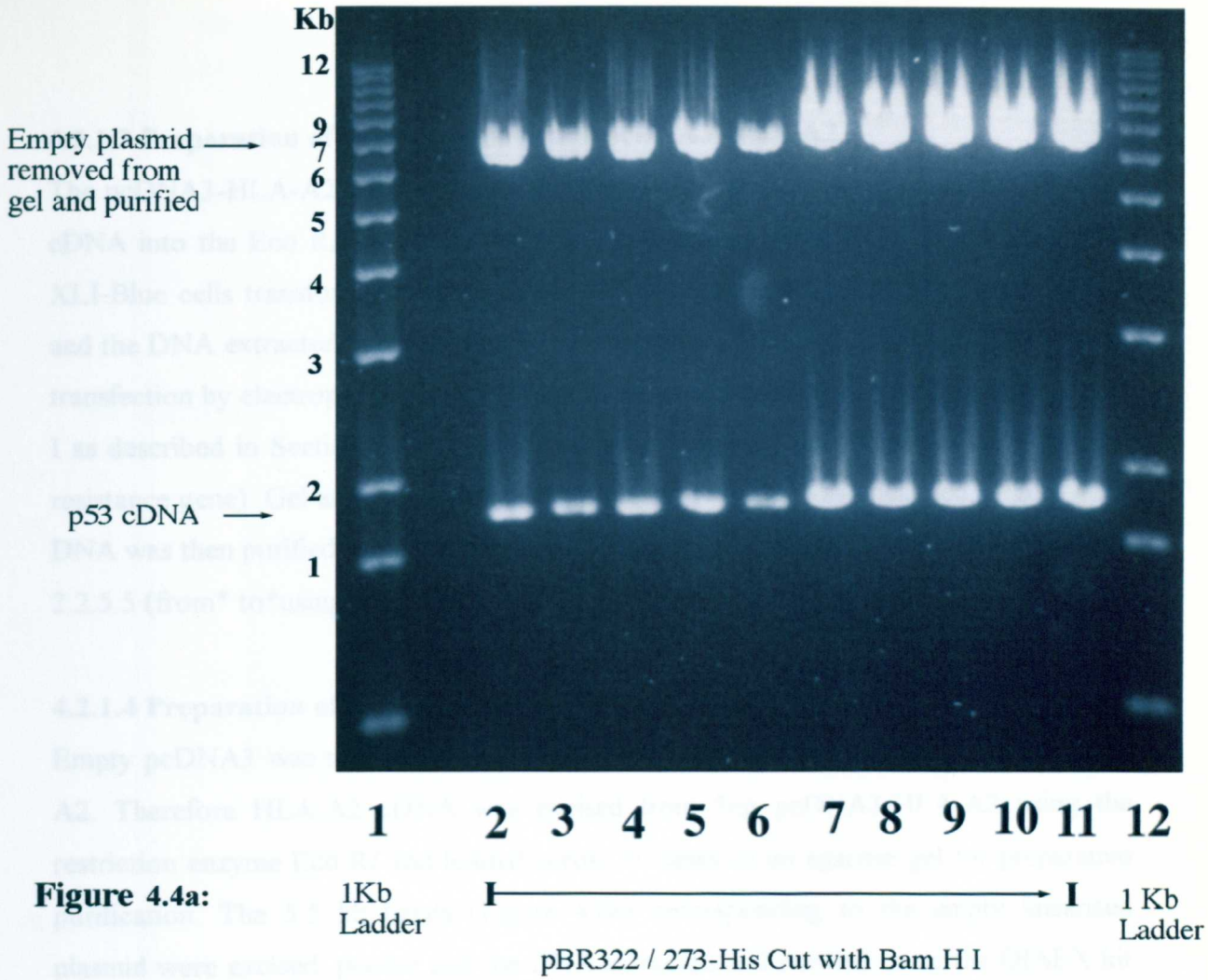


Figure 4.4a:

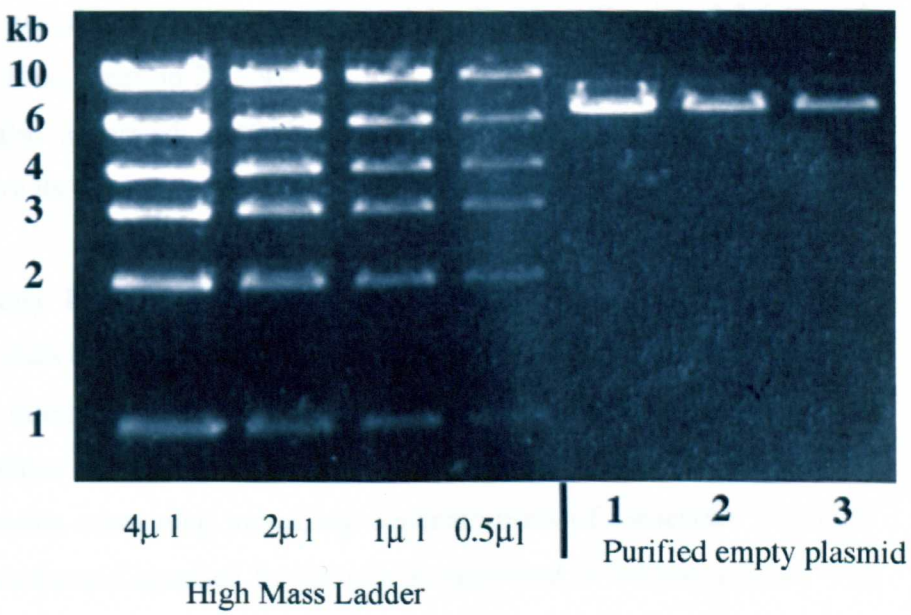


Figure 4.4b:

4.2.1.3 Preparation of Transfection Grade pcDNA3-HLA-A2

The pcDNA3-HLA-A2 construct was previously generated by insertion of the HLA-A2 cDNA into the Eco R*I* site of the multiple cloning sites of pcDNA3 (see Figure 4.1). XLI-Blue cells transformed with pcDNA3-HLA-A2 were grown in large scale culture and the DNA extracted using the maxiprep procedure (Section 2.2.5.10). Prior to use in transfection by electroporation, the DNA was linearised using the restriction enzyme Sca I as described in Section 2.2.5.6, which cuts the construct only once (in the ampicillin resistance gene). Gel analysis confirmed that the entire DNA was linearised (Figure 4.5). DNA was then purified using the phenol and ethanol precipitation as described in Section 2.2.5.5 (from* to*using sterile TE).

4.2.1.4 Preparation of Transfection Grade Empty pcDNA3

Empty pcDNA3 was required as a vector control in transfection using pcDNA3-HLA-A2. Therefore HLA-A2 cDNA was excised from 5ng pcDNA3-HLA-A2 using the restriction enzyme Eco R*I* and loaded across 10 lanes of an agarose gel for preparative purification. The 5.5 kb bands (Figure 4.6a) corresponding to the empty linearised plasmid were excised, pooled and the DNA extracted and purified using the QIAEX kit (Section 2.2.5.8). To determine the concentration of the purified DNA, 2, 1 and 0.5 µl of the solution was electrophoresced on an agarose gel and compared with serial dilutions of a high mass ladder (Figure 4.6b). The concentration of the purified DNA was estimated to be 0.44 µg/µl, which was circularised by ligation (Section 2.2.5.4) and transformed into XLI-Blue (Section 2.25.4).

Empty plasmid was also linearised using the Sca I reaction as for pcDNA3-HLA-A2 (Section 2.2.5.6) before its use in transfection by electroporation.

4.2.2 Geneticin Toxicity Titration for Cell Lines Selection

Before cells could be stabely transfected it was necessary to determine the concentration of the selective agent (Geneticin) required selecting for the transfected cells. Cells were plated into 24-well plates to obtain ~10% confluency and 24hr later, the media was replaced with fresh media containing increasing concentrations of Geneticin. The cells were cultured for 2 weeks and assessed for growth as described in Section 2.2.6.1. The minimum concentration of Geneticin which resulted in 100% cell death was chosen as

Figure 4.5: Gel electrophoresis of the linearised pcDNA3-HLA-A2 purified DNA

pcDNA3-HLA-A2 DNA was grown in large scale, purified, using a maxiprep, and then linearised using Sca I restriction enzyme.

As can be seen by the picture of the gel all DNA was completely linearised and therefore ready to be used for the transfection of A431 and SKBr3 cells.

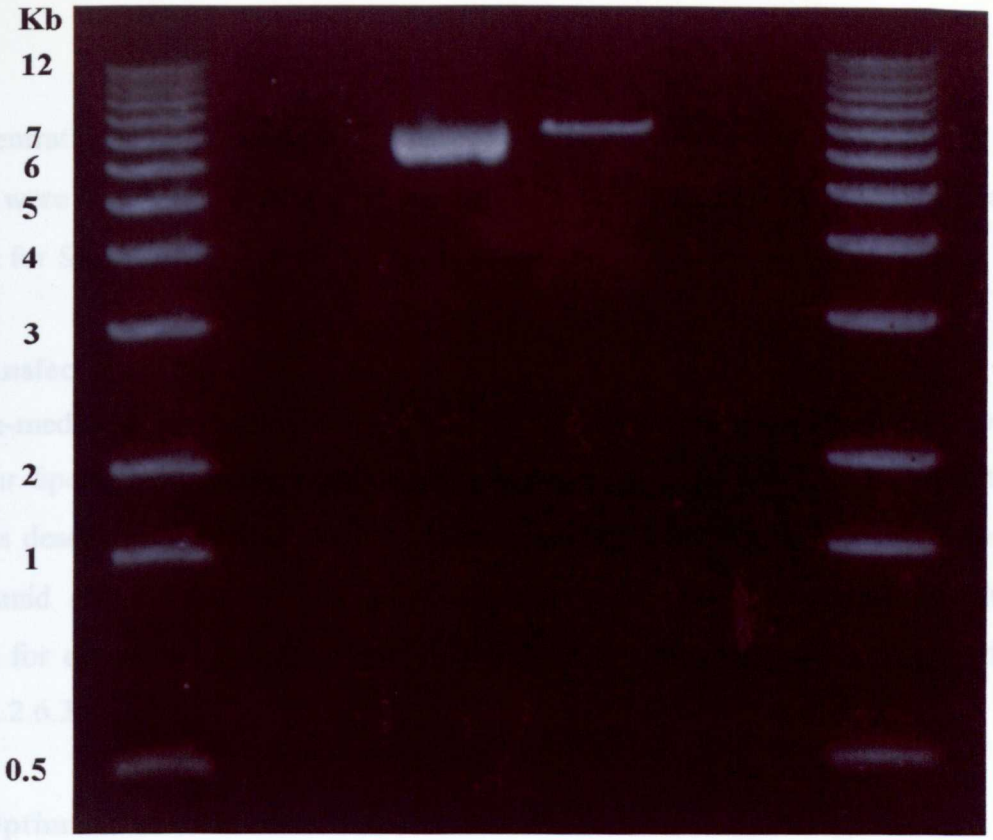

Kb
12
7
6
5
4
3
2
1
0.5
1
2
3
4
5
6
1 Kb Ladder
/
Uncut
Linearised with Sca I
/
1 Kb Ladder
pcDNA3 -HLA-A2

Figure 4.5

the concentration for transfection of that cell line. The concentrations required for selection were found to be 500μg/ml for SaOs-2 cells, 200μg/ml for A431 cells and 300μg/ml for SKBr3 cells.

4.2.3 Transfection of SaOs-2 Cells

Liposome-mediated transfection was assessed for its effectiveness in transfecting SaOs-2 cells. Four liposome reagents were screened in order to determine the most effective reagent as described in Section 2.2.6.2.1 using plasmid pCMV-β as a reporter plasmid. This plasmid encodes the E. coli β-galactosidase gene under the control of CMV promoter for expression in mammalian cells. Expression was assessed as described in Section 2.2.6.3.

4.2.3.1 Optimisation of Transfection Conditions

The four Liposome reagents used, only the cells incubated with the Lipofectamine could be transfected. Cells incubated with the three other reagents were either killed by the procedure or were unable to be transfected. The optimal concentration of DNA/Lipofectamin was determined (Section 2.2.6.2.2) and found to be 1.66 μl of Lipofectamine and 0.42 μg of DNA per well of 9.6cm^2. Following the determination of the optimal DNA/Lipofectamine concentration, the incubation time required for efficient transfection was then determined.

Cells were incubated for 2, 3, 4, 5, 6 hrs with the DNA/Lipofectamine mixture in a 24-well plate, at 37°C before replacing the mixture with 1ml of normal pre-warmed growth medium. The cells were then cultured for 24hr and stained for β-gal expression (Section 2.2.6.3). Optimal transfection efficiency was found with an incubation time of 2hr with the percentage of cells staining positive for β-gal greater than 20%.

4.2.3.2 Stable Transfection of SaOs-2 Cells

Since the optimal conditions for transfection of SaOs-2 cells were now established, cells were grown in a 9.6cm^2 well (6-well plate) and transfected with 0.42μg of pBR322-273 mixed with 1.66μl Lipofectamine (amount given for one well) for 2hrs. SaOs-2 cells were transfected in parallel with pBR322-175 or pBR322 alone. Twenty-four hours after transfection the medium was replaced with fresh medium and cells were subsequently

Figure 4.6a: Separation of empty pcDNA3 vector from HLA-A2 cDNA

Pure DNA was cut using Eco RI restriction enzyme and run in an agarose gel. The 5+Kb bands correspond to the empty plasmid.

Figure 4.6b: Determination of the quality and quantity of pcDNA3 empty removed from the gel (Bands figure 4.5a) and purified.

4µl of high mass ladder contained 200ng of 10Kb fragment, 120ng of 6Kb fragments, 80ng of 4Kb fragment and 60ng of 2Kb fragments. 2 µl (lane 1), 1µl (lane 2) and 0.5µl (lane 3) of solution was electrophoresced on an agarose gel and compared with serial dilutions of a high mass ladder. Purified empty fragment band on lane 1 compared with 6Kb band intensity of high mass ladder. The brightness of the band compared with 120ng of high mass ladder giving a concentration of 0.44µg/µl of purified empty pcDNA3.

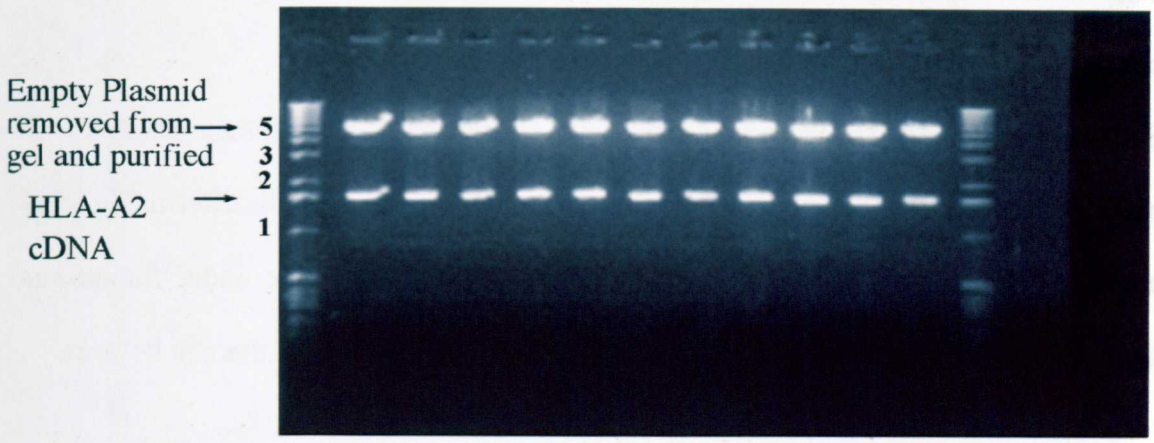
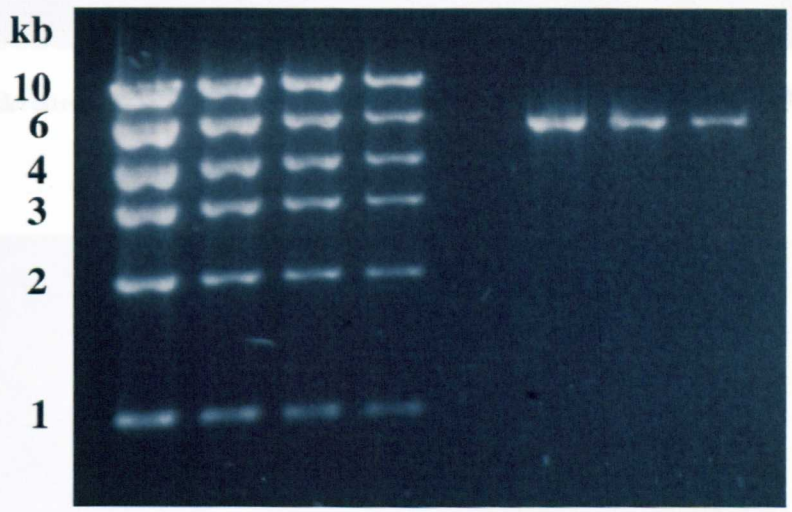


Figure 4.6a:

1 2 3 4 5 6 7 8 9 10 11 12 13

1 Kb Ladder — pcDNA3 - HLA-A2 cut with EcoR I — 1 Kb Ladder



4µl 2µl 1µ l 0.5µl | 1 2 3

High Mass Ladder

Purified empty plasmid

Figure 4.6b:

Figure 4.7a: p53 expression in Saos-2 cells transfected with pBR322-273 before cloning

Saos-2 cells were transfected with pBR322-273, grown on slides and then labelled with p53 antibody, after fixation with paraformaldehyde and permeabilisation using 70% ethanol. Nuclei were counterstained with DAPI which appear Blue under fluorescent light. The nuclei of all cells and therefore the location of all cells can thereby be seen.

Figure 4.7b: p53 expression in Saos-2 cells transfected with pBR322-273 before cloning

The expression of p53 protein, in green fluorescence, can be seen within the same cells as mentioned above. Only two cells express the protein.

FIGURE 4.7: SAOS-2 CELLS TRANSFECTED WITH pBR322-273, BEFORE CLONING.

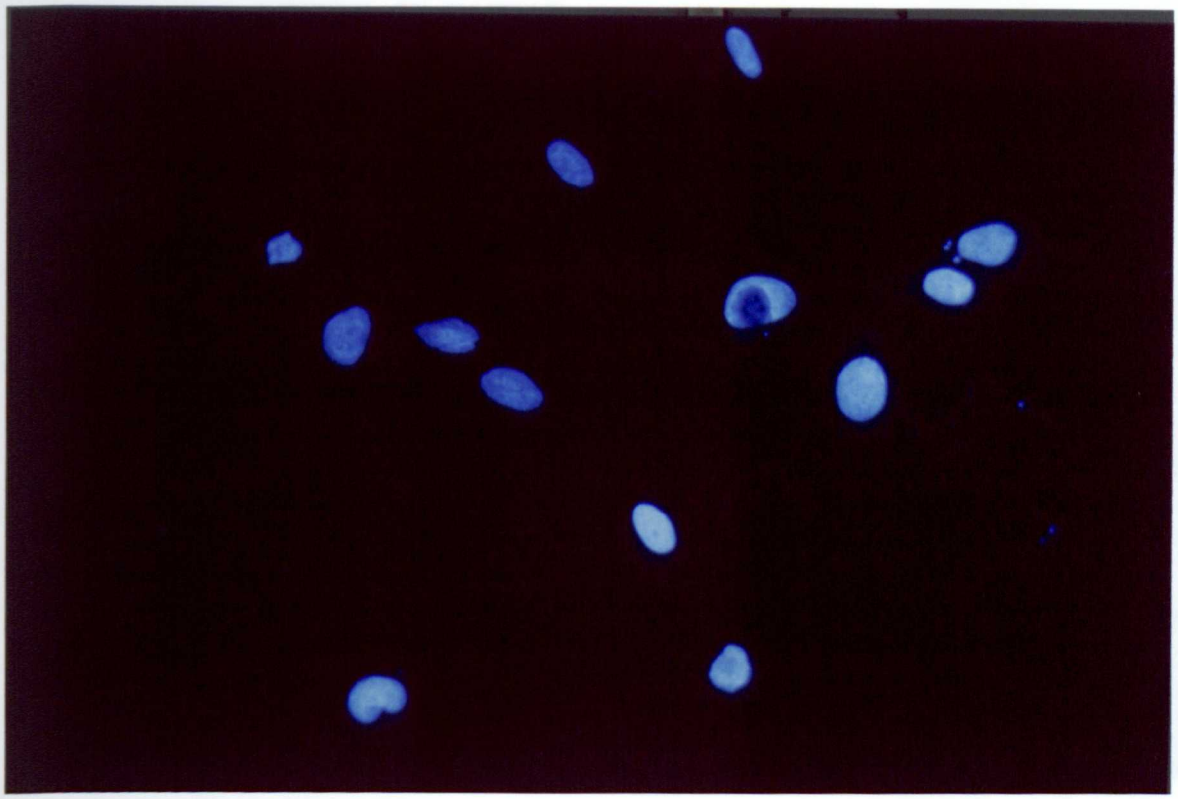


Figure 4.7a: Nuclei Staining

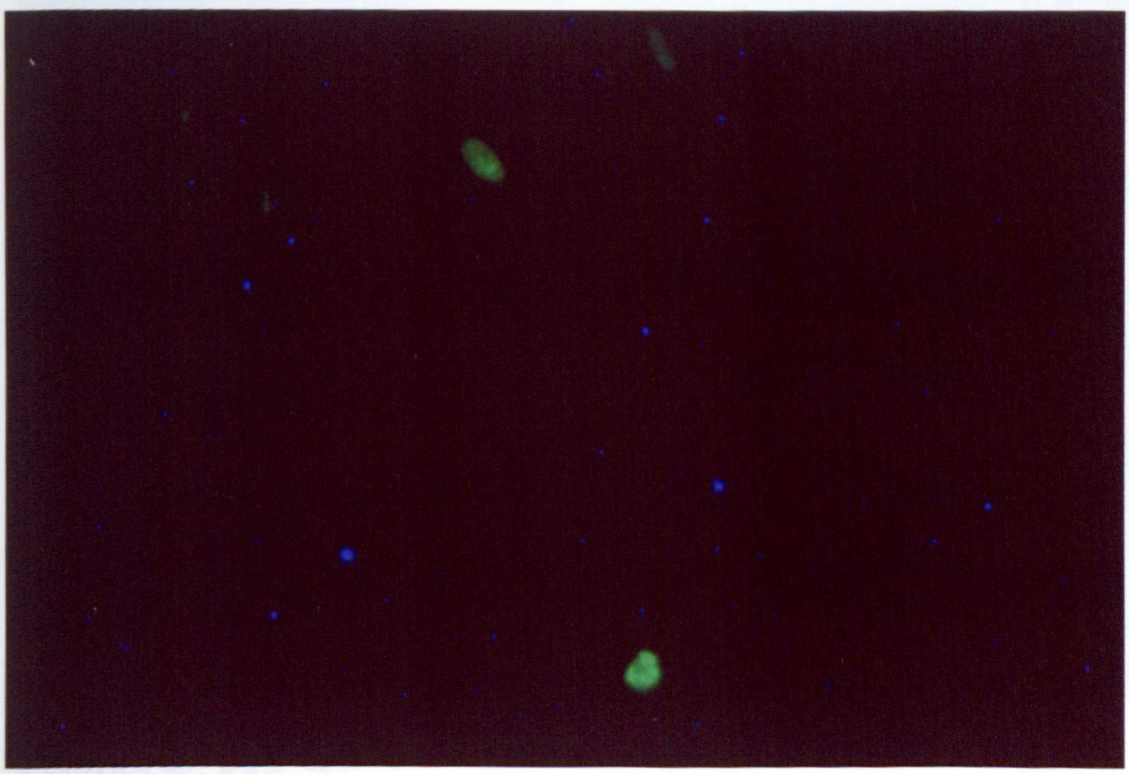


Figure 4.7b: p53 expression (same cells)

Figure 4.8a: Saos-2 cells transfected with pBR322-273 after cloning

Saos-2 cells were transfected with pBR322-273, grown on slides and then labelled with p53 antibody, after fixation with paraformaldehyde and permeabilisation using 70% ethanol. Nuclei were counterstained with diluted ethidium bromide which appear red under fluorescent light. The nuclei of all cells, and therefore the location of all cells, can thereby be seen.

Figure 4.8b: p53 expression in Saos-2 cells transfected with pBR322-273 after cloning

The expression of p53 protein, in green fluorescence, can be seen within the same cells as mentioned above. All cells express the protein.

FIGURE 4.8: SAOS-2 CELLS TRANSFECTED WITH pBR322-273, AFTER CLONING.

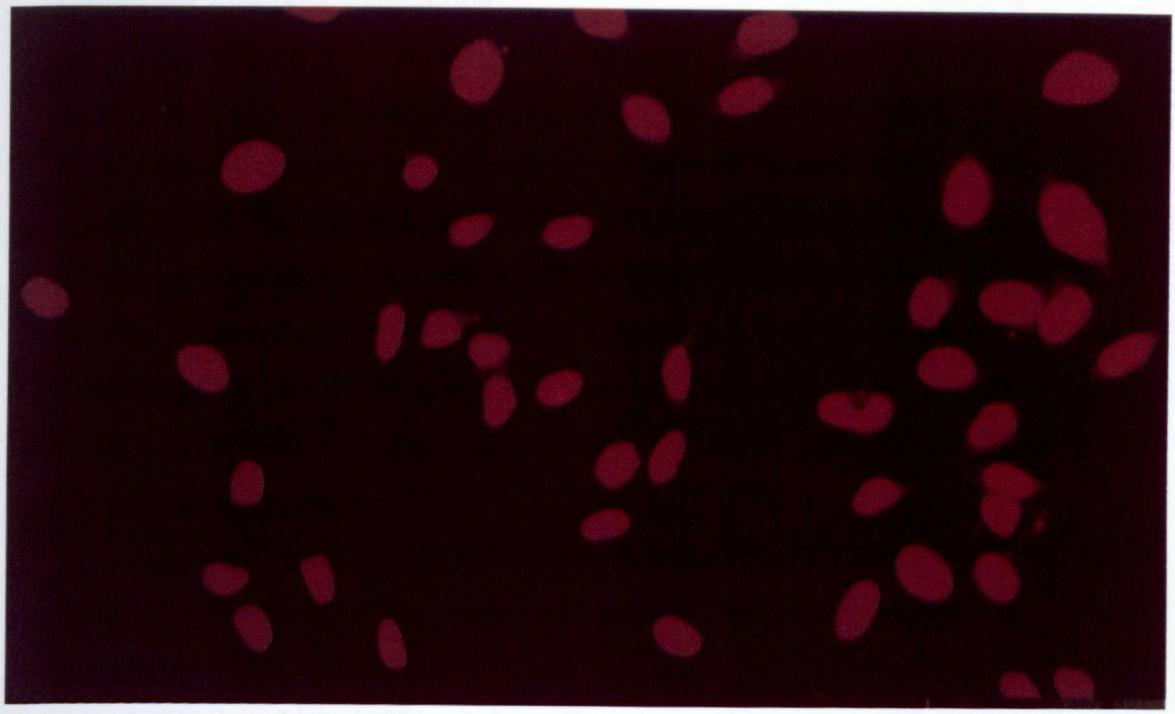


Figure 4.8a: Nuclei Staining

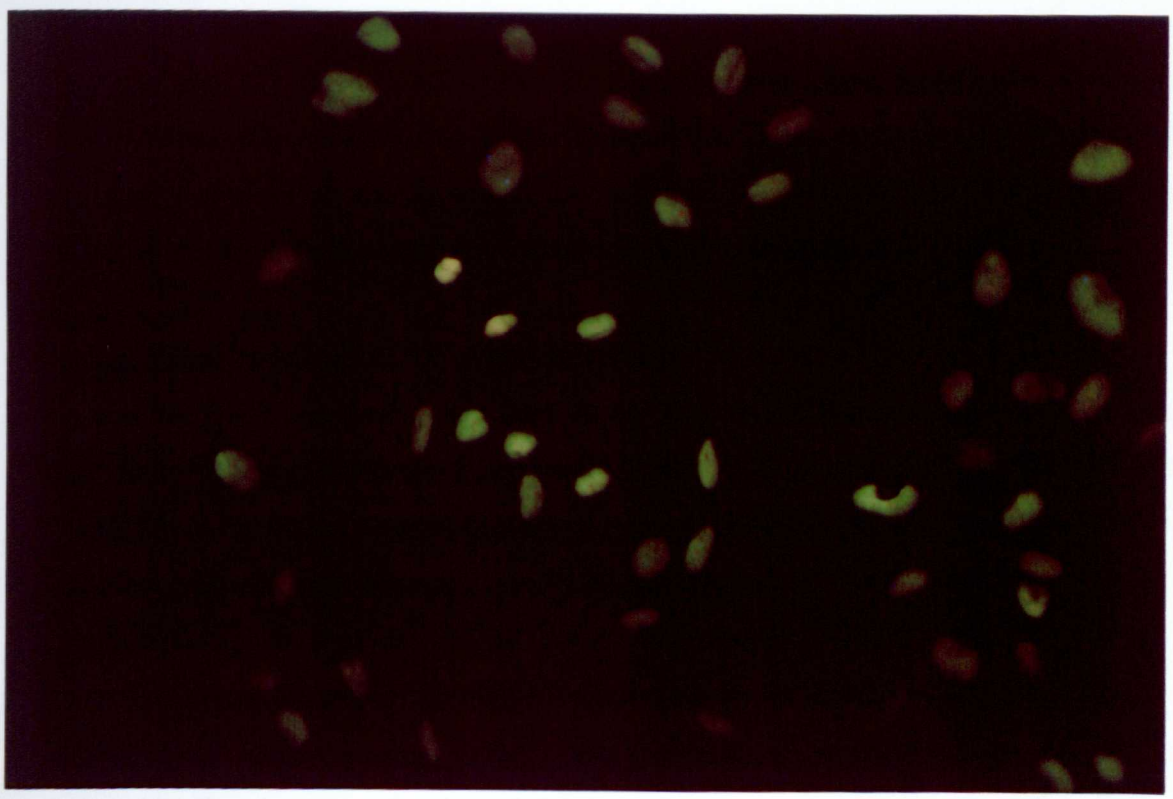


Figure 4.8b: p53 expression (same cells)

passaged 1:10 into selective medium (normal growth medium containing 500µl/ml of Geneticin) 72hr post-transfection.

4.2.3.3 Analysis of p53 Expression in the Stable Transfectants

Stably transfected cells were initially grown as bulk cultures and expanded to $25cm^2$ or $75cm^2$ flasks. To determine whether the p53 mutants proteins were expressed following Geneticin selection, cells were grown in 8-well chamber slides and stained using an indirect immunofluorescence procedure (Section 2.2.7) with p53 specific antibody DO-7, which binds to the 35-45 amino-acids of the N-terminus of the protein and recognises both mutant and wild-type conformations. Stained cells were observed under a fluorescent microscope.

As can be seen from figures 4.7a and 4.7b not all SaOs-2 cells stably transfected with pBR322-273 expressed the mutant p53 protein. The same was found with cells stabely transfected with pBR322-175. Bulk cultures were therefore cloned to obtain a population in which 100% of the cells express the mutant p53 protein. Clones were expanded and tested for p53 protein expression by indirect immunofluorescence as described above.

For the pBR322-273 transfectants, 100% of cells from clone 6 were shown to express p53 protein (figure 4.8a and 4.8b). These results were confirmed by FACS analysis (Figure 4.9) which also indicated the range of intensities of p53 protein expression. Similar results were obtained for the pBR322-175 transfectants (Figure 4. 10).

4.2.4 Transfection of A431 and SkBr3 Cells

Since the A431 and SKBr3 cell lines do not express HLA-A2 at their surface as shown by flow cytometry (Figures 4.13c and 4.13e) it was necessary to transfect these cells with HLA-A2 cDNA to enable them to present the HLA-A2 p53 peptides. Initial pilot studies showed that liposome-mediated transfection of A431 and SKBr3 cells was inefficient, in comparison to its ability to transfect SaOs-2 cells (Section 4.2.3). Lipofectamine, lipofectin, DMRIE-C and CellFectin were unable to mediate transfection of pCMVβ reporter plasmid to these cells (no cells stained positive when incubated with the X-gal substrate; Section 2.2.6.3). Electroporation-mediated transfection was therefore used as an alternative method of transfection.

4.2.4.1 Optimisation of Electroporation Procedure

As described in Section 4.1.1.2, voltage is the most critical parameter to obtain efficient transfection by electroporation. The capacitance was set at 960μFD and various voltages were tested to determine which voltage was the optimum for transfection of these cell types. With the apparatus available the pulse-time could not be pre-set, but rather varied according to the voltage, volume, or ionic strength of the solution used. To optimise transfection conditions for A431 and SKBr3 cells, the pcDNA3-HLA-A2 plasmid was itself used as a reporter plasmid since it was readily detectable at the cell surface by flow cytometry. 48 hrs after transfection cell surface expression of transfected HLA-A2 was detected by indirect immunofluorescence staining using the HB-82 antibody as described in Section 2.2.2.

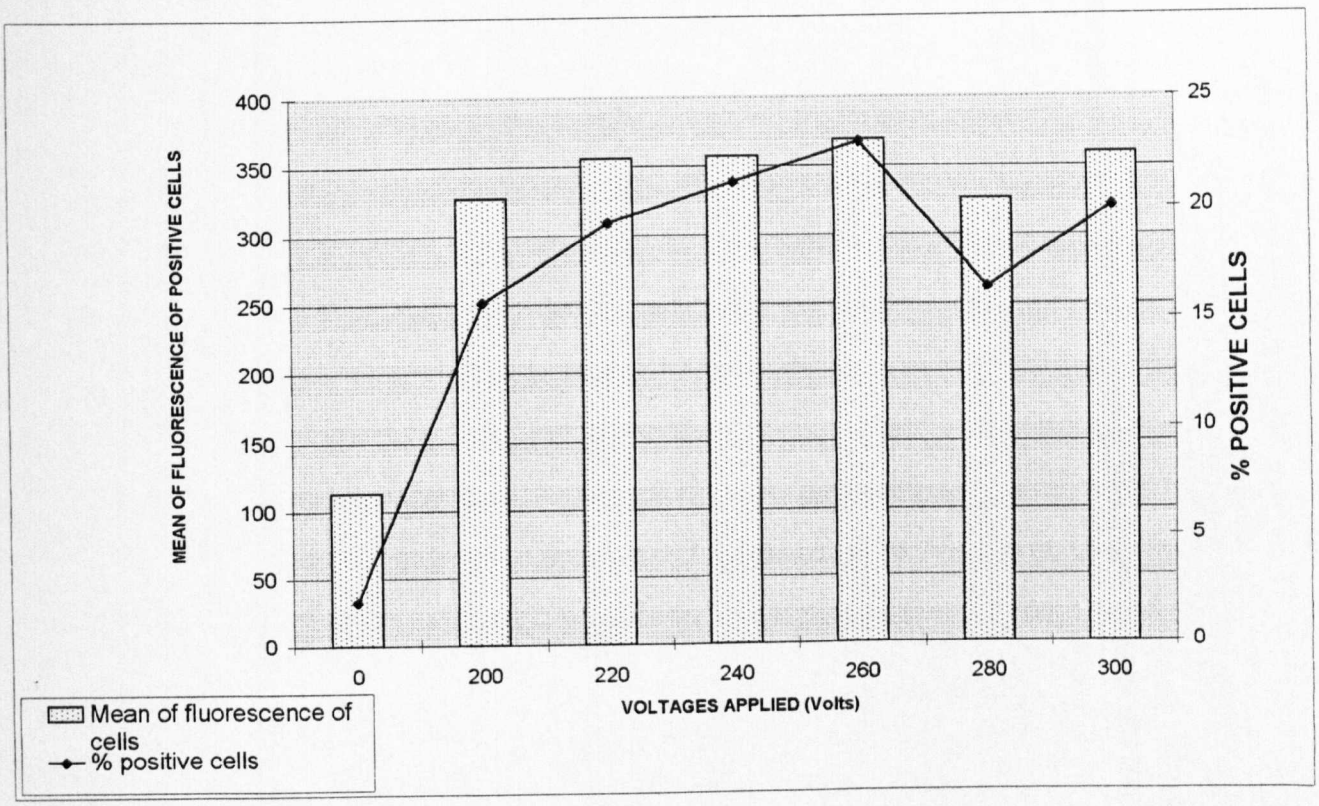


Figure 4.11: Effect of electroporation voltage on the transfection of A431 cells using pcDNA3-HLA-A2.

Cells labelled with HB-82 antibody were subsequently incubated with FITC conjugated goat anti-mouse IgG and analysed by flow cytometry. In order to discriminate between alive and dead cells, propidium iodide was added to the cells after staining just before being analysed by flow cytometry.

As seen from Figure 4.11, the number of transfected A431 cells peaked at a voltage of 260 volts. The level of transgene expression in the transfected cell population indicated by the mean channel fluorescence intensity of cells alive, not stained by prodium iodide (greater than 70%), did not significantly change with the voltage applied. Therefore, since 260 volts was shown to mediate transfection of the highest percentage of cells, this voltage was chosen to stably transfect the A431 cell line.

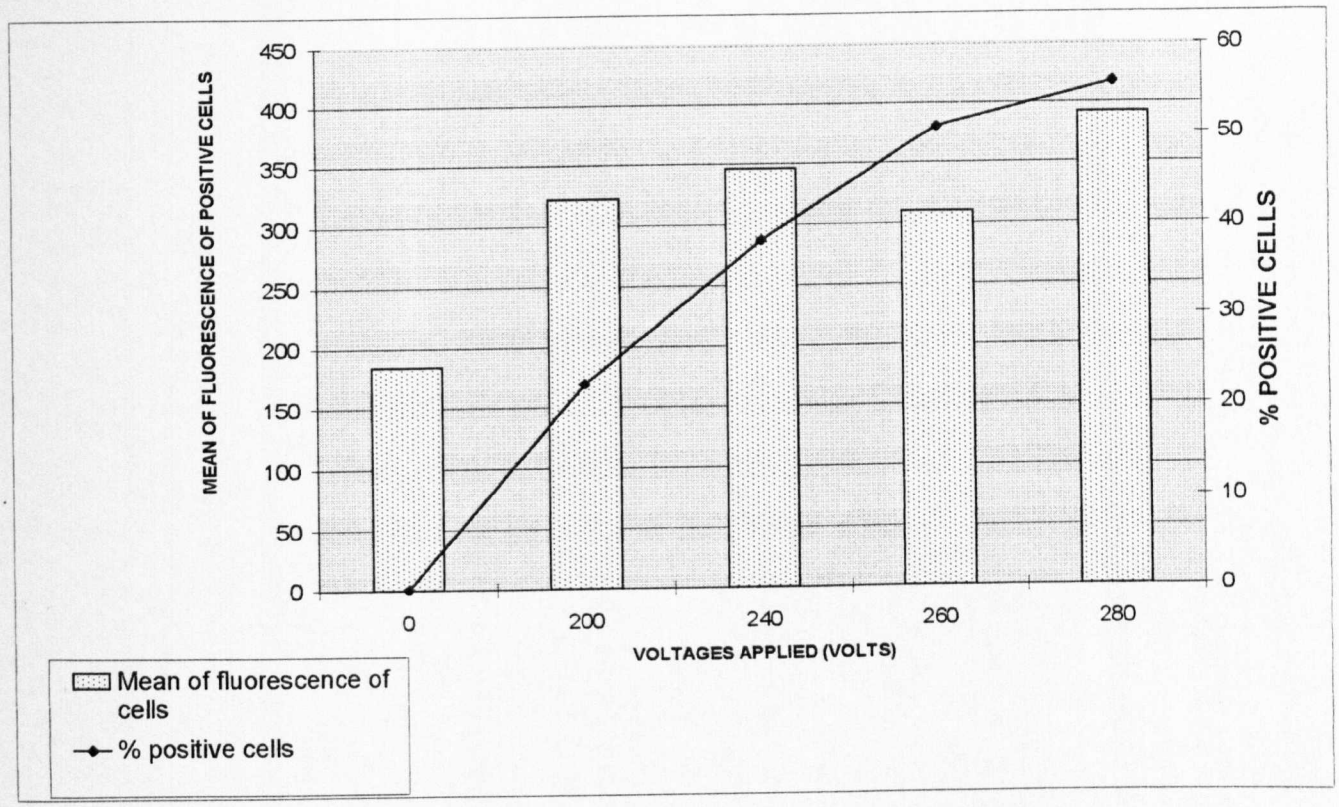


Figure 4.12: Effect of electroporation on the transfection of SKBr3 cells with pcDNA3-HLA-A2.

Cells labelled with HB-82 antibody were subsequently incubated with FITC conjugated goat anti-mouse IgG and analysed by flow cytometry. Untreated cells were used as a negative control for background staining by HB-82. In order to discriminate between alive and dead cells, propidium iodide was added to the cells after staining just before being analysed by flow cytometry.

As can be seen from the results presented in Figure 4.12 the number of transfected SKBr3 cells peaked at a voltage of 280 volts. The level of transgene expression in the transfected cell population, indicated by the mean channel fluorescence intensity of cell

alive (greater than 70%), did not significantly change with voltage applied. Therefore, since 280 volts was shown to mediate transfection of the highest percentage of cells, this voltage was chosen to stably transfect the SKBr3 cell line.

Table 1: Revised Summary Table

Cell Line	P53 Status	HLA-A2 Expression	Transfected with
SaOs-2	Null	+++	1)pBR322-273 2)pBR322-175 3)pBR322 (empty)
A431	Point Mutation 273 (R->H)	-	1)pcDNA3-HLA-A2 2)pcDNA3 (empty)
SKBr3	Point Mutation 175 (R->H)	-	1)pcDNA3-HLA-A2 2)pcDNA3 (empty)

4.2.4.2 Stable Transfection

A431 and SKBr3 cells were stably transfected by electroporation with pcDNA3-HLA-A2 using the voltages determined in Section 4.2.4.1. Immunostaining of cells for HLA-A2 expression 48hr post-transfection indicated that 76.45% of SKBr3 cells and 37.82% of A431 cells expressed the transfected HLA-A2 cDNA (Figures 4.13d and 4.13f). Cells expressing high levels of HLA-A2 were sorted, using a Vantage FACSsort (Section 2.2.2.2) and subsequently selected for stable transfectants by culture in medium containing 200 μg/ml or 300μg/ml Geneticin for A431 and SKBr3 respectively (see Section 4.2.2). Stably transfected cultures were regularly assessed for HLA-A2 transgene expression. A summary of all generated transfectants is presented on Table 1.

Figure 4.9: p53 protein expression in Saos-2 cells untransfected (1), after transfection with pBR322-273 before cloning (2) and after cloning (3).

Cells were fixed with paraformaldehyde and permeabilised with 70% ethanol before the addition of either isotype control (IgG2b) antibody (A) or p53 antibody (DO-7) (B). Cells were then incubated with FITC conjugated goat anti-mouse IgG and analysed by flow cytometry.

Graphs show no p53 protein expression in Saos-2 cells (B1), different population of cells expressing p53 protein in Saos-2 /273 cells before cloning (B2) and a single population of Saos-2 /273 cells expressing p53 protein after cloning.

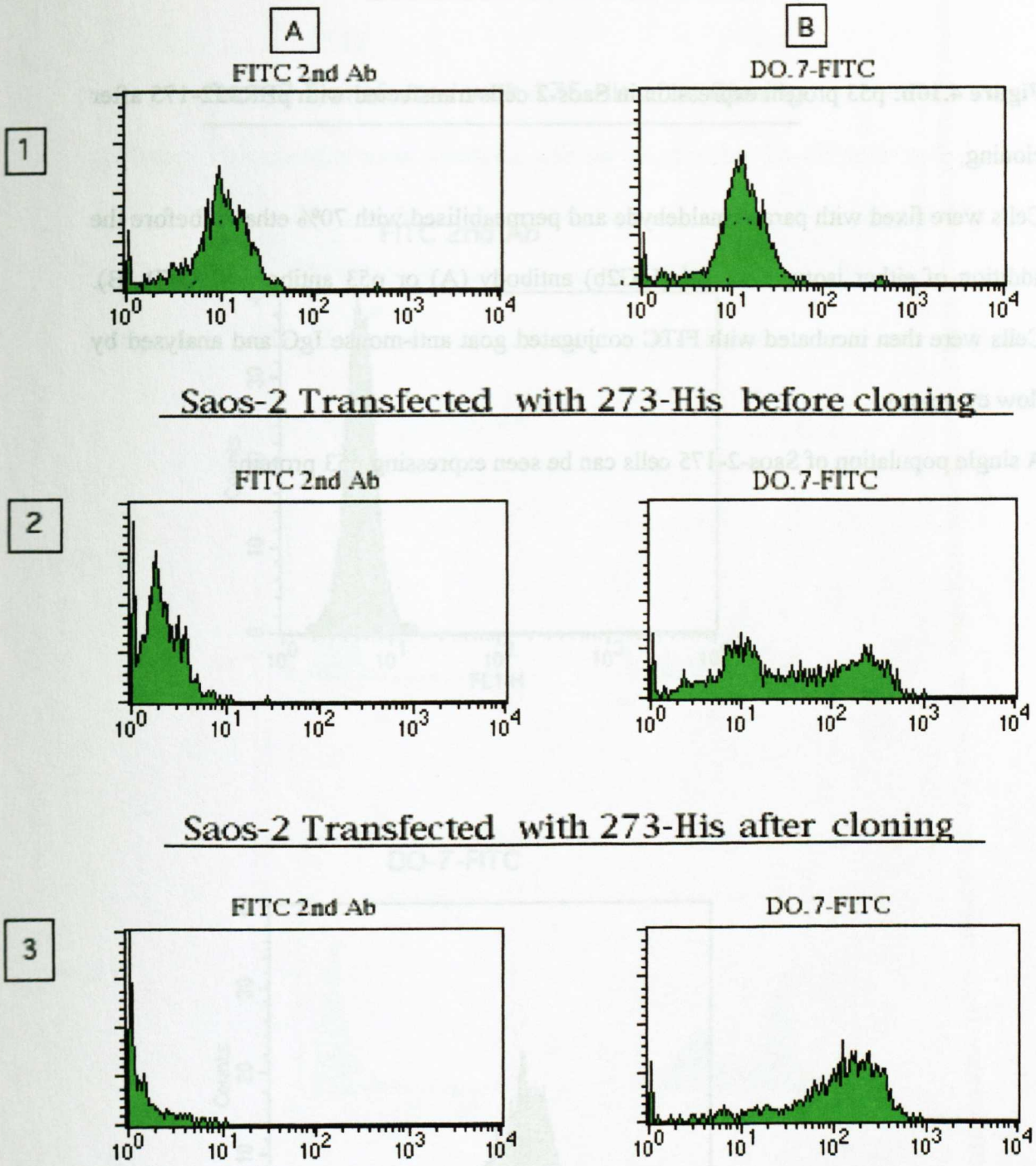

Saos-2 untransfected
A
B
1
FITC 2nd Ab
DO.7-FITC
Saos-2 Transfected with 273-His before cloning
2
FITC 2nd Ab
DO.7-FITC
Saos-2 Transfected with 273-His after cloning
3
FITC 2nd Ab
DO.7-FITC

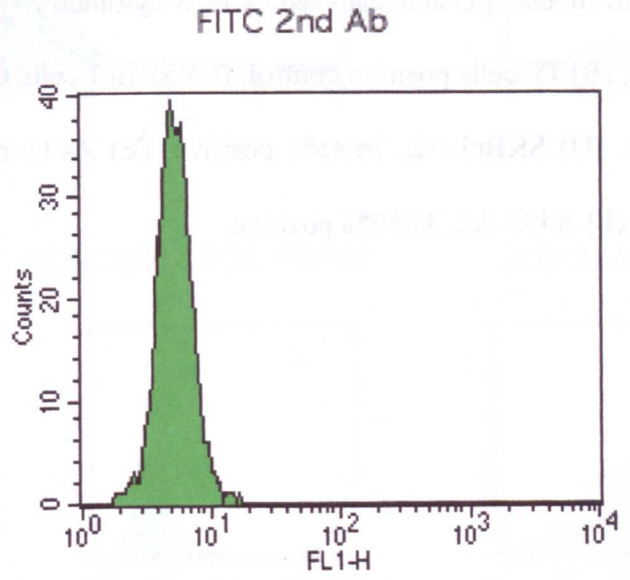
Figure 4.9 :

Figure 4.10b: p53 protein expression in Saos-2 cells transfected with pBR322-175 after cloning.

Cells were fixed with paraformaldehyde and permeabilised with 70% ethanol before the addition of either isotype control (IgG2b) antibody (A) or p53 antibody (DO-7) (B). Cells were then incubated with FITC conjugated goat anti-mouse IgG and analysed by flow cytometry.

A single population of Saos-2-175 cells can be seen expressing p53 protein.

Saos-2 Transfected with 175-His after Cloning



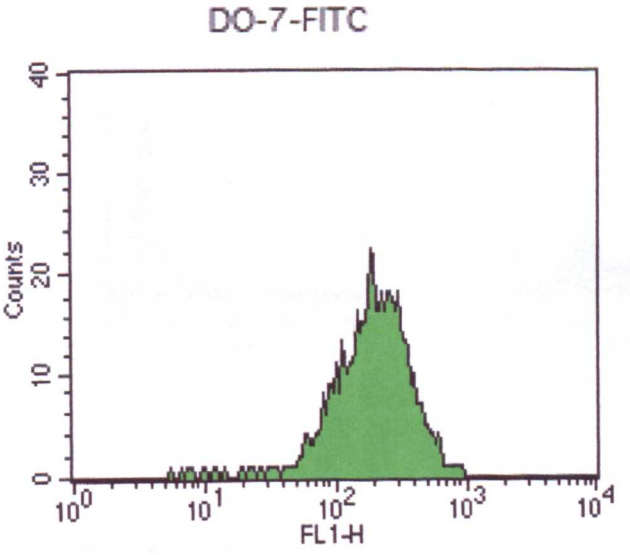


Figure 4.10 :

Figure 4.14: Cell surface HLA-A2 expression on transfected A431 and SKBr3 cells. Cells labelled with HB-82 antibody or no first antibody were subsequently incbated with FITC conjugated goat anti-mouse IgG and analysed by flow cytometry. (A) JY cells no first antibody, (B) JY cells positive control, (C) SKBr3 cells transfected with HLA-A2 vector control, (D) SKBr3-A2, 76.45% positive, (E) A431 transfected with HLA-A2 vector control, (F) A431-A2, 37.82% positive.

FACS SORT FOR HIGH EXPRESSING HLA-A2 CELLS

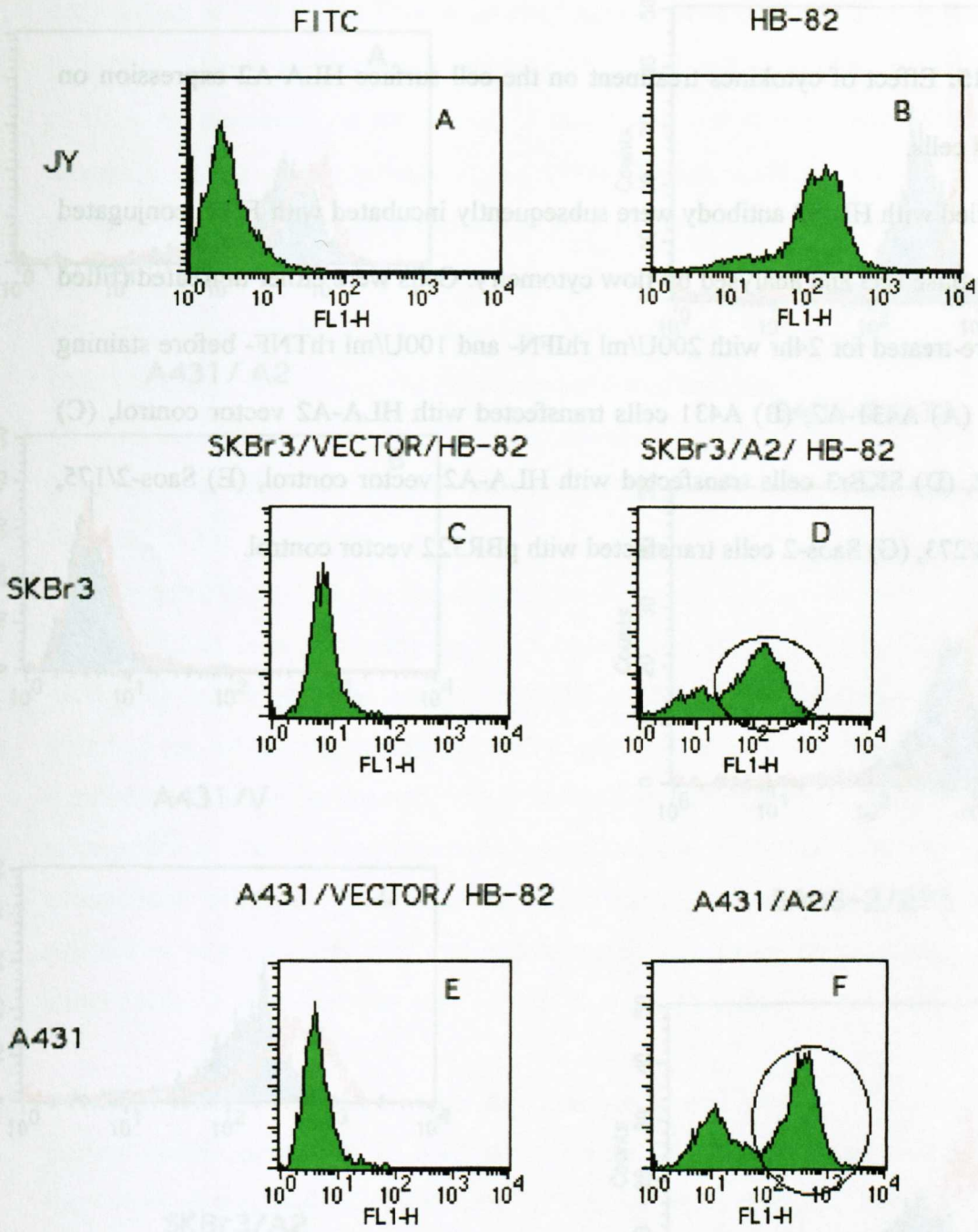


Figure 4.13 :

Figure 4.15: Effect of cytokines treatment on the cell surface HLA-A2 expression on transfected cells.

Cells labelled with HB-82 antibody were subsequently incubated with FITC conjugated goat anti-mouse IgG and analysed by flow cytometry. Cells were either untreated (filled blue) or pre-treated for 24hr with 200U/ml rhIFN- and 100U/ml rhTNF- before staining (red line). (A) A431-A2, (B) A431 cells transfected with HLA-A2 vector control, (C) SKBr3-A2, (D) SKBr3 cells transfected with HLA-A2 vector control, (E) Saos-2/175, (F) Saos-2/273, (G) Saos-2 cells transfected with pBR322 vector control.

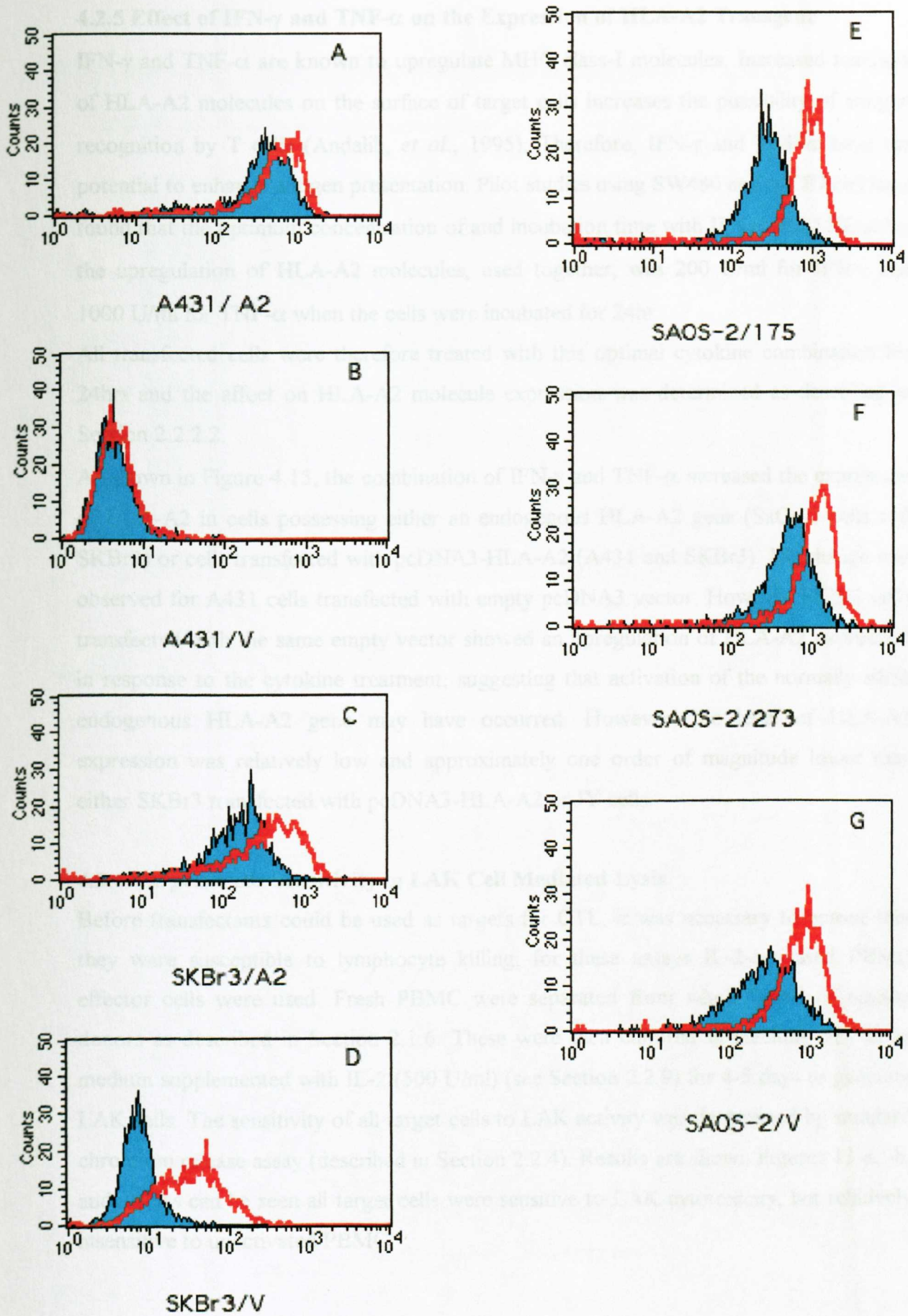

LEVEL OF HLA-A2
A
A431 / A2
B
A431/V
C
SKBr3/A2
D
SKBr3/V
E
SAOS-2/175
F
SAOS-2/273
G
SAOS-2/V
Counts

Figure 4.14

4.2.5 Effect of IFN-γ and TNF-α on the Expression of HLA-A2 Transgene

IFN-γ and TNF-α are known to upregulate MHC class-I molecules. Increased numbers of HLA-A2 molecules on the surface of target cells increases the possibility of antigen recognition by T cells (Andalib, *et al.*, 1995). Therefore, IFN-γ and TNF-α have the potential to enhance antigen presentation. Pilot studies using SW480 and MCF7 cell lines found that the optimum concentration of and incubation time with IFN-γ and TNF-α for the upregulation of HLA-A2 molecules, used together, was 200 U/ml for IFN-γ and 1000 U/ml for TNF-α when the cells were incubated for 24hr.

All transfected cells were therefore treated with this optimal cytokine combination for 24hrs and the affect on HLA-A2 molecule expression was determined as described in Section 2.2.2.2.

As shown in Figure 4.15, the combination of IFN-γ and TNF-α increased the expression of HLA-A2 in cells possessing either an endogenous HLA-A2 gene (SaOs-2 cells and SKBr3) or cells transfected with pcDNA3-HLA-A2 (A431 and SKBr3). No change was observed for A431 cells transfected with empty pcDNA3 vector. However SKBr3 cells transfected with the same empty vector showed an upregulation of HLA-A2 expression in response to the cytokine treatment, suggesting that activation of the normally silent endogenous HLA-A2 gene may have occurred. However, the level of HLA-A2 expression was relatively low and approximately one order of magnitude lower than either SKBr3 transfected with pcDNA3-HLA-A2 or JY cells.

4.2.6 Targets Cells Sensitivity to LAK Cell Mediated Lysis

Before transfectants could be used as targets for CTL, it was necessary to ensure that they were susceptible to lymphocyte killing; for these assays IL-2-activated PBMC effector cells were used. Fresh PBMC were separated from whole blood of healthy donors as described in Section 2.1.6. These were then cultured in medium only or in medium supplemented with IL-2 (500 U/ml) (see Section 2.2.9) for 4-5 days to generate LAK cells. The sensitivity of all target cells to LAK activity was determined by standard chromium release assay (described in Section 2.2.4). Results are shown Figures 13-a, -b, and –c. As can be seen all target cells were sensitive to LAK cytotoxicity, but relatively insensitive to unactivated PBMC.

Figure 4.15: Sensitivity of all target cells to LAK activity

All target cells were labelled with 51Cr and mixed with either IL-2 activated PBMC (noted +) or with non-activated PBMC (noted-) at different effector: target ratios. These were done in duplicate for at least two different donors.

Figure 4.15a: Column blue, yellow and red show the sensitivity of the Saos-2 cells transfected with pBR322 empty plasmid, pBR322-273 and pBR322-175 respectively to IL-2 activated PBMC (+).

Figure 4.15b: Column blue and yellow show the sensitivity of the A431 cells transfected with pcDNA3-HLA-A2 and pcDNA3 empty plasmid respectively to IL-2 activated PBMC (+).

Figure 4.15c: Column blue and yellow show the sensitivity of the SKBr3 cells transfected with pcDNA3-HLA-A2 and pcDNA3 empty plasmid respectively to IL-2 activated PBMC (+).

FIGURE 4.15: Transfected Cells Sensitivity to LAK Activities

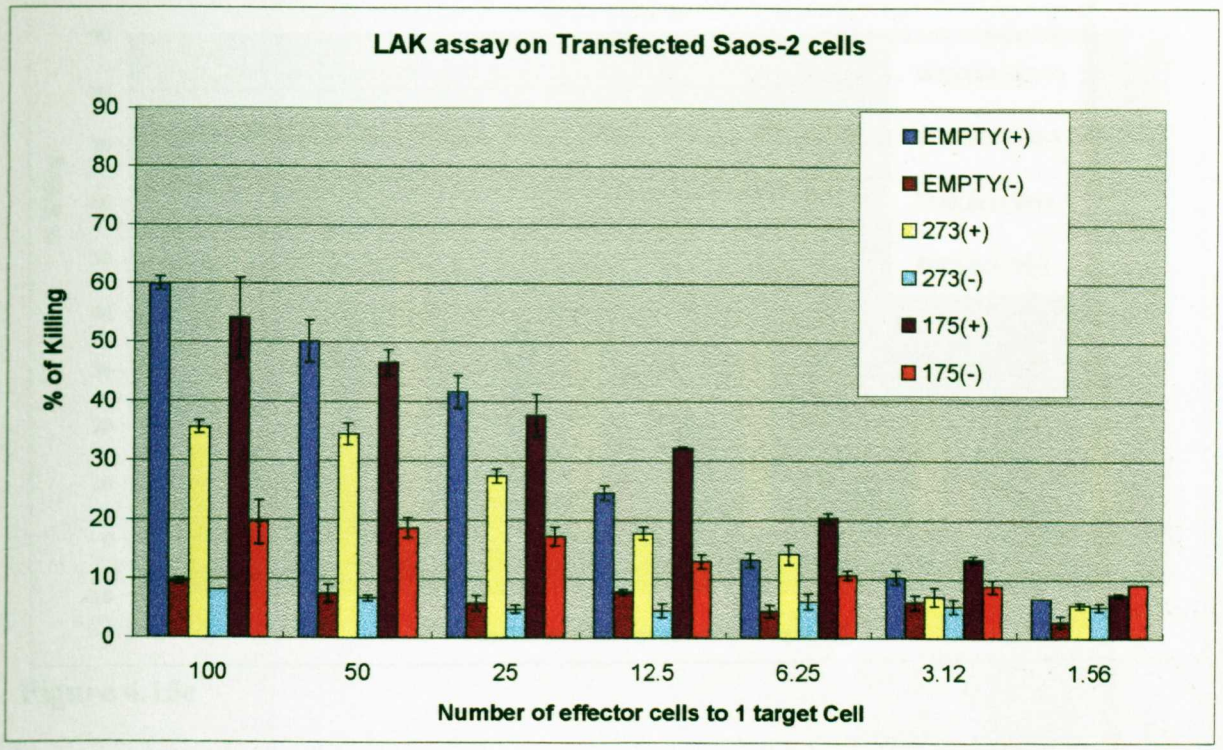


Figure 4.15a

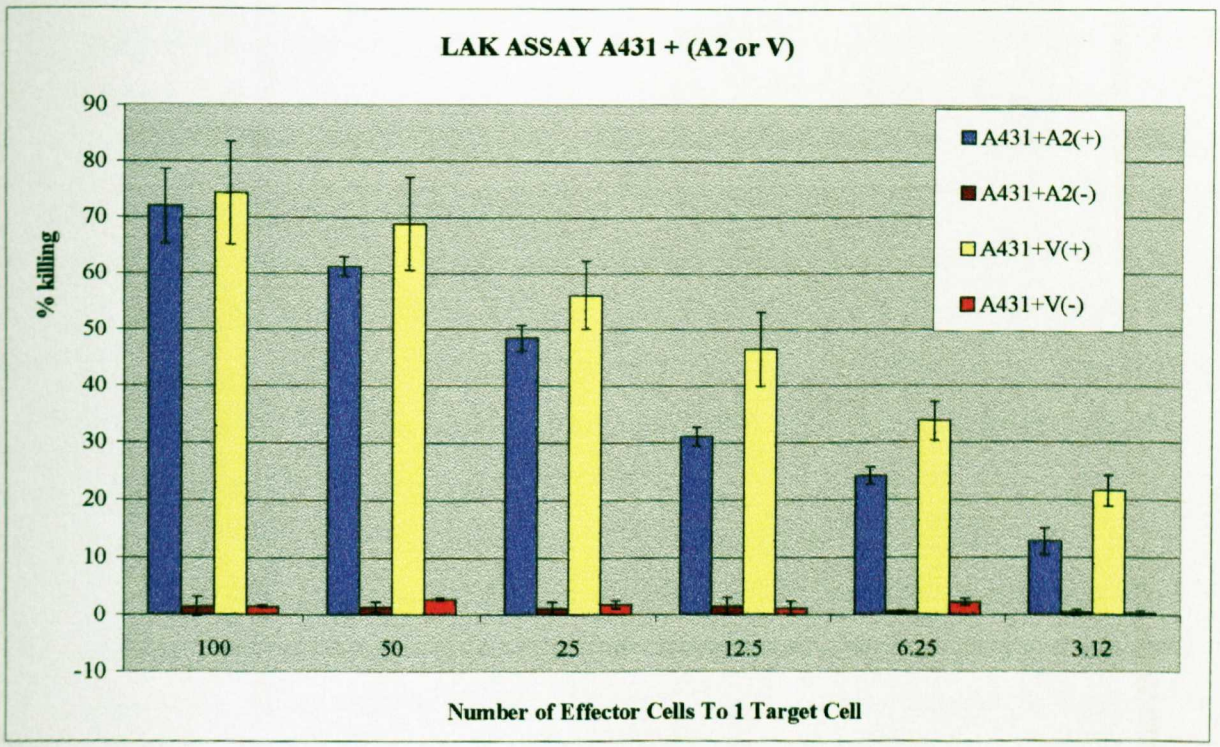


Figure 4.15b

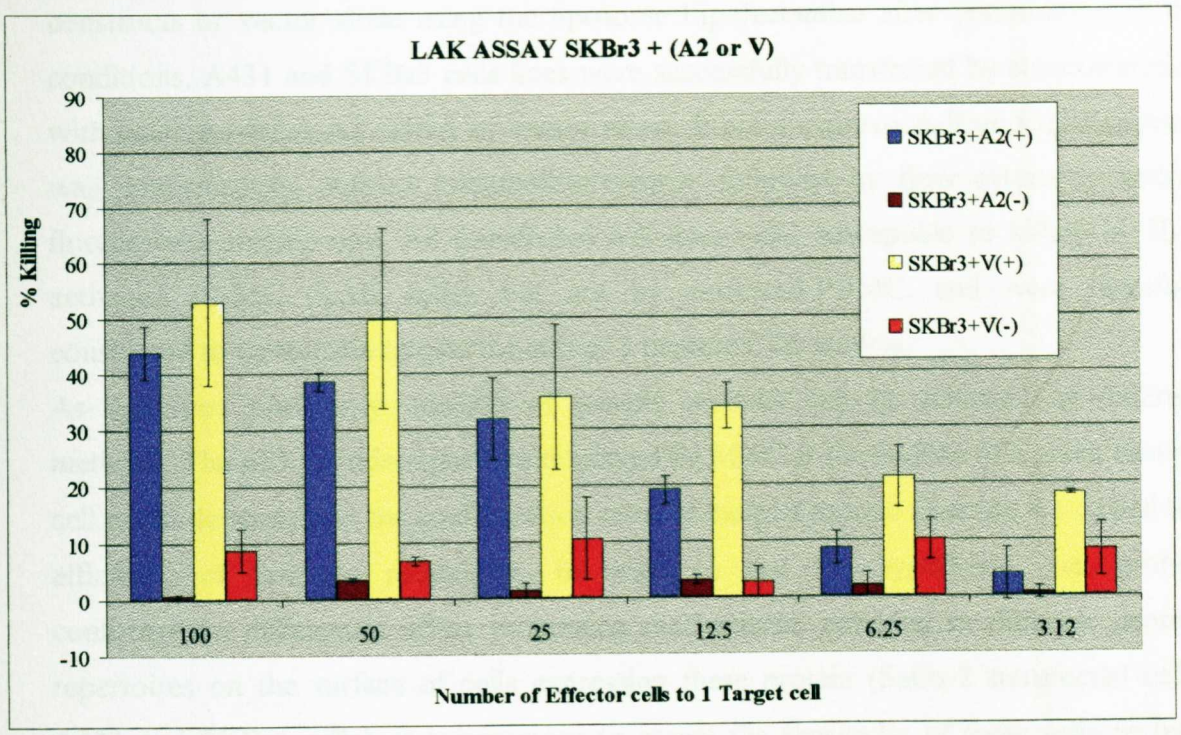

LAK ASSAY SKBr3 + (A2 or V)
% Killing
90
80
70
60
50
40
30
20
10
0
-10
100
50
25
12.5
6.25
3.12
Number of Effector cells to 1 Target cell
SKBr3+A2(+)
SKBr3+A2(-)
SKBr3+V(+)
SKBr3+V(-)

Figure 4.15c

4.3 Conclusion and Discussion

SaOs-2 cells were successfully transfected with pBR322-175 and pBR322-273 p53 constructs or vector alone using the liposome Lipofectamine after optimisation of the conditions. A431 and SKBr3 cells lines were successfully transfected by electroporation with pcDNA3-HLA-A2 cDNA or vector alone. Protein expression from the transgenes was confirmed by indirect immunofluorescence followed by flow cytometry and/or fluorescence microscopy. All transfected cell lines were susceptible to killing by IL-2 activated PBMC (LAK cells), but not by untreated-PBMC, and were therefore considered to be suitable targets for anti-p53 peptide CTL studies.

As described previously, transfer of genetic material can be achieved by different methods. The p53 peptide repertoire displayed by MHC at the surface of a given tumour cell might depend upon the conformation state of the p53 protein (Section 4.1.3) and the efficiency of protease processing. In order to test the hypothesis, that protein conformation differences affect processing mechanisms, resulting in different peptide repertoires on the surface of cells expressing these protein (SaOs-2 transfected cells, A431 and SKBr3 cells), it is necessary to assess the sensitivity of these cells to lysis induced by p53 peptide-specific CTL. This chapter details the preparation and characterisation of these targets.

SaOs-2 cells were successfully transfected with either mutated p53 cDNA or vector only using the liposome-mediated method with Lipofectamin reagent. Wild-type p53 gene could not be transfected into the p53 null SaOs-2 cells because of its detrimental effect on the cell cycle. The p53 protein was expressed to high levels in a cloned line (clone 6) as assessed by immunofluorescence and flow cytometry, and A431 and SKBr3 cells expressing mutant p53 protein (see Section 2.2.6) were successfully transfected with HLA-A2 cDNA using the electroporation since liposome-mediated transfection did not prove to be a successful method for transfecting these cells. Although, non-viral methods can be used to transduce genes for the permanent expression of a given transgene the optimal method remains cell type dependent.

Transfection is a powerful technique, but it is essential to standardise the procedure for a given cell line. A number of parameters need to be determined before the transfer of genetic material can be applied *in vivo*. It is essential to define the target cells as well as efficiency of gene delivery, protein modification, immunological status, and accessibility. In contrast to *in vivo* gene delivery systems, *in vitro* methodology can and has been used

for the successful transduction of genetic information. Antigen presentation capability of tumour cells is very often either impaired or inefficient. By fusing tumour cells with allogeneic, activated dendritic cells, *in vitro*, Geng, *et al.*, were able to stimulate naïve T cells in the primary mixed lymphocyte reaction (MLR) and induce a tumour-specific CTL *in vivo*. These resulting hybrid cells showed to present tumour antigen via MHC class-I molecules to cytotoxic T cells, and at the same time to activate efficiently T helper cells, via allogeneic MHC class-II molecules. More over the first clinical trials with hybrid cell vaccination have provided evidence of tumour regression in renal cell carcinoma and melanoma patients (Kugler, *et al.*, 1998). Therefore by increasing tumour antigen presentation capabilities one improve their immunogenicity. The transfection of dendritic cells with tumour-associated antigens and / or cytokine genes have shown to induce tumour specific cellular immunity both *in vitro* and *in vivo* (Bakker, *et al.*, 1995; Tüting, *et al.*, 1997).

PAGES
MISSING
IN
ORIGINAL

CHAPTER 5: GENERATION OF CTL *IN VITRO*

5.1 Introduction

5.1.1 Cell Mediated Immunity

Immune responses are mediated by a variety of cells and by the soluble molecules they secrete. Although leukocytes are central to all immune responses, other cells in the tissues also participate by signalling to the lymphocytes and by responding to the cytokines released by T-lymphocytes and macrophages. Leukocytes, white cells of the blood, include three groups:

- Lymphocytes: B-cells producing antibodies, T-cells (Th1 and Th2) producing cytokines, Large granular like lymphocytes amongst which the Natural Killer cells (NK cells) producing cytokines.
- Phagocytes: Mononuclear phagocytes (Dendritic cells, Monocytes, Macrophages), Neutrophils and Eosinophils.
- Auxiliary cells: Basophils, Mast cells and Platelets, all producing inflammatory molecules.

One more group can be added: Tissue cells, producing IFN and other cytokines.

Different pathogens will elicit different immune responses involving different cell types. Extra-cellular pathogens and toxins are generally eliminated by antibodies, whereas virally or bacteria infected cells are eliminated by activated-T-lymphocytes: Cytotoxic T-Lymphocytes (CTL).

Virally infected cells and tumour cells bear some resemblance in the way they present antigen to the immune system and one would assume that they would therefore generate similar immune reaction following their appearance in the body. This is, however, not the case and is explained below.

5.1.1.1 Viral Immunity

Viruses are a group of organisms that must enter a host cell to proliferate, since they lack the necessary biochemical machinery to manufacture proteins and metabolise sugars. Some viruses also lack the enzymes required for nucleic acid replication, and are dependent on the host cell for these functions as well. Once inside the cell they take over the cell's own biosynthetic machinery to produce new virus particles that are, for some of them, endogenously processed and presented on the cell surface as peptides complexed with MHC class-I molecules. In doing so, the virus often kills the cell whose content, poured out, may cause some tissue damage. This provides the danger signal

necessary to trigger an immune response. Tumour cells, however, which arise from inappropriate (anarchic) activation or inactivation of genes involved directly, or indirectly, in the control of cellular growth, differentiation, and cell death, grow, as a result, in an uncontrolled manner but with very little, if any, cell damage. Therefore no danger signals are provided to the immune system and leukocytes are neither attracted nor stimulated. As a result of tissue damaged phagocytes are attracted to the injury site and clear up any potentially harmful molecules as well as virus proteins and virus themselves. These are processed and presented to T-helper cells through class-II molecules and to pre-cytotoxic T-cells through the MHC class-I molecules. The two different processing pathways giving rise to peptides associated with either MHC class-I or class-II molecules are explained in detail in Section 1.1.2. Once activated the T-helper cells secrete many important cytokines such as IL-4 and IL-5, which stimulate the pre-activated B-cells to produce antibodies, and IL-2, which is required for activation of CTL. However, resting T-cells which recognise antigen without any 'help' signal become tolerant (Guerder, *et al.*, 1992), and cytokine secretion by T-helper cells into an environment containing no cytotoxic T cells would be redundant. Therefore, for effective CTL priming, this 'help' must be provided in a cognate manner such that both T-helper cell and CTL recognise antigen on the same antigen-presenting-cell (APC) (Keene, *et al.*, 1982; Bennett, *et al.*, 1997). The chance of a rare T-helper cell and equally rare CTL coming in contact with the same APC is very low indeed and moreover this would not explain why some immune responses can be elicited without the need for T helper cells (Buller, *et al.*, 1987).

Matzinger and co-worker (Ridge, *et al.*, 1998), suggested instead a sequential activation mechanism where by T-helper cells deliver a signal that activates the APC by recognising antigen on them. The activated APC would then directly stimulate the CTL. These professionals APC have been identified as being dendritic cells. They take up antigen in the whole body tissues and migrate to lymph nodes where they present antigen to T cell (Steinman, *et al.*, 1991). The molecules responsible for the interaction between the T-helper and dendritic cells are CD40L, a membrane molecule expressed by T cells that have been stimulated, and CD40, a surface receptor expressed on dendritic cells (Banchereau, *et al.*, 1998). Their interaction results in the activation of dendritic cells. As a result their capacity to present antigen and co-stimulatory capacity are increased, rendering them capable of signalling directly, avoiding the requirement for T-helper cells

(Sallusto, *et al.*, 1994; Caux, *et al.*, 1994; Cella, *et al.*, 1996). However, in the case of tumour where tumour-specific-antigens are normally encountered outside any cell damaged or inflammatory context this concept opens new possibilities to elicit potent immune responses. For example by stimulating dendritic cells, using CD40 antibodies, or pulsing them with peptides recognised by specific-T-helper cells, one could boost a normally weak response to tumour-specific antigen (Bennett, *et al.*, 1998; Schoenberger, *et al.*, 1998).

How CTL then kill the infected cells or tumour target is discussed below.

5.1.1.2 How Do CTL Kill?

Two molecular mechanisms of cellular cytotoxicity appear to have evolved independently in lymphocytes. The first one is the granule exocytosis pathway. In this pathway, upon interaction with the target cell, the cytotoxic effector cell releases the content of its cytoplasmic granules in a direct manner into the intercellular space between lymphocyte and target cell. These cytoplasmic granules contain granzymes and perforin. Perforin is a glycoprotein of 534 amino-acids with sequence homology to the membrane attack complex-forming complement C9. In analogy to C9, perforin is able to integrate into the target cell membrane and polymerase in the presence of Ca^{++} forming polyperforin pores comprising 12-18 monomers of 10-20nm diameter. The permeabilisation of the target cell membrane finally leads to the death of the target cell.

Perforin expression is mainly confined to $CD8^+$ T cells, NK cells and $\gamma\delta$ T cells (Smyth, *et al.*, 1990; Nakata, *et al.*, 1990), and the expression level of perforin *in vivo* during a viral interaction correlates with the kinetics of the cytotoxic activity (Mueller, *et al.*, 1989; Young, *et al.*, 1989). The granules-associated serine esterases, called granzymes, and especially granzymes B, enter the target cells through the perforin channel and then initiates apoptosis by cleaving and activating members of the ICE/ Ced-3 family of cell death proteases (Hudig, *et al.*, 1981/1993).

Other effector mechanisms account for lymphocyte-mediated cytotoxicity. These include effector molecules such as TNF (Liuu, *et al.*, 1987) and induction of cell death in the target cell by the interaction of Fas ligand expressed on activated T cells with Fas receptor expressed on the surface of the target cells (Kägi, *et al.*, 1994a/1994b; Lowin, *et al.*, 1994). Fas, also called Apo-1 or CD95, was originally isolated with two independent antibodies that induced apoptosis in various human cell lines (Trauth, *et al.*,

1989). Fas belongs to the tumour necrosis factor (TNF) receptor and nerve growth factor receptor family, and it shares with TNF receptor a conserved extracellular region and a cytoplasmic domain, which is essential for the induction of apoptosis (Nagata, *et al.*, 1995). Dependent on the target cell, $CD8^+$ T cells confer lysis by either a perforin-dependent pathway alone or by the concomitant action of the perforin-dependent and the Fas-dependent pathway.

Perforin-dependent granule exocytosis pathway has been found however to be the main cytotoxic pathway (Kägi, *et al.*, 1994a).

5.1.1.3 Cellular Responses against Tumour *in Vivo*

It is now clear that at least some human tumours can elicit a cellular immune response (Boon, *et al.*, 1994). Lymphocytes derived directly from tumours lesions and draining lymph nodes have been isolated and used in adoptive immune transfer protocols with some success in patients with melanoma and with significant clinical responses in patients with renal cell carcinoma (Rosenberg, *et al.*, 1988; Topalian, *et al.*, 1988; Kradin, *et al.*, 1989). These CTL recognised antigens that are tumour specific and MHC restricted. This restricted specificity is the result of the *in vivo* antigenic priming of the $CD8^+$ cells with antigen expressed on the tumour they infiltrate (Barth, *et al.*, 1990).

Autologous tumour-specific CTL have indeed been found among TIL, lymph node lymphocytes or PBL-T cells in patients with metastatic melanoma (Topalian, *et al.*, 1989); ovarian cancer (Ionnides, *et al.*, 1991); renal cell carcinoma (Finke, *et al.*, 1992) and many other cancers. Their isolation and manipulation ex-vivo has allowed the discovery of many antigens (see Section 1.2). Immunotherapies using the patients' own immune system to participate in the elimination of the tumour cells can therefore be used to complement conventional therapies that are unsuccessful for many cancers. Cervical cancer is one example where after optimal primary treatment of low-risk early-stage disease, recurrent disease is found in 15% of the patients (Larson, *et al.*, 1988). More over treatment results of recurrent disease are relatively poor (Lawhead, *et al.*, 1989).

Some tumour variants resistant to TIL have been shown to be present in the tumour population (Topalian, *et al.*, 1990). This represents a critical finding since CTL resistant tumour variants can escape TIL therapy. In addition, freshly isolated TIL have a low cytolytic potential because of the micro-environment of the tumour which profoundly affects the cytotoxic capacity of these immune cells (Miescher, *et al.*, 1986).

Many strategies have since been developed to expand or stimulate *in vitro* these immune cells derived either from TIL or PBMC of the patient. These include the cloning and expansion *in vitro* of therapeutic CTL found in TIL, the stimulation of PBMC from patients using different antigens recognised by more than one HLA-allele (to avoid escape) or using combined MHC class-I and class-II antigens. The search for tumour-specific antigens is still ongoing and a number of potential candidates have already been found, especially in melanoma, but also in other tumours. These are reviewed in detailed in Section 1.2.

5.1.1.4 Cancer Vaccines Prospects

A tumour vaccine is aimed at activating or boosting weak immune responses against tumour-antigens to which the immune system has, in most cases, already been exposed, and should lead to tumour rejection. It must be safe, must produce immunity in a very high proportion of the people to whom it is given and must be cheap. A successful vaccine will generate long-live immunological memory, meaning that the vaccine must prime both B and T-cells.

There are, mainly, two types of tumour vaccines, (1) cell-based cancer vaccines, or (2) peptide-based vaccines. These can either be used to stimulate the patient's own immune system to eradicate the tumour (Active immunotherapy) or to directly fight the tumour (Adoptive immunotherapy). Active immunotherapy requires that the patient is immunologically competent and that tumours can be selectively recognised by the immune system. On the other hand adoptive immunotherapy main problem is to generate sufficient numbers of cells with the correct homing ability. Cell based-vaccines have originally consisted of killed tumour cells or tumour lysates mixed with adjuvants (Berd, *et al.*, 1990). In most cases these did not induce strong tumour-specific immunity due to the inadequate co-stimulation on activation of tumour-specific T-helper cells (Kern, *et al.*, 1986). It was therefore thought that their antigen presentation could be enhanced by genetically modifying them. So by transfecting irradiated tumour cells with co-stimulatory molecules such as B7 molecules or with cytokines genes such as IL-2 or GM-CSF, $CD8^+$ cells could directly be stimulated without the need of APC or T-helper cells (van Elsas, *et al.*, 1997; Gansbacher, *et al.*, 1990; Fearon, *et al.*, 1990; Townsend, *et al.*, 1993). In this approach, however, the antigen content as well as cytokine level produced and secreted are unknown and there is the problem of inherent genetic and

phenotypic diversity resulting from the *in vitro* culture of tumour cells. In addition the method is patient-specific and therefore costly and labour intensive. Since MHC compatibility between patient and tumour is not required, one way to obviate the need for culture or transduction of each patient's tumour cells, is to use allogeneic tumour cell lines as vaccines (Toes, *et al.*, 1996).

When tumour antigens are known, peptide-based vaccines can be administrated together with appropriate vehicle (adjuvant) to the patient. This has indeed proved very promising with patients with advanced melanoma (Marchand, *et al.*, 1995). Many different adjuvants have been tried, amongst which are complete or incomplete Freund's adjuvant, peptide conjugated with KLH (Keyhole Limpet Hemocyanin) or BCG, all of them aiming at boosting the immune response by stimulating the T-helper cell. More recently, approaches to immunise without the use of adjuvants include the use of dendritic cells. These have successfully been used as vehicle to transport and present tumour antigen directly to CTL *in vivo*. Dendritic cells were either pulse with known immunogenic peptide, unfractionnated acid-eluted peptides or with tumour lysates. Peptide-pulsed dendritic cells resulted in the generation of CTL-mediated cytoxicity against peptide-positive tumour targets providing a complete and long lasting protection against challenge (e.g. epitope from the E7 protein, De Bruijn, *et al.*, 1998; mutated p53 peptide, Gabrilovich, *et al.*, 1996). These were also effective at rejecting tumour, even when the tumour was already established such as 3LL lung cancer (Mayordomo, *et al.*, 1998). As stated before active immunotherapy depends upon the intact host immune system, which is frequently not the case. In adoptive immuntherapy however CTL are generated *in vitro* and their clear sensitisation and specific cytotoxic abilities towards tumour cells only can be demonstrated. Successful adoptive immunotherapy have been reported in immuno-suppressed persons where EBV-specific CTL were generated and a response to the virus for up to eighteen month was detected (Heslop, *et al.*, 1996). Good clinical response rates (33%) was observed in patients with advanced melanoma when TIL (Tumour Infiltrating Lymphocytes) were cultured with low doses of IL-2 before re-infusion (Arienti, *et al.*, 1993). These responses were found to correlate with tumour-lysis by TIL *in vitro*. The way TIL are cultured before reinfusion may greatly influence their composition and function, and therefore therapeutic efficacy. IL-2 in combination with IL-12 *in vitro* strongly enhanced the development of tumour-specific $CD8^+$ CTL and prevented overgrowth of non-specific, LAK cells as reported for TIL derived from

breast cancer, renal cancer and neuroblastoma (Kuge, *et al.*, 1995).

Combined sequential active then adoptive immunotherapy may be another approach to boast responses the tumour. Chang, *et al*, 1997, started by irradiated autologous tumour cells and injected them to the patients with BCG. A week later, draining lymph node cells were isolated and reactivated by culturing them with CD3 mAb and IL-2 and finally reinfused to the patients, partial and complete responses were so obtained.

Different APC have been used for the generation of CTL *in vitro* and these are described below.

5.1.2 Generation of CTL *in Vitro*

Peptides that have been found to bind to HLA-A2 molecules, with high affinity, need to be tested for their capability of inducing a CTL response *in vitro* before they can be considered as potential candidate for immunotherapy. The primary stimulation of CTL *in vitro* is important and requires correct presentation of the peptide as explained in Section 5.1.1.2. Many different techniques have taken advantage of different cell types for the induction of CTL *in vitro* some of them are reviewed below.

5.1.2.1 Using T2 cells

T2 cells are lymphoblastoid, HLA-$A2^{+}$, cells that are deficient in antigen processing (see Section 3.1.2.2). Their inability to process and present their own peptides but on the other hand their capacity to present empty HLA-A2 molecules that can be stabilised by exogenously added peptides has been used to study *in vitro* binding capacity of different peptides. Their potentially high number of MHC class-I molecules on their surface made them good APC candidate for the induction of CTL *in vitro*. T2 cells loaded with high concentration of p53-peptide were indeed capable of generating stable peptide-specific CTL clones (Houbiers, *et al.*, 1993). Using the same protocol more peptides from the p53 protein capable of inducing CTL and a novel conserved CTL epitope in an HIV-1-seronegative donor were identified *in vitro* (Nijman, *et al.*, 1993; Gnjatic, *et al.*, 1995; van der Burg, *et al.*, 1995). More over CTL generated with the 264-272 peptide from the p53 protein were showed to kill not only peptide-pulsed target as previously reported but also human carcinoma cells overexpressing p53 protein (Röpke, *et al.*, 1996). These results were important since they meant that, as far as p53 protein is concern, using *in vitro* stimulation of CTL with T2 cells as APC natural tolerance could be broken.

As mentioned earlier the main advantages in using T2 cells as APC is their potentially high number of MHC molecules on their surface and the certainty that peptide chosen for the induction of CTL does bind to HLA-A2 molecules. However these cells lack some important co-stimulatory molecules that are necessary for the activation of CTL especially for weaker antigen.

5.1.2.2 Using B Cell Blasts or Whole PBMC

Alternatively B cells, either alone (B cell Blasts) or as part of leukocyte mixture (PBMC) can be used as APC. These have the advantage of expressing co-stimulatory molecule B7.1, which stimulates the capacity of the responder cells to produce IL-2 (van der Bruggen, *et al.*, 1994). B-cells or PBMC pulsed with p53 or MAGE-3 peptides were showed to induce CTL that recognised tumour cells expressing p53 protein and MAGE-3 respectively (van der Bruggen, *et al.*, 1994; Röpke, *et al.*, 1995; Gnjatic, *et al.*, 1998). It seemed therefore unnecessary to specifically isolate B cell Blasts for the induction of CTL *in vitro*.

5.1.2.3 Using Dendritic Cells

It is now clear that dendritic cells play a strategic role in the control of immunity and their use in the induction of CTL *in vitro* has become crucial. They, indeed, combine advantages of both T2 cells, with their large number of MHC-peptide complexes at their surface, and B cells, expressing many accessory molecules that enhance adhesion and signalling (co-stimulation) of T-cells (Inaba, *et al.*, 1997; Caux, *et al.*, 1994). In addition they secrete high levels of IL-12, known to increase both innate (natural killer) and acquired (B and T cells) immunity (Cella, *et al.*, 1996). The presence of dendritic cells in small numbers, within whole blood as well as the lack of specific markers has, until recently, limited their use. Advances in methods to generate large numbers of DC from precursors in blood or bone marrow have overcome these limitations. Several methods of maintenance and *in vitro* expansion of DC have been described involving culture in GM-CSF and/ or other cytokines such as IL-4, TNF and stem cell factor (Romani, *et al.*, 1994). DC represent the most potent APC known so far and one dendritic cell is capable of stimulating up to 3000 T-cells (See Section 5.2.2.2). Another important characteristic of dendritic cells is their ability not only to present exogenously added peptides but also actively pick up and process exogenous antigen, and presenting them through the MHC-

class-I pathway (Shen, *et al.*, 1997). More over it has been shown that they can acquire antigen from apoptotic cells and induce class-I restricted CTL (Albert, *et al.*, 1998); explaining the correlation with the number of dendritic cells found in tumour and good prognosis for the patient (Becker, 1993).

Dendritic cells pulsed with either peptide or unfractionated tumour-derived peptide induced not only *in vitro* antigen-specific immunity but also clinical responses (Hsu, *et al.*, 1996; Nair, *et al.*, 1997; Nestle, *et al.*, 1998).

5.1.3 Aims

The aim of this study was to assess the ability of the p53 peptide 217-225 to generate peptide-specific CTL *in vitro* capable of killing first peptide-pulsed target then tumour cells expressing HLA-A2 'naturally' or transfected with the gene and overexpressing mutated p53 protein either 'naturally' or transfected with mutated p53 gene.

Of particular interest was the potentially different processing pathways of the p53 protein depending on its conformation (mutant or wild-type) and the expression and recognition by these CTL of peptide 217-225 and 264-272.

p53 peptide 264-272 was used as positive control for the generation of CTL *in vitro* once optimum conditions were found.

5.2 Results

5.2.1 Generation of CTL Using Whole PBMC

PBMC isolated from healthy donors (see Section 2.2.3.1) were separated and cultured as described in Section 2.2.3.3. Briefly, PBMC were cultured with 50μg of peptide in AIM-V medium containing IL-7 and KLH. On day 3 IL-2 was added to the cultures. On days 7 and 14 responder cells were restimulated with peptide-pulsed and Mitomycin C treated autologous PBMC in AIM-V medium containing IL-2. On day 21 the cytolytic activity of the generated CTL cultures was assessed in a standard 4hr ^{51}Cr release assay as described in Section 2.2.4.

The first experiment to generate peptide specific CTL utilised whole PBMC. A number of conditions (culture and cyto-assay) were then assessed, in order to improve the sensitivity of detection of peptide specific lysis. These conditions included the recognition of target cells by CTL, initial number of potential CTL and promoting the development of killer cells.

5.2.1.1 Cytotoxicity Enhancement Using MA2.1 Antibody

Monoclonal antibodies against HLA-A2 molecules normally inhibit the recognition of target cells by specific CTL. Antibody MA2.1 however not only does not inhibit this recognition but has also been shown to actually enhance it. When target cells were pre-incubated with MA2.1 antibody, followed by incubation with peptide, higher percentage of cells were killed and less peptide were required for the same effect than when targets were not treated (Bodmer, *et al.*, 1989). The antibody seems to enable HLA-A2 molecules to bind peptide at the cell surface more efficiently, thereby stabilising the MHC-peptide interaction. Preliminary experiments were performed in order to determine whether inclusion of the MA2.1 antibody in the cyto-assay could increase the sensitivity of lysis using peptide pulsed T2 or JY cells as targets. MA2.1 was added to the target cells at the time of ^{51}Cr labelling, the cells were washed twice and incubated with the Flu matrix peptide (M58-68). These cells were then added the responder cells at different Effector: Target ratio. The responder cells were CTL generated with the Flu peptide. As can be seen from Figure 5.1, which is a representative of two donors with each ratio performed in triplicate, the level of cytotoxicity was increased by almost 25% when target cells were treated with the antibody. Therefore, in further experiments with T2 or

Figure 5.1: Effect of pre-treatment of target cells (JY) with MA2.1 antibody on the level of cytotoxicity of CTL.

CTL were generated after following stimulation of PBMC from healthy HLA-$A2^+$ donors with Flu peptide and two subsequent restimulations with peptide-pulsed and mitomycin C treated autologous PBMC.

Cytotoxicity of effector CTL was determined by percentage release of ^{51}Cr from ^{51}Cr labelled JY target cells pre-treated with MA2.1 and peptide-pulsed (Red column), pre-treated with MA2.1 but no peptide-pulsed (Yellow column), peptide-pulsed JY cells (Pink column) and JY alone (Blue column).

Figure 5.2: Effect of $CD8^+$ enrichment on the level of cytotoxicity of CTL against JY pre-treated with MA2.1 and +/- peptide-pulsed.

CTL were generated after following stimulation of PBMC from healthy HLA-A2+ donors with Flu peptide and two subsequent restimulations with peptide-pulsed and mitomycin C treated autologous PBMC.

Cytotoxicity of effector CTL was determined by percentage release of ^{51}Cr from ^{51}Cr labelled JY target cells pre-treated with MA2.1 and peptide-pulsed (Red column), pre-treated with MA2.1 but no peptide-pulsed (Yellow column), peptide-pulsed JY cells (Pink column) and JY alone (Blue column).

Cytotoxicity of CTL enriched in $CD8^-$ cells against JY cells pre-treated with MA2.1 and peptide-pulsed (Yellow column), against JY cells pre-treated with MA2.1 (Pink column).

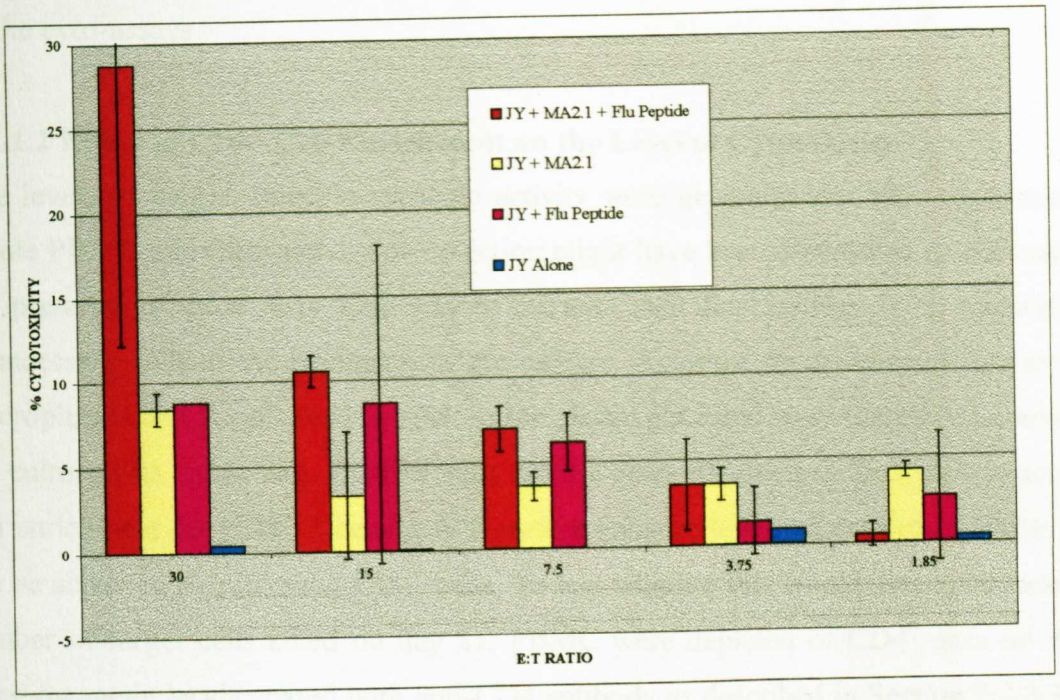


Figure 5.1

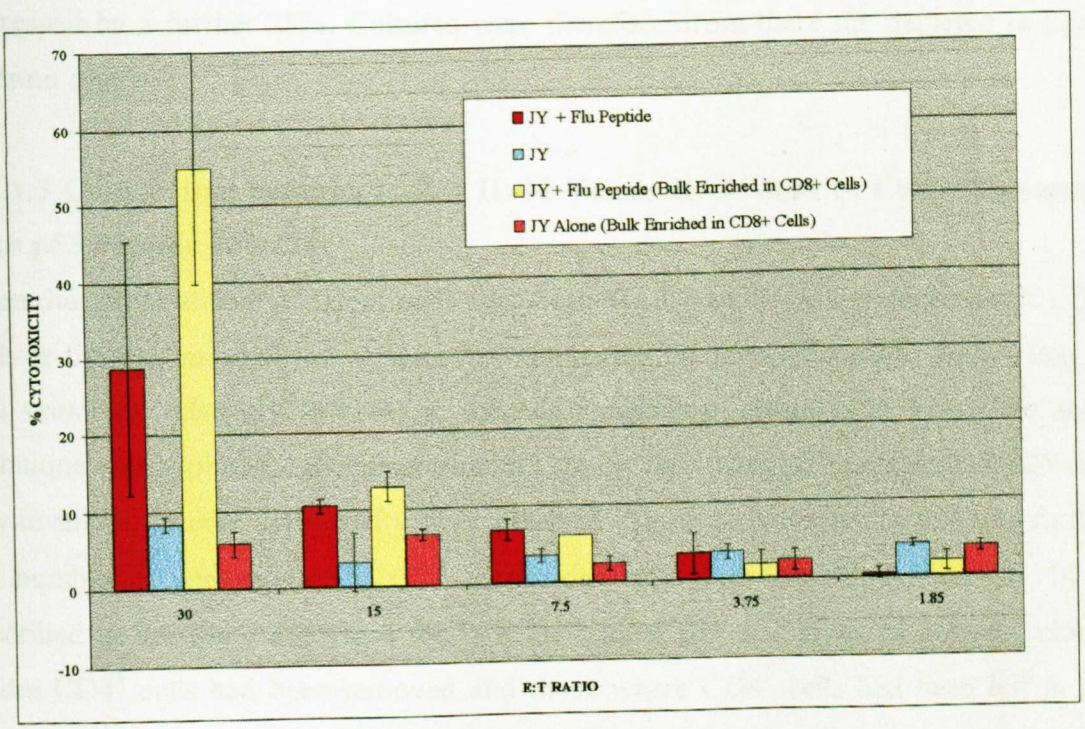


Figure 5.2

JY cells, they were pre-incubated with MA2.1 antibody prior to incubation with peptides in the cyto-assays.

5.2.1.2 Effect of $CD8^+$ Cell Enrichment on the Level of Cytotoxicity

The levels of peptide-specific cytolytic activity were generally low after stimulation of whole PBMC and their activity or detection might have been diminished or obscured by the presence of other cells. One way to increase their final number is by removing all unnecessary cells at the beginning of the culture. Amongst the leukocytes, monocytes, macrophages and B cells tend to stick to the plastic plate and were therefore removed as the culture was expanding. $CD8^+$ T cells are the main effector cell for cytolytic activity, and enrichment for $CD8^+$ T cells may therefore enhance levels of cytolytic activity. This can be achieved by removing $CD4^+$ cells. To test whether this would indeed increase the number of target cells killed on day 21, PBMC were depleted of $CD4^+$ cells on day 7 using magnetic beads coated with anti-CD4 antibody as described in Section 2.2.3.3 day 14. The rest of the CTL generation procedure was followed unchanged as described in Section 2.2.3.3. As can be seen on Figure 5.2, which is a representative of two experiments with each ratio performed in triplicate, the level of cytotoxicity was increased by a further 25%. Cultures were therefore, from there on, depleted in CD4+ cells on day 7.

5.2.1.3 Comparison between IL-2 + IL-12 Versus IL-2 Alone in Clones Generated with p53 Peptide 217-225

Even though treatment of target cells with both MA2.1 antibody and depletion of $CD4^+$ cells at the beginning of the cultures have increased the level of specific cytotoxicity, it still remained relatively low for a secondary response. More over when the same technique was applied for the generation of CTL *in vitro* using p53 peptide 217-225 very low levels of specific cytotoxicity were detected. So, in order to try to increase further the number of peptide-specific CTL, responder cells were semi-cloned on day 14 as described in Section 2.2.3.3 and the level of cytotoxicity was assessed between clones where $CD4^+$ cells had been removed and those where $CD4^-$ cells had been left in the culture. By cloning and removing $CD4^-$ cells, $CD8^-$ cells were, most certainly, lacking the beneficial effects of an important cytokine, interleukin-12, produced mainly by APC

Figure 5.3a: Cytotoxicity of CTL generated in clones against JY cells +/- peptide-pulsed using the 217-225 peptide.

CTL were generated after following stimulation of PBMC from healthy HLA-A2+ donors with 217-225 peptide and two subsequent restimulations with peptide-pulsed and mitomycin C treated autologous PBMC. Responder cells were semi-cloned on day 14. Cytotoxicity of CTL against peptide-pulsed JY cells (Red cloumn), against JY alone (Yellow column). * Clone with specific cytotoxicity (% cytotoxicity of CTL against peptide-pulsed JY cells - % cytotoxicity of CTL against JY cells alone) ≥ 10.

Figure 5.3b: Effect of CD4 depletion on cytotoxicity of CTL generated in clones against JY cells +/- peptide (217-225)-pulsed.

Same as for Figure 5.3a, with however CD4 depletion from the cultures on day 7.

Figure 5.3c: Effect of IL-12 addition in cultures on cytotoxicity of CTL generated in clones against JY +/- pulsed with 217-225 peptide.

CTL were generated after following stimulation of PBMC from healthy HLA-$A2^+$ donors with 217-225 peptide and two subsequent restimulations with peptide-pulsed and mitomycin C treated autologous PBMC. IL-12 was added on day 3 and at every restimulation, responder cells were semi-cloned on day14.

Cytotoxicity of CTL against peptide-pulsed JY cells (Blue column), against JY alone (Red column). * Clone with specific cytotoxicity (% cytotoxicity of CTL against peptide-pulsed JY cells - % cytotoxicity of CTL against JY cells alone) ≥ 10.

Figure 5.3d: Effect of IL-12 addition and CD4+ cells depletion on cytotoxicity of CTL generated in clones against JY +/- pulsed with 217-225 peptide.

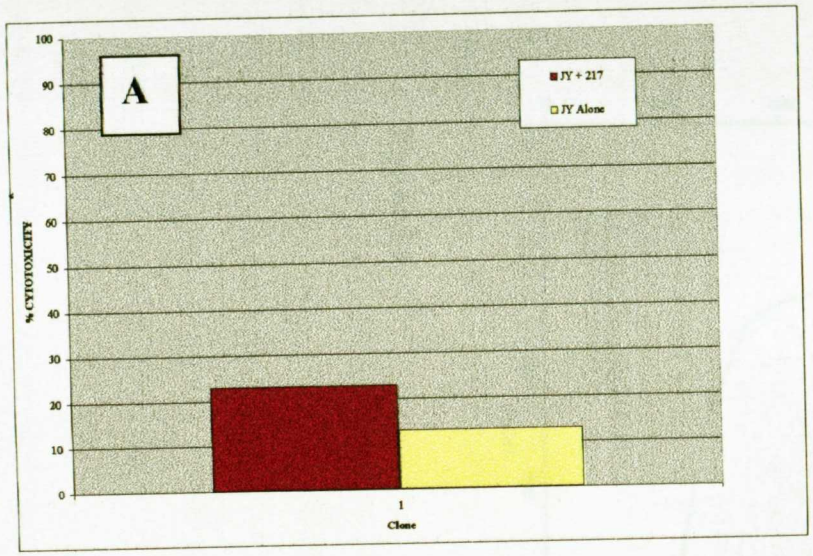
Same as for Figure 5.4a, with however depletion in $CD4^+$ cells from the cultures on day 7.

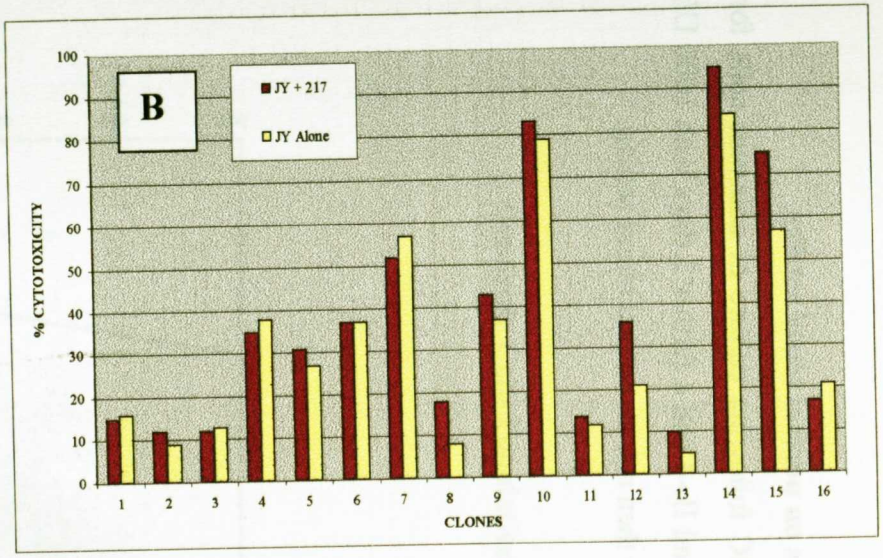
Only 48 wells, instead of 96 (1/2 plate), were viable and tested for cytolytic activities.

No CD4 Depletion

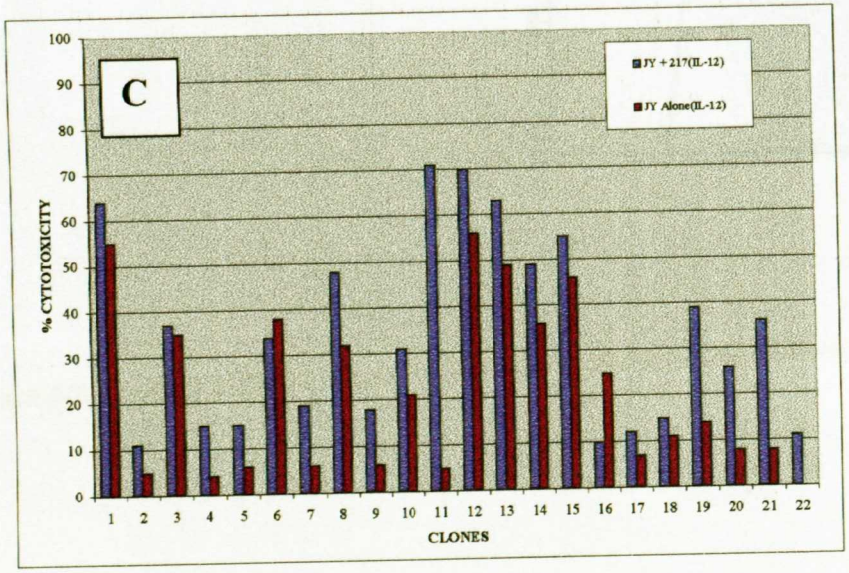
CD4 Removed from cultures

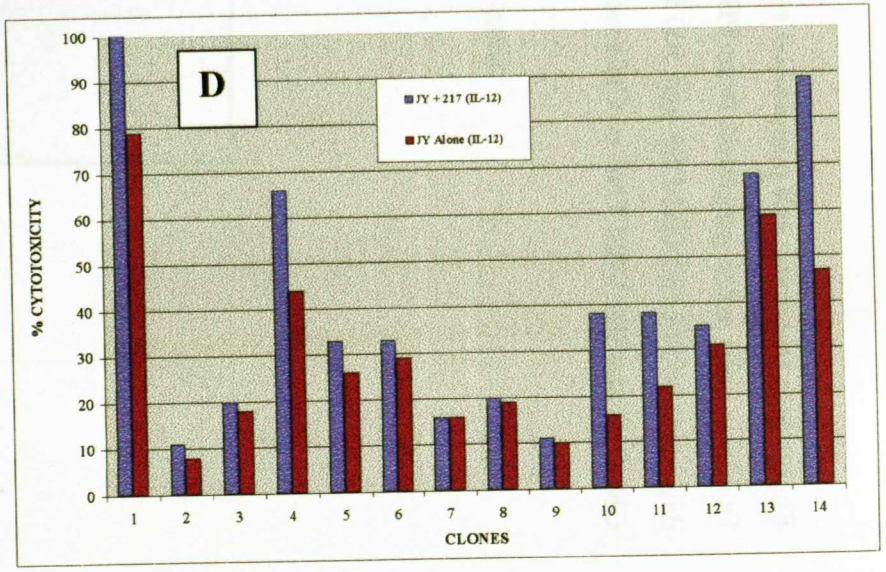
+ IL-2





+ IL-2
+ IL-12





(out of 48 wells only)

Figure 5.3:

Figure 5.4: Dendritic cell population runs on normal blood setting.

Dendritic cells were generated from PBMC following culture of adherent cells for 7 days with 1000 U/ml GM-CSF and 500 U/ml IL-4 and a further 3 days with 1000 U/ml GM-CSF and 100 U/ml TNF-α. Cells were then run on normal blood setting.

Dendritic Cells Population Gated

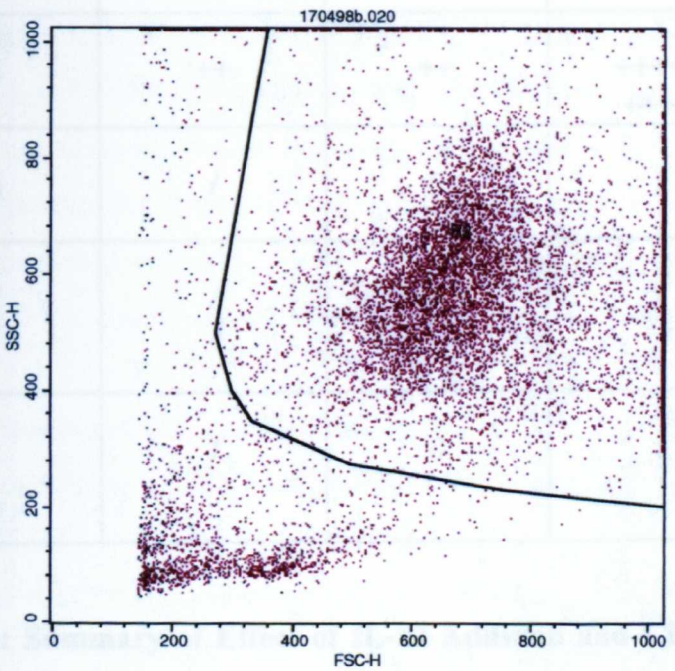


Figure 5.4:

	CD4$^+$ Cells Not Depleted		CD4$^+$ Cells Depleted	
CTL No	**+IL-2**	**+IL-2+IL-12**	**+IL-2**	**+IL-2+IL-12**
12	+	++++++ +++++	++++	+++++ ½ Plate
13	0	0	+	0
14	0	0	0	+++++
15	++	++	+++++ ++++	+++++
16	/	0	/	+
17	/	/	/	+
21	/	0	/	++++ ++

Table 1: Summary of Effect of IL-12 Addition and CD4$^+$ Cell Depletion on Cytolytic Activities of CTL Generated from Different HLA-A2$^+$ Donors PBMC.

CTL were generated after stimulation of PBMC with 217-225 peptide and two subsequent restimulations with peptide-pulsed and Mitomycin C treated autologous PBMC. Responder cells were semi-cloned on day 14.

IL-12 was added to some cultures on day 3 and at each stimulation. CD4$^+$ cells were removed from some of the cultures on day 7 with anti-CD4 coated magnetic beads.

Results show number of positive semi-clones generated.

cells but also by B cells (Stern, *et al.*, 1990; D'andrea, *et al.*, 1992). This cytokine has been shown to directly affect the differentiation of CD4-expressing T-helper cells, both naïve and memory, into IFN-γ-producing Th1 cells when present in the first few days of culture of the clones (Manetti, *et al.*, 1995). IFN-γ together with IL-12 then promotes the differentiation of T-cells into cytotoxic T-lymphocytes (CTL). The effect of the addition of IL-12 on day 3 of the cultures was therefore also assessed.

Using this method it was possible using the p53 217-225 peptide to induce a CTL response as can be seen from Figures 5.3. These CTL were capable of specifically killing peptide-pulsed target cells. More positive clones were generated when cultures were depleted in $CD4^+$ cells (Figures 5.3B and 5.3D and Table 1). The addition of IL-12 not only increased further the number of positive clones (Figure 5.3C in comparison with Figure 5.3A and Table 1) but also increased the level of cytotoxicity (Figure 5.3D).

The results of CTL generation for several donors are summarised in Table 1. Taking all these results together optimum conditions were established when target cells (T2 or JY) were pre-incubated with MA2.1, and when cultures were depleted in $CD4^+$ cells as well as when cells were semi-cloned and IL-12 was added at the beginning of the cultures.

5.2.2 Generation of CTL Using Dendritic Cells

While the conditions optimised above led to the generation of peptide-specific CTL, the numbers of CTL obtained by the semi-cloning method were low and not sufficient to assess their specificity against a wide panel of target cells. In order to generate a large number of peptide-specific CTL it was decided to utilise dendritic cells as APC. During the course of my thesis, methods to generate CTL using dendritic cells were being developed in other laboratories indicating their potential (Bakker, *et al.*, 1995; van Elsas, *et al.*, 1996; Nair, *et al.*, 1997). These represent the most potent APC known so far. Following stimulation they, indeed, express high level of MHC molecules and co-stimulatory molecules and secrete IL-12.

5.2.2.1 Generation and Characterisation of Dendritic Cells

Dendritic cells were generated following Romani, *et al.*, (1994) method (see Section 2.2.3.3). Briefly PBMC were isolated from HLA-A2 positive healthy donors and cultured in RPMI + 10% FCS in T125 flask for 2 hours at 37C. Nonadherent cells were

Figure 5.5a: Dendritic cells: 7 days of culture.

Dendritic cells were generated from PBMC following culture of adherent cells for 7 days with 1000 U/ml GM-CSF and 500 U/ml IL-4. Observed at x100.

Figure 5.5b: Dendritic cells: 10 days of culture.

Dendritic cells were generated from PBMC following culture of adherent cells for 7 days with 1000 U/ml GM-CSF and 500 U/ml IL-4 and a further 3 days with 1000 U/ml GM-CSF and 100 U/ml TNF-α. Observed at x100.

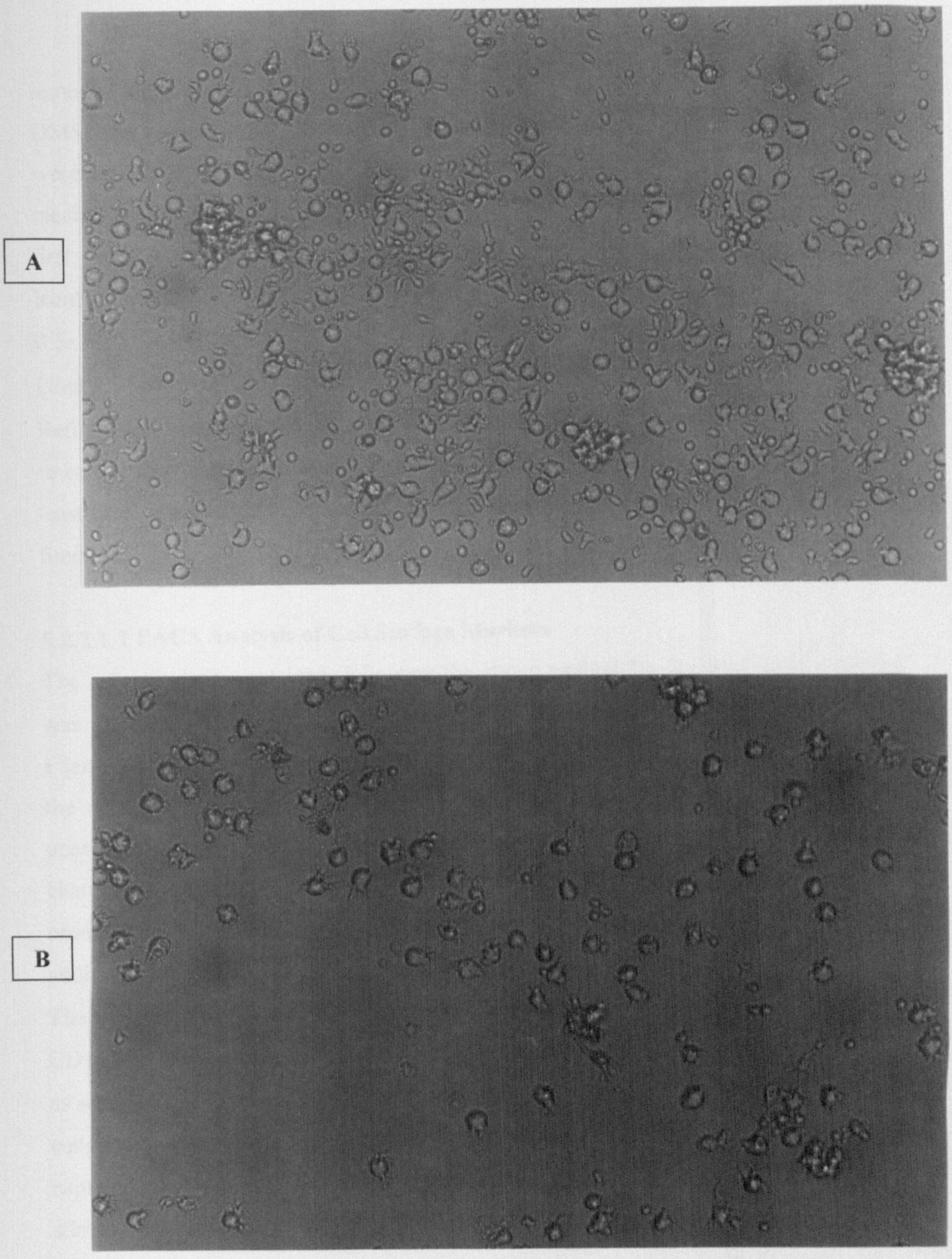


Figure 5.5: Dendritic Cells after A) 7 Days of Cultures and B) 10 Days of Cultures. Magnification x100.

removed and frozen at $1x10^7$ cells/ml in AIM-V medium containing 60% FCS and 10% DMSO (to be used as effector cells in the generation of CTL). The adherent cells, which were used to generate dendritic cells were cultured in RPMI + 10% FCS for 24hrs. Fresh medium containing 500 U/ml of IL-4 and 1000 U/ml of GM-CSF were added to the cells first plated at a high density (day 1) and then plated at a lower density (day 2), and incubated at 37°C (see Section 2.2.3.3). On the fourth day, cells were then plated at $0.5x10^6$ cells/well in 1ml of RPMI +10% FCS containing 1000 U/ml GM-CSF and 100 U/ml TNF-α. Cells were incubated for another three days.

Before these cells could be use as APC in the generation of CTL *in vitro* it was necessary to certify their identity as dendritic cells. Characterisation of dendritic cells is based on at least three criteria, (1) morphology, (2) expression of cell surface markers, and (3) their function.

5.2.2.1.1 FACS Analysis of Cell Surface Markers

The cell population obtained, following the above method for dendritic cell generation, was analysed on the FACS by running on normal blood setting. As can be seen from Figure 5.4 the generated cell population constituted mainly of large cells (cells shifted to the right) and granular cells (cells shifted towards the top of the panel). These also possessed many dendrites and showed typical morphological and phenotypic characteristics of dendritic cells (Figures 5.5). No real morphological differences were observed between dendritic cells observed on day 7 (Figure 5.5a) and on day 10 after addition and culture with maturation factor, TNF-α (Figure 5.5b).

The generated cells were stained for cell surface markers CD3, CD19, CD14, CD54, CD1a, CD86, MHC Class-I and MHC Class-II using FITC labelled goat anti-mouse IgG as second layer (Section 2.2.2.2). A typical result is shown in Table 2. As can be seen the cells did not express T-cell markers (CD3 negative) or B-cell markers (CD19), but did express high levels of CD1a molecules, MHC Class-I and Class-II molecules, co-stimulatory molecules (CD86 and CD80) but no or low level of CD14 molecules.

Markers	All Gated	
	Fluorescence Intensity	**% of cells stained**
FITC Control	8.88	56.11
Isotype Control	41.29	52.52
Isotype Control*	13.36	52.62
CD3*	**13.93**	**58.30**
CD19*	**18.57**	**51.50**
CD1a*	**337.52**	**56.92**
CD14 Control	10.80	53.04
CD14	**10.57**	**50.12**
CD86*	**94.56**	**58.12**
CD80	80.41	54.96
CD83	72.93	57.80
CD54*	**144.66**	**48.74**
Class-I (ABC)	**1952.06**	**56.02**
Class-II (DR)	**2043.18**	**56.52**

Table 2: Cell surface characterisation of dendritic cells.

Dendritic cells were generated from PBMC following culture of adherent cells for 7 days with 1000 U/ml GM-CSF and 500 U/ml IL-4 and a further 3 days with 1000 U/ml GM-CSF and 100 U/ml TNF-α. Cells were labelled for 30 minutes with antibodies specific for cell surface markers, washed and labelled with FI conjugated anti-mouse IgG for a further 30 minutes and analysed by flow cytometry.

* Antibodies referred to Isotype Control (*)

5.2.2.1.2 Mixed Lymphocytes Reactions (MLR)

To test the functionality of the dendritic cells a mixed lymphocyte reaction was performed as described in Section 2.2.11. Briefly autologous or allogeneic nonadherent cells were mixed with different numbers of dendritic cells in RPMI containing 17% AIM-V and 5% autologous serum and cultured for four days at 37°C. PBMC cultured on their own and medium only served as negative controls. Thymidine was then added to all wells and 18hr later plates were harvested and level of thymidine incorporated by cells was detected using a gamma counter-Topcount ^{TM}.

MLR results for two different donors are shown in Figure 5.6. As can be seen dendritic cells generated following the method described in Section 2.2.3.3 were capable of stimulating both autologous and allogeneic T-cells with a greater response obtained with allogeneic cells.

The morphological, cell marker expression and MLR results suggest that the cells generated by the culture method described above are dendritic cells, in agreement with previous reports and therefore suitable for use as APC in the generation of CTL.

5.2.2.2 Generation of CTL

Peptide specific CTL using either the p53 217 or 264 peptides and dendritic cells as APC were generated following the method described in Section 2.2.3.3. Briefly, on day 0, nonadherent cells, stored as frozen stock from day one, were thawed, washed, counted and depleted of $CD4^+$ cells using anti-CD4 dynabeads. Effector cells were co-cultured with peptide-pulsed dendritic cells at an effector: APC ratio of 10:1 (Figures 5.7) with IL-2 and IL-7. Effector cells were restimulated seven days later with peptide-pulsed and mitomycin C treated autologous whole PBMC. Every subsequent seven days, effector cells were restimulated with a mixture of peptide-pulsed and mitomycin C treated autologous PBMC, allogeneic HLA-$A2^+$ PBMC and/ or LCL and Rosi cells at a ratio of effector: APC of 10:1 in AIM-V containing 5% autologous serum and IL-2. Cytotoxic activities of CTL thereby generated were tested on day 38 in a standard 4hrs chromium release assay (Section 2.2.4).

Figure 5.6 (a&b): Two Mixed Lymphocytes Reactions using dendritic cells as stimulator cells from two donors.

Dendritic cells were generated from PBMC following culture of adherent cells for 7 days with 1000 U/ml GM-CSF and 500 U/ml IL-4 and a further 3 days with 1000 U/ml GM-CSF and 100 U/ml TNF-α. On day 10 cells were mixed with non-adherent autologous or allogeneic PBMC at different ratio for 5 days with addition of ^{3}H Thymidine for the last 18hrs.

Proliferation of autologous non-adherent PBMC as reported by ^{3}H Thymidine incorporated (Red columns), proliferation of allogeneic non-adherent PBMC (Blue columns), medium only (Yellow column).

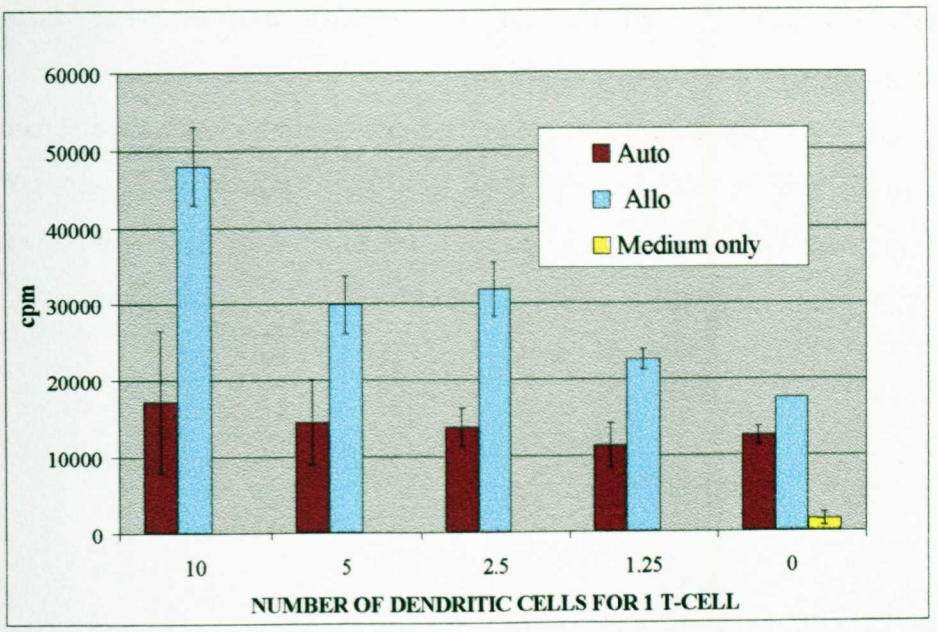


Figure 5.6a:

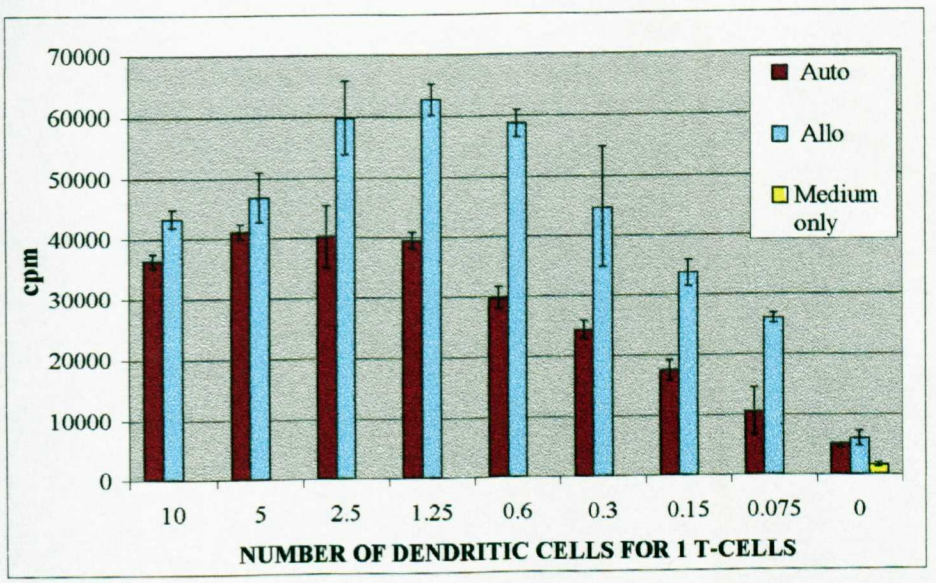


Figure 5.6b:

Figure 5.7a: Picture of dendritic cells mixed with non-adherent, CD4+ cells depleted autologous PBMC. Observed x10.

Dendritic cells were generated from PBMC following culture of adherent cells for 7 days with 1000 U/ml GM-CSF and 500 U/ml IL-4 and a further 3 days with 1000 U/ml GM-CSF and 100 U/ml TNF-α. On day 10 dendritic cells were mixed with non-adherent, $CD4^-$ cells depleted autologous PBMC at an APC: responder cells ratio of 1:10.

Figure 5.7b: Picture of dendritic cells mixed with non-adherent, CD4+ cells depleted autologous PBMC. Observed x100.

Dendritic cells were generated from PBMC following culture of adherent cells for 7 days with 1000 U/ml GM-CSF and 500 U/ml IL-4 and a further 3 days with 1000 U/ml GM-CSF and 100 U/ml TNF-α. On day 10 dendritic cells were mixed with non-adherent, $CD4^-$ cells depleted autologous PBMC at an APC: responder cells ratio of 1:10.

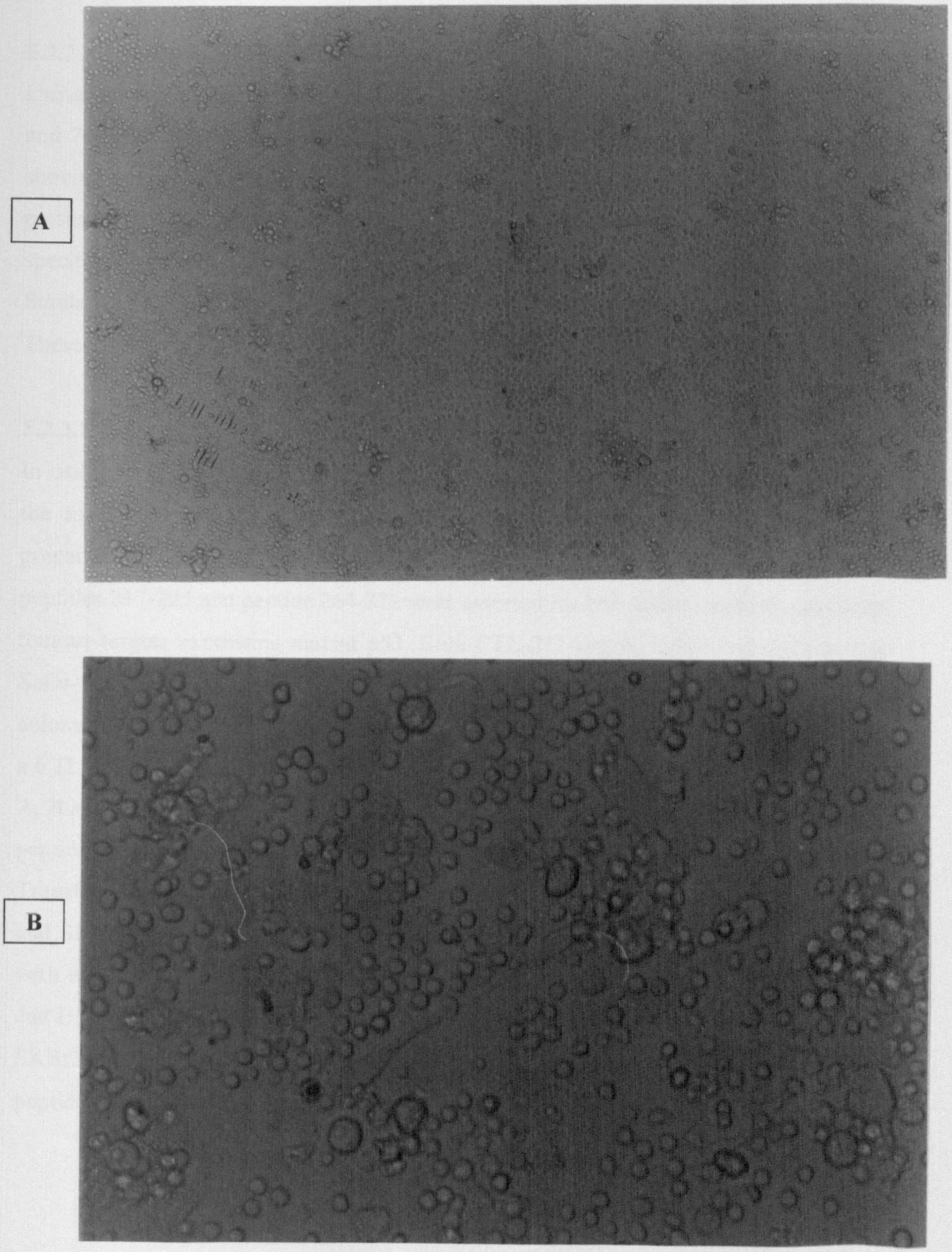


Figure 5.7: Dendritic Cells Obtained after 10 Days of Cultures and Mixed with non-Adherent $CD4^-$ autologous PBMC. A) Magnification x10, B) x100.

5.2.2.2.1 Induction of Peptide Specific CTL

Using the above method, CTL could successfully be generated to p53 peptides 217-225 and 264-272. Peptide 264-272 was used as a positive control as it has previously been shown to induce CTL. Figure 5.8, which is representative of three donors with each ratio performed in triplicate, shows that T2 cells pulsed with either 217-225 or 264-272 were specifically killed by CTL-217 (Figure 5.8a) and CTL-264 (Figure 5.8b) respectively. Similarly, JY pulsed with the relevant peptide were lysed by CTL-217 (Figure 5.9). These CTL lines did not however kill cells expressing wild-type p53 (T2 and JY).

5.2.2.2.2 Recognition of Tumour Targets Expressing Mutant p53 Protein

In order to assess whether the 217-225 was endogenously processed and presented at the surface of tumour cells a number of cells expressing mutated p53 protein were prepared (see Sections 4.2.3 and 4.2.4) and used as targets. CTL lines generated to peptides 217-225 and peptide 264-272 were assessed for lytic activity against transfected tumour targets expressing mutant p53. Both CTL-217 and CTL-264 were able to lyse SaOs-2/175 but not SaOs-2 cells transfected with control vector (Figures 5.10, red columns compared with green columns). In contrast, SaOs-2/273 was only recognised by a CTL line generated to the 217-225 peptide from one out of three donors (Figures 5.10, A, B and C yellow colomns) and was not killed by CTL lines generated with the 264-272 peptide (Figures 5.10, D, E and F, Yellow columns).

Transfected cell lines A431-A2 (expressing p53 protein mutated at codon 273 Arg->His) and SKBr3-A2 (expressing p53 protein mutated at codon 175 Arg->His) when pulsed with either the 217-225 or 264-272 peptide were killed by CTL-217 (Figures 5.11, A and B, blue columns) and CTL-264 (Figures 5.11, C and D, blue columns) respectively. SKBr3-A2, but not A431-A2 cells were also sensitive to lysis by both CTL lines without peptide pulsing (Figures 5.11 A and 5.11 C, red columns).

Figure 5.8: Cytotoxicity of CTL lines CTL-217 and CTL-264 against T2 cells.

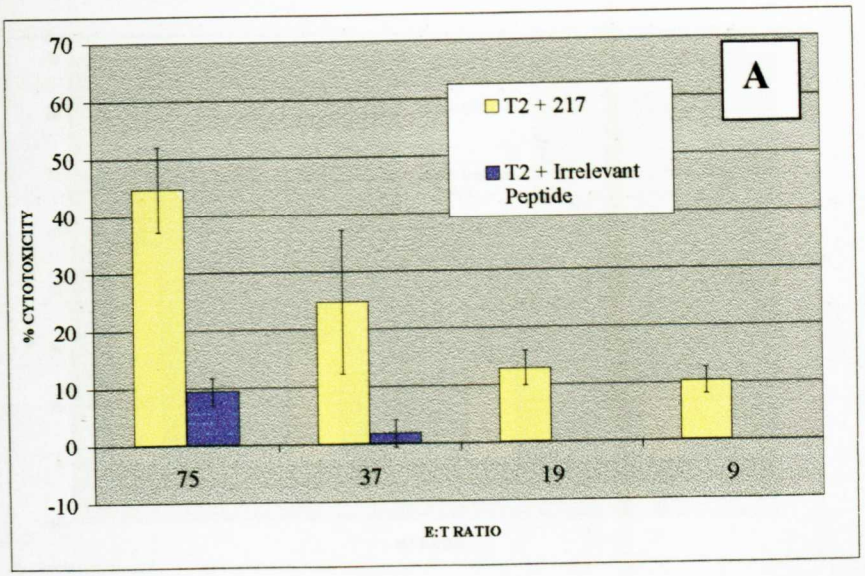
CTL lines were generated following initial stimulation with peptide pulsed autologous dendritic cells and three subsequent rounds of restimulation with first peptide-pulsed and mitomycin C treated autologous PBMC, then for the next two restimulation with peptide-pulsed and mitomycin C treated autologous PBMC, allogeneic HLA-$A2^+$ PBMC and/or LCL, and Rosi cells.

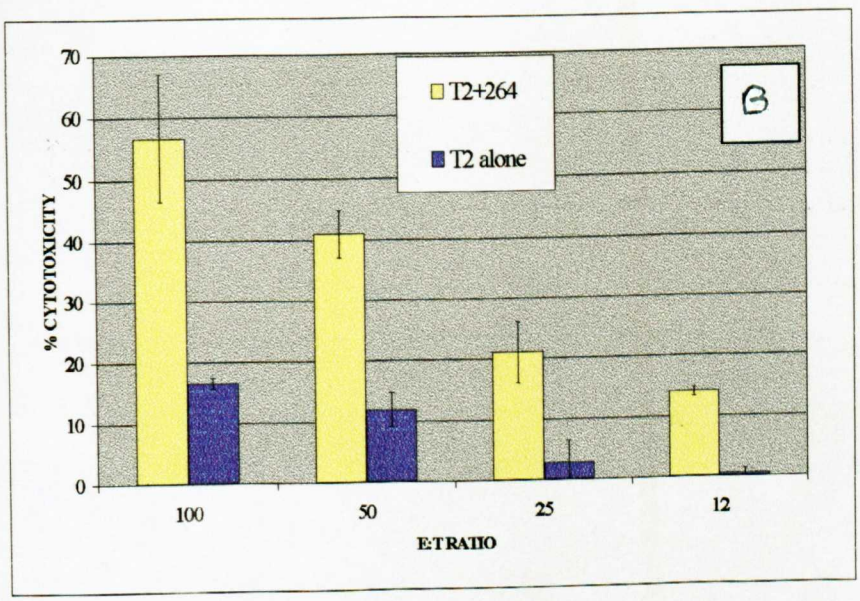
Cytotoxicity of effector CTL lines was determined by percentage release of ^{51}Cr from ^{51}Cr labelled T2 target cells pulsed with appropriate peptides: (A) 217-225 peptide (Yellow columns) or T2 cells alone (Blue columns); (B) cytotoxicity of CTL-264 against T2 cells pulsed with 264-272 peptide (Yellow columns) T2 cells alone (Blue columns).

Figure 5.9: Cytotoxicity of CTL lines CTL-217 against JY cells.

CTL lines were generated following initial stimulation with peptide pulsed autologous dendritic cells and three subsequent rounds of restimulation with first peptide-pulsed and mitomycin C treated autologous PBMC, then for the next two restimulation with peptide-pulsed and mitomycin C treated autologous PBMC, allogeneic HLA-$A2^+$ PBMC and/or LCL, and Rosi cells.

Cytotoxicity of effector CTL lines was determined by percentage release of ^{51}Cr from ^{51}Cr labelled JY target cells pulsed with 217-225 peptide (Yellow columns) or JY cells alone (Blue columns).


A
T2 + 217
T2 + Irrelevant Peptide
% CYTOTOXICITY
70
60
50
40
30
20
10
0
-10
75
37
19
9
E:T RATIO


B
T2+264
T2 alone
% CYTOTOXICITY
70
60
50
40
30
20
10
0
100
50
25
12
E:T RATIO

Figures 5.8:

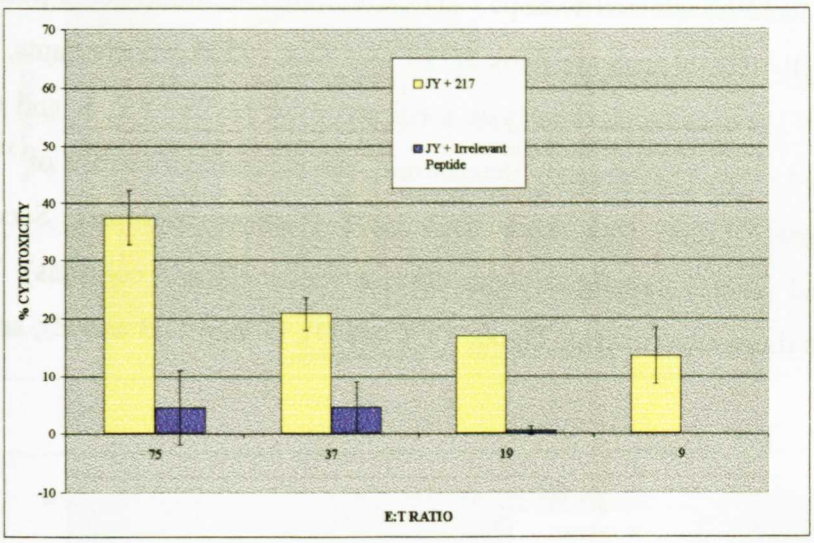
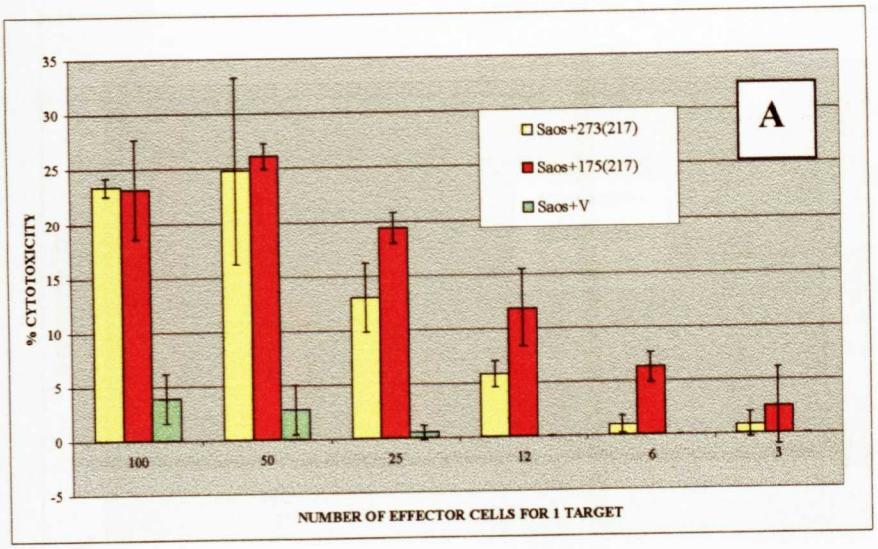
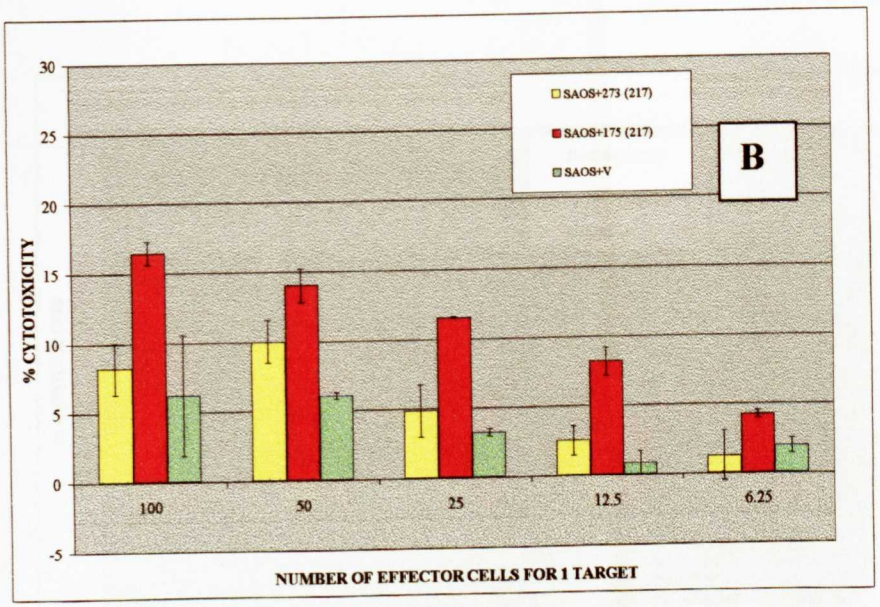

70
60
50
40
30
20
10
0
-10
% CYTOTOXICITY
JY + 217
JY + Irrelevant Peptide
75
37
19
9
E:T RATIO

Figure 5.9:

Figure 5.10: Sensitivity of Saos-2 transfectants to lysis by CTL-217 and CTL-264. Saos-2 cells were transfected with p53 cDNA mutated at amino-acids position 175 (R->H) or 273 (R->H) to generate Saos-2/175 and Saos-2/273 transfectants, respectively. Sensitivity of the transfectants to lysis mediated by CTL-217 (A, B and C) and CTL-264 (D, E and F) was assessed by determining the percentage release of ^{51}Cr from ^{51}Cr labelled targets. Target cells were Saos-2/273 (Yellow columns), Saos-2/175 (Red columns) and Saos-2 transfected with control vector (Green columns). Three donors were used in these experiments: Donor 1 (A and D); Donor 2 (B and E); and Donor 3 (C and F).


A
Saos+273(217)
Saos+175(217)
Saos+V
% CYTOTOXICITY
35
30
25
20
15
10
5
0
-5
100
50
25
12
6
3
NUMBER OF EFFECTOR CELLS FOR 1 TARGET


B
SAOS+273 (217)
SAOS+175 (217)
SAOS+V
% CYTOTOXICITY
30
25
20
15
10
5
0
-5
100
50
25
12.5
6.25
NUMBER OF EFFECTOR CELLS FOR 1 TARGET

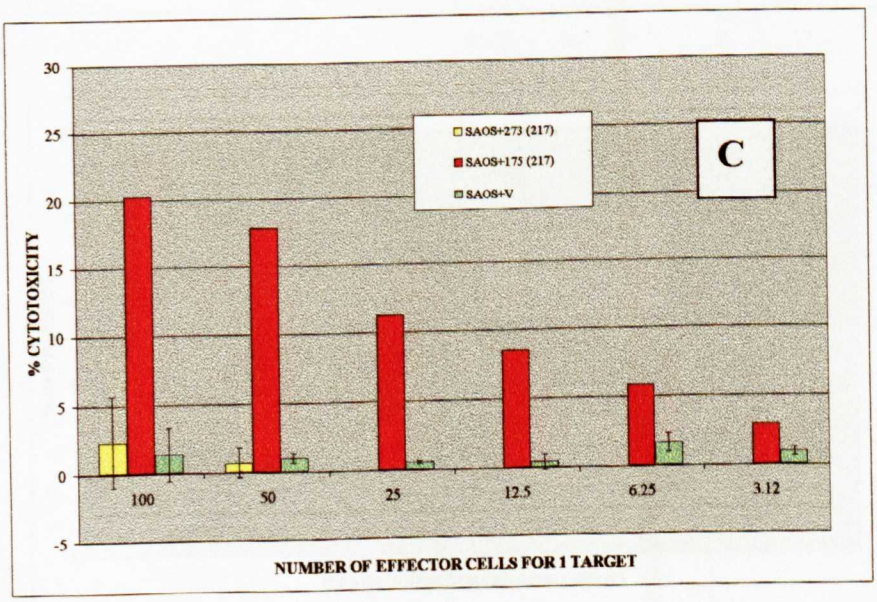
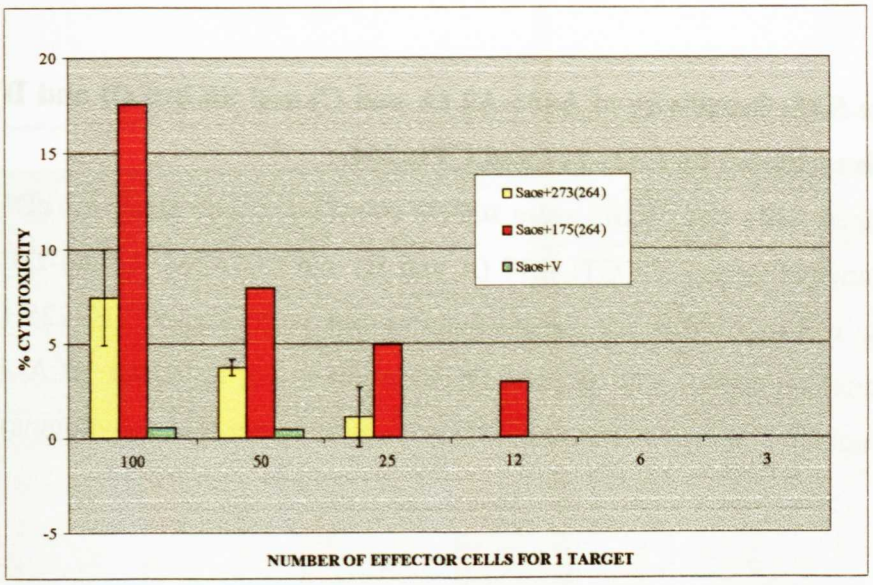
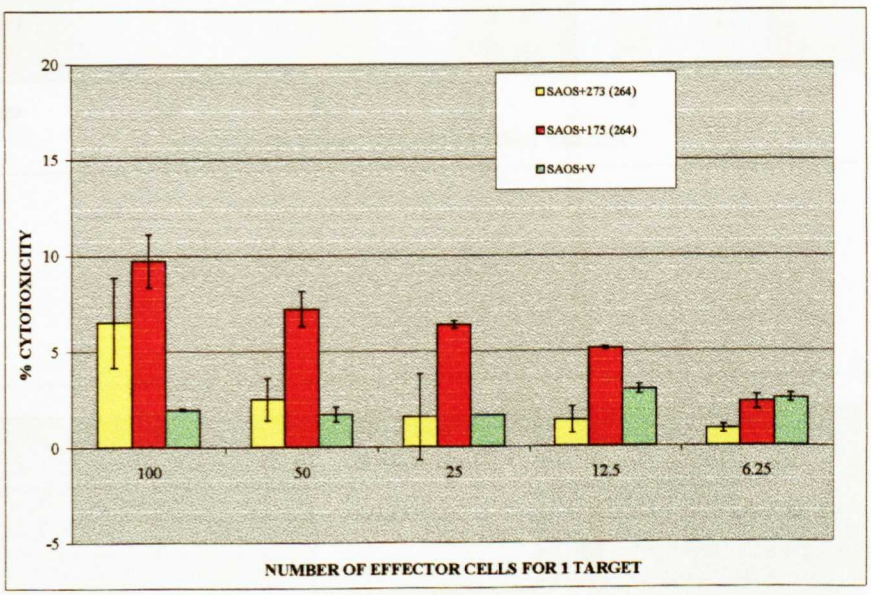

C
SAOS+273 (217)
SAOS+175 (217)
SAOS+V
% CYTOTOXICITY
30
25
20
15
10
5
0
-5
100
50
25
12.5
6.25
3.12
NUMBER OF EFFECTOR CELLS FOR 1 TARGET

Figure 5.10:

D


20
15
10
5
0
-5
% CYTOTOXICITY
Saos+273(264)
Saos+175(264)
Saos+V
100
50
25
12
6
3
NUMBER OF EFFECTOR CELLS FOR 1 TARGET

E


20
15
10
5
0
-5
% CYTOTOXICITY
SAOS+273 (264)
SAOS+175 (264)
SAOS+V
100
50
25
12.5
6.25
NUMBER OF EFFECTOR CELLS FOR 1 TARGET

F

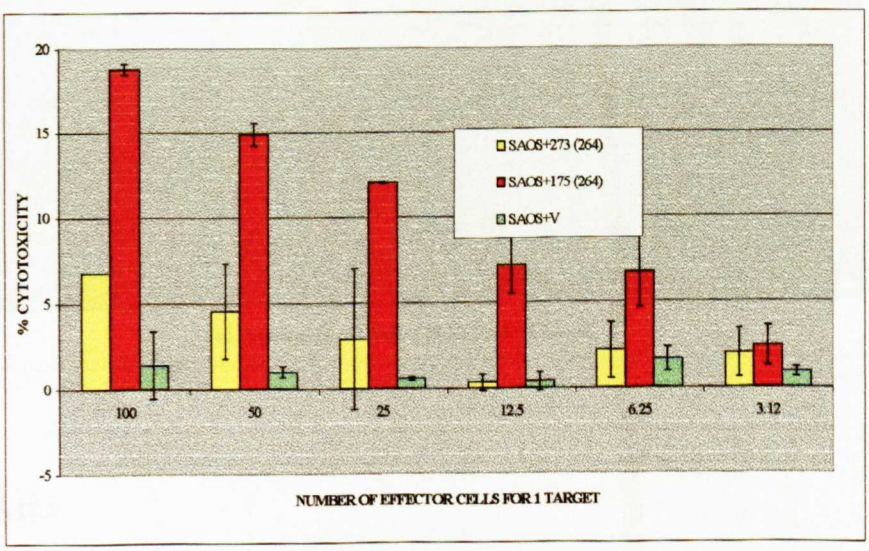
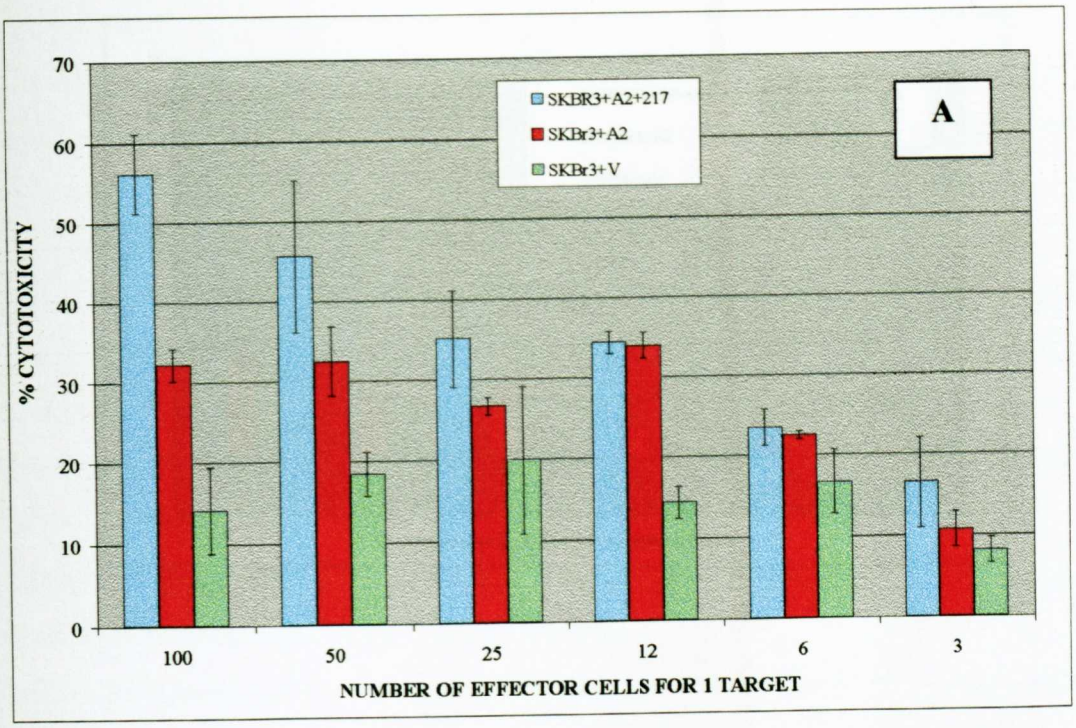

20
15
10
5
0
-5
% CYTOTOXICITY
SAOS+273 (264)
SAOS+175 (264)
SAOS+V
100
50
25
12.5
6.25
3.12
NUMBER OF EFFECTOR CELLS FOR 1 TARGET

Figure 5.11: Sensitivity of A431-A2 (A and C) and SKBr3 (B and D) transfectants to lysis mediated by CTL-217 and CTL-264.

Cell lines A431 and SKBr3 were stabely transfected with HLA-A2 cDNA and used as ^{51}Cr labelled targets for CTL-217 (A and B) and CTL-264 (C and D) generated from Donor 1. Target HLA-A2 transfectants pulsed with peptide 217-225 (A and B; Blue columns) or peptide 264 (C and D; Blue columns), unpulsed HLA-A2 transfectants (Red columns) and cells transfected with control vector (Green columns).


A
SKBR3+A2+217
SKBr3+A2
SKBr3+V
% CYTOTOXICITY
70
60
50
40
30
20
10
0
100
50
25
12
6
3
NUMBER OF EFFECTOR CELLS FOR 1 TARGET

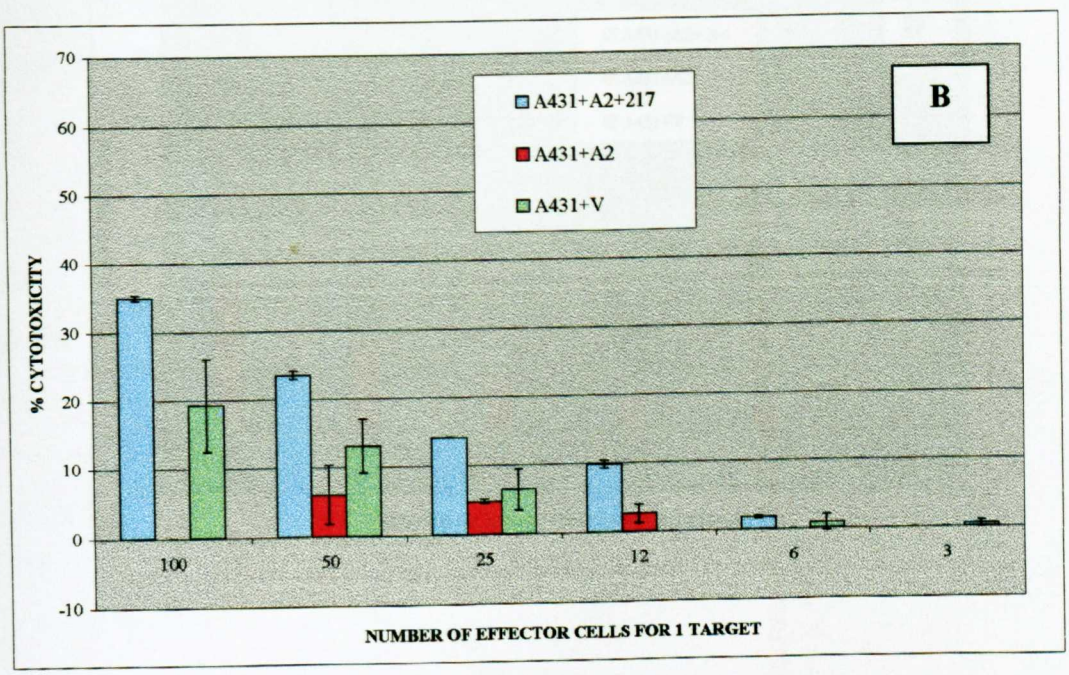
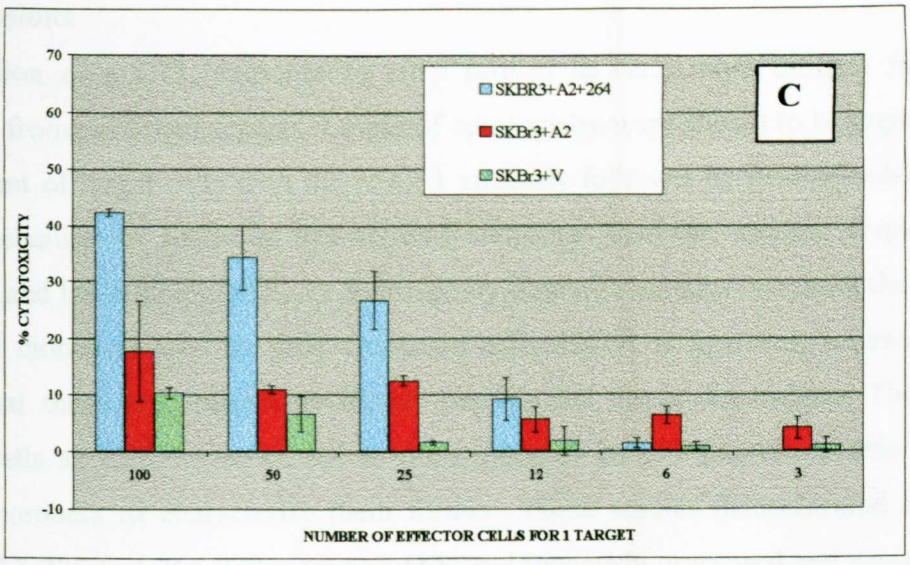
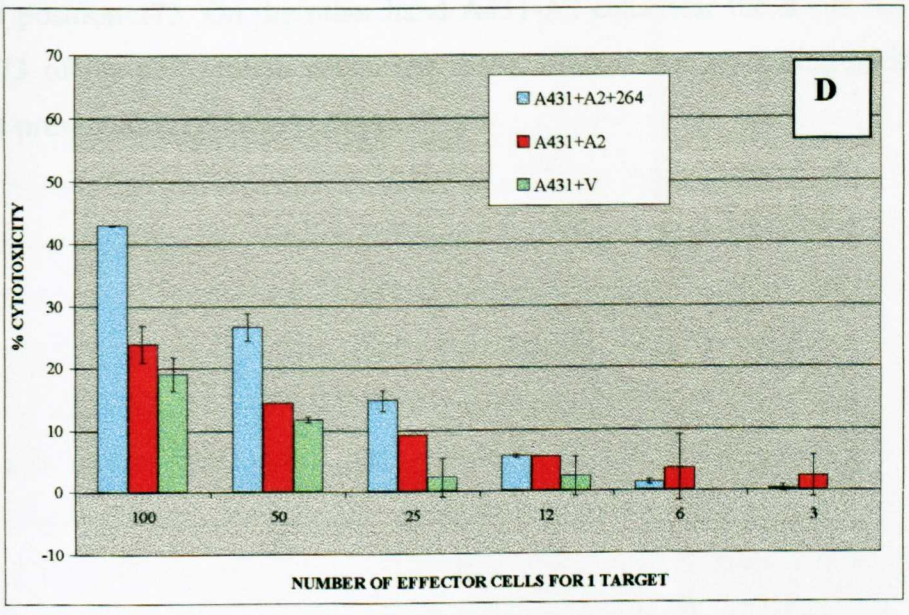

B
A431+A2+217
A431+A2
A431+V
% CYTOTOXICITY
70
60
50
40
30
20
10
0
-10
100
50
25
12
6
3
NUMBER OF EFFECTOR CELLS FOR 1 TARGET

Figure 5.11 :


C
SKBR3+A2+264
SKBr3+A2
SKBr3+V
% CYTOTOXICITY
70
60
50
40
30
20
10
0
-10
100
50
25
12
6
3
NUMBER OF EFFECTOR CELLS FOR 1 TARGET


D
A431+A2+264
A431+A2
A431+V
% CYTOTOXICITY
70
60
50
40
30
20
10
0
-10
100
50
25
12
6
3
NUMBER OF EFFECTOR CELLS FOR 1 TARGET

5.3 Conclusions

The induction of a CTL response *in vitro* proved to be a more difficult task than anticipated from published reports. Levels of cytotoxicity were shown to be improved by the treatment of target cells with the MA2.1 antibody followed by peptide-pulsing. The precise mechanism of action of this antibody remains, however, unclear. Depletion of $CD4^+$ cells and the addition of IL-12 followed by semi-cloning also increased the number of positive clones as well as their cytotoxic activities. It is however, noteworthy to mention that these responses were mostly peptide and donor dependants. The use of dendritic cells as APC however, led to the generation of p53 peptide-specific CTL in sufficient numbers to characterise them further. These studies demonstrated that p53 peptides 217-225 and 264-272 were naturally endogenously processed and presented on the surface of the SaOs-2/175 and SKBr3-A2 cells.

Transfected HLA-A2 cDNA was functional since transfected cells A431-A2 and SKBr3-A2 were killed by CTL lines when pulsed with relevant peptide.

SaOs-2/175 cells and SKBr3-A2 cells bear the same R->H mutation of the p53 gene at amino-acid position 175. On the other hand A431-A2 cells bear the R->H mutation at position 273 of the p53 protein which may have affected the processing pathway and subsequent presentation of these epitopes.

5.4 Discussion

The use of peptide vaccines derived from T-cell epitopes of tumour associated antigens or overexpressed molecules in tumour cells is now an acceptable approach to elicit anti-tumour immunity and a number of peptide vaccine clinical trials are in progress (Nestle, *et al.*, 1998; Hsu, *et al.*, 1996). The p53 suppressor gene is mutated in 50-60% of human cancers (Hollstein, *et al.*, 1991) and results in the stabilisation and accumulation of the inactivated protein within the cancer cell. Conformational changes in the p53 protein, generated as a result of point mutations, are frequently recognised by the monoclonal antibody Pab240, due to the exposure of the 240 epitope, RHSVV (amino-acids 212-217) (Milner, *et al.*, 1990). It is possible that peptides in this exposed region could be processed in a different manner to the wild-type protein, presented on the tumour cells surface in the context of MHC class-I and therefore, have the potential to be recognised by tumour specific CTL.

The p53 gene is a tumour suppressor gene present in all cells, and mutations within the gene represent the single most common genetic alterations observed in human cancers (Hollstein, *et al.*, 1991). The majority of mutations of p53 result in the stabilisation and accumulation of the protein in tumour cells (Bodmer, *et al.*, 1992) making p53 an ideal candidate for immunotherapy against a wide variety of cancers. Mutations, commonly point missense mutation, are mainly clustered around four hot spots (175, 248, 249, 273) in highly conserved domains in the central region of the protein (Hollstein, *et al.*, 1991). These can further be classified into two groups according to the effect they have on the protein conformation of p53. One class of mutants (273, 248) does not significantly affect the conformational structure of the protein (Hinds, *et al.*, 1990), does not associate with heat shock protein 70 (hsp-70), and are Pab240-/Pab1620+ (Milner, 1995). The other class of mutants (175, 249) results in the unfolding of the protein, and exposure of the 240 epitope which is normally cryptic and inaccessible to antibodies; these mutant proteins are associated with the hsp70 and were found to be highly sensitive to proteolytic enzymes (Lee, *et al.*, 1994). The frequency of p53 mutations is strongly correlated with the presence of antibodies against the p53 protein in several types of cancers (Agelopoulou, *et al.*, 1994; Lubin, *et al.*, 1995), as a result of an active secondary self-immunisation. Moreover Davidoff and co-workers (Davidoff, *et al.*, 1992) showed that tumours which elicit an antibody response contain HSP70 complexed to mutant p53. These findings and the fact that several HSPs have been shown to have a

role in antigen processing (Vanbuskirk, *et al.*, 1989; Srivastava, *et al.*, 1994), suggest that antigen processing of the mutant p53 conformational protein could be different from mutations which do not affect the conformation of the p53 protein which may result in markedly different peptide repertoires being displayed on the surface of the cells.

Peptides 217-225 and 264-272 were successfully used for the generation of CTL lines, which were able to specifically kill peptide-pulsed targets as well as tumour targets overexpressing the p53 protein. Tumour targets expressing mutant p53 conformation were preferentially killed as opposes to tumour targets expressing wild-type conformational mutants providing evidence for the hypothesis of differential processing. These results confirm and extend the recent findings of Theobald, *et al.*, 1998, who demonstrated that the R->H mutation at residue 273 of human p53 alters protesomal processing of the protein due to inhibition of proteolytic cleavage between residue 272 and 273. Target cells bearing this mutation were not recognised by CTL generated to the wild-type p53 peptide 264-272 *in vitro* or *in vivo*, as this epitope was not generated. However, a HLA-A2 restricted CTL line specific for human wild-type p53 peptide 149-157 lysed transfected cell expressing this mutation, indicating efficient processing of other p53 epitopes. Here, target cells with the R->H mutation at position 273 were not lysed by CTL-264 lines and in addition, were only lysed by CTL lines to wild-types p53 217-225 peptide in one out of three donors. Whilst the former observation is in agreement with that of Theobald et al (Theobald, *et al.*, 1998), their results suggested that the 273 mutation may only affect a flanking peptide epitope and a precursor peptide. Here, the lack of recognition of the 273 mutated p53 observed with CTL lines generated to 217 in some donors could be due to differences in the affinities of the TCR of the CTL lines generated or relative expression of p53 and HLA-A2 molecules; alternatively, regions of the protein distant to the mutation may fail to donate peptides to the processing pathway.

All these conclusions, however, were drawn after analysis of standard 4hr chromium release assay results. The relevance of this assay as a surrogate immunologic measurement of anti-tumour activity has, however, been questioned after a lack of correlation between CTL induction *in vitro* and clinical response was found in two vaccine trials using HLA-A2-restricited MAGE-3 or gp100 peptides (Marchand, *et al.*, 1995; Rosenberg, *et al.*, 1998). New assays are now being used as a replacement of or in combinations with *in vitro* standard 4hr-chromium release assay. These include ELISA

and ELISPOT techniques, detecting cytokines released, such as IL-2, IFN-γ or TNF-α, as a result of CTL induction.

As for the induction of CTL *in vitro* and their use *in vivo* many methods have been developed as described in Section 5.1.2, among them methods using dendritic cells as APC have obtained the most promising results. Clinical trials, using dendritic cells from patients with B cell lymphoma pulsed with idiotypic protein demonstrated measurable anti-tumour cellular responses in four patients with one complete tumour regression (Hsu, *et al.*, 1996). More recently a clinical pilot study using DC pulsed with tumour lysate or a mixture of peptides, known to induce CTL, showed that antigen-specific immunity could be induced in patients with advanced melanoma. No physical signs of autoimmunity was found and in 5 out of 16 immunised patients clinical responses were observed with two complete responses and three regression of metastases in various organs (Nestle, *et al.*, 1998). In addition a phase I clinical trial using dendritic cells pulsed with melanoma cells lysate showed lymphocytes infiltration at the vaccination site by $CD8^+$ T-cells. These were shown to release TNF-α upon encounter with specific antigen and cytotoxicity towards cells expressing MAGE peptides (Chakraborty, *et al.*, 1998). Future immunisations may not need whole dendritic cells since peptide-pulsed DC have been shown to secrete antigen-presenting vesicles (exosomes) which were capable of priming CTL *in vivo* and resulted in tumour rejection (Zitovel, *et al.*, 1998). DC number obtained by *in vitro* methods may still be a problem, one possible alternative might be to fuse them with tumour cell lines. Gong, *et al.*, 1997, fused MC38 carcinoma cells with DC and showed induction of CTL *in vivo* by the fusion products. Vaccination with these hybrid cells induced CTL, which protected even against established metastases.

Here they have successfully been used as stimulators for the generation of human anti-p53 specific CTL which has potential clinical application. DC pulsed with mutant p53 peptide have been shown to be effective at immunising mice carrying tumours expressing that p53 mutant (Gabrilovich, et al., 1996). It would be interesting to try to generate CTL using p53-overexpressing-cell-lysate-loaded DC as APC.

Key questions are whether patients whose tumours harbour a p53 mutation have demonstrable precursor CTL or whether they can be induced, and whether their is an association between the p53 gene mutation, HLA type and disease progression.

CHAPTER 6: P53 AS A POTENTIAL TARGET FOR IMMUNOTHERAPY AGAINST HEAD AND NECK CANCER

6.1 Introduction

6.1.1 Head and Neck Cancer Background

6.1.1.1 Epidemiology

Head and neck cancer represents one of the most common cancers with 500 000 new cases per year (Parkin, *et al.*, 1988). Most of them are of the squamous cell type, and therefore only head and neck squamous cell carcinoma (HNSCC) will be discussed here. The majority of HNSCC patients are male over 40 years of age, with a male: female ratio of 4-1 (Sloan, *et al.*, 1991). However with the number of young smokers increasing as well as the number of women smoking the incidence of HNSCC is changing. Incidence also varies among different parts of the world, due to environmental differences as well as different habits of the populations (Sloan, *et al.*, 1991).

6.1.1.2 Head and Neck Cancer Risk Factors

Excessive absorption of alcoholic beverages and smoking are clearly associated with an increase risk in a number of cancers, but are particularly associated with head and neck cancers representing the two major causes of HNSCC (Copper, *et al.*, 1991). More over the combination of alcohol and smoking seem to exert a greater effect than either alone and account for about 75% of all oral pharyngeal cancers. Other causes such as bad oral hygiene, exposure to environmental risk factors like nickel or wood dust and textile fibres, chronic and viral diseases and genetic pre-disposition have all been associated with increased risk in HNSCC (Sloan, *et al.*, 1991; Vokes, *et al.*, 1993; Scully, *et al.*, 1993; Schantz, *et al.*, 1994).

6.1.2 Diagnosis and Treatments

Tumours from the head and neck region very often arise without any obvious symptoms and tumours are, therefore, generally large by the time they are reported by HNSCC patients. Prognosis depends on the size and site of primary tumour, the number of lymph nodes involved, as well as distant spread, grade of differentiation and the presence of aneuploidy. A TNM, from the International Union against Cancer, classification is used to stage the extent of the disease. T stage defining the extent of the primary tumour, N stage the extent of regional lymph node metastases and M stage the measurement of distant metastasis. Stages are then divided into IV with Stage I representing an early disease whereas a Stage IV represents an advanced and disseminated one. Although

surgery and radiation, given singly or in combination are the best treatment, especially when the disease is treated early, the overall survival rate remains low. 50% of HNSCC patients die within 5 years of diagnosis as a consequence of local or regional recurrences and second primary tumours (Cooper, *et al.*, 1989). One possible reason for the development of multiple primary tumours has been related to the 'field of cancerisation' phenomena (Slaughter, *et al.*, 1953). The hypothesis is that the mucosa of the upper aerodigestive tract from predisposed individuals, has been exposed to the same carcinogens, leading to the accumulation of genetic alterations which in turn result in the induction of multiple, independent malignant loci. This said, local and distant metastasis to the lung, for example, can also frequently develop synchronously or metachronously (Schwartz, *et al.*, 1994). The diagnostic differentiation between metastatic disease and second primary tumour, during the follow-up period, is, as a consequence, extremely difficult and the outcome of such a diagnosis is very important for the therapeutic decision. Since conventional histology is in the case of HNSCC of no use, other markers are needed. Indeed, if a biomarker was available and able to identify patients at high risk of developing second primary tumours or recurrence, it would have profound implications for therapy decisions and especially for the follow-up of these patients. Since chemotherapy is a long and costly treatment, radiotherapy and surgery remain the best treatment for HNSCC. The development of biomarkers to determine which patients are most likely to develop recurrence of primary disease or second primary tumours and therefore most likely to benefit from it, would save time and money.

P53 gene mutations and expression in HNSCC appear to be associated with shorter relapse-free and overall survival. Therefore, p53 has been proposed as a biomarker for identifying individuals at high risk of developing a recurrence of primary disease and second primary tumours who may benefit from adjuvant therapy and chemoprevention after definitive local therapy (Shin, *et al.*, 1996).

6.1.2 p53 and Head and Neck Cancer

6.1.2.1 Role of p53 in Head Neck Cancer

The evolution from hyperplasia and dysplasia to malignancy and metastasis in the oral region as for many cancers, is, as mentioned earlier, probably the result of accumulation of genetic damage such as proto-oncogene mutations, gene amplification, chromosome breakage, translocation, proto-oncogene overexpression and loss of tumour suppressor genes. Although specific molecular events accompanying head and neck cancer formation are not well defined for HNSCC, the p53 gene is most certainly involved. Indeed, mutations of the p53 gene have been reported in 53-93% of HNSCC and have been shown to be associated with rapid tumour cell proliferation in this type of cancer (Boyle, *et al.*, 1993). These appear to be an early event since they have been detected in pre-malignant lesions (Pavelic, *et al.*, 1994). Consistently the same mutation has been found in premalignant lesions, in recurrent disease and metastasis (Zariwala, *et al.*, 1994). Tumours that develop from the progression of genetically distinct premalignant processes in the surrounding epithelial field will have different p53 mutation whereas tumours with the same p53 mutation will have come from the same origin (Chung, *et al.*, 1993). The possibility that different tumours share exactly the same p53 mutation is, indeed, extremely remote. Patients who have a p53 alteration in their primary tumours and/or in the resected margins have been shown to be at a greater risk to develop a recurrence and thus need more aggressive adjuvant therapy than those who have no p53 alterations or no p53 mutations present in their resected margins (Brennan, *et al.*, 1995).

6.1.2.2 p53 as Biomarker of Head and Neck Cancer

Mutations of the p53 tumour suppressor gene, as well as overexpression of the p53 protein in tumour tissues, and the occurrence of serum antibodies to p53 have been demonstrated in a variety of human malignancies. The presence of mutations and the detection of p53 antibody have been shown to be associated with increased risk of recurrence and overall survival of HNSCC, making p53 a good molecular marker (Werner, *et al.*, 1997). Since most of p53 mutations lead to the synthesis of stabilised protein it has been possible to study the accumulation of mutated, inactive p53 protein by the use of immunohistochemical techniques (Coggi, *et al.*, 1997). Strong variations of intratumoral p53 staining as well as their clinical relevance, have, however, been found in head and neck tumours (Shin, *et al.*, 1994). Accumulation of mutant p53 protein can

result, maybe through release during cell necrosis, in the production of anti-p53 antibody and a strong correlation between the presence of p53 antibodies and the frequency of p53 mutations has been found in many cancers (Lubin, *et al.*, 1995; Angelopoulou, *et al.*, 1994). The proportion of patients with HNSCC who have serum p53 antibodies was, however, found to be smaller than that of patients exhibiting tumour cell accumulation of p53 protein (Bourhis, *et al.*, 1996). Interestingly, only 50% of patients with p53 mutations in their tumours, had serum p53 antibodies whereas, serum p53 antibodies were not detected in patients bearing no p53 mutation in their tumours (Cough, *et al.*, 1994). So despite the fact that a strong correlation was found between p53 accumulation and the presence of p53 antibodies, and the presence of antibodies and poor clinical outcome, no association was found between p53 protein accumulation and clinical outcome (Bourhis, *et al.*, 1996). This suggests that the humoral response to p53 could be a good marker for identifying, among patients with p53 accumulation, those with a high probability of failure.

6.1.2.3 p53 as a Potential Tumour Target for HNSCC Patients

Mutations within the p53 tumour suppressor gene represent the single most common genetic alterations in human cancers and are detected in a wide variety of cancers (Soussi, *et al.*, 1994). The recognition of the p53 alteration is an important characteristic in clinical diagnosis since it is an independent, unfavourable prognostic factor in many cancers, including breast, colon and gastric carcinomas (Remvikos, *et al.*, 1992; Thor, *et al.*, 1992; Starzynska, *et al.*, 1992). More over, tumour cells bearing p53 mutations have been shown to be resistant to radiation or chemotherapy, sensitivity which was restored when the wild-type p53 gene was introduced in the cells (Gallardo, *et al.*, 1996). Immunhistochemical analyses have been extensively used for the screening of p53 alterations in a wide variety of cancers (Soussi, *et al.*, 1994). Accumulation of p53 protein within tumour cells of cancer patients had, however, no association with clinical outcome. The level of p53 antibodies found in patient's sera has, on the other hand, beenproposed for diagnosis and prognosis of patients with HNSCC and was shown to be dependent on the protein accumulation (Bourhis, *et al.*, 1996). In addition in lung cancers, p53 antibodies were found several years before clinical diagnosis of the tumour (Lubin, *et al.*, 1995). Serological analysis of p53 offers the advantage that no tumour sample is required, and it can be easily used for follow-up of patients.

6.1.3 Aim

The aim of this study was to determine the immunological status of HNSCC patients with respect to p53, by analysis of p53 serum antibody, and analysis of p53 protein expression by immunohistochemistry, when tumour samples were available. It was also intended to assess the cellular immune response to p53 protein by CTL and proliferation assay and to determine if there was a correlation between the cellular and humoral responses and protein expression.

6.2 Results

6.2.1 Patient Sample Collection

The day before their operation, patients were asked to fill in a consent form, approved by the South Sheffield Ethics Committee, authorising the use of blood and tumour sample for research. PBMC isolated from whole blood were used for the proliferation assay as well as for the generation of CTL in vitro for patients HLA-A2$^-$.

A total of 14 patients entered the study with, blood sample from all of them but tumour sample from only 10 of them.

On the day of the operation, 60mls of blood were collected with or without a tumour sample. The blood was separated as described in Section 2.2.3.1, briefly blood was diluted in sterile PBS and cells were laid onto J-prep solution. Tubes were spun for 35 minutes at room temperature with breaks off. Thereafter some plasma was removed and stored immediately in 1ml tubes at -80°C until required for p53 antibody analysis. Leukocyte layers were then removed and washed twice in ice-cold PBS. Cells were counted and frozen in cryovial tubes at $1x10^7$ cells/ml in AIM-V containing 60% of FCS and 10% DMSO. Tubes were stored in liquid Nitrogen until needed with the exception of few used straight away for HLA-typing. For HLA-typing cells were washed with PBS and HLA-A2 antibody was added to them for 30 minutes as described in Section 2.2.2.2. Cells were then washed twice with PBS and a goat anti-mouse antibody was used as second layer. Cells were incubated for a further 30 minutes followed by another wash. Cell surface expression of HLA-A2 molecules was analysed by flow cytometry.

Only four patients were found to be HLA-A2 positive, Table 1.

6.2.2 Serological Analysis

Plasma from each patient was sent to France to Prof. Soussi laboratories for p53 antibody analysis. The method used was briefly as follows: p53-Abs in the sera of patients were measured using an enzyme-linked immunosorbent assay (ELISA). The assay was based on the results obtained by Lubin, *et al.*, 1993. Major sites of antigen recognition by p53-Abs detected in human serum are the amino- and carboxyl-terminal segments of the protein (Lubin, *et al.*, 1993). This epitope restriction has been demonstrated by binding sera to a series of 77 overlapping peptides that encompass the

entire p53 molecules. The majority (98%) of positive sera reacted with an amino-terminal peptide, and 48% reacted with a carboxyl-terminal peptide (Lubin, *et al.*, 1993). Sera were therefore tested in an ELISA that employed five peptides as follows:

- EPPLSQETFSDLWKLLPENNVLSPL (Amino-terminal)
- DDLMLSPDDIEQWFT (Amino-terminal)
- SPDDIEQWFTEDPGP (Amino-terminal)
- EALELKDAQAGKEPGGSRAHSSHLK (Carboxyl-terminal)
- GSRAHSSHLKSKKGQSTSRHKKLMF (Carboxyl-terminal)

The ELISA was performed with sterptavidin-coated plates, biotinylated peptides, and direct binding of human p53-Abs in serum that was diluted 1: 100 (with phosphate-buffered saline, pH 7.6, plus 5% nonfat dried milk) for the assay. The negative control employed a human serum albumin peptide. Sera were considered positive if a ratio of 1.5 or greater was obtained, with a difference in optical density (at 450nm) of at least 0.15.

Antibodies against p53 were found in two of the patient's sera, Table 1.

Patient	Tumour Sample	HLA-A2 Expression	P53 Antibodies
1	No	-	-
2	Yes	-	-
3	No	-	-
4	No	+	-
5	Yes	-	-
6	Yes	-	-
7	Yes	+	-
8	Yes	-	-
9	Yes	+	+
10	Yes	-	-
11	Yes	-	+
12	Yes	-	-
13	No	+	-
14	Yes	-	-

Table 1: HLA-A2 Expression and p53 Antibody Presence in HNSCC Patients Blood or Sera Respectively.

6.2.3 p53 Expression in HNSCC Patient's Tumour Sections

p53 protein expression was analysed on frozen sections from 10 HNSCC patients. Sections of 7μm were cut and stained following a three-stage immunoperoxidase technique, using commercially available ABC Elite kit. The technique described in Section 2.2.8 uses primary antibody (IgG2b or DO7 titrated in our laboratories using p53 overexpressing cell line SW480) followed by a biotynilated second antibody and then performed avidin and biotinylated horseradish peroxidase macromolecular complex. The bound peroxidase was visualised by DAB. Endogenous peroxidase activity was blocked by pre-incubating sections in a solution of hydrogen peroxidase in methanol. Tumour tissue stained with isotype-matched antibody control (IgG2b) and normal tissue were used as negative control. Positive control cytospins of MCF7 cells were also used in staining runs to ensure that any negative result was not due to failure of the immunohistochemical staining procedure.

Slides were graded independently by 2 viewers, and then viewed together to discuss, compare and give final grade.

As can be seen in Table 2, 6 out of 10 tumour sections stained positive for p53 protein expression. 5 expressed very high level of the protein (75-100%). Metastatic tissue obtained from patient 11 also stained positive. The staining was localised in the nucleus. Many of the specimens showed a 'diffuse' or 'basal' distribution of positive cells but significant data could not be obtained for the distribution of positive cells because many biopsies were too small to determine their type of distribution. Staining intensity and the percentage of positive cells varied (Table 2, Figures 6.1-6.4).

A summary of results obtained in this study is presented in Table 3.

% of Cells Stained / Patient No	0-5	6-25	26-50	51-75	76-100
2					+++
5	+				
6		+++			
7	+				
8					++
9					+++
10	-				
11					+++
11 (met)					+++
12	+				
14				+	

Table 2: p53 Staining Results in HNSCC Tumour Specimens

(+++): Positive cells stained black or dark brown

(++): Positive cells stained brown distinct from background unstained cells

(+): Positive cells stained light but apparently stained compared with background cells

(-): Cells remained unstained

Patient Number	Date of Surgery	Age at Surgery	Sex	Tumour Specimen Site	HLA-A2	P53+ve	P53 Abs
1	17.09.97	68	M	No Sample	-	Nd	-
2	24.09.97		M		-	+	-
3	15.10.97	48	F	No sample	-	Nd	-
4	29.10.97	38	M	No Sample	+	Nd	-
5	12.11.97	39	M	Metastasis Lymph Node	-	-	-
6	10.12.97	38	M	Right COMMANDO Anterior trimmed margins		-	-
7	14.01.98	57		Right Neck Dissectomy	+	-	-
8	21.01.98				-	+	-
9	04.02.98	27	M	Total Glossectomy	+	+	+
10	11.02.98	50	M	COMMANDO Specimen	+	-	-
11	18.02.98	70	M	Total Laryngectomy	-	+	+
12	25.02.98	54	M	Laryngectomy	-	-	-
13	01.04.98	72	M	No Sample	+	Nd	-
14	29.04.98	67	M	Pharungo-Laryngo Oesophagectomy	-	+	-

Table 3: Summary of results obtained with HNSCC patients regarding p53.

6.2.4 Cellular Immune Response in Patient with HNSCC to p53

Tilkin, *et al.*, 1995, showed that only patients with p53 gene mutation, p53 protein accumulation and anti-p53 antibodies had T cells, which could proliferate when incubated *in vitro* with human wild-type p53. However HLA expression in these patients was not assessed. Here a similar study was carried out in patients with HNSCC including HLA expression and CTL generation.

CTL were generated as described in Section 2.2.3.3 using PBMC from all HLA-A2+ patients (patient 9 and 10) and two HLA-A2$^-$ patients, both overexpressing p53 protein, as detected by immunohistochemistry, Section, with one positive for p53-Abs (patient 11) and one negative for p53-Abs (patient 8) and peptide 264-272 (Table 4). During the course of the stimulation however these became contaminated and the experiment was aborted and due to the too small PBMC number obtained from each patient no other CTL could be generated.

Patient Number	HLA-A2 expression	p53 Expression	p53 antibody
4	+	Nd	-
7	+	-	-
9	+	+	+
10	+	-	-
13	+	-	-
8	-	+	-
11	-	+	+

Table 4: Summary of HLA-A2 and p53 Protein Expression and p53-Abs of Patients whom CTL have been generated.

The aim was to compare the immunological status of HNSCC patients regarding p53 and to link it with HLA expression.

The cellular immune response to p53 protein using a proliferation assay was also intended. However due to the lack of p53 protein no assay could be performed.

6.3 Conclusion and Discussion

In this study p53 protein accumulation was detected in 60% of HNSCC patients (6/10) and p53 antibodies in 20% (2/10) of these patients sera, and 14% in total (2/14 sera tested). Interestingly the two patients with p53 antibodies were also found to have p53 protein accumulation. Four patients were HLA-A2. One patient, patient 9, HLA-A2+, was found to have p53 antibodies and p53 accumulation. mRNA was extracted from all tumour samples available (see Section 2.2.10) and sent to France for sequencing to determine if there was a relationship between p53 mutation, protein expression and development of a humoral immune response. Unfortunately the samples were unsuitable for sequencing due to the mRNA being degraded. This was probably caused by the long gap period between surgery and delivery by the pathologist and freezing the tumour sample, and as a consequence no results were obtained. However, it is likely that the p53 gene would have been mutated in many of them since intense p53 immunostain-positive cells were shown to exhibit p53 genetic abnormalities (Kim, *et al.*, 1997). Although not all patients with p53 alterations develop p53 antibodies, patients who develop antibodies will have a mutated p53 gene. More over only mutations occurring in exons 5 and 6, which produce a conformational mutant p53 protein which bind to HSP70, will produce antibodies according to Davidoff *et al.*, (1992). One can therefore assume that patient 9 has a p53 gene that is mutated within these exons. It is also possible that, for an identical mutation, the humoral response is dependent on the MHC class-I or class-II molecule expressed by each individual. It is possible that patients 9 and 14 might have the same p53 mutation, but being of different HLA haplotype, one did not develop antibodies against the protein. A larger study would be necessary to test this hypothesis.

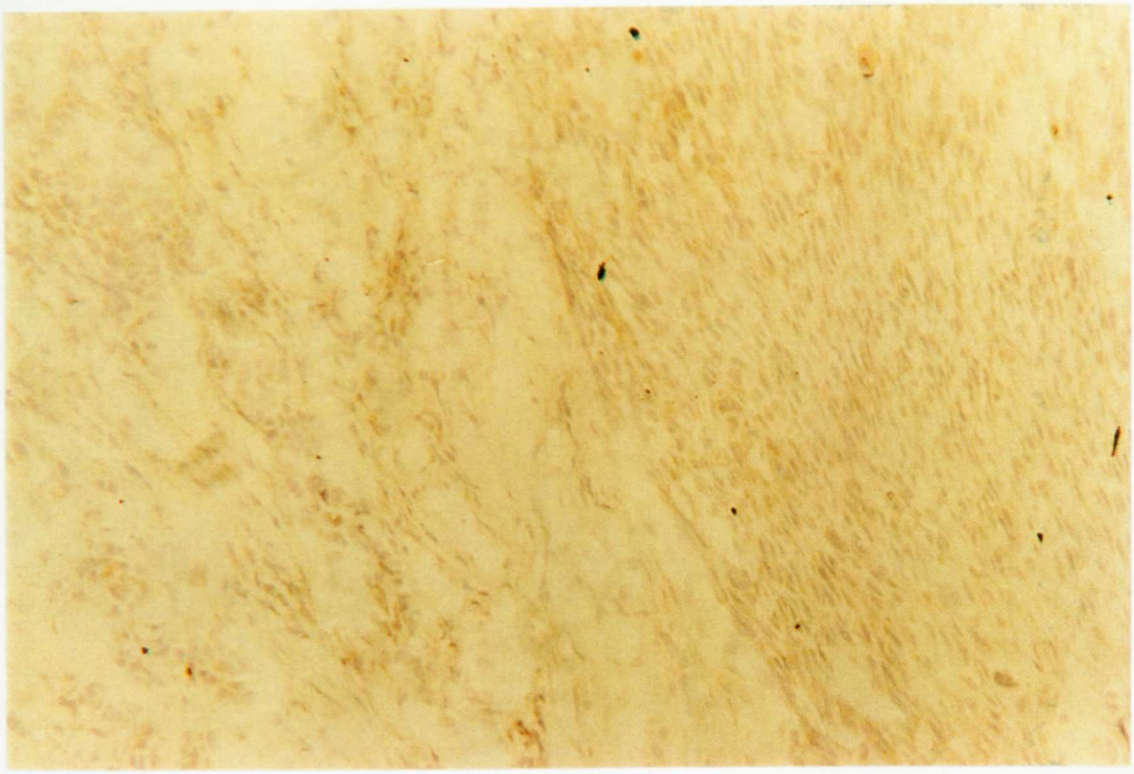
p53-Antibodies are likely to be the result of self-immunisation process toward the p53 protein which is normally expressed in minute quantities in the body (Lubin, *et al.*, 1993). Interestingly immunoglobulin subclasses of these antibodies have been found to be of IgG1 and IgG2 in all sera of HNSCC patients suggesting an active immunisation, these being the result of a second immune response (Lubin, *et al.*, 1995).

The aim of this study was to compare the cellular immune response to p53 protein, the humoral response to p53 and the p53 protein expression in patients with HNCSS in relationship with HLA expression. This type has not previously been reported for HNSCC patients. However, due to time constraints, the lack of p53 protein and the small amount of lymphocytes obtained from these patients, it was not possible to pursue this. Primary proliferative T-cell responses to wild-type p53 protein have been reported in patients with breast cancer (Tilkin, *et al.*, 1995). A T-cell proliferative response was carried out in six breast cancer patients, five bearing a p53 mutation, three demonstrated significant lymphoproliferative responses to wild type p53 protein. These patients also had p53 antibodies in their sera accompanied by p53 accumulation of mutated protein in tumour cell nuclei. The other three patients and healthy donors did not however have any humoral or cellular immune responses to p53 protein. Interrestingly one of the patient negative for p53 antibody showed a strong p53 protein accumulation. Therefore this study showed that p53 can prime a p53-specific T cell response *in vivo*, and that wild-type p53 is processed at least *in vitro* by APC and recognised by T-cells of certain breast cancer patients.

Studies assessing the proliferative response to wild-type p53 in other patient groups have not yet been reported. p53 Antibodies recognise immunodominant epitopes in the linear carboxyl- and amino-terminal domains of the protein. These epitopes do not correspond to any mutational hot spots (Schlichtholz, *et al.*, 1992). Yet while no antibody response can be detected in healthy individuals or in most patients with no mutated p53 gene, if a mutation occurs disrupting the protein conformation such as mutation at codon 175, a humoral response will follow (Davidoff, *et al.*, 1992). This indicates that protein accumulation is necessary but not always sufficient to generate an antibody response toward p53 protein and no explanations have yet been found to explain this phenomena.

Future studies need to address whether a correlation exists between antibody response, protein accumulation, mutation and proliferative response to the protein or CTL activity. The results of these studies may provide information regarding the prognosis of a p53-Abs response.

A



B

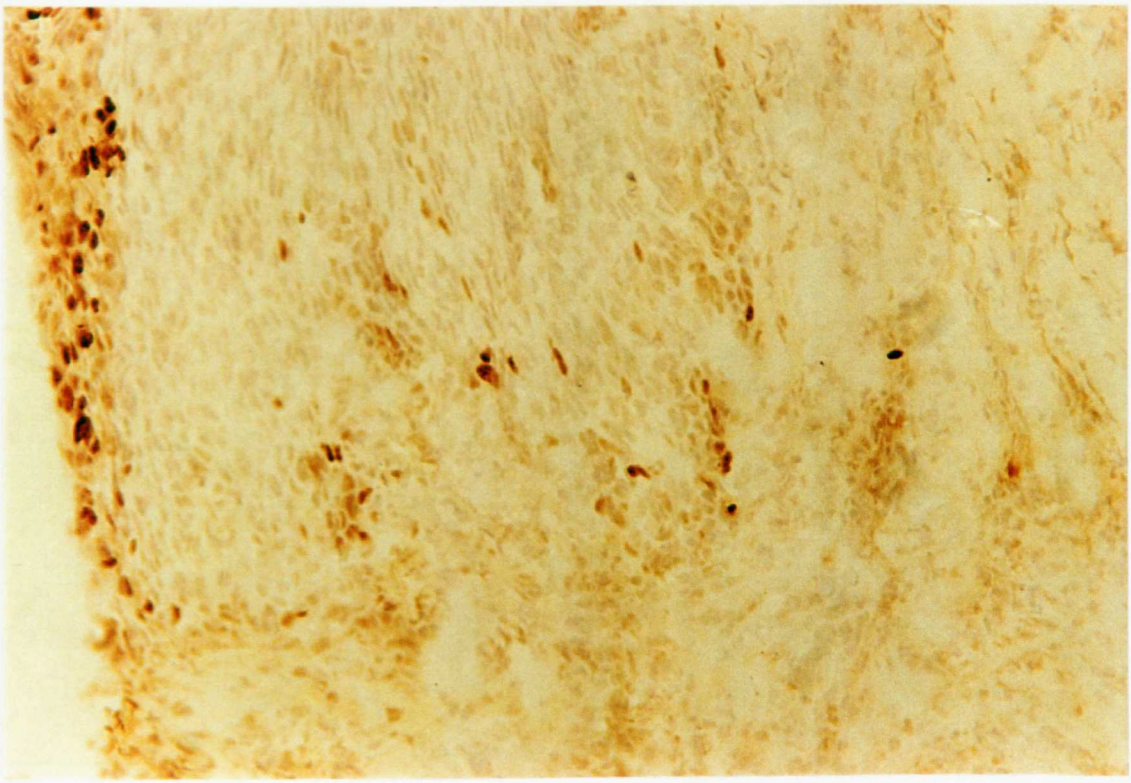
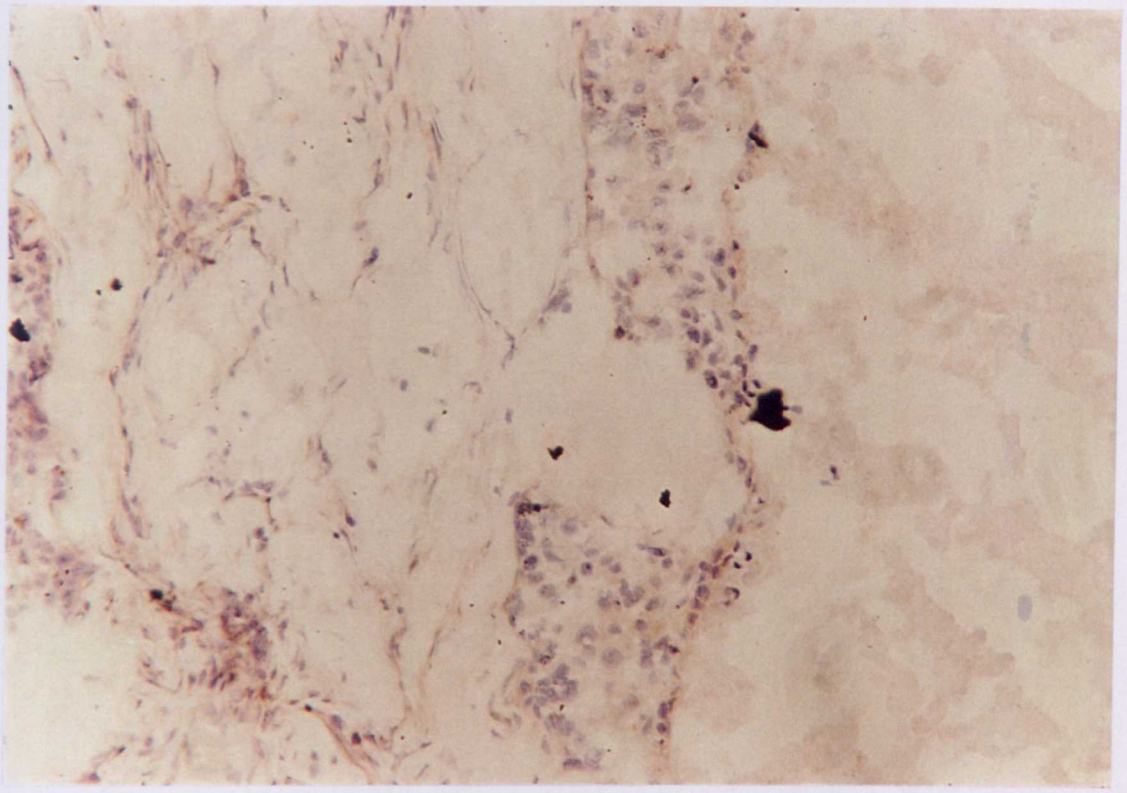


Figure 6.1: HNSCC patient 6.

Section stained with: A= Isotype control. B= p53 antibody DO-7. Magnification x 100.

A



B

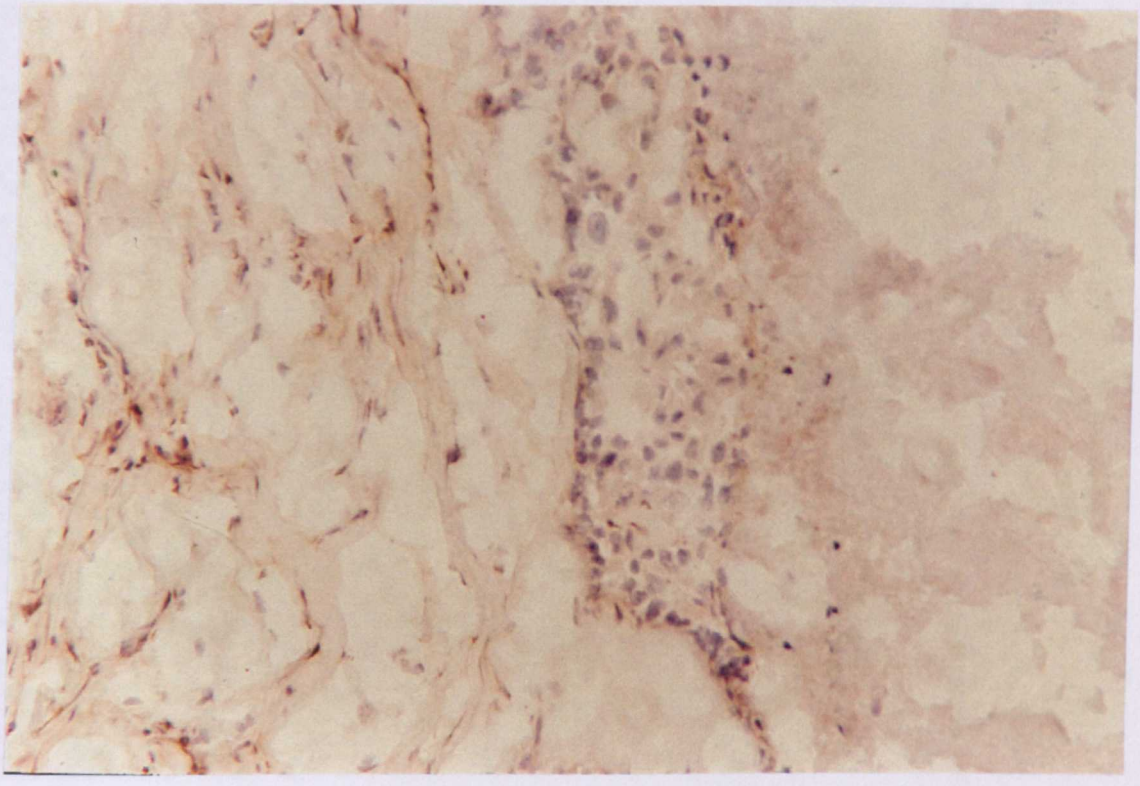
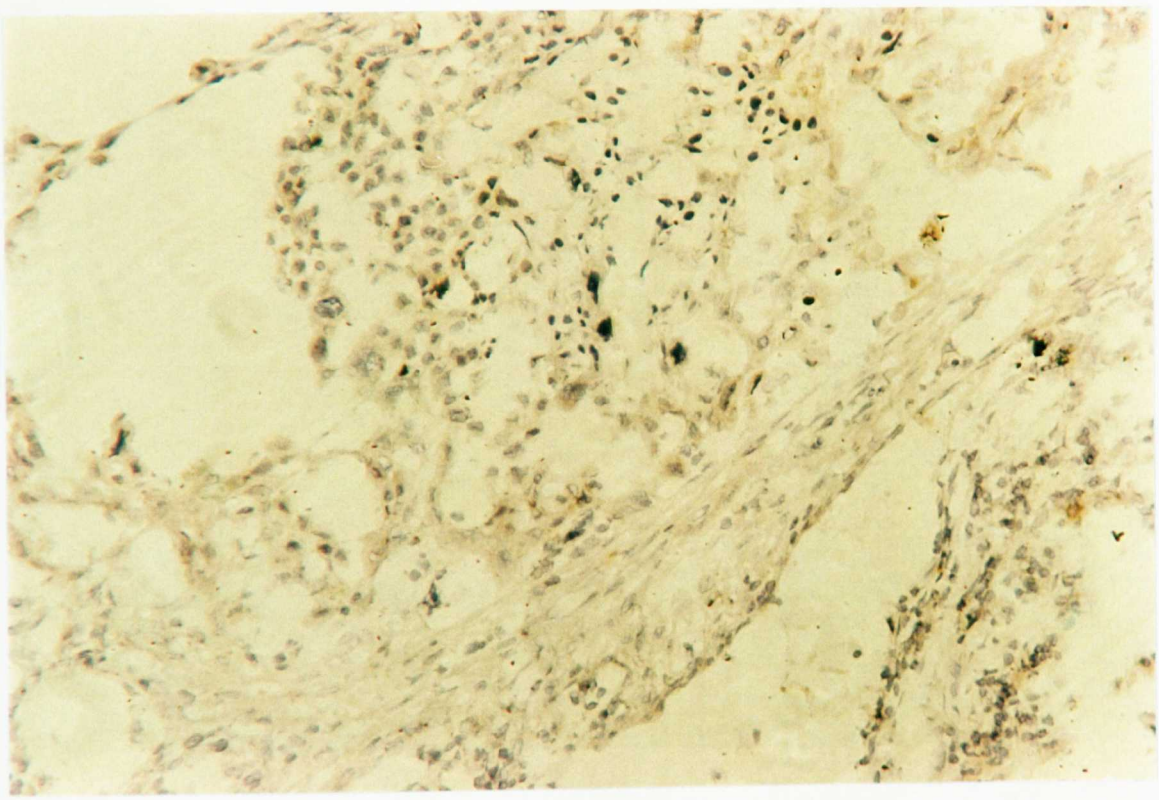


Figure 6.2: HNSCC patient 7.

Section stained with: A= Isotype control. B= p53 antibody DO-7. Magnification x 100.

A



B

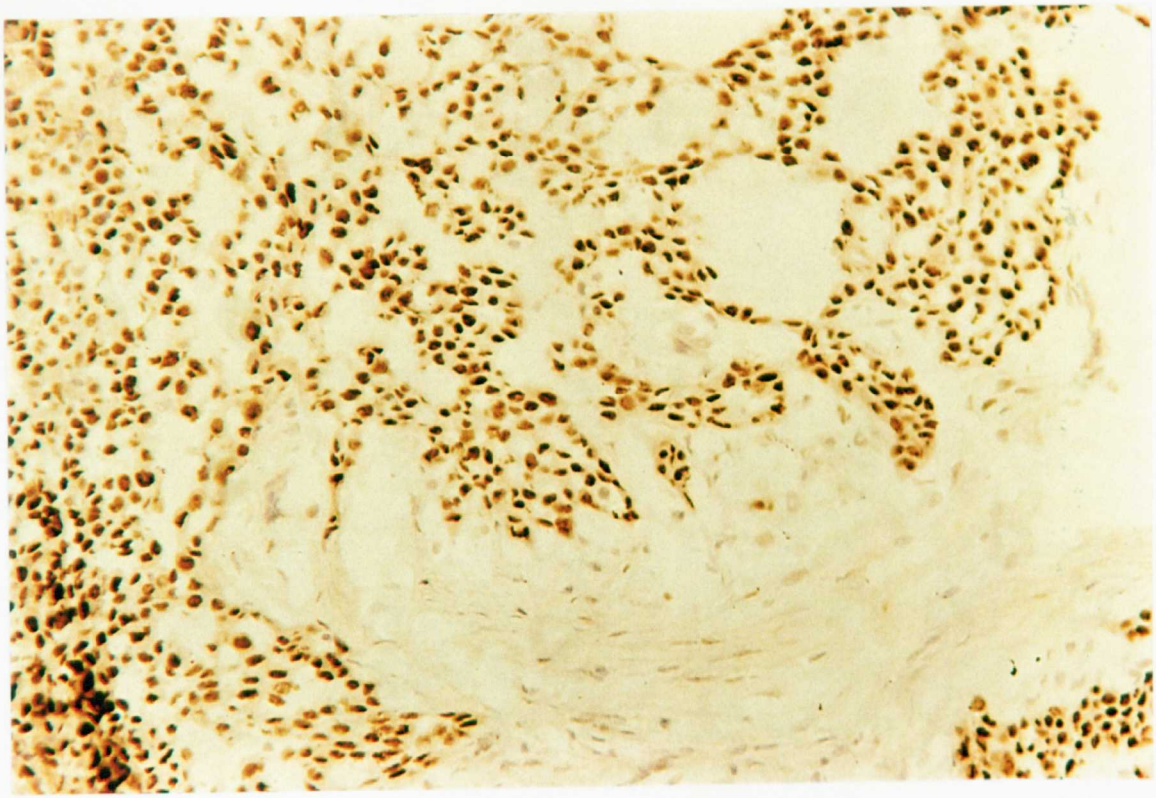


Figure 6.3: HNSCC patient II .

Section stained with: A= Isotype control. B= p53 antibody DO-7. Magnification x 100.

A

B

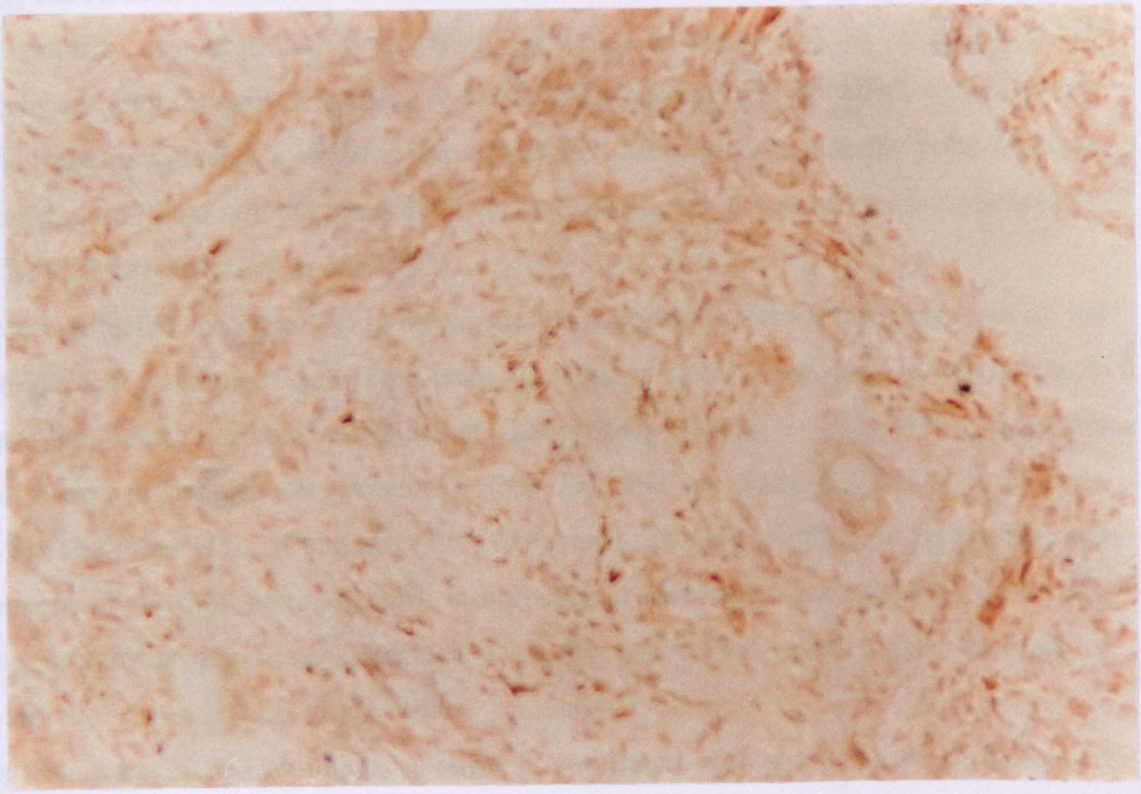


Figure 6.4: HNSCC patient 15

Section stained with: A= Isotype control. B= p53 antibody DO-7. Magnification x 100.

CHAPTER 7: DISCUSSION

The p53 suppressor gene is mutated in 50-60% of human cancers (Hollstein, *et al.*, 1991) and results in the stabilisation and accumulation of the inactivated protein within the cancer cell. Conformational changes in the p53 protein, generated as a result of point mutations, are frequently recognised by the monoclonal antibody Pab240, due to the exposure of the 240 epitope, RHSVV (amino-acids 212-217) (Milner, *et al.*, 1990). It is possible that peptides in this exposed region are processed in a different manner to the wild-type protein, presented on the tumour cells surface in the context of MHC class-I and therefore, have the potential to be recognised by tumour specific CTL. The p53 gene is a tumour suppressor gene present in all cells, and mutations within the gene represent the single most common genetic alterations observed in human cancers (Hollstein, *et al.*, 1991; Caron de Fromental and Soussi, 1992).

The majority of mutations of p53 result in the stabilisation and accumulation of the protein in tumour cells (Bodner, *et al.*, 1992) making p53 an ideal candidate for immunotherapy against a wide variety of cancers. Mutations, commonly point missense mutation, are found within the core domain and are clustered around four hot spots (175, 248, 249, 273 (Hollstein, *et al.*, 1991).

These can further be classified into two groups according to the effect they have on the protein conformation of p53. One class of mutants (273, 248) does not significantly affect the conformational structure of the protein (Hinds, *et al.*, 1990), does not associate with heat shock protein 70 (hsp-70), and are Pab240-/Pab1620+ (Milner, 1995). The other class of mutants (175, 249) results in the unfolding of the protein, and exposure of the 240 epitope which is normally cryptic and inaccessible to antibodies; these mutant proteins are associated with the hsp70 and were highly sensitive to proteolytic enzymes (Lee, *et al.*, 1994).

The frequency of p53 mutations is strongly correlated with the presence of antibodies against the p53 protein in several types of cancers (Agelopoulou, *et al.*, 1994; Lubin, *et al.*, 1995), as a result of an active secondary self-immunisation. Moreover Davidoff and co-workers (Davidoff, *et al.*, 1992) showed that tumours which elicit an antibody response contain HSP70 complexed to mutant p53. These findings and the fact that several HSPs have been shown to have a role in antigen processing (Vanbuskirk, *et al.*, 1989; Srivastava, *et al.*, 1994), suggest that antigen processing of the mutant p53 conformational protein could be different from mutations which do not affect the

conformation of the p53 protein which may result in markedly different peptide repertoires being displayed on the surface of the cells. These peptides remain to be identified.

The elution of such peptides on the surfaces of tumour cells, however attractive is the idea remains extremely difficult due to their small amount and the large number of cells required. Therefore the problem of their identification had to be approached from the other end. This implied using the amino-acid protein sequence to select HLA-A2 peptides, which would then have to be tested for their ability to bind to HLA-A2 molecules and generate CTL *in vitro* and then determine whether they are naturally endogenously processed and presented at the surface of cancer cells.

Here new HLA-$A2^+$ p53 peptides (8-11 amino-acids long) were selected from the p53 protein sequence and investigated for their potential use in future immunotherapy programmes against cancer.

Peptides were first tested for their ability to stabilise HLA-A2 molecules at the surface of T2 cells. The results of this assay were used to select high binder peptides to be used in the generation of CTL *in vitro* (Chapter 3). Peptides 217-225 and 188-197 were thereby selected. Peptide 217-225, chosen to test the hypothesis of different processing pathway depending on the protein conformation, was used for the generation of CTL generation, which was first performed using whole PBMC. Dendritic cells were then successfully generated and used as APC in the assay. Then dendritic cells pulsed with peptide 217-225 were used to generate CTL *in vitro*. Peptide 264-272 was first used as positive control in the generation of CTL but was then used to test the hypothesis that p53 processing pathway might be different depending on its conformation (Chapter 5). In order to test this hypothesis a panel of target cells expressing either a conformational mutated p53 protein (mutation at amino-acid position 175) or 'wild-type' conformational mutated p53 protein (mutation at amino-acid position 273) and HLA-A2 molecules were prepared (Chapter 4). CTL generated with peptide 217-225 or peptide 264-272 were both shown to kill preferentially target cells bearing a p53 mutation at amino-acid position 175, indicating that the protein conformation might indeed have an influence in the outcome of which peptides are presented at the surface of the cell.

Fifteen wild-type p53 peptides were selected and tested for their ability to stabilise HLA-A2 molecules at the surface of the T2 cells. The T2 binding assay is a very easy, quick and non-hazardous (no radio-labelled isotope involved) assay for testing the ability of a given peptide for its ability to bind to HLA-A2 molecules. The results obtained with this assay using well-known peptides correlated with published work (Chapter 3). Peptides containing no anchor residue were not found to stabilise the HLA-A2 molecules at the surface of the T2 cells whereas peptides containing two preferred anchor residues showed strong binding properties (FR >1.5). Peptides with only one tolerated anchor residue did not show high binding capability. However, peptides with one preferred anchor residue at position 9 and either one or none tolerated anchor residue at position 2 did show strong affinity for HLA-A2 molecules (FR > 1.5) with the exception of two peptides. This illustrates the need of two anchor residues for peptide binding even though peptide can bind with only one anchor residue. However factors other than size and anchor residues are also critical in determining whether or not a peptide will bind HLA-A2 molecules. Indeed the presence of other residues at non-anchor positions can either increase or abolish completely the binding affinity of peptide bearing them (Drijfhout, *et al.*, 1995). Despite the presence, in a given antigen, of peptides with both anchor residues only a few will generate CTL *in vitro*. One other important parameter is the determination of the efficiency of peptide to bind and remain bound to MHC molecules. Indeed a strong correlation between immunogenicity and the stability of MHC-peptide complex has been reported (van der Burg, *et al.*, 1996). A peptide with a poor ability to bind to HLA-A2 molecules was also found to be non-immunogenic. By improving its binding ability it was also possible to enhance its immunogenicity (Lipford, *et al.*, 1995). This however does not automatically imply that peptides with moderate binding affinity are immunologically non-important. This was the case of the WT p53 149 peptide found to have a moderate binding affinity (Nijman, *et al.*, 1994) and yet capable of eliciting CTL *in vitro* capable of killing peptide-pulsed target as well as tumour targets (Theobald, *et al.*, 1995/1997). However if a peptide possess a too strong an affinity for a given MHC molecule it is likely that CTL reacting with it it would have be deleted as a saveguard mechanism to avoid later autoimmunity. This has indeed been reported for peptide 187-197 (Theobald, *et al.*, 1997).

It is therefore likely that a therapeutic window of binding affinities exists for the generation of a CTL response, beyond which very low or very high affinity binding peptides, would for different reasons, fail to elicit a CTL response. The level of HLA molecules expressed on the surface of tumour cells is also a determining influence, and different CTL responses have been observed with target cells expressing different levels of HLA class-I surface molecules (Rivoltini, *et al.*, 1995).

Quantitative and qualitative differences in peptide and MHC class-I expression will determine the efficiency with which antigen-specific CTL will be generated. For peptides, this depends mainly on how the cell has processed the protein, from which they derived. The p53 peptide repertoire displayed by MHC at the surface of a given tumour cell might therefore depend upon the conformation state of the p53 protein and the efficiency of protease processing. In order to test this hypothesis, tumour cells expressing either, 'naturally' or transfected with, conformational p53 mutants or wild-type conformation p53 protein and expressing HLA-A2 molecules or transfected with the HLA-A2 gene were prepared and used as target for CTL generated with either 217-225 or 264-272 p53 peptides. Wild-type p53 gene could not be transfected into the p53 null SaOs-2 cells because of its detrimental effect on the cell cycle. Induction of CTL response *in vitro* proved to be a difficult task, especially before dendritic cells were used as APC. Dendritic cells, indeed, play a crucial role in initiating cellular immune responses and have been proposed as an adjuvant for cancer therapy (Grabbe, *et al.*, 1995). Here they have successfully been used as stimulators for the generation of human anti-p53 specific CTL which has potential clinical application.

These CTL lines were able to specifically kill peptide-pulsed targets as well as tumour targets overexpressing the p53 protein. Tumour targets expressing mutant p53 conformation were preferentially killed as compared with tumour targets expressing wild-type conformational mutants providing evidence for the hypothesis of differential processing. However protein overexpression appears not to be prerequisite for the presentation of p53 epitopes and recognition by the CTL since Röpke, *et al.*, 1996 and more recently Chikamatsu, *et al.*, 1999, showed that SCC4, a HNSCC cell line HLA-$A2^+$, bearing a missense mutation at codon 155 and overexpressing p53 protein was not killed by CTL generated with the 264-272 peptide, whereas SCC9 cells, HLA-$A2^+$, bearing a deletion at position 275-285, and which do not overexpress p53 protein was killed by the same CTL. Interestingly CTL clones but not bulks, generated with dendritic

cells pulsed with p53 peptide 264-272 or 149-157 by Chikamatsu, *et al.*, 1999 were able to kill non-peptide-pulsed tumour cells. In addition only bulk CTL populations generated with peptide 264-272 were able to generated CTL, from two out of five HLA-$A2^+$ donors, which, were able to kill peptide-pulsed tumour target as well as T2 cells. In this study however bulk CTL generated with either the 217-225 or 264-272 peptides were able to kill both peptide-pulsed and non-peptide pulsed target. It is possible that the method used to generate dendritic cells may influence their ability to present to and activate $CD8^+$ cells. Chikamatsu, *et al.*, 1999 produced dendritic cells CD1a negative whereas in this study the denritic cells used were CD1a positive.

Results obtained in this study confirm and extend the recent findings of Theobald, *et al.*, 1998. They demonstrated that the R->H mutation at residue 273 of human p53 alters proteosomal processing of the protein due to inhibition of proteolytic cleavage between residue 272 and 273. Target cells bearing this mutation were not recognised by CTL generated to the wild-type p53 peptide 264-272 *in vitro* or *in vivo*, as this epitope was not generated. However, a HLA-A2 restricted CTL line specific for human wild-type p53 peptide 149-157 lysed transfected cell expressing this mutation, indicating efficient processing of other p53 epitopes. Here, target cells with the R->H mutation at position 273 were not lysed by CTL-264 lines and in addition, were only lysed by CTL lines to wild-types p53 217-225 peptide in one of three donors. Whilst the former observation is in agreement with that of Theobald et al (Theobald, *et al.*, 1998), their results suggested that the 273 mutation may only affect a flanking peptide epitope and a precursor peptide. Here, the lack of recognition of the 273 mutated p53 observed with CTL lines generated to 217 in some donors could be due to differences in the affinities of the TCR of the CTL lines generated or relative expression of p53 and HLA-A2 molecules; alternatively, regions of the protein distant to the mutation may fail to donate peptides to the processing pathway.
Key questions are whether patients whose tumours harbour a p53 mutation have demonstrable precursor CTL or whether they can be induced, and whether there is an association between the p53 gene mutation, HLA type and disease progression.

The study of HNSCC patients performed here demonstrated a p53 protein accumulation in 60% of these patients (6/10) and p53 antibodies in 20% (2/10) of sera, and 14% in

total (2/14 sera tested). Interestingly the two patients with p53 antibodies were also found to have p53 protein accumulation. However p53 accumulation is not always sufficient for antibody production (Tilkin, *et al.*, 1995). Four patients were found to be HLA-A2$^+$. One patient, patient 9, HLA-A2$^+$, was found to have both p53 antibodies and p53 accumulation. Only 50 % of patients with p53 mutation will develop antibodies (Cough, *et al.*, 1994) and the type of mutation itself may influence the production of antibodies (Davidoff, *et al.*, 11992). It is also possible that for the same mutation one individual might have p53 antibodies whereas an another one might not. This might be due to different MHC class-I or class-II molecules expressed by these individuals.
p53-antibodies are likely to be the result of self-immunisation process toward the p53 protein which is normally expressed in minute quantities in the body (Lubin, *et al.*, 1993). Interestingly immunoglobulin subclasses of these antibodies have been found to be of IgG1 and IgG2 in all sera of HNSCC patients suggesting an active immunisation, these being the result of a second immune response (Lubin, *et al.*, 1995). p53 can prime a p53-specific T cell response *in vivo*, and is processed at least *in vitro* by APC and recognised by T cells of certain breast cancer patients (Tilkin, *et al.*, 1995).

In summary new p53 epitope (peptide 217-225) from the 240 region of the protein was shown to bind with high affinity to HLA-A2 molecules and to generate CTL capable of killing peptide-pulsed targets as well as tumour cells overexpressing p53 protein. This peptide was endogenously processed and presented at the surface of tumour cells expressing a conformational mutant p53 suggesting that p53 protein processing is influenced by its conformation.

This study concentrated in the assessement of new class-I restricted antigenic epitopes from the p53 protein for their potential use in future immunotherapy programme. It is now however becoming clear that in order to obtain optimal immunisation both MHC class-I and class-II antigenic epitopes will be necessary for recognition by CD4$^+$ and CD8$^+$ cells. Many groups have already started the search for p53 class-II epitopes and results obtained in this study concerning class-I epitopes will be carried on and extended to class-II epitopes in the laboratory of Professor Rees, Trent University, Nottingham.

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PUBLICATIONS ARISING FROM THIS WORK

- "P53 as a potential tumour target for immunotherapy" (Poster)

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- "p53 conformational mutants are killed preferentially by cytotoxic T lymphocytes generated in vitro using dendritic cells pulsed with new wild-type p53 peptide" (Poster)

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