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Amino Acid Modifications in the Wild Type Sequence p53 232–240 Overcome the Poor Immunogenicity of this Self Tumour Epitope

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Abstract: A major limitation in antigen-specific cancer vaccines is that most of the tumour antigens that are potent candidates for broad applicability originate from self proteins. The peptides presented by tumour cells are derived from tissue-specific differentiation proteins, from proteins altered by genetic mutation or by non mutated proteins that are normally silent in most adult tissues. As a consequence, T-cell responses elicited against those antigens are rather weak. Several data showed that amino acid modifications could enhance the immunogenicity of such antigens by priming T-cells that have escaped central tolerance based on a poor avidity. In this regard, this strategy could be powerful for inducing immunity against tumours. The present report focuses on the murine wild type epitope p53 232–240 that is poorly immunogenic. It shows that substitution of the two cysteine residues by serine or amino butyric acid derivatives and substitution of the two methionine residues by norleucine residues resulted in enhanced stability of the MHC/peptide complex. The MHC binding affinity of analogue peptides was enhanced between 10 and 100 fold. They were also potent immunogens, stronger than was the original wild type epitope; T-cell responses were increased up to 50 times. Moreover, the effector T-cells elicited by three of these peptides cross reacted with the natural epitope. These observations have important implications for strategies that use the modified-peptide epitope.

Keywords: peptide analogues; MHC/peptide stability; immunogenicity; adoptive transfer; tumour immunity

INTRODUCTION

CD8+ lymphocytes have been shown to play a major role in controlling tumour development [1,2]. However, most tumour antigens characterized so far are shared by both malignant and normal cells [3]. In other words, immunity against cancer should target self antigens. Therefore cancer vaccines must be designed to break immune tolerance or ignorance in order to prime an efficient immune response. The low immunogenicity of a defined self antigen can be due to negative selection of high affinity clones during T-cell maturation or to a weak peptide presentation to T cells which can result from a low affinity of the peptide for the MHC molecule [4]. Such

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epitopes remain invisible to naive T lymphocytes but they can be targeted by differentiated cytotoxic T effectors. The potential repertoire specific to those antigens must remain unaltered because they must not induce tolerance. To stimulate this repertoire, peptide variants have been designed in order to enhance peptide/MHC formation. Such a strategy would be effective when T-cell effectors primed by the altered peptide are able to cross react with the original peptide present on the tumour cell surface [5–8].

Because they are found in many cancers, some antigens are of major interest as targets in the design of a new cancer vaccine. The p53 protein is a good candidate since its expression is altered in more than 50% of all human cancers [9] and since it is associated with an immune response *in vivo*. Overexpression of p53 has been associated with humoral, cytotoxic and proliferative responses in cancer patients [10–12]. Moreover, tumour-reactive cytotoxic responses directed against wild type (WT) and mutated p53 epitopes have been induced in mice and from human peripheral blood mononuclear cells [13–16]. Considering the highly variable nature of p53 mutations in diverse cancers, it would be easier to design immunotherapy against WT p53 epitopes in order to target a broad range of tumours. However, animal studies have highlighted the poor immunogenicity of such epitopes.

In this context, we have previously described the WT p53 cytotoxic epitope 232–240 that is restricted to the H2-D^b molecule [14]. Colon carcinoma, fibrosarcoma and hepatocarcinoma cells were demonstrated to express the endogenous determinant naturally [14,17]. However, efficient induction of a specific T-cell response has been fraught with difficulties due to some characteristics of the epitope; it is a poor binder to its MHC molecule and the methionine and cysteine residues that are present in its sequence hamper its stability in solution. In the present study we attempted to address both problems by replacing these residues by chemically related derivatives that are not sensitive to oxidation. This strategy increased the formation of peptide/MHC complexes, resulting in higher immunogenicity of the corresponding analogues. Moreover T-cell effectors induced against these analogues cross-reacted with the natural p53 peptide. However, such cells were inefficient at inducing tumour destruction. These results show that a modified peptide strategy may not be as efficient as previously suggested when the density of the target peptide on the tumour cell remains below a certain threshold.

METHODS

Mice

C57BL/6 mice were purchased from Harlan (Lyon, France).

Transgenic ASV-B carry SV40 early genes controlled by the antithrombin III liver-specific promoter on the Y chromosome and these mice die from hepatic tumours before 36 weeks of age [18]. The mice used in this study were back-crossed with C57BL/6 mice (>25 generations).

Peptide Synthesis

Hepatitis B virus core protein (HBVc) 128–140 (TPPAYRPPNAPIL) helper epitope was obtained from Neosystem (Strasbourg, France).

Wild type p53 cytotoxic T-cell epitope 232–240 (KYMCNSSCM) and peptide analogues were synthesized in Fmoc chemistry by stepwise solid-phase methodology using a multichannel peptide synthesizer [19]. Protected amino acids were coupled by *in situ* activation with (benzotriazol-1-yloxy)*tris*-(dimethylamino)phosphonium hexafluorophosphate and *Nα*-Fmoc deprotection was performed as previously described [19]. Side chain deprotection and cleavage of peptides from the solid support was performed by treatment with reagent K (82.5% TFA, 5% phenol, 5% water, 5% thioanisole, 2.5% 1,2-ethanedithiol) for 2 h 30 at 20 °C [20].

The peptides were purified by reversed-phase HPLC (RP-HPLC) using a Perkin-Elmer preparative HPLC system on an Aquapore ODS 20 µm column (100 × 10 mm). The elution was achieved with a linear gradient of aqueous 0.1% TFA (A) and 0,08% TFA in 80% acetonitrile, 20% water (B) at a flow rate of 6 ml/min with UV detection at 220 nm.

All peptides were identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and their homogeneity was assessed by C18 RP-HPLC.

Cell Lines

RMA-S lymphoma cells were maintained on RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplied with 10% FCS, 20 mM HEPES, 2 mM glutamine, antibiotics and 5×10^{-5} M 2-ME (Sigma, St Louis, MO).

Cytotoxicity Assay

Cytotoxic activity was detected by a standard ^{51}Cr assay. Target cells were labelled with 0.1 mCi

sodium ^{51}Cr chromate in the presence of 10^{-5} M peptide (or irrelevant peptide), washed and adjusted to 5×10^4 cells/ml. Target cells (0.1 ml) were mixed with effector cells (0.1 ml) in duplicate in 96-well round bottom microplates (Costar, Cambridge, MA) at various effector to target cell ratios (E:T). These cultures were incubated for 4 h. Aliquots (0.1 ml) of supernatants were harvested and the radioactivity was determined in a gamma counter (Packard, Meriden, CT). Peptide recognition by the CTL lines was titrated by incubating ^{51}Cr labelled RMAS cells with peptides at various dilutions. The percent cytotoxicity was calculated as follows:

$$\% \text{ lysis} = 100 \times (\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release}).$$

ELISPOT Assay for Single Cell IFN-γ Secretion

The ELISPOT assay for detecting epitope-specific T-cells that secrete IFN-γ was adapted from Miyahira *et al.* [21]. Nitrocellulose microplates (Millipore, Bedford, MA) were coated with 4 μg/ml anti-mouse IFN-γ (R4-6A2, PharMingen, San Diego, CA). A total of 3×10^5–3.3×10^3 lymph node cells were tested in the presence of 30 U/ml human rIL2. CD8+ T-cells were stimulated with 5×10^4 RMAS cells pulsed with 10^{-5} M peptide. After 24 h, the plates were washed, incubated with 4 μg/ml biotinylated anti-mouse IFN-γ antibody (XMG1.2, PharMingen, San Diego, CA), and subsequently incubated with alkaline phosphatase-labelled extravidin. After adding chromogenic alkaline phosphatase substrate (Bio-Rad, Hercules, CA), IFN-γ spot forming cells (SFC) were counted using a stereomicroscope.

Evaluation of Binding Affinity and Stability

Binding affinity and stability assays were adapted from Ljunggren *et al.* [22]. RMA-S cells were cultured overnight at 26°C. For the binding assay, 3×10^5 cells were incubated with the peptide at various concentrations (between 1×10^{-4} M and 1×10^{-10} M) at 26°C for 1 h, then at 37°C for 4 h. For the stability assay, 2×10^6 RMA-S cells were incubated with 10^{-5} M peptide at 26°C for 1 h, then at 37°C for 4 h. The cells were washed three times, then re-incubated at 37°C. Aliquots of 0.3 ml were harvested after 0, 0.5, 1, 2, 4 and 6 h incubation and left at 4°C until staining. Two controls were performed: minimal H2D^b stabilization was obtained on RMA-S cells incubated without peptide at 37°C; maximal H2D^b expression was obtained on RMA-S cells incubated without peptide at 26°C. Cell surface H2D^b molecules were revealed by incubating cells at 4°C with monoclonal antibody 28.14.8s (HB27, ATCC), then with FITC-conjugated goat anti-mouse IgG antibody. The mean fluorescence (MF) was detected by flow cytometry on a Facscan cytofluorimeter.

The relative percentage of expression of H2D^b molecules was calculated as follows:

$$\% \text{ of H2D}^b \text{ expression} = 100 \times (\text{experimental MF} - \text{minimal MF})/(\text{maximal MF} - \text{minimal MF}).$$

Induction of Peptide Specific CTL and Adoptive Transfers

Mice were s.c. injected in the tail with 50 μg of CTL epitope mixed with 140 μg of HBVc 128–140 helper epitope peptide (TPPAYRPPNAPIL) in IFA. After 11 days, the responses of the cells from the draining lymph node were tested *ex vivo* by IFN-γ enzyme-linked immunospot (ELISPOT). To induce CTL lines, these cells (6×10^6 cells/well) were seeded in 24-well plates with 5 μM peptide for 6 days in culture medium. They were maintained weekly with peptide-pulsed C57BL/6-irradiated splenocytes. Human rIL-2 (10 U/ml, Boehringer Mannheim, Mannheim, Germany) was added every 3 days. CTL cell lines were used in cytotoxic and ELISPOT assays after two to three *in vitro* stimulations.

ASV-B transgenic mice (20 weeks old) were injected i.v. with 5×10^6 CTL line cells that are specific for (i) p53 modified peptides or (ii) for the epitope 223–231 of large T antigen of SV40 as a positive control [23].

RESULTS AND DISCUSSION

According to several reports, increasing the peptide binding affinity to the MHC molecule often resulted in stronger immunogenicity [5–8]. Therefore, amino acid substitutions were introduced in a WT p53 epitope in order to enhance the specific immune response toward this epitope. This peptide is nine amino acids long and it contains dominant residues at both H2-D^b binding anchor positions (Asn in position 5 and Met in position 9), but it is a poor binder to the MHC molecule. Moreover, the presence in the sequence of four residues that are

readily oxidized (two methionine and two cysteine residues) is detrimental to the chemical stability of the peptide in solution at neutral or basic pH. In an attempt to solve both issues, we engineered seven peptide analogues (A1–A7) in which both Met residues (except A1 where only one Met was substituted) were replaced by a steric analogue, norleucine (Nle), and Cys residues were either not replaced or were replaced by serine or aminobutyric acid (Abu) derivatives (see Table 1). A recent study by Kang *et al.* suggested that the cysteine residues of a peptide must be free from disulphide bonding for efficient stimulation of T cells [24].

Since peptide analogues contain steric analogues at anchor and non anchor residues, substitutions are likely to alter both MHC binding and TCR recognition. Thus the peptide/H2-D^b binding was assessed first by using an assay based on the RMA-S cell line [22]. This cell line is TAP2 deficient, and therefore lacks the capability to transport peptides from the cytosol to the endoplasmic reticulum to be loaded on to the newly synthesized MHC molecules. The MHC molecules that lack bound peptides are very unstable at 37 °C. Their loading by exogenous peptide delivery allowed them to be stabilized and thus to be detected by cytofluorometry. In these conditions, the MHC class I molecule expression at the RMA-S cell surface reflects the peptide binding affinity when maintained at 37 °C. Figure 1A shows that the replacement of Met by Nle, at anchor position 9, was sufficient to enhance H2-D^b binding affinity at least 10 fold (A1 compared with WT). Additional modification of Cys at position 4 slightly reduced this characteristic (A1, A2, A3 compared with A4, A5, A6 and A7). Moreover, the rate of H2-D^b loss from the RMA-S cell surface over time was greatly reduced in the modified epitopes (Figure 1B). At 4 h, WT epitope/H2-D^b complexes had totally disappeared from the cell surface, while the level of peptide analogues/H2-D^b complexes was generally maintained, except in A4 and A5 for which the mean fluorescence intensity was reduced to 10% by that time. This indicates that substitutions of Met residues by Nle residues, even

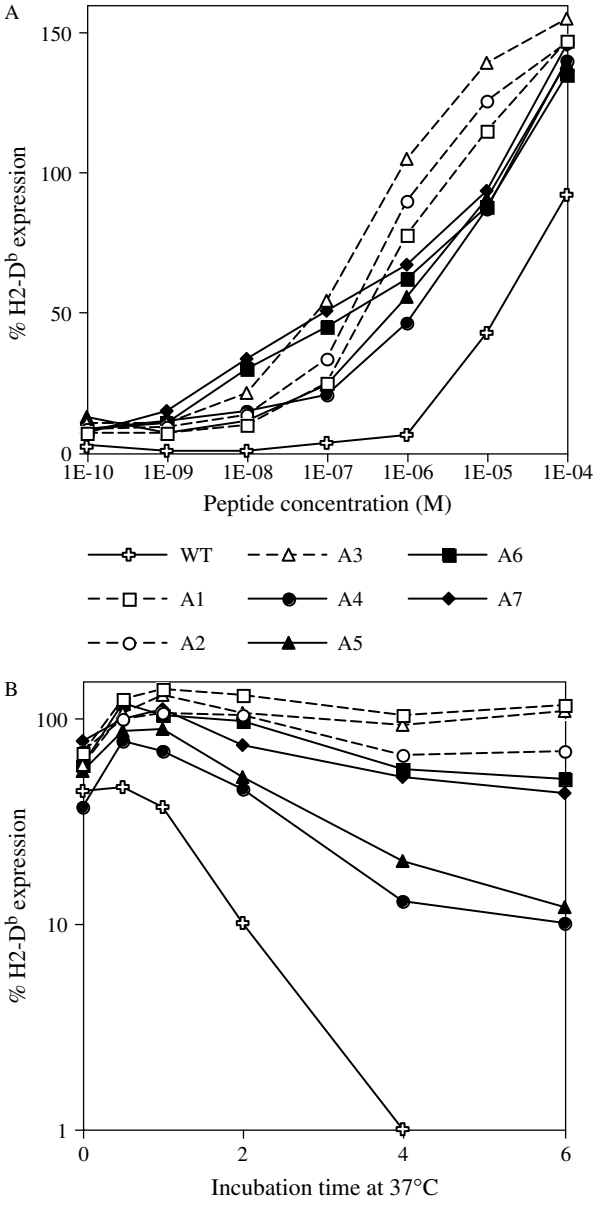

Figure 1 H2-D^b expression on the RMA-S cell surface was evaluated at 37 °C after stabilization by addition of exogenous WT or modified peptides. The results are expressed as a percentage of H2-D^b expression at 26 °C. (A) represents % of H2-D^b expression for peptide concentrations ranging from 10^{-11} to 10^{-4} M. (B) represents % of H2-D^b expression over time.

Table 1 Peptide Analogues Sequences

WT	K	Y	M	C	(N)	S	S	C	(M)
A1	K	Y	M	C	N	S	S	C	Nle[a]
A2	K	Y	Nle	C	N	S	S	C	Nle
A3	K	Y	Nle	C	N	S	S	S	Nle
A4	K	Y	Nle	S	N	S	S	S	Nle
A5	K	Y	Nle	Abu[b]	N	S	S	S	Nle
A6	K	Y	Nle	S	N	S	S	Abu	Nle
A7	K	Y	Nle	Abu	N	S	S	Abu	Nle

(N) (M) anchor residue.
[a] Nle (Norleucine).
[b] Aminobutyric acid.

at an anchor position, do not reduce the binding affinity but in fact increase it. On the other hand, blocking possible disulphide bond formation by introducing Abu or Ser derivatives instead of Cys residues does not dramatically affect the binding affinity.

The immunogenicity of peptide analogues was evaluated after immunization of C57BL/6 mice with each of the single peptides in IFA. The I-Ad-restricted helper peptide HBVc 128–140 was included in the immunizing formulation as previously described [25]. Specific CD8+ T-cell responses were quantified *ex vivo* by IFN-γ ELISPOT conducted on uncultured lymph node cells (Figure 2). The responses assessed by this method ranged generally, in our hands, from 100 to 1000 SFC/10^6 cells, the maximum being reached with a strong epitope such as peptide 404–411 of Tag or peptide 366–374 of the influenza virus nucleoprotein [23]. Except for analogue A1 that did not induce a significant response, all peptide analogues were more immunogenic than the original WT epitope. The number of IFN-γ secreting cells was from 10 to more than 50 times higher than the number obtained after WT epitope immunization. Interestingly, the less immunogenic peptides (WT, A1 and A2) were those that still contained both Cys residues. In the same way, A3, in which only one Cys residue was replaced, did not induce a very strong response either. On the contrary, the presence of Abu was associated with strong immunogenicity since immunization with A5, A6 and A7 generated more than 500 IFN-γ-secreting spots for 10^6 lymph node cells. To determine if the increase in stability and immunogenicity due to the replacement of Met by Nle and of Cys by Abu is a general feature, it would be informative to assess the impact of these modifications with several other tumour or virus epitopes.

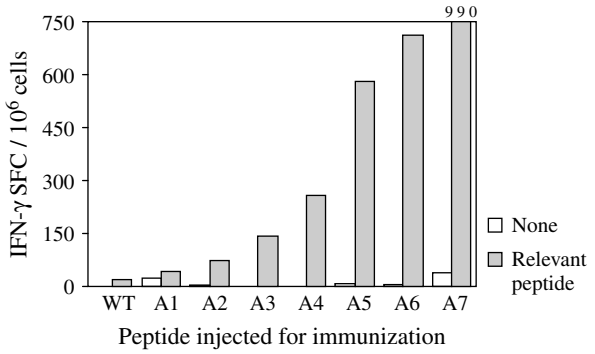

Figure 2 C57BL/6 mice were immunized with WT or modified peptides as indicated in Materials and Methods. Epitope-specific T-cell responses were assayed *ex vivo* by IFN-γ ELISPOT. Uncultured lymph node cells were exposed for 24 h to RMA-S cells pulsed with each peptide. The results are expressed as the number of IFN-γ SFC per 10^6 cells.

Cytotoxic T-cell (CTL) lines were established *in vitro* in order to evaluate their ability to recognize the natural epitope (Figure 3). No CTL lines specific for WT, A1 and A7 could be obtained. The frequency of A1- and WT-specific precursors was probably too low and, in our hands, we were never able to amplify these precursors in culture. In contrast, the frequency of A7 precursors was very high. Moreover, A7 binding affinity to the H2-D^b molecule was strong. This might indicate that the *in vitro* stimulation protocol used had deleterious effects. Alexander-Miller *et al.* suggested that stimulation with a relatively high peptide concentration could result in effector apoptosis rather than in the induction of proliferation [26]. CTL lines directed against A2, A4 and A6 also were reactive with the natural peptide, as evaluated by the ^{51}Cr release assay. However, lysis of target cells loaded with the WT epitope by effectors that were specific to the peptide analogues was generally reduced two fold compared with the lysis of target cells loaded with the relevant peptide. No rules could be defined to associate amino acid modifications with cross reactivity since the three peptides have different types of modification.

The main goal of this study was to induce T-cell effectors with anti-tumoral potency. Therefore, we explored the impact of adoptive transfers of A2-, A4- and A6-specific CTL lines on the hepatic tumour that develops in SV40 large T antigen (Tag)-transgenic mice. In these animals, most hepatocytes express Tag and accumulate WT p53 [18]. Transgenic mice with fully developed hepatic tumours were injected with 5×10^6 T cells reactive against A2, A4, A6 or against the Tag 223–231 epitope as a positive control. Indeed, we have previously shown that the transfer of CTLs specific for this last epitope reduced the tumour weight by 23%–58% [23]. Two days after the transfers, the animals were killed and liver biopsies were performed to analyse the histological alterations in the tumours (Plate 1). The Tag-specific CTL line induced a large destruction of malignant cells; large foci of apoptosis were regularly distributed throughout the liver tissue sections. In sharp contrast, no alteration was observed in the liver of mice treated with CTL lines that are specific for the p53 peptide analogues. Since tumour

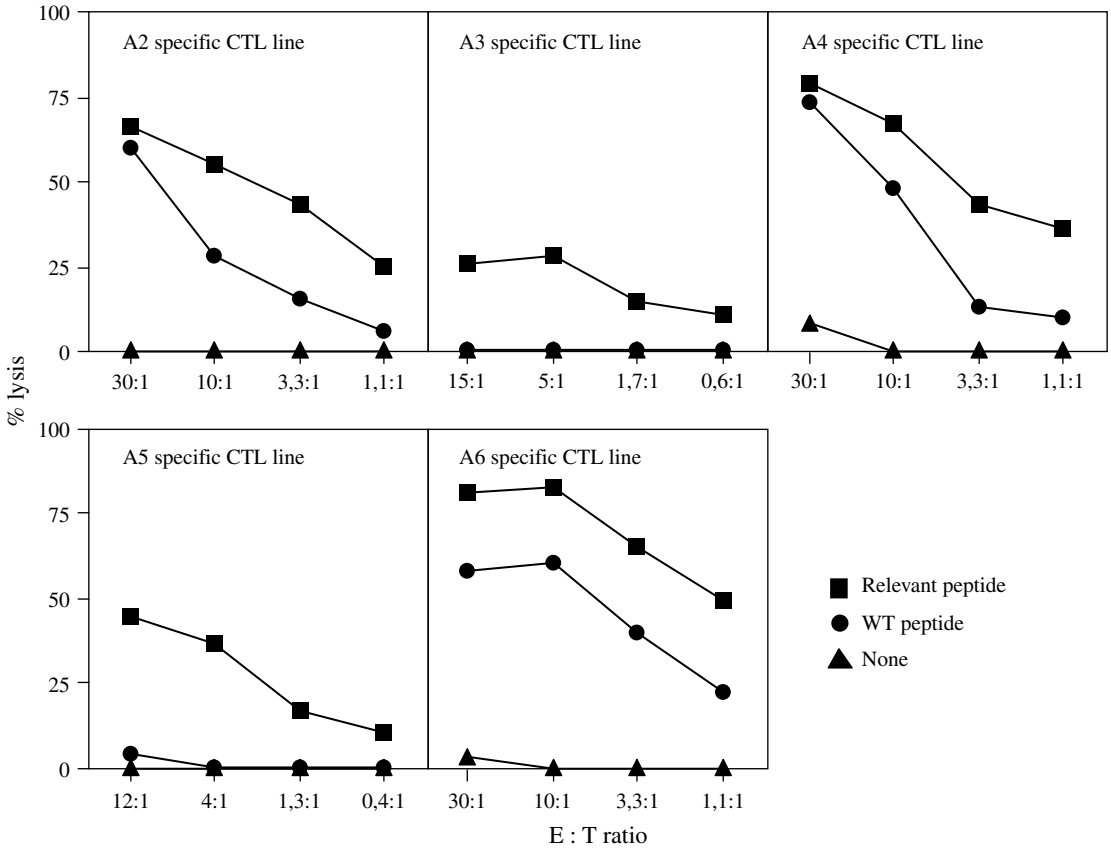

Figure 3 CTL lines that are specific for the modified peptides were induced in C57BL/6 mice. Lysis of RMA-S cells pulsed with WT or with relevant peptides by these CTL lines was evaluated by the ^{51}Cr release assay. Cytolytic activity was assessed at various effector: target ratios.

access was not dependent on T-cell specificity (data not shown), the absence of an objective response could not be explained by a restricted CTL entry in the tumour site. However, this may reflect a lower level of MHC expression, of antigenic peptide or co-stimulatory molecules on the tumour cell surface compared with RMA-S cells. As shown in Figure 4, the peptide concentrations required for efficient IFN-γ production ranged from 10^{-8} to 10^{-7} M for the peptide used to induce and amplify the CTL lines and only from 10^{-6} to 10^{-5} M for the natural peptide. These observations suggest that the level of MHC/peptide complexes at the tumour cell surface, required for efficient recognition, was probably not reached. Moreover preliminary *in vitro* studies on the degradation by proteasome of a long peptide containing the WT epitope suggested that this peptide was not efficiently generated (data not shown). Thus, this epitope would not constitute an efficient molecular target to induce killing, even by fully activated effectors. However, since CTL lines were not cloned, they should contain cells with differing avidity and fine specificity by which some of them could recognize the peptide on the tumour cell. Enrichment of these effectors could be promoted by *in vitro* stimulation with the original WT epitope.

CONCLUSION

Taken together, these results show that Met and Cys residues that often reduce the stability of peptides in solution could be replaced by chemically related derivatives without affecting the binding affinity for the relevant MHC molecule. Moreover, the aminobutyric acid derivative should be further evaluated as an efficient cysteine substitute to potentiate immunogenicity. However, even if the CTL lines that cross react with the parental peptide could be generated with peptide analogues, no recognition of the naturally processed epitope on the tumour

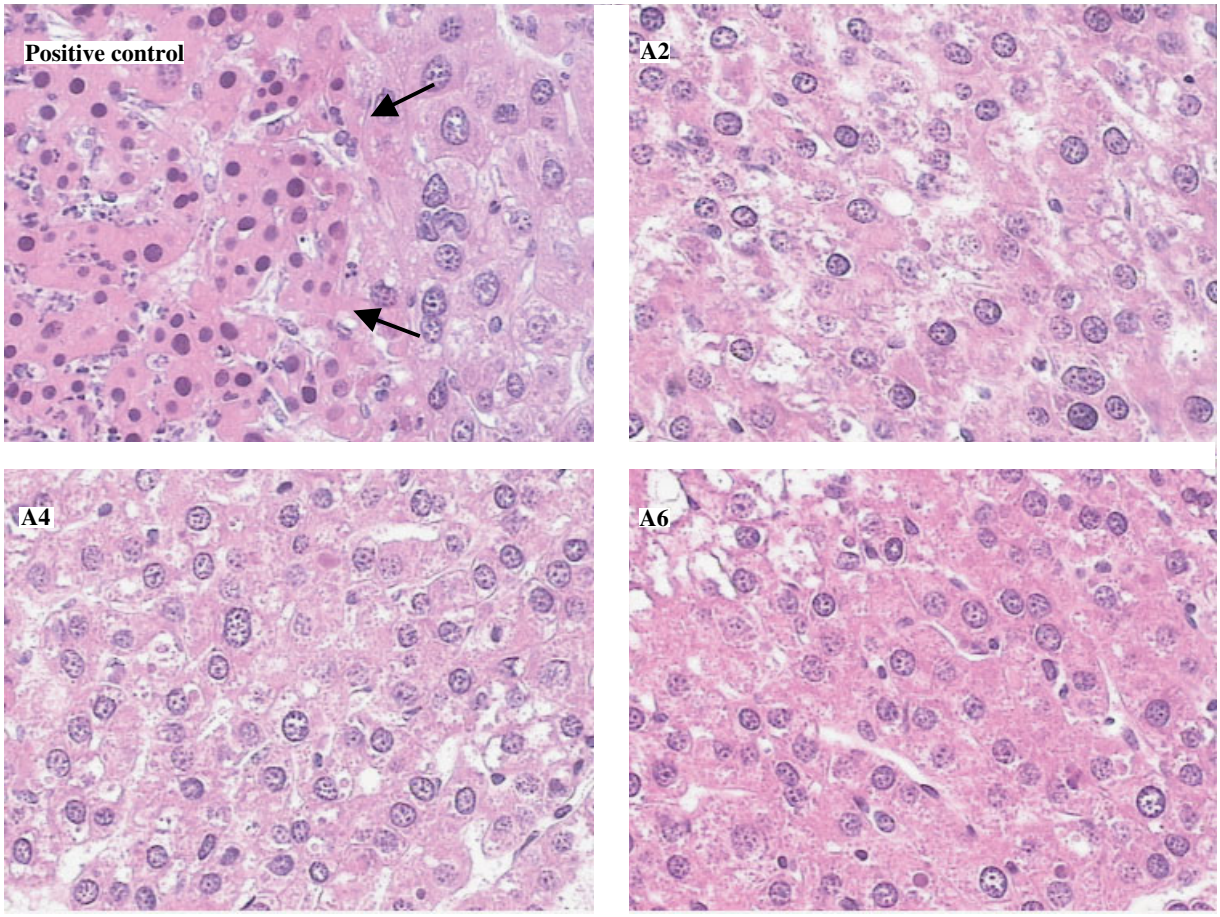

Plate 1 ASV-B mice transgenic for large T antigen of SV40 (Tag), that develops a hepatic tumour that accumulates p53, were injected i.v. with 5×10^6 CTL cells specific for A2, A4, A6 or for Tag epitope 223–231 as a positive control. Tumour destruction was evaluated 48 h after the adoptive transfer by histological examination of haematoxylin- and eosin-stained liver sections (original magnification X40). Arrows point to destroyed foci.

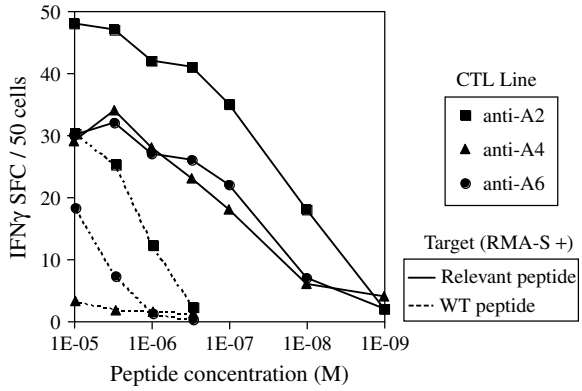

Figure 4 Avidity of CTL lines anti- A2, A4 and A6 for H2-D^b/WT epitope complex or H2-D^b/relevant peptide complex was evaluated. 50 cells of each CTL line were exposed for 24 h to RMA-S cells pulsed with WT or relevant peptide at concentrations ranging from 10^{-9} to 10^{-5} M. T-cell responses were assessed by IFN-γ ELISPOT.

cell was observed. The same kind of results were obtained by Clay *et al.* in human clinical trials in which patients were immunized with a modified epitope of the tumour melanoma antigen, gp100. Immunization with this epitope affected the T cell repertoire by expanding an array of T cells that were reactive to the parental epitope but only very few of them recognized effectively melanoma cells [27]. In this context, the major drawback seems to be the level of MHC/peptide complexes present at the tumour cell surface.

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