Cancer Immunol Immunother (2000) 49:417–425

ORIGINAL ARTICLE

Stephanie E. B. McArdle · Robert C. Rees
Kevin A. Mulcahy · Jeanine Saba
Catherine A. McIntyre · Anna K. Murray

Induction of human cytotoxic T lymphocytes that preferentially recognise tumour cells bearing a conformational p53 mutant

Received: 23 March 2000 / Accepted: 22 June 2000

Abstract The tumour-suppressor gene *p53* is pivotal in the regulation of apoptosis, and point mutations within *p53* are the commonest genetic alterations in human cancers. Cytotoxic T lymphocytes (CTL) recognise peptide-MHC complexes on the surface of tumour cells and bring about lysis. Therefore, p53-derived peptides are potential candidates for immunisation strategies designed to induce antitumour 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), which may be processed differently from the wild-type protein resulting in an altered MHC-associated peptide repertoire recognised by tumour-specific CTL. In this study 42 peptides (37 overlapping nonameric peptides, from amino acids 193–237 and peptides 186–194, 187–197, 188–197, 263–272, 264–272, possessing binding motifs for HLA-A2) derived from the wild-type p53 protein sequence were assayed for their ability to stabilise HLA-A2 molecules in MHC class I stabilisation assays. Of the peptides tested, 24 stabilised HLA-A2 molecules with high affinity (fluorescence ratio >1.5) at 26 °C, and five (187–197, 193–200, 217–224, 263–272 and 264–272) also stabilised the complexes at 37 °C. Peptides 188–197, 196–203 and 217–225 have not previously been identified as binders of HLA-A2 molecules and, of these, peptide 217–225 stabilised HLA-A2 molecules with the highest fluorescence ratio. Peptide 217–225 was chosen to generate HLA-A2-restricted CTL in vitro; peptide 264–272 was used as a positive control. The two primary CTL thus generated (CTL-217 using peptide 217–225; and CTL-264 using peptide 264–272) were capable of specifically killing peptide-pulsed T2 or JY cells. In order to determine whether these peptides were endogenously processed and to test the hypothesis that mutants expressing different protein conformations would generate an alternative peptide repertoire at the cell surface, a panel of target cells was generated. HLA-A2$^+$ SaOs-2 cells were transfected with p53 cDNA containing point mutations 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 mutations at positions 273 and 175 respectively, were transfected with a cDNA encoding HLA-A2. The results showed that primary CTL generated in response to both peptides were capable of killing SaOs-2/175 and SKBr3-A2 cells, which possess the same mutation, but not SaOs-2/273, A431-A2 or SKBr3 cells transfected with control vector. This suggests that these peptides are presented on the surface of SaOs-2/175 and SKBr3-A2 cells in a conformation-dependent manner and represent potentially useful target peptides for immunotherapy.

Key words p53 · Conformational mutants · CTL · HLA-A2 peptides · Dendritic cells

S. E. B. McArdle · K. A. Mulcahy · J. Saba
C. A. McIntyre · A. K. Murray
Division of Oncology and Cellular Pathology (Cancer Studies), University of Sheffield Medical School, Sheffield, UK

S. E. B. McArdle (✉) · R. C. Rees
Nottingham Trent University, Department of Life Sciences, Clifton Lane, Nottingham NG11 8NS, UK
e-mail: stephanie.mcardle@ntu.ac.uk
Tel.: +115-848-3544
Fax.: +115-948-6636

Introduction

Cancer cells arise from the inappropriate activation or deactivation of genes involved directly or indirectly in the control of cellular growth, differentiation and cell death [15, 38, 16] resulting in uncontrolled cell proliferation. Viral proteins and tumour antigens are normally processed via the 26s proteasome. The resulting peptides are then presented on the surface of malignant cells in association with MHC class I molecules for recognition by cytotoxic T lymphocytes (CTL). Following the pioneering work of Boon and co-workers, definitive evidence for tumour-specific or associated antigens was obtained [4] and a number of tumour antigens capable

of eliciting CTL responses were identified. These included melanoma differentiation antigens [4], mutant ras [25], Epstein Barr virus [31], human papilloma virus [30], HER-2/neu [40], mucin [14], and p53 [24].

Of particular interest is the *p53* tumour-suppressor gene, which is mutated in 50–60% of human cancers [11]. Genes containing missense mutations often result in the stabilisation and accumulation of the inactivated protein within the cancer cell. CTL generated to self-peptides can, in some circumstances, undergo clonal expansion and mediate cytolysis upon recognition of appropriate peptide MHC class I complexes. Indeed, it has been shown that mutant p53 protein overexpression results in the processing and presentation of p53 peptides by MHC class I molecules on the surface of tumour cells [28]. This represents a "break of immune tolerance" against wild-type p53 peptides sequences. CTL responses against tumours bearing a mutant p53 protein have been obtained following either vaccination in vivo using murine models [39, 19, 17] or stimulation of peripheral blood mononuclear cells (PBMC) from healthy human donors in vitro using synthetic peptides [24, 28, 9].

"Hotspot" mutations of the *p53* gene have been classified into two categories: (i) those which induce a conformational change in p53 protein to a mutant conformation, for example a point mutation at amino acid 175, and (ii) those which retain the wild-type conformation, for example a point mutation at position 273 [21, 22]. Conformational changes in the p53 protein are frequently recognised by the mAb PAb240, owing to unfolding of the protein and exposure of the 240 epitope, RHSVV (residues 212–217). It is, therefore, possible that some peptides could be derived from conformational mutants instead of from the wild-type protein. If these peptides are endogenously processed and presented at the cell surface of tumour cells, they could be used as targets for CTL tumour-specific immunotherapy.

In the present study overlapping peptides from the wild-type p53 protein were synthesised and assessed for binding to HLA-A2 molecules in an MHC stabilisation assay. We identified a new p53 peptide that bound HLA-A2 antigen with high affinity (peptide 217–225) and could induce primary CTL from healthy donors, capable of killing either targets pulsed with the 217–225 peptide or tumour cells overexpressing mutant p53 protein. Our results also indicate that mutations affecting the p53 protein conformation (175-like mutants) were more susceptible to CTL-mediated lysis than cells expressing a p53 mutation that does not result in a conformational change in the protein (273-like mutants), suggesting that p53 antigen processing, and hence the peptide repertoire, might be conformation-dependent.

Materials and methods

Peptides

Peptides were synthesised by Alta Bioscience, UK, and shown to be at least 80% pure by HPLC and mass spectrometry. These peptides consisted of three groups: 37 overlapping nonameric peptides derived from the 240 region of the wild-type p53 protein, amino acids 193–237; 5 peptides, nine or ten amino acids in length, situated close to this region, which possessed binding motifs for HLA-A2: 186–194, 187–197, 188–197, 263–272 and 264–272; and a control peptide, derived from the matrix protein of influenza virus, previously shown to bind to HLA-A2molecules with high affinity (M58–66) [8], which was used as a positive control. Peptides were dissolved in dimethylsulfoxide (DMSO) and stored in small aliquots at −20 °C until required.

Antibodies

The mAb HB-82, which recognises HLA-A2 and HLA-B7 alleles, was obtained as supernatant (a gift from Dr. J. Bartholomew, Cancer Research Campaign Paterson Institute, Manchester, UK). The mAb DO-7, which recognises both wild-type and mutant p53 protein, and the corresponding isotype control were purchased from Dako, UK. Fluroscein-isothiocyanate (FITC)-conjugated goat anti-(mouse Ig) antibody, used as a top layer for flow cytometry, was purchased from TCS, UK.

MA2.1 is a mAb specific for HLA-A2 and HLA-B17 molecules, and incubation of T2 cells with this antibody enables HLA-A2 to bind peptide at the cell surface more efficiently [3]. The hybridoma cell line producing this antibody was purchased from the ATCC.

Cytokines

Recombinant human (rh) interferon γ (IFNγ) was a gift from NCI Biogen, UK; rh tumor necrosis factor α (TNFα) was a gift from Strangeways Laboratories (Cambridge, UK); rh interleukin-2 (IL-2) was a gift from Glaxo Pharmaceuticals (Geneva, Switzerland); rhIL-7 and rhIL-4 were purchased from Peprotech (London, UK) and (rh) granulocyte/macrophage-colony-stimulating factor (GM-CSF) from Roche (Lewes, UK).

Cell lines

The 174CEM.T2 (T2) cell line is a lymphoblastoid cell line defective in antigen processing (TAP-1 mutation) [5]. T2 cells and JY cells, a lymphoblastoid cell line (HLA-A2$^+$), were a gift from Dr. J. Bartholomew (Paterson Institute, Manchester, UK). K562 is an erythroleukaemia cell line that is HLA-class-I-negative and sensitive to natural-killer (NK)-cell-mediated lysis (a gift from ICRF, London, UK). T2, K562 and JY cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS). Rosi, an HLA-A1$^+$/HLA-A2$^-$ lymphoblastoid cell line was obtained from the Ludwig Institute (Brussels, Belgium) and cultured in Iscove's modified Dulbecco's medium supplemented with 10% FCS. The SaOs-2 cell line is an HLA-A2$^+$ osteosarcoma cell line in which the p53 alleles are deleted [10]. SaOs-2 cells were obtained from the Department of Human Metabolism and Clinical Biochemistry, Sheffield, UK, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. The A431 breast carcinoma cell line (HLA-A2$^-$), bearing a point mutation in the *p53* gene within the codon for amino acid 273, was purchased from ECACC and cultured in MEM supplemented with 10% FCS and 8 mg/ml insulin. The SKBr3 cell line (genetically HLA-A2$^+$ but phenotypically HLA-A2$^-$) is a breast carcinoma cell line bearing a point mutation in the *p53* gene within the codon for amino acid 175, and was purchased from ATCC and cultured in McCoy's 5A medium (Gibco) supplemented with 10% FCS.

cDNAs

Full-length cDNA for p53 mutated at either amino acid position 175 (R → H) or 273 (R → H), subcloned into a pBR322-derived plasmid and under the control of the human cyclomegalovirus promoter, were gifts from Professor T. Soussi (Marie-Curie, Paris,

France). The HLA-A2 cDNA subcloned into pcDNA3 (Invitrogen) was a gift from Dr. P.F. Robbins, NIH, USA.

Isolation of peripheral blood mononuclear cells (PBMC)

The collection of blood for this study was approved by South Sheffield Research Ethics Committee. Venous blood from healthy volunteers, was added to heparin and PBMC were isolated by standard gradient centrifugation on J Prep.

MHC stabilisation assay

Peptides were tested for their ability to stabilise HLA-A2 molecules on the surface of T2 cells in a MHC class I stabilisation assay, at 26 °C and 37 °C, as previously described [20]. Briefly 40 µl T2 cells (4×10^6 cells/ml) was incubated with 10 µl relevant peptide for at least 18 h at 26 °C and 37 °C. The influenza matrix peptide, M58–66, was used as a positive control and the background expression of the HLA-A2 molecule determined using DMSO as a negative control. Each peptide was tested on at least three separate occasions at a final concentration of 100, 10 and 1 µM. Peptides bound to the surface of the T2 cells were determined by indirect immnuofluorescence using HB-82 as primary antibody and an FITC-conjugated goat anti-(mouse Ig) as the top layer. The fluorescence ratio (FR) for each peptide was calculated by comparing the median channel of fluorescence of the experimental sample with that of the DMSO (negative) control. Peptides were considered to stabilise HLA-A2 molecules with high affinity if the FR was greater than or equal to 1.5 and low affinity if the FR was above 1.1 and less than 1.49 at a peptide concentration of 100 µM.

Transfection with p53 cDNA

SaOs-2 cells were seeded in a six-well plate at 0.5×10^6/well and cultured for 15 h at 37 °C. The cells were gently rinsed with serum-free growth medium and then overlaid with 200 µl/well transfection mix. The transfection mix consisted of serum-free growth medium containing 2 µg p53 plasmid DNA, purified on Qiagen Maxi-prep, and 1.66 ml Lipofectamin (Life Technologies, UK) previously incubated for 45 min at room temperature. Following incubation at 37 °C, in 5% CO_2 in air for 2 h, the transfection mix was replaced with 1 ml/well DMEM + 10% FCS. The plate was then incubated at 37 °C, for 15 h, at which time the medium was replaced with 2 ml DMEM + 10% FCS. After a further 72 h culture, cells were subcultured (1:20) into selective medium containing Geneticin (Life Technologies, Paisley, UK) at 0.5 mg/ml. Proliferating colonies were subsequently cloned and p53 protein expression was determined, after fixation and permeabilisation of the cells, by indirect staining with DO-7 antibody followed by FITC-labelled IgG, and analysed in a flow cytometer.

Transfection with HLA-A2 cDNA

Two cell lines bearing "naturally" mutated *p53* genes, changing an amino acid at either position 175 (SKBr3) or position 273 (A431), were used for comparison. Since these cells do not express HLA-A2 molecules at their surface they were transfected with HLA-A2 cDNA subcloned into pcDNA3 (pcDNA3-HLA-A2). Linearised pcDNA3-HLA-A2 (4 µg) was mixed with 2×10^6 cells in a total volume of 0.4 ml complete growth medium, placed in an electroporation cuvette (Equibio Ltd., UK) and electroporated at 280 V (SKBr3) and 260 V (A431) and a capacitance of 960 mF. After 24–48 h, cells were stained for HLA-A2 molecule expression using mAb HB-82. Cells expressing high levels of HLA-A2 were sorted, in a Vantage FACSsort cytometer (Becton Dickinson). Sorted cells were then cultured in the presence of geneticin (0.3 mg/ml for SKBr3 and 0.2 mg/ml for A431). HLA-A2 molecule expression was subsequently determined using the HB-82 mAb, followed by FITC-conjugated goat anti-(mouse IgG), and the fluorescence intensity measured by flow cytometry.

Effect of cytokine treatment on transfected cells

In order to determine the effect of cytokine treatment on expression of HLA-A2 molecules, transfected cells were treated with 200 U/ml rhIFNγ and 1000 U/ml rhTNFα for 24 h and expression levels were assessed by flow cytometry. Previous work in our laboratory had demonstrated that these concentrations of cytokines were optimal for increasing HLA-A2 molecule expression.

Generation of CTL

Generation of dendritic cells

Dendritic cells were first generated by culturing adherent PBMC with GM-CSF (1000 U/ml) and IL-4 (500 U/ml) for 7 days followed by 3 days of culture with TNFα. The cells generated did not express T cell (CD3) or B cell (CD19) markers, but did express high levels of CD1a molecules, MHC class I and class II antigens and the co-stimulatory molecule CD86 (data not shown). They expressed low levels of CD14 molecules, and were capable of stimulating allogeneic PBMC in mixed lymphocyte reaction (MLR) assay (data not shown). Mature dendritic cells were pulsed with relevant peptide before addition of $CD8^+$-enriched T cells.

$CD8^+$-enriched T cells and CTL generation

Non-adherent cells, stored as frozen stock from day 1, were thawed, washed, counted and depleted of $CD4^+$ cells using anti-CD4 Dynabeads (Dynal). Samples containing 2×10^6 of these effector cells were then co-cultured with 2×10^5 peptide-pulsed dendritic cells (dendritic cells were incubated with 50 µg peptide and β_2-microglobulin for 2 h at 37 °C, in 2 ml of RPMI-1640 containing 5% heat-inactivated autologous plasma) together with 10 U/ml rhIL-2 and 10 ng/ml rhIL-7. Effector cells were restimulated every 7 days with a mixture of peptide-pulsed and mitomycin-C-treated (8 µg/ml for 2 h followed by extensive washing) autologous PBMC, allogeneic HLA-$A2^+$ PBMC, and Rosi cells (unpulsed and used as feeder cells) at a ratio of 1:10 (antigen-presenting cells: effectors). On day 28, CTL were tested for their cytolytic activity as outlined below.

Cytotoxicity assay

The cytolytic activity of the CTL cultures generated were assessed in a standard 4-h ^{51}Cr-release assay. Briefly, 10^6 tumour targets were labelled with 3.7 MBq ^{51}Cr for 1 h in the presence (JY and T2 targets) or absence (transfected targets) of MA2.1 supernatant. Targets were incubated for an additional 1 h in the presence or absence of 10 µg/ml synthetic peptide, 217–225 or 264–272 as appropriate. Varying numbers of effector cells were added to 2×10^3 target cells, in triplicate, in the presence of a forty fold excess of unlabelled K562 cells to block non-specific lysis, in a final volume of 200 µl in a microtitre plate. Maximum lysis was determined in 1% sodium dodecyl sulfate and spontaneous release did not exceed 20% of the maximum. After a 4-h incubation, 50 µl supernatant was harvested onto a Lumaplate and ^{51}Cr release determined using a Topcount (Canberra Packard, UK). The percentage cytotoxicity was determined using the formula: Cytotoxicity (%) = [(test ^{51}Cr release − spontaneous release)/(maximum ^{51}Cr release − spontaneous release)] × 100.

Results

p53 peptides binding to HLA-A2 molecules

In order to determine whether peptides could bind to HLA-A2 molecules an MHC class I stabilisation assay

was performed at 37 °C and 26 °C. When tested at 37 °C, five peptides (peptides 187, 193, 217, 263, and 264) were shown to stabilise HLA-A2 molecules with high affinity at 100 μM (data not shown). In the light of previous work [20] demonstrating that a difference in HLA-A3 stabilisation could occur at 37 °C and 26 °C, p53 peptide stabilisation assays were also conducted at 26 °C. The results are shown in Fig. 1. Out of 24 peptides tested, 7 stabilised HLA-A2 molecules with high affinity (FR > 1.5) and 8 with low affinity (1.1 < FR < 1.5) when tested at 26 °C.

Three peptides, 188–197, 193–201 and 217–225, were shown to stabilise HLA-A2 molecules consistently in this assay, with peptide 217–225 possessing the highest FR score. This peptide has not previously been studied and was therefore chosen, along with peptide 264–272 (known to be capable of generating CTL in vitro) [28, 9], to generate CTL in vitro.

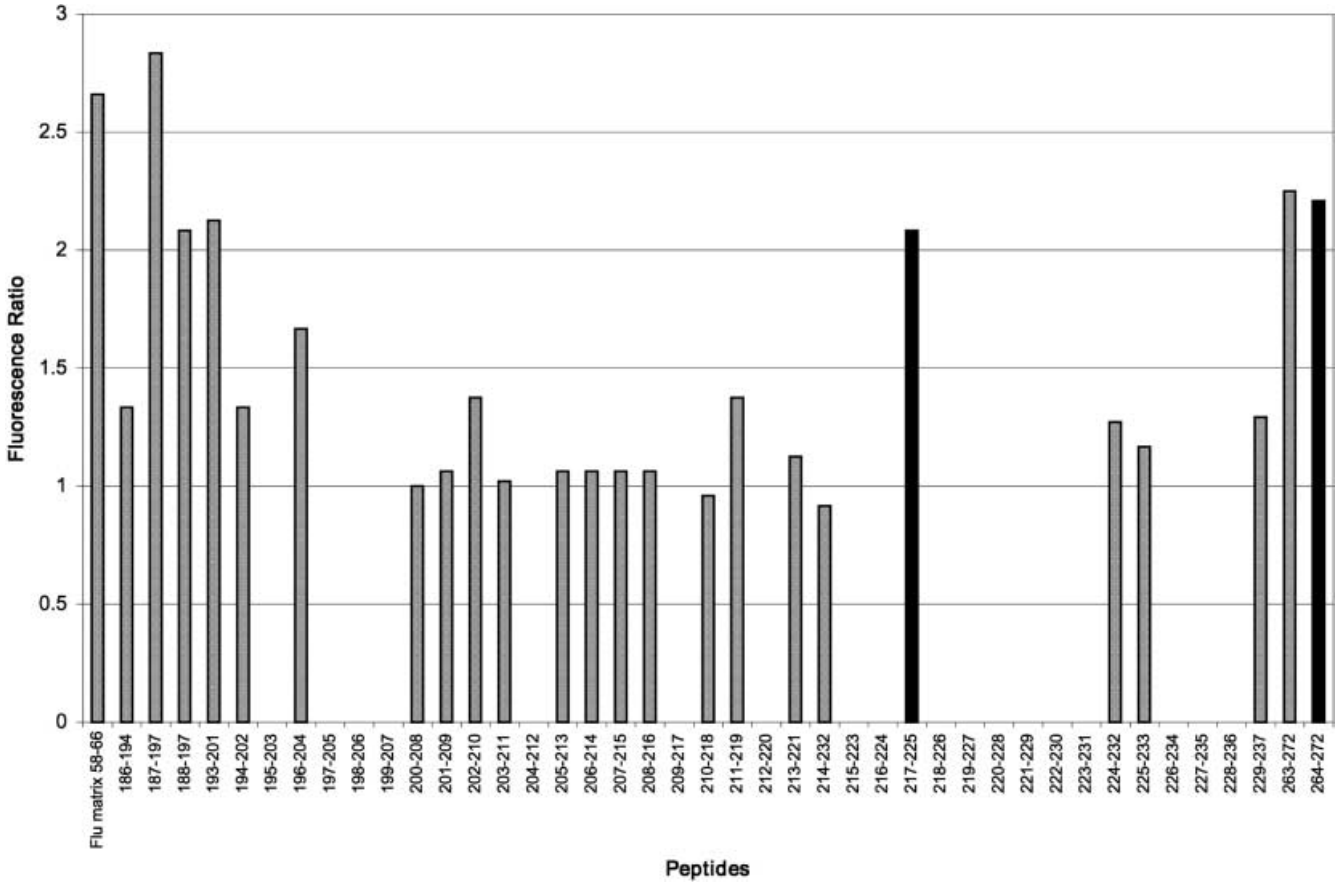
Fig. 1 Stabilisation of HLA-A2 by p53-derived peptides. Peptides were tested for their ability to stabilise HLA-A2 at 26 °C in an MHC class I stabilisation assay. T2 cells were incubated overnight in the presence of each peptide and stained for the presence of stabilised surface HLA-A2 molecules by dual-layer monoclonal antibody staining. The fluorescence ratio (FR) was determined by comparing the median channel of fluorescence of samples in the presence of peptide with that in the presence of dimethylsulfoxide (negative control). Peptides with FR < 1.1 were considered unable to stabilise HLA-A2, peptides with 1.49 > FR > 1.11 and FR < 1.49 were considered to stabilise HLA-A2 with low affinity and peptides with FR > 1.5 were considered to have high affinity [25]

Induction of peptide-specific CTL

In order to determine whether the 217–225 encoded peptide could elicit CTL capable of recognising targets expressing mutant p53, co-cultures of peptide-pulsed stimulators and autologous lymphocytes were established in vitro. CTL-217 was generated using the 217–225 peptide and CTL-264 was generated using the 264–272 peptide. Cytotoxicity assays were performed after three successive stimulations, as shown in Fig. 2 (results representative of three experiments). Pre-treatment of T2 cells with the MA2.1 antibody was shown to increase the level of killing (data not shown) and was therefore used to pre-treat T2 cells routinely prior to the assay for cytotoxicity. T2 cells pulsed with peptide 217–225 or 264–272 were specifically killed by CTL-217 (Fig. 2A) and CTL-264 (Fig. 2B) respectively. Similarly, only JY cells pulsed with the relevant peptide were lysed by these primary CTL (data not shown), whereas cells expressing wild-type p53 (T2 and JY cells) were not killed.

Transfection and characterisation of target cells

SaOs-2 cells (HLA-A2-positive) were transfected with p53 cDNA mutated at position 273 (SaOs-2/273) or 175 (SaOs-2/175), and stable, high-expression clones were selected (Table 1). As shown, only the SaOs-2 cells transfected with the mutated p53 cDNA expressed p53



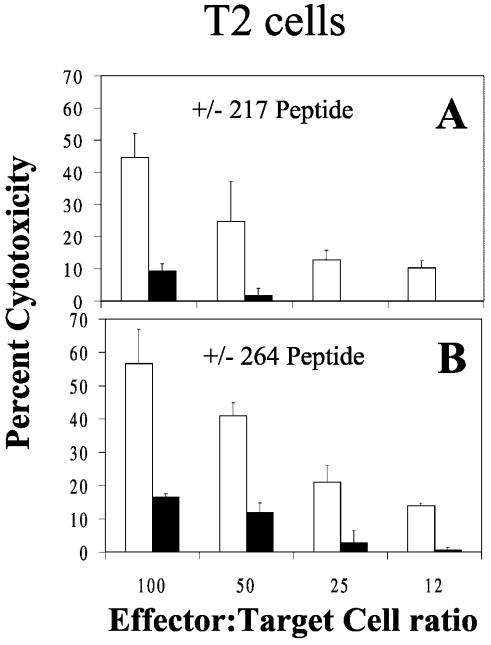


Fig. 2A, B Cytotoxicity of primary CTL CTL-217 and CTL-264 against T2 cells. Primary cytotoxic T lymphocytes (CTL) were generated following initial stimulation of peripheral blood mononuclear cells (PBMC) with peptide-pulsed autologous dendritic cells and two subsequent rounds of restimulation with peptide-pulsed mixtures of autologous PBMC, HLA-A2$^+$ allogeneic PBMC and Rosi cells. Cytotoxicity of effector CTL was determined by the percentage release of ^{51}Cr from ^{51}Cr-labelled T2 target cells pulsed with the appropriate peptides. **A** Cytotoxicity of CTL-217 against T2 cells pulsed with 217–225 peptide (*white bars*) or irrelevant peptide (*black bars*); **B** cytotoxicity of CTL-264 against T2 cells pulsed with 264–272 peptide (*white bars*) or irrelevant peptide (*black bars*)

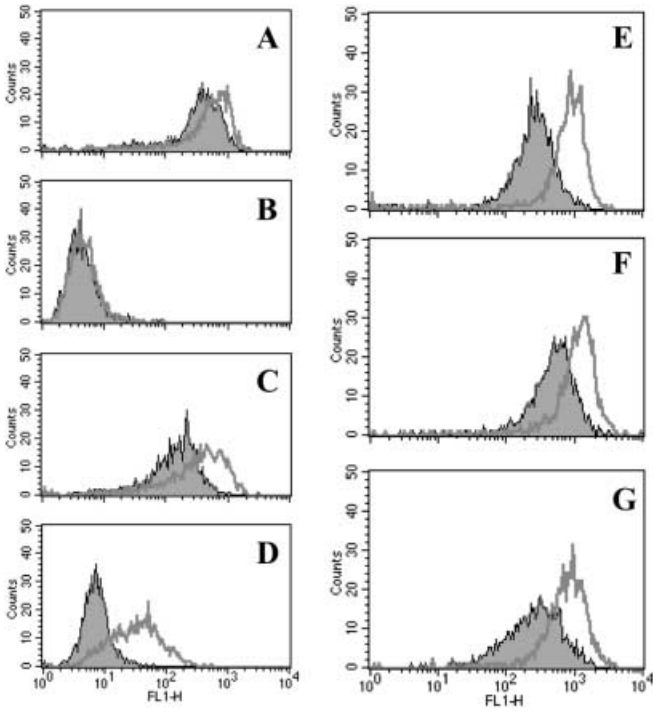


Fig. 3A–G Cell-surface HLA-A2 expression on transfected cells. Cells labelled with HB-82 antibody were subsequently incubated with fluorescein-isothiocyanate-conjugated goat anti-(mouse IgG) and analysed by flow cytometry. Cells were either untreated (*shaded profile*) or treated for 24 h with 200 U/ml recombinant human interferon γ (rhIFNγ) and 1000 U/ml recombinant human tumour necrosis factor α (TNFα) (*open profile*). **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 transfected with p53 vector control

Table 1 Expression of p53 protein in cells used as targets for lysis mediated by cytotoxic T lymphocytes. Figures in parentheses represent the mean fluorescence ratio of the cells after cytokine treatment. Cells were fixed in 1% paraformaldehyde, permeabilised in 70% methanol, labelled with either anti-p53 antibody DO-7 or the appropriate isotype control antibody for 30 min, washed and subsequently labelled with fluorescein-isothiocyanate-conjugated goat anti-(mouse IgG) for 30 min. Labelled cells were analysed by flow cytometry

Median channel fluorescence intensity		Cell line
Isotype control antibody	DO-7 antibody	
22.45 (21.16)	112.39 (99.06)	A431/A2
9.02 (9.21)	50.95 (72.26)	A431/vector
17.06 (19.08)	79.44 (95.9)	SKBR3/A2
18.51 (13.47)	124.75 (118.34)	SKBR3/vector
19.59 (19.85)	1319.33 (2070.72)	SaOs-2/175
18.83 (17.39)	473.02 (706.39)	SaOs-2/273
12.49 (12.58)	48.13 (47.83)	SaOs-2/vector

protein to high levels. A431 and SKBr3 cells (naturally expressing mutant p53) were stably transfected with HLA-A2 cDNA (A431-A2 and SKBr3-A2 respectively) and lines expressing high levels of cell-surface HLA-A2 molecules selected for (Fig. 3). Following cytokine treatment, with rhIFNγ and rhTNFα for 24 h, expression of the HLA-A2 molecule was up-regulated in all cell lines containing transfected HLA-A2 cDNA or the endogenous gene, and tumour cells used as targets in CTL assays were therefore pre-treated with these cytokines. In addition all target cells were shown to be sensitive to killing by rhIL-2-treated PBMC, but were relatively resistant to spontaneous NK activity (data not shown).

Recognition of tumour targets expressing mutant p53 protein

Primary CTL generated to peptide 217–225 and peptide 264–272 were assessed on three separate occasions for lytic activity against tumour targets expressing mutant p53. Both CTL-217 and CTL-264 could lyse SaOs-2/175 but not SaOs-2 cells transfected with control vector (Fig. 4). In contrast, SaOs-2/273 was only recognised by CTL generated to the 217–225 peptide from one out of three donors (Fig. 4C) and was not killed by CTL generated with the 264–272 peptide. These results show that both peptides 217–225 and 264–272 were naturally (endogenously) processed and presented on the surface of the SaOs-2/175 cells. While rhIFNγ and rhTNFα were used to maximise the HLA-A2 antigen expression on the

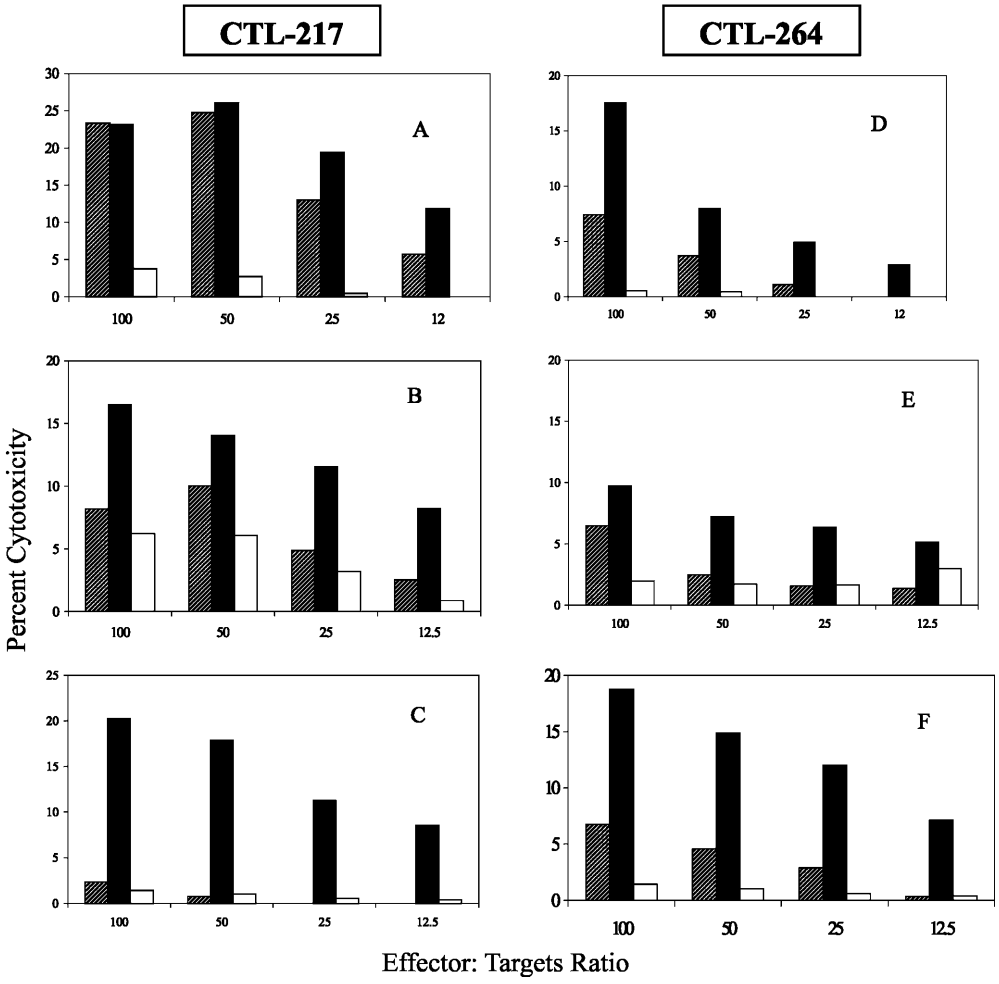


Fig. 4A–F Sensitivity of SaOs-2 transfectants to lysis by CTL-217 and CTL-264. SaOs-2 cells were transfected with p53 cDNA mutated at amino acid position 175 (R → H) or 273 (R → H) to generate SaOs-2/175 and SaOs-2/273 transfectants respectively. Sensitivity of the transfectants to lysis mediated by 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 are SaOs-2/273 (*hatched bars*), SaOs-2/175 (*black bars*) and SaOs-2 cells transfected with control vector (*white bars*). Three donors were used in these experiments: donor 1 (**A**, **D**); donor 2 (**B**, **E**) and donor 3 (**C**, **F**)

surface of target cells, untreated, transfected SaOs-2 cells were also susceptible to killing by these CTL (data not shown).

Transfected cell lines A431-A2 and SKBr3-A2, when pulsed with either the 217–225 or the 264–272 peptide, were killed by CTL-217 (Fig. 5A, B) and CTL-264 (Fig. 5C, D) respectively, demonstrating the functionality of the transfected HLA-A2 gene. SKBr3-A2 cells, but not A431-A2 cells nor SKBr3 (not pulsed with peptide) were lysed by both 217 and 264 primary CTL (Fig. 5). These results indicate that peptides 217–225 and 264–278 are naturally processed and presented on the surface of the SKBr3-A2 cells but not on the surface of SKBr3 (negative for HLA-A2 protein expression), and demonstrate that the increase in HLA-A2 expression observed after cytokine treatment was insufficient for CTL recognition of target peptides.

SKBr3 cells and SaOs-2/175 possess the same hotspot mutation at amino acid 175 of the p53 protein, whereas A431-A2 cells carry an R → H mutation at position 273 of the p53 protein. These results suggest that the processing of the p53 protein is influenced by the site of mutation and consequently the conformation of the protein, which may influence the peptide repertoire presented by HLA antigens at the cell surface.

Discussion

The use of peptide- or DNA-based vaccines derived from T cell epitopes of tumour-associated antigens overexpressed in tumour cells is now an acceptable approach to elicit antitumour immunity, and a number of clinical trials using peptide vaccines are currently in progress [23, 13]. p53 represents a potential target for specific immunotherapy, and identification of p53-encoded CTL epitopes is essential for peptide vaccine strategies. In this study 37 overlapping nonameric peptides derived from the wild-type p53 protein, amino acids 193–237, as well as peptides 186–194, 187–194, 188–197, 263–272 and 264–272 possessing HLA-A2 binding motifs, were assayed for their ability to stabilise HLA-A2 molecules in an MHC class I stabilisation assay. Of the 42 peptides tested, 5 also stabilised HLA-A2 at 37 °C at 100 μM concentration. In a recent publication comparing the MHC stabilisation characteristics

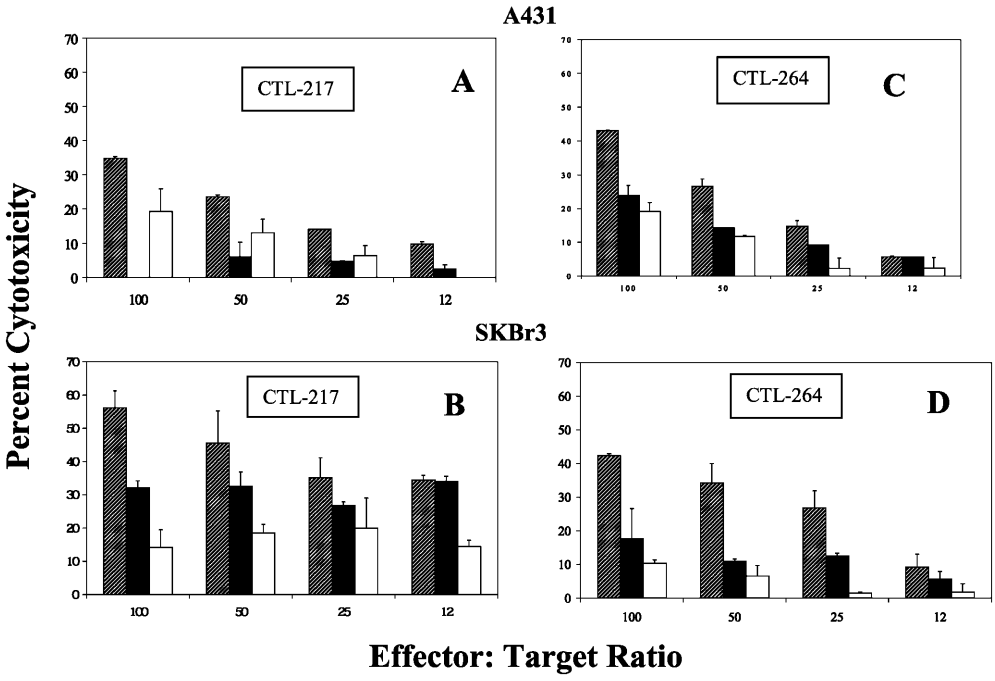


Fig. 5A–D Sensitivity of A431-A2 (A, C) and SKBr3-A2 (B, D) transfectants to lysis mediated by CTL-217 and CTL-264. Cell lines A431 and SKBr3 were stably transfected with HLA-A2 cDNA, treated with 200 U/ml rhIFNγ and 1000 U/ml rhTNFα for 24 h to up-regulate HLA-A2 transgene expression, and used as ^{51}Cr labelled targets for CTL-217 (**A**, **B**) and CTL-264 (**C**, **D**) generated from donor 1. Target cells were HLA-A2 transfectants pulsed with peptide 217–225 (**A**, **B**; *hatched bars*) or peptide 264–272 (**C**, **D**; *hatched bars*), unpulsed HLA-A2 transfectants (*black bars*) and cells transfected with control vector (*white bars*)

of peptides derived from MAGE-1, -2 and -3 proteins we demonstrated an enhanced sensitivity when the assay was performed at 26 °C [20].

Some of these peptides have subsequently been used to generate MAGE-1-specific CTL in vitro (McIntyre et al. submitted for publication). A selection of p53 peptides were therefore re-tested at 26 °C. Out of the 24 peptides tested at this temperature, 7 stabilised HLA-A2 molecules with high affinity (FR > 1.5) and 8 with low affinity (1.1 < FR < 1.5). Three peptides, 188–197, 193–200 and 217–225, not previously reported in the literature, were shown consistently to stabilise HLA-A2 molecules in this assay. These peptides have one preferred anchor residue for HLA-A2 at position 9 with or without one tolerated anchor residue at position 2. Peptides 187–197, 229–237, 263–272 and 264–272 have previously been shown to bind to HLA-A2 [12, 32, 33] with similar affinities to those reported here, whereas the binding affinity for peptides 193–201 and 210–218 was inconsistent with previous findings [12, 35]. As anticipated, peptides containing no known anchor residues (peptides 211–219 and 224–231) failed to stabilise HLA-A2 molecules on T2 cells.

Despite the presence, in a given antigen, of peptides with both preferred anchor residues, not all will be capable of eliciting a CTL response in vitro. Peptides that fail to bind and remain associated with MHC class I molecules for a minimum time are generally unable to elicit an immune response, whereas a strong correlation between immunogenicity and MHC-peptide complex stability has been reported [37]. Similarly, peptides binding with weak affinity have been reported to be non-immunogenic, whereas peptides with strong binding affinity for MHC class I molecules can result in the induction of tolerance or elimination of peptide-specific naive T cells in the thymus. Using p53-deficient ($p53^{-/-}$) and $p53^{+/+}$ HLA-A2.1/K^b transgenic mice as a model to assess tolerance to self proteins, it was demonstrated that the CTL response to the p53 wild-type peptide 187–197 was undetected in $p53^{+/+}$ mice but could be found in $p53^{-/-}$ transgenic mice. Whilst CTL could be generated to a second p53 epitope, 261–269, in both types of mouse, the avidity of the CTL generated was tenfold higher in $p53^{-/-}$ transgenic mice than in $p53^{+/+}$ mice, suggesting that high-avidity CTL are eliminated when the protein is expressed naturally throughout life [34]. It is therefore likely that a therapeutic window of binding affinities exists for the generation of a CTL response, beyond which very low- or high-affinity binding peptides, would, for different reasons, fail to elicit a CTL response.

The level of HLA class I 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. This has been observed here with SKBr3 cells (weakly HLA-A2-positive), which were not killed unless an HLA-A2 gene was transfected and expressed in the cells [26]. Quantitative and qualitative differences in peptide and MHC class I expression will determine the efficiency with which antigen-specific CTL are generated. For peptides, this depends mainly on how the protein from which they are derived has been processed by the cell.

The majority of mutations of p53 result in the stabilisation and accumulation of the protein in tumour cells, making p53 an ideal candidate for immunotherapy against a wide variety of cancers. Mutations, commonly

point missense mutation, are in the main clustered around four hot spots (amino acids 175, 248, 249, 273) in highly conserved domains in the central region of the protein [11]. One class of mutants (273, 248) do not significantly affect the conformational structure of the protein [10], do not associate with heat-shock protein 70 (hsp70), and are $Pab240^{-}/Pab1620^{+}$ [6, 10]. In the other class of mutants (175, 249), there is unfolding of the protein, and exposure of the 240 epitope, which is normally cryptic and inaccessible to antibodies; these mutant proteins are associated with hsp70 and are highly sensitive to proteolytic enzymes [6, 10]. The frequency of p53 mutations has been reported to be strongly correlated with the presence of antibodies against p53 in several types of cancer [1, 18], as a result of an active secondary self-immunisation. Moreover, Davidoff and co-workers [7] showed that tumours that elicit an antibody response contain hsp70 complexed to mutant p53. These findings, together with the fact that several heat-shock proteins have been shown to have a role in antigen processing [36, 29], suggest that antigen processing of the mutant p53 conformational protein could be different from that of mutants where the conformation of p53 protein is not affected; this could result in markedly different peptide repertoires being displayed by MHC class I antigens on the surface of the cells.

In the present study we have shown that two wild-type p53 peptides, 217–225 and 264–272, were successfully used for the generation of primary CTL that were able to specifically kill peptide-pulsed targets as well as tumour targets overexpressing the p53 protein. Tumour targets expressing a mutant p53 with an altered conformation were more readily killed than tumour targets expressing p53 mutants with the wild-type conformation, suggesting that the two proteins generate a different class I peptide repertoire, thus confirming and extending the recent findings of Theobald et al. [35]. They demonstrated that the R $\rightarrow$ H mutation at residue 273 of human p53 alters proteosomal processing of the protein by inhibiting proteolytic cleavage between residues 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, an HLA-A2-restricted CTL line specific for human wild-type p53 peptide 149–157 lysed transfected cells expressing this mutation, indicating that efficient processing and presentation of other p53 epitopes occurs. In the present study, target cells with the R $\rightarrow$ H mutation at position 273 were not lysed by CTL-264 and were only lysed by CTL-217 generated from one of three donors. Whilst the former observation is in agreement with that of Theobald et al. [36], their results suggested that the 273 mutation may only affect a flanking peptide epitope and a precursor peptide. In our study, the lack of recognition of p53 mutated at position 273, observed with CTL generated to peptide 217, could be due to differences in the affinities of the T cell receptor of the CTL generated or to relative expression of p53 and HLA-A2 molecules; alternatively, regions of the protein distant from the mutation may fail to donate peptides to the processing pathway. Further evidence suggests that overexpression of p53 protein is not obligitary for CTL recognition [28]. The SCC9 cell line has a deletion in p53, at position 274–285, resulting in low or undetectable levels of p53 in the tumour cells; however, these targets were susceptible to killing by CTL specific for the wild-type p53 264–272 peptide. In contrast, the SCC-4 cell line, which has a missense mutation in p53 codon 151 leading to overexpression of p53 molecules, did not present this epitope. These results imply that differences exist in the processing and presentation of p53 epiotpes, depending on the mutation and its effect on the protein conformation, which would influence the effective implementation of p53-based cancer vaccine immunotherapy.

Dendritic cells play a crucial role in initiating cellular immune responses and have been proposed for use as an adjuvant for cancer therapy [27]. We have shown that dendritic cells can be successfully used as stimulators for the generation of "bulk" anti-p53-specific CTL populations, which recognise naturally processed p53 epitopes on tumour targets. Appella et al. [2] recently demonstrated the generation of anti-p53 CTL from human peripheral blood using dendritic cells, but only cloned populations could recognise tumour targets. Interestingly, these dendritic cells were CD1a-negative whereas the dendritic cells generated here were CD1a-positive, suggesting that the method used to generate dendritic cells may influence their ability to present to and activates $CD8^{+}$ cells. Key questions that remain are (1) whether patients whose tumours harbour a p53 mutation have demonstrable precursor CTL or whether they can be induced, and (2) whether there is an association between the p53 gene mutation, HLA type and disease progression. Wild-type p53 peptides 217–225 and 264–272 may be candidate peptides that could be included in vaccines for immunisation of HLA-A2-positive cancer patients. A better understanding of the processing pathways for mutant genes of cancer-associated proteins and the peptide rerpertoire generated will indicate the peptides that could be important targets for immunotherapy.

Acknowledgements We wish to acknowledge Olivia Smith for the flow cytometry and Professor J. Milner for her helpful discussions during the early stages of this project. This work was funded by Yorkshire Cancer Research, the John and Lucille van Geest Foundation and the Medical Research Council.

References

1. Angelopoulou K, Diamandis EP, Sutherland DJA, Kellen JA, Bunting PS (1994) Prevalence of serum antibodies against the p53 suppressor gene protein in various cancers. Int J Cancer 58: 480
2. Appella E, Lotze M, Whiteside TL, Deleo AB (1999) Generation of anti-p53 cytotoxic T lymphocytes from human peripheral blood using autologous dendritic cells. Clin Cancer Res 5: 1281

3. Bodmer H, Ogg G, Gotch F, McMichael A (1989) Anti-HLA-A2 antibody-enhancement of peptide association with HLA-A2 as detected by cytotoxic T lymphocytes. Nature 342: 443
4. Boon T, Gajewski TF, Coulie PG (1995) From defined human tumour antigens to effective immunisation. Immunol Today 16: 334
5. Cerundolo V, Elliott T, Elvin J, Bastin J, Rammenssee H-G, Townsend A (1991) The binding affinity and dissociation rates of peptides for class I major histocompatibility complex molecules. Eur J Immunol 21: 2069
6. Cho Y, Gorina S, Jeffrey PD, Pavletich N (1994) Crystal structure of a p53 tumor suppressor DNA complex: understanding tumorigenic mutations. Science 265: 346
7. Davidoff AM, Iglehart JD, Marks JR (1992) Immune response to p53 is dependent upon p53 HSP70 complexes in breast cancers. Proc Natl Acad Sci USA 89: 3439
8. Di Brino M, Tsuchida T, Turner RV, Parker KC, Coligan JE, Biddison WE (1993) HLA-A1 and HLA-A3 T cell epitopes derived from influenza virus proteins predicted from peptide binding motifs. J Immunol 151: 5930
9. Gnjatic S, Cai ZZ, Viguier M, Chouaib S, Guillet J-G, Choppin J (1998) Accumulation of the p53 protein allows recognition by human CTL of a wild-type p53 epitope presented by breast carcinomas and melanomas. J Immunol 160: 328
10. Hinds PW, Finlay CA, Quartin RS, Baker SJ, Fearon ER, Vogelstein B, Levine AJ (1990) Mutant p53 DNA clones from human colon carcinomas cooperate with ras transforming primary rat-cells. A comparison of the hot spot mutant phenotypes. Cell Growth Differ 1: 571
11. Hollstein M, Sidransky D, Vogerlstein B, Harris CC (1991) p53 mutations in human cancer. Science 253: 49
12. Houbiers JG, Nijman HW, Van der Burg SH, Drijfhout JW, Kenemans P, Van der Velde CJ, Brand A, Momburg F, Kast WM, Melief CJ (1993) In vitro induction of human cytotoxic T lymphocyte responses against peptides of mutant and wild-type p53. Eur J Immunol 23: 2072
13. Hsu FJ, Benike C, Fagnoni F, Liles TM, Czerwinsi D, Taidi B, Engelman EG, Levy R (1996) Vaccination of patients with B cell lymphoma using autologous antigen pulsed dendritic cells. Nat Med 2: 52
14. Jerome KR, Barnd DL, Bendt KM, Boyer CM, Taylor-Papadimitriou J, McKenzie IFC, Bast RC, Finn OJ (1991) Cytotoxic T-lymphocytes derived from patients with breast adenocarcinoma recognise an epitope present on the protein core of a mucin molecule preferentially expressed by malignant cells. Cancer Res 51: 2908
15. Kerr JFR, Winterford CM, Harmon BV (1994) Apoptosis: its significance in cancer and cancer therapy. Cancer 73: 2013
16. Ko JJ, Prives C (1996) p53: puzzle and paradigm. Genes Dev 10: 1054
17. Lacabanne V, Viguier M, Guillet J-G, Choppin J (1996) A wild-type p53 cytotoxic T cell epitope is presented by mouse hepatocarcinoma cells. Eur J Immunol 26: 2635
18. Lubin R, Schlichtholz B, Teillaud J-L, Garay E, Bussel A, Wild CP, Soussi T (1995) P53 antibodies in patients with various types of cancer: assay, identification, and characterisation. Clin Cancer Res 1: 1463
19. Mayordomo JI, Loftus DJ, Sakamoto H, De Cesare CM, Appasamy PM, Lotze MT, Storkus WJ, Appella E, DeLeo AB (1996) Therapy of murine tumors with p53 wild-type and mutant sequence peptide-based vaccines. J Exp Med 183: 1357
20. McIntyre CA, Rees RC, Platts KE, Cooke CJ, Smith O, Mulcahy KA, Murray AK (1996) Identification of peptide epitopes of MAGE 1–3 human tumour antigens which demonstrate HLA-A3 specific binding. Cancer Immunol Immunother 42: 246
21. Milner J (1995) Flexibility: the key to p53 function? Trends Biol Sci 20: 49
22. Milner J, Medcalf EA (1990) Temperature-dependent switching between 'wild-type' and 'mutant' forms of p53-Val135. J Mol Biol 216: 481
23. Nestle FO, Alijagic S, Gillet M, Grabbe S, Dummer R, Burg, Shanendorf D (1998) Vaccination of melanoma patients with peptide or tumor lysate pulsed dendritic cells. Nat Med 3: 328
24. Nijman HW, Van der Burg SH, Vierboom MPM, Houbiers JGA, Kast WM, Melief CJM (1994) P53, a potential target for tumour directed T cells. Immunol Lett 40: 171
25. Peace DJ, Chen W, Nelson H, Cheever MA (1991) T-cell recognition of transforming proteins encoded by mutated *ras* proto-oncogenes. J Immunol 146: 2059
26. Rivoltini L, Barrachini KC, Viggiano V, Kawakami Y, Smith A, Mixon A, Restifo NP, Topalian SL, Simonis TB, Rosenberg SA (1995) Quantitative correlation between HLA class-I allele expression and recognition of melanoma cells by antigen specific cytotoxic T lymphocytes. Cancer Res 55: 3149
27. Romani N, Grumer S, Brang D, Kampgen E, Lenz A, Trockenbacher B, Konwalinka G, Fritsch P, Steinman RM, Schuler G (1994) Proliferating dendritic cell progenitors in human blood. J Exp Med 180: 83
28. Röpke M, Hald J, Guldberg P, Zeuthen J, NO-rgaard L, Fugger L, Svejgaard A, Van Der Burg S, Nijman HW, Melief CJM, Claesson MH (1996) Spontaneous human squamous cell carcinomas are killed by a human cytotoxic T lymphocyte clone recognising a wild-type p53-derived peptide. Proc Natl Acad Sci USA 93: 14704
29. Srivastava PK, Udono H, Blachere NE, Li Z (1994) Heat shock proteins transfer peptides during antigen processing and CTL priming. Immunogenetics 39: 93
30. Stauss HJ, Davies H, Sadovnikova E, Chain B, Horowitz N, Sinclair C (1992) Induction of cytotoxic T lymphocytes with peptides in vitro: identification of candidate T-cell epitopes in human papilloma virus. Proc Natl Acad Sci USA 89: 7871
31. Steven NM, Leese AM, Annels NE, Lee SP, Rickinson AB (1996) Epitope focusing in the primary cytotoxic T cell response to Epstein-Barr virus and its relationship to T cell memory. J Exp Med 184: 1801
32. Stuber G, Leder GH, Storkus WT, Lotze MT, Modrow S, Szekely L, Wolf H, Klein E, Karre K, Klein G (1994) Identification of wild-type p53 peptides binding to HLA-A2 assessed by a peptide loading deficient cell line assay and a novel major histocompatibility complex class I binding assay. Eur J Immunol 24: 765
33. Theobald M, Biggs J, Dittmer D, Levine AJ, Sherman LA (1995) Targeting p53 as a general tumor antigen. Proc Natl Acad Sci USA 92: 1193
34. Theobald M, Biggs J, Hernandez J, Lustgarten J, Labadie C, Sherman LA (1997) Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. J Exp Med 185: 833
35. Theobald M, Ruppert T, Kuckelkorn U, Hernandez J, Haussler A, Ferreira EA, Liewer U, Biggs J, Levine AJ, Huber C, Koszinowski GH, Kloetzel P-M, Sherman LA (1998) The sequence alteration associated with a mutational hotspot in p53 protects cells from lysis by cytotoxic T lymphocytes specific for a flanking peptide epitope. J Exp Med 188: 1017
36. Vanbuskirk A, Crump BL, Margoliash E, Pierce SK (1989) A peptide binding protein having a role in antigen presentation is a member of the hsp 70 to heat shock family. J Exp Med 170: 1799
37. Van der Burg SH, Visseren MJW, Brandt RMP, Kast WM, Melief CJM (1996) Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. J Immunol 156: 3308
38. Yang E, Korsmeyer S (1996) Molecular thanatopsis: a discourse on the bcl-2 family and cell death. Blood 88: 386
39. Yanuck M, Carbone DP, Pendleton CD, Tsukui T, Winter SF, Minna JD, Berzofsky JA (1993) A mutant p53 tumour suppressor protein is a target for peptide-induced $CD8^+$ cytotoxic T-cells. Cancer Res 53: 3257
40. Yoshino I, Peoples GE, Goedegebuure PS, Maziarz R, Eberlein TJ (1994) Association of HER2/neu expression with sensitivity to tumor-specific CTL in human ovarian cancer. J Immunol 152: 23930