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Identification of NY-ESO-1 Peptide Analogues Capable of Improved Stimulation of Tumor-Reactive CTL[1]

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Expression of NY-ESO-1 in a high proportion of different human tumors makes this protein a very attractive vaccine target. NY-ESO-1 peptides, recognized by HLA-A2-restricted CTL, have recently been described. However, it remains unclear how efficiently tumors generate these epitopes, and whether peptide analogues can be used for optimal expansion and activation of NY-ESO-1-specific HLA-A2-restricted CTL. By generating unique CTL clones, we demonstrate that NY-ESO-1-positive tumor cells are efficiently killed by HLA-A2-restricted CTL specific for the peptide epitope NY-ESO-1 157–165. Presentation of this epitope is not affected by the presence or absence of the proteasome subunits low molecular proteins 2 and 7 and is not blocked by proteasome inhibitors, while it is impaired in the TAP-deficient cell line LBL 721.174. NY-ESO-1 157–165 peptide analogues were compared for their antigenicity and immunogenicity using PBL from melanoma patients. Three peptides, containing the carboxyl-terminal cysteine substituted for either valine, isoleucine, or leucine, were recognized at least 100 times more efficiently than the wild-type peptide by specific CTL. Peptide analogues were capable of stimulating the expansion of NY-ESO-1-specific CTL from PBL of melanoma patients much more efficiently than wild-type peptide. These findings define the processing requirements for the generation of the NY-ESO-1 157–165 epitope. Identification of highly antigenic NY-ESO-1 peptide analogues may be important for the development of vaccines capable of expanding NY-ESO-1-specific CTL in cancer patients. ***The Journal of Immunology*****, 2000, 165: 948–955.**

There is now considerable evidence that human tumors often express Ags that render them susceptible to lysis by CTL (1). Some of the Ags recognized by CTL have been defined at the molecular level, by cloning tumoricidal CTL, and using tumor gene expression libraries to find their targets. Most of these Ags were discovered in melanomas, where tumor cell lines can be generated relatively easily for assays of cytolytic activity. However, recent data suggest that other tumors may also be susceptible to immune attack. In particular, the repertoire of tumor Ags has been rapidly expanded by the application of serological analysis of recombinant cDNA expression libraries (SEREX)[3] from human tumors using autologous serum (2). The SEREX technique has identified a host of new Ags recognized by Abs in cancer patients' sera. Since many of these Ags occur in a wide variety of tumors, they offer the prospect of broad spectrum anti-cancer vaccines aimed at inducing CTL attack.

One of the most promising of these new SEREX Ags is NY-ESO-1, which is found in 30% of breast, prostate, and ovarian cancer, as well as melanoma, but not in normal tissues, with the exception of testis (3). Expression of NY-ESO-1 by tumor cells in melanoma patients stimulates a combined humoral and cellular response in a significant percentage of patients (4). The high immunogenicity of NY-ESO-1 and its broad tumor expression make this protein a very promising target for tumor-specific vaccination strategies. If NY-ESO-1 epitopes were presented by different tumor cells and recognized by CTL, then vaccines designed to boost CTL responses against NY-ESO-1 epitopes may be useful in the treatment of these tumors.

HLA-A2-binding NY-ESO-1 peptides, capable of being recognized by CTL, have recently been described (5). However, very little is known about the processing requirements for the generation of these epitopes, and whether tumor cells are efficiently lysed by NY-ESO-1-specific CTL. As some tumor epitopes may be generated so poorly by tumor cells that CTL fail to kill them efficiently (6), we sought to analyze the processing and presentation of a defined NY-ESO-1 epitope. Attention was focused on the role of the proteasome subunits low molecular protein (LMP) 2 and LMP7, as a recent report raised the possibility that processing of certain tumor target proteins by LMP-positive cells may result in a poor presentation of tumor CTL epitopes (7). Since this work requires the development of highly specific CTL lines, we used MHC class I tetramers to define and clone a population of NY-ESO-1-specific CTL, using previously described protocols (8). These clones allowed confirmation of the identity of an HLA-A2-restricted NY-ESO-1 epitope presented by NY-ESO-1-positive tumors and analysis of its processing requirements. This epitope, NY-ESO-1 157–165, contains a cysteine at carboxyl terminus of the peptide. Since it is known that cysteinylation and dimerization of cysteine residues may reduce antigenicity of synthetic peptides

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[3] Abbreviations used in this paper: SEREX, serological analysis of recombinant cDNA expression libraries; β_2m, β_2-microglobulin; LMP, low molecular protein; TCEP, tris(2-carboxyethyl)phosphine hydrochloride.

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binding to MHC class I molecules (9), we sought to study whether modification of cysteine 165 in the NY-ESO-1 157–165 epitope would increase its immunogenicity.

Materials and Methods

Synthetic peptides

Peptides were synthesized by standard solid-phase chemistry on a multiple peptide synthesizer (Genosys, The Woodlands, TX) by using F-moc for transient NH_2-terminal protection. All peptides were >90% pure, as indicated by analytical HPLC. Lyophilized peptides were diluted in DMSO and stored at −20°C.

Cells

The NY-ESO-1-positive tumor line NW 37 was cultured in Dulbecco medium (Life Technologies, Grand Island, NY) supplemented with 10% FCS. NY-ESO-1-specific CTL clones were stimulated with a mixture of allogeneic irradiated PBL- and EBV-transformed B cells, and PHA (5 μg/ml), and cultured in 5% human serum with IL-2 (200 U/ml; Chiron, Emeryville, CA), as described (8).

PBL from melanoma patients were stimulated with either 100 or 10 nM peptide in the presence or absence of 200 μM TCEP (Pierce, Rockford, IL). Peptides were added to total PBL, and cells were cultured in Iscove's medium supplemented with 5% human serum and IL-2 (200 U/ml). After 1 wk in culture, cells were stimulated with irradiated T2 cells pulsed with either 100 or 10 nM'm peptide in the presence or absence of 200 μM tris(2-carboxyethyl)phosphine hydrochloride (TCEP). T2 cells and PBL were mixed at a ratio of 1:1. Cells were stained with HLA-A2 tetramers containing the peptide NY-ESO-1 157–165.

MHC class I tetramers

Tetrameric MHC class I/peptide complexes were synthesized as described (10, 11). Briefly, purified HLA heavy chain and β_2-microglobulin (β_2m) were synthesized by means of a prokaryotic expression system (pET; R&D Systems, Minneapolis, MN). The heavy chain was modified by deletion of the *trans*-membrane and cytosolic tail and COOH-terminal addition of a sequence containing the Bir-A enzymatic biotinylation site. Heavy chain, β_2m, and peptide were refolded by dilution. The 45-kDa refolded product was isolated by FPLC and then biotinylated by Bir-A (Avidity) in the presence of biotin, adenosine 5′-triphosphate, and Mg^{2+} (all from Sigma, St. Louis, MO). Streptavidin-PE conjugate (Sigma) was added in 1:4 molar ratio.

mAbs and flow cytometry immunofluorescence analysis

Cells were stained with tetramers for 15 min at 37°C, then washed in PBS/1% FCS at 37°, before incubating with TriColor anti-CD8 (Caltag, Burlingame, CA) for 30 min on ice. Cells were washed three times in ice-cold PBS/1% FCS and analyzed by flow cytometry using CellQuest software. Cloning of tetramer-positive $CD8^+$ cells was conducted, as described (8), from a patient with an NY-ESO-1 positive melanoma (NW 14), after pulsing the PBL with NY-ESO-1 157–165 peptide 10 μM, and culturing in IL-2 200 U/ml for 5 days. HLA-A2 surface expression in T2 cells was measured by staining T2 cells with the HLA-A2-specific Ab BB7.2. T2 cells were incubated with peptides overnight at 37°C in RPMI without FCS. Cells were then washed and stained with 10 μg/ml of BB7.2, and washed and stained with FITC-labeled goat anti-mouse Ig.

Chromium release assay

Ag recognition was assessed using target cells (T2 or melanoma) labeled with ^{51}Cr for 90 min at 37°C and washed twice. Labeled target cells (5000 cells in 100 μl) were then added to varying numbers of effector cells (100 μl) in U-bottom microwells in presence or absence of peptides at different concentrations. Target cells were incubated with peptides for 30 min at 37°C before the addition of effector cells. Reducing agents DTT and TCEP were added together with target cells and peptides before adding effector cells. Chromium release was measured after incubation for 4 h at 37°C. The percent specific lysis was calculated as: $100 \times$ [(experimental − spontaneous release)/(total − spontaneous release)].

Immunoprecipitation of metabolically labeled HLA-A2

T2 cells were resuspended at 2×10^7/ml in methionine and cysteine-free R10 (RPMI 1640 with added glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and FCS (10% v/v)), for 1 h at 37°C. Promix (143 μCi; 70% [^{35}S]methionine and 30% [^{35}S]cysteine; Amersham, Arlington Heights, IL) was then added, and the mixture was incubated for 60 min. Cells were lysed in 0.5 ml ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% Nonidet P-40, 2 mM PMSF, and 5 mM iodoacetamide), followed by centrifugation of pellet nuclei. Lysates were precleared overnight with Pansorbin at 4°C and immunoprecipitated with 15 μg/ml of BB7.2 and protein A-coated Sepharose beads. Following extensive washing, proteins were eluted from the beads using standard SDS-PAGE loading sample buffer and heated at 95°C for 5 min.

Vaccinia infection

Target cells were infected with vaccinia at a multiplicity of infection of 5 for 90 min and, after washing, suspended in R10. Infected cells were grown either overnight or for 4 h. The vaccinia expressing the full-length NY-ESO-1 was made by cloning NY-ESO-1 cDNA into the thymidine kinase gene using the vector pSC11, as previously described (12). An influenza matrix vaccinia (Matrix-Vac) was used as a specificity control. This vaccinia has been described previously (12).

Lactacystin treatment of target cells

A total of 10^6 cells was suspended in 50 μl of R10 media containing 100 μM lactacystin for 1 h before addition of vaccinia in 50 μl at multiplicity of infection of 5. After 90-min infection, cells were washed and suspended in 5 ml of R10 containing 1 μM lactacystin and grown overnight to allow expression of the NY-ESO-1 and influenza matrix genes.

Results

Intracellular processing of the tumor Ag NY-ESO-1 and its presentation to HLA-A2-restricted CTL

HLA-A2 tetramers containing the peptide NY-ESO-1 157–165 were used to sort and clone $tetramer^+$ $CD8^+$ cells from melanoma patients' PBL (4, 8). Four NY-ESO-1-specific clones were expanded from a melanoma patient (Fig. 1*A*) and tested for killing specificity. NY-ESO-1-positive melanoma cells were efficiently killed, demonstrating that intracellular processing of the NY-ESO-1 protein results in the generation of a peptide recognized by NY-ESO-1 157–165-specific CTL (Fig. 1*B*). Similar results were obtained with NY-ESO-1-specific CTL lines expanded from another melanoma patient (data not shown). Three overlapping HLA-A2-binding NY-ESO-1 peptides (NY-ESO-1 157–165; NY-ESO-1 157–167; and NY-ESO-1 155–163; see Table I) were previously shown to be seen by polyclonal CTL lines derived from melanoma patients (5). It remained to be established whether lysis of target cells pulsed with these overlapping peptides was due to different CTL clones, each with different TCR specificity, or whether all three peptides were recognized by a single CTL clone. The use of tetramer-sorted NY-ESO-1-specific CTL clones allowed confirmation of the identity of the CTL determinant recognized by a single NY-ESO-1-specific TCR. We demonstrated that the 9-mer peptide NY-ESO-1 157–165, 10-mer peptide NY-ESO-1 157- 166, and the 11-mer peptide 157–167 were all recognized by NY-ESO-1-specific CTL clones, while the overlapping peptide NY-ESO-1 155–163 and the 8-mer peptide NY-ESO-1 157–164 were not seen (Figs. 1*C* and 4*A*). These results demonstrate that TCR recognition of the peptide NY-ESO-1 157–165 by a defined CTL clone requires the presence of either or both glutamine 164 and cysteine 165, hence suggesting that modification of these two residues may alter the efficiency of CTL recognition.

To analyze the processing requirements for the generation of the NY-ESO-1 157–165 epitope, experiments were conducted using processing mutant cells, which were previously characterized for the presence of defined blocks in the MHC class I-processing pathway (12–14). These studies demonstrated that expression of vaccinia-encoded NY-ESO-1 in the TAP-deficient cell line LBL 721.174 (.174) failed to sensitize them for lysis by NY-ESO-1 157–165-specific CTL. In contrast, the presence or absence of the proteasome subunits LMP2 and LMP7 did not impair efficient presentation of the NY-ESO-1/HLA-A2 epitope 157–165 (Fig. 2*A*). As a control for these results, we showed that the LMP-negative

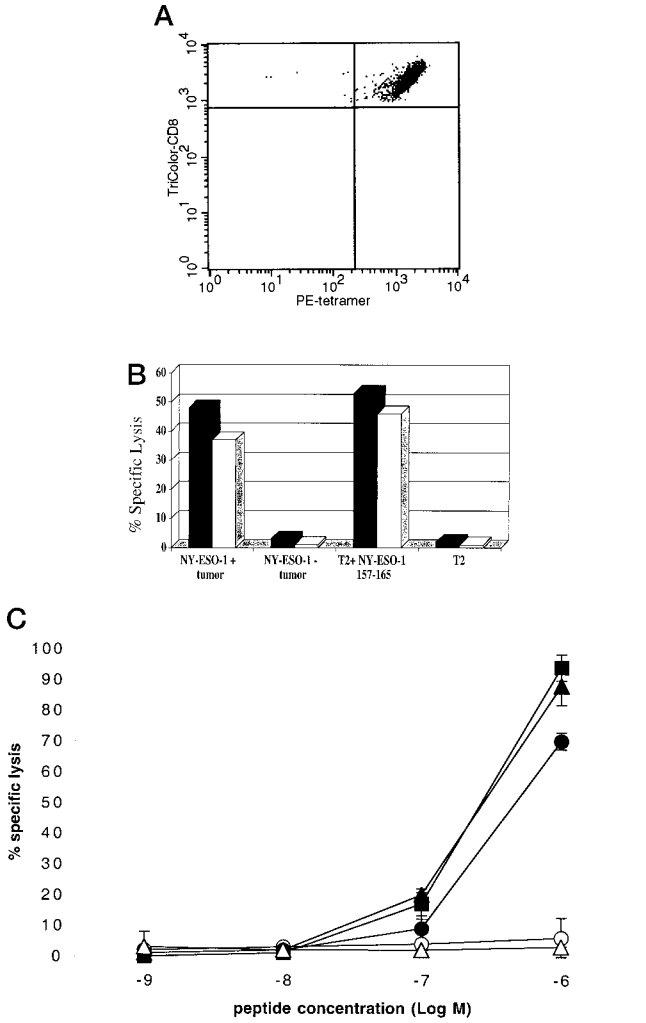

FIGURE 1. NY-ESO-1-positive tumors are killed by NY-ESO-1 specific CTL. *A*, Tetramer staining of a NY-ESO-1 157–165-specific CTL clone. Staining of NY-ESO-1 CTL clone with anti-CD8 Ab and PE-labeled HLA-A2 tetramer refolded around the NY-ESO-1 peptide SLLMWITQC. *B*, Killing of NY-ESO-1 positive tumor cells. A CTL clone specific for the NY-ESO-1 157–165 epitope was used against the following targets: NW-37 (NY-ESO-1 positive tumor cell line), SK-MEL-29 (NY-ESO-1 negative tumor cell line), T2 pulsed with 1 μM peptide NY-ESO-1 157–165 ($T2^+$ NY-ESO-1 157–165), and unpulsed T2 cells (T2). Black and white bars correspond to 1:1 and 0.3:1 E:T ratio. These results were confirmed in four separate experiments, using four different NY-ESO-1 157–165 specific CTL clones. *C*, Lysis of target cells pulsed with different length NY-ESO-1 157–165 peptides. T2 cells were pulsed with different peptides at concentration shown in the figure. Peptides used were: NY-ESO-1 157–165 (▲), NY-ESO-1 157–166 (■), NY-ESO-1 157–167 (●), NY-ESO-1 157–164 (○), and Flu matrix 58–66 (△). SD values at each peptide concentration were added in the figure. These results were confirmed in three separate experiments.

Table I. *List of peptides used in this paper*

NY-ESO-1 Analogues	
Peptide	Sequence
NY-ESO-1 155–163	QLSLLMWIT
NY-ESO-1 157–164	SLLMWITQ
NY-ESO-1 157–165	SLLMWITQC
NY-ESO-1 157–166	SLLMWITQCF
NY-ESO-1 157–167	SLLMWITQCFL
NY-ESO-1 157–165 (V)	SLLMWITQV
NY-ESO-1 157–165 (L)	SLLMWITQL
NY-ESO-1 157–165 (I)	SLLMWITQI
NY-ESO-1 157–166 (CV)	SLLMWITQCV
NY-ESO-1 157–166 (CI)	SLLMWITQCI
NY-ESO-1 157–166 (AL)	SLLMWITQAL
NY-ESO-1 157–166 (AI)	SLLMWITQAI
NY-ESO-1 157–166 (AF)	SLLMWITQAF

cells .174/TAP failed to present the LMP7-dependent influenza matrix epitope 58–66, while transfection of LMP7 relieved its block in presentation (12). Consistent with these findings, we showed that presentation of the NY-ESO-1 157–165 epitope was not altered by the addition of the proteasome inhibitor lactacystin (Fig. 2*B*), while proteasome inhibition by lactacystin in .174/TAP cells relieved the presentation of the LMP7-dependent influenza matrix epitope 58–66 (Fig. 2*B*) (12). These results, while confirming that generation of the influenza matrix epitope 58–66 is impaired in LMP7-negative cells (12), demonstrate that generation of the NY-ESO-1 157–165 epitope is resistant to the effect of proteasome inhibitors and is not dependent on the presence or absence of LMP proteasome subunits.

Identification of NY-ESO-1 157–165 peptide analogues with an increased immunogenicity

It is known that modification of cysteine residues contained within antigenic peptides may affect the immunogenicity of MHC class I-restricted epitopes (9, 15). As the peptide NY-ESO-1 157–165 contains a cysteine at position 165, we sought to address whether modification of cysteine 165 would reduce the immunogenicity of the peptide NY-ESO-1 157–165. CTL recognition of different doses of the peptide NY-ESO-1 157–165 was analyzed in the presence or absence of 200 μM of the reducing agents DTT and TCEP (Fig. 3 and data not shown). The results of these experiments demonstrated that the presence of reducing agents increased by 10-fold the antigenicity of the peptide NY-ESO-1 157–165, while no effect was observed for the recognition of different peptides lacking cysteine residues (Fig. 3 and data not shown). These results are consistent with previous published data (9), and suggest that a modification of the cysteine 165 in the NY-ESO-1 peptide 157–165 may result in a reduced recognition by specific CTL. Stabilization of HLA-A2 molecules expressed on the surface of T2 cells by different concentrations of the NY-ESO-1 157–165 peptide (13) revealed that this peptide has a low binding affinity, as compared with the binding affinity of the influenza matrix peptide 58–66 (Fig. 4, *B* and *C*). Binding of the NY-ESO-1 157–165 peptide to HLA-A2 molecules did not significantly change in the presence and absence of 200 μM TCEP (data not shown). It is worth noting that, despite the low binding affinity of peptide NY-ESO-1 157–165, HLA-A2 tetramers containing this peptide were stable and capable of staining NY-ESO-1-specific CTL (4) (Figs. 1*A* and 5).

The effect of reducing agents on the antigenicity of the peptide NY-ESO-1 157–165 prompted us to study the binding and cytotoxicity of peptide analogues in which cysteine 165 was substituted for different amino acids (Table I). Two distinct approaches were followed. First, 9-mer peptide NY-ESO-1 157–165 was synthesized with cysteine 165 replaced by the stronger HLA-A2 anchor residue amino acids (16) valine, isoleucine, and leucine. Second, 10-mer peptides were synthesized containing at the carboxyl terminus anchor residue amino acids, more often observed at the

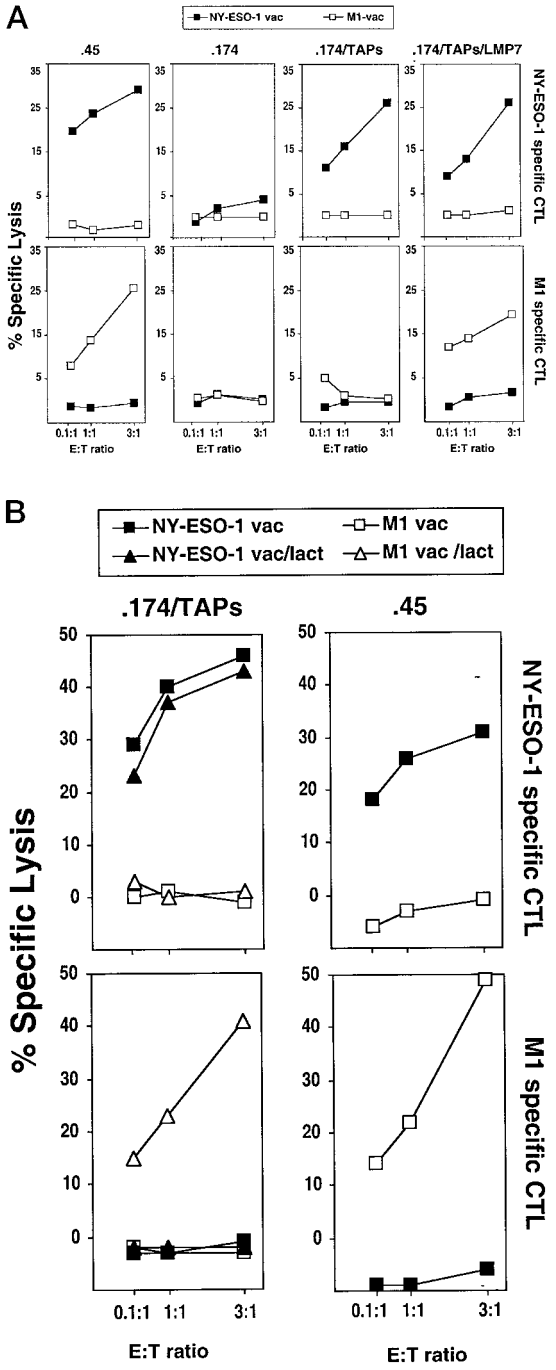

FIGURE 2. Generation of the NY-ESO-1 epitope 157–165 is not dependent on the presence or absence of LMP2 and LMP7 proteasome subunits. *A*, CTL clone specific for either the NY-ESO-1 157–165 epitope (*top panel*) or influenza matrix epitope 58–66 (*bottom panel*) was used against the following infected targets: the parental line 45; the TAP-negative, LMP2- and LMP7-negative cell line .174; .174 transfected with TAP1 and TAP2 (.174/TAPs); .174 transfected with TAP1 and TAP2 genes and LMP7 (.174/TAPs/LMP7). Cells were infected with recombinant vaccinia virus containing either the NY-ESO-1 protein (NY-ESO-1 vac) (■) or the influenza matrix protein (M1-vac) (□). The results of these experiments were confirmed in three separate experiments. *B*, CTL clone specific for either the NY-ESO-1 157–165 epitope (*top panel*) or influenza matrix epitope 58–66 (*bottom panel*) was used against .174 transfected with TAP1 and TAP2 (.174/TAPs) and the parental line .45. The target cells .174/TAPs were treated (triangles) or mock treated (squares) with lactacystin. The results of these experiments were confirmed in two separate experiments.

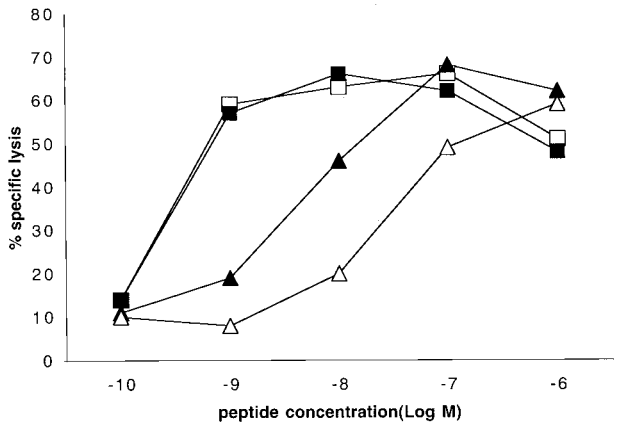

FIGURE 3. Increased antigenicity of the peptide NY-ESO-1 157–165 in the presence of reducing agents. Recognition of peptide NY-ESO-1 157–165 (triangle) and peptide NY-ESO-1 157–165(V) (square) in the presence (filled symbols) or absence (open symbols) of 200 μM DTT. Similar results were generated with TCEP (data not shown). E:T ratio was 1:1. The results of this experiment were confirmed in two separate experiments.

carboxyl terminus of HLA-A2-binding peptides (16). Cysteine 165, at position 9 of these peptides, was either conserved (NY-ESO-1 157–166 (CV), NY-ESO-1 157–166 (CI)), or substituted for an alanine (NY-ESO-1 157–166 (AL), NY-ESO-1 157–166 (AI), NY-ESO-1 157–166 (AF)). In addition, peptide NY-ESO-1 157–165 was synthesized containing the SH group of cysteine 165 blocked by a $-NH-CO-CH_2$ side chain. While recognition of the latter peptide was not significantly better than the wild-type peptide 157–165 (data not shown), substitution of cysteine 165 with either valine (NY-ESO-1 157–165 (V)), isoleucine (NY-ESO-1 157–165 (I)), or leucine (NY-ESO-1 157–165 (L)) increased by at least 100-fold recognition by NY-ESO-1 157–165-specific CTL, as compared with the recognition of NY-ESO-1 157–165 (Fig. 4*A*). In contrast, 10-mer peptide analogues, with the exception of peptide NY-ESO-1 157–166 (CV), were not recognized more efficiently than the wild-type peptide NY-ESO-1 157–165 (Fig. 4*A*). Addition of TCEP to peptide NY-ESO-1 157–165 (V) did not increase the percentage of specific lysis, while TCEP increased by 10-fold recognition of wild-type peptide NY-ESO-1 157–165 (Fig. 3).

The increased recognition of peptides NY-ESO-1 157–165 (V), NY-ESO-1 157–165 (I), and NY-ESO-1 157–165 (L) can be accounted for by a combination of higher peptide-binding affinity to HLA-A2 molecules and TCR. Binding of peptide analogues to HLA-A2 molecules was compared with the binding of the wild-type peptide NY-ESO-1 157–165. Peptides NY-ESO-1 157–165 (V) and NY-ESO-1 157–165 (I) were capable of stabilizing HLA-A2 molecules more efficiently than the wild-type peptide NY-ESO-1 157–165 (Fig. 4*B*). These results were further confirmed by measuring peptide binding to metabolically labeled HLA-A2 molecules in T2 cells (as first described in Ref. 13) (Fig. 4*C*). Addition of TCEP during the assay did not increase surface expression of HLA-A2 molecules, while there was a small shift for peptide NY-ESO-1 157–166 (CI) (data not shown).

Stimulation of NY-ESO-1-specific CTL from melanoma patients' PBL

To study the ability of different peptide analogues to stimulate proliferation of NY-ESO-1-specific CTL, PBL from two melanoma patients with a high titer of NY-ESO-1 specific Abs were

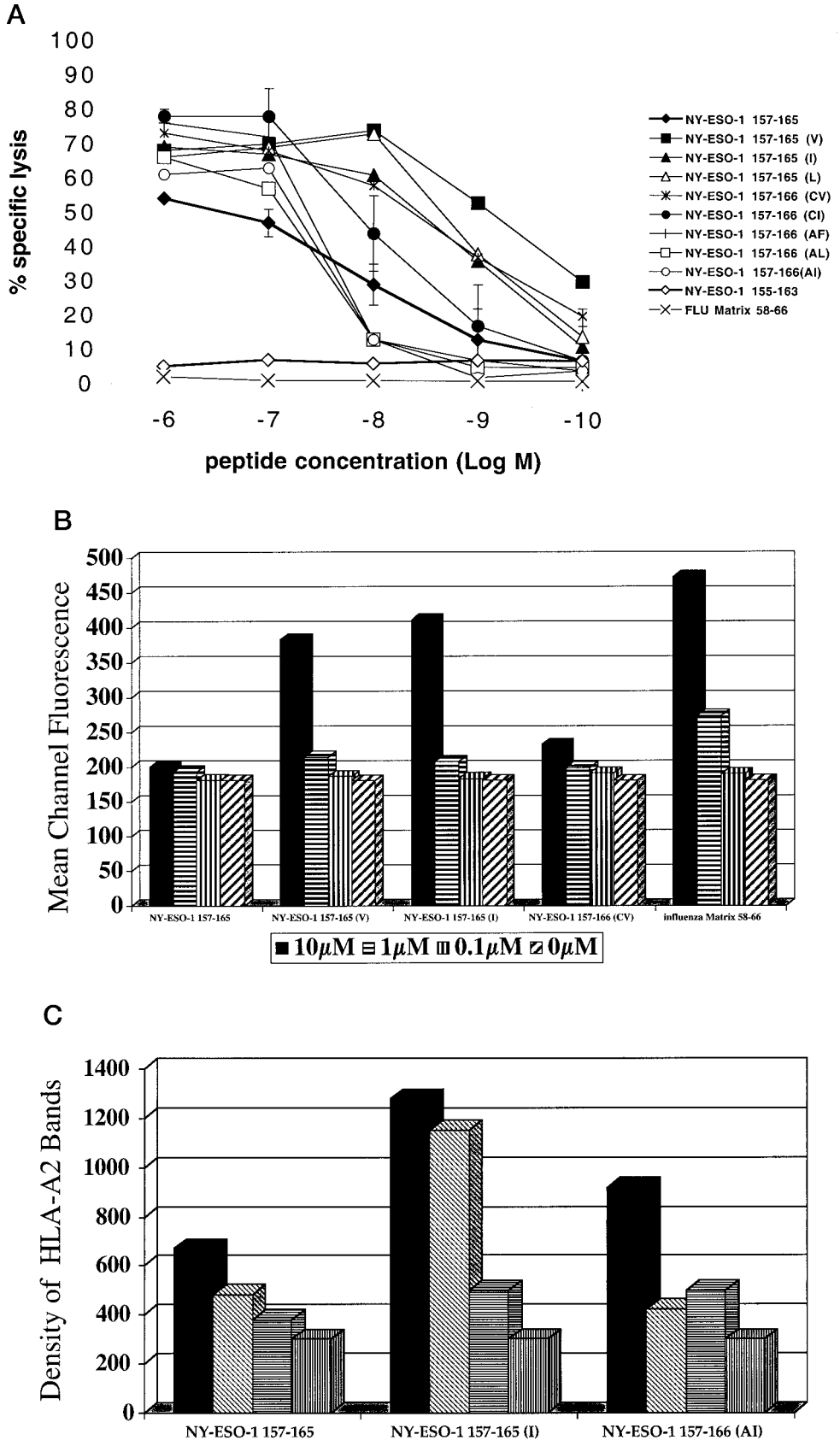

FIGURE 4. Substitution of cysteine 165 significantly increases antigenicity of NY-ESO-1 peptide analogues. *A*, Recognition of NY-ESO-1 peptide analogues by CTL clones specific for NY-ESO-1 157–165 peptide. T2 cells were pulsed with peptide concentrations shown in the *x*-axis of the figure. Symbols corresponding to each peptide used in the experiment are shown. The wild-type peptide NY-ESO-1 157–165 titration curve has a thicker black line. E:T ratio was 1:1. SD values at each peptide concentration were added in the figure. The results of this experiment were confirmed in three separate experiments. *B*, Stabilization of HLA-A2 surface expression on T2 cells by NY-ESO-1 peptide analogues. Each bar shows mean channel fluorescence of T2 cells stained with the HLA-A2-specific Ab BB7.2 after overnight incubation with different peptides at concentrations shown in the figure. *C*, The effect

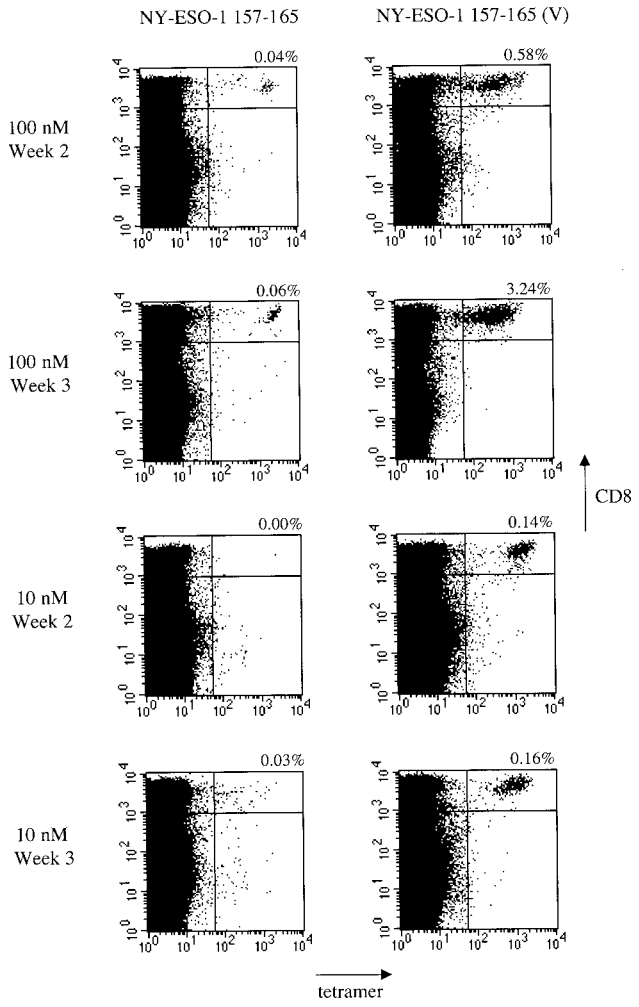

FIGURE 5. Peptide NY-ESO-1 157–165 (V) stimulates the expansion of NY-ESO-1-specific CTL from melanoma patients' PBL more efficiently than the peptide NY-ESO-1 157–165. Tetramer staining of a melanoma patient's PBL with anti-CD8 Ab and PE-labeled HLA-A2 tetramer refolded around the NY-ESO-1 wild-type peptide NY-ESO-1 157–165. PBL were stimulated with either peptide NY-ESO-1 157–165 (*left column*) or peptide NY-ESO-1 157–165 (V) (*right column*) in the presence of 200 μM TCEP. PBL samples were stimulated with either 100 or 10 nM of each peptide for either 2 wk or 3 wk. Percentage of tetramer$^+$ CD8$^+$ cells is shown in each panel.

stimulated with different doses of wild-type peptide NY-ESO-1 157–165 and peptide NY-ESO-1 157–165 (V). To minimize the effect of modification of the cysteine 165 contained in the peptide NY-ESO-1 157–165, PBL were maintained either in the presence or absence of 200 μM TCEP. Stimulation of PBL samples with peptide NY-ESO-1 157–165 (V) in the presence of reducing agents resulted in a 14-fold greater expansion of NY-ESO-1-specific CTL over 2 wk, and 54-fold expansion over 3 wk (Fig. 5), as compared with CTL expansion driven by the wild-type peptide NY-ESO-1 157–165. Similar results were obtained after stimulating PBL in the absence of reducing agents (data not shown). Specific CTL were stained with HLA-A2 tetramers containing the NY-ESO-1 157–165 peptide, hence demonstrating the ability of these cells to recognize the wild-type peptide NY-ESO-1 157–165. A dose of 10 nM of peptide NY-ESO-1 157–165 (V) was capable of stimulating expansion of NY-ESO-1-specific CTL, while identical doses of the wild-type peptide NY-ESO-1 157–165 failed to expand NY-ESO-1-specific CTL. These results confirmed the enhanced immunogenicity of the peptide analogue NY-ESO-1 157–165 (V).

Discussion

NY-ESO-1 gene was isolated from an esophageal squamous cell carcinoma by SEREX, and has been shown to be expressed in 20–40% of several common tumor types, including breast cancer, lung cancer, prostate cancer, bladder cancer, head and neck cancer, and melanoma (3). In vitro stimulation of patients' PBL with NY-ESO-1-derived peptides led to the identification of three overlapping peptides (NY ESO-1 157–165, NY-ESO-1 157–166, and NY-ESO-1 155–63) recognized by the patient's PBL in association with HLA-HLA-A2 molecules (5). We have recently demonstrated that ~40–50% of melanoma patients with advanced tumors expressing NY-ESO-1 make a simultaneous Ab and CTL response against NY-ESO-1 (4), hence demonstrating that NY-ESO-1 is to date the only cancer-testis Ag capable of eliciting both a humoral and cellular response in a large proportion of patients. However, it remained to be established how efficiently tumor cells are capable of presenting NY-ESO-1-derived peptides to NY-ESO-1-specific CTL, and whether antigenicity of NY-ESO-1 synthetic peptides can be increased by substituting amino acid residues that do not impair TCR recognition. To address these questions, we generated NY-ESO-1-specific CTL clones using HLA-A2 tetramers containing the peptide NY-ESO-1 157–165.

In the first part of this work, we showed that NY-ESO-1 can be efficiently processed by melanoma cells and recognized by NY-ESO-1 157–165-specific CTL clones (Fig. 1*B*). These results confirm previously published data (5) and extend them by further defining the processing pathway responsible for the presentation of the NY-ESO-1/HLA-A2 epitope. Although the nature of the naturally processed NY-ESO-1/HLA-A2 peptide remains to be determined, we demonstrated in this study that peptides NY ESO-1 157–165, NY-ESO-1 157–166, and NY-ESO-1 157–167 are recognized by clonal CTL, while peptide NY-ESO-1 157–164 was not seen (Fig. 1*C*). These results provide insights into the definition of the minimal-length NY-ESO-1 HLA-A2 peptide presented by tumor cells. Since peptide NY-ESO-1 155–163 was not recognized (Fig. 4*A*), while peptide NY-ESO-1 157–165 was efficiently seen, our results are consistent with the possibility that glutamine 164 and cysteine 165 of the peptide NY-ESO-1 157–165 contribute to the antigenicity of the peptide NY-ESO-1 157–165. This conclusion is supported by the findings that some NY-ESO-1 peptide analogues, in which cysteine 165 was substituted for certain amino acids, were more antigenic than the peptide NY-ESO-1 157–165.

The observation that certain viral epitopes are not generated in cells lacking the IFN-γ-inducible proteasome subunits LMP2 and LMP7 raises the question of whether the generation of certain melanoma epitopes can be impaired by a down-regulation of LMP2 and LMP7 (12–14, 17, 18). As these proteasome subunits are not required for cell viability, it is to be expected that a strong CTL response will select for melanoma cells with mutations or

of NY-ESO-1 157–165 peptide analogues on stability of metabolically labeled HLA-A2 molecules. Stable HLA-A2/β_2m complexes were precipitated with the conformation-specific mAb BB7.2 after overnight preclearing of T2 lysate in the presence of 1 μM (■), 10 nM (▨), 1 nM (▤), and no peptide (▥). *y*-axis shows density of HLA-A2 heavy chain bands.

deletion of LMPs, resulting in a functional deficiency of the Ag-processing pathway. Indeed, human spontaneous lung carcinoma cell lines with down-regulation of LMPs have been described (19). A recent report has suggested that dendritic cells may fail to generate defined tumor CTL epitopes, as a result of their destruction by LMP-positive proteasomes (7). This reasoning led us to study the role of LMP gene products and proteasome proteolytic activity for the generation of the NY-ESO-1 epitope 157–165. Our results demonstrate that presentation of NY-ESO-1/HLA-A2 epitope is not dependent on the presence of LMP2 and LMP7, as we showed efficient lysis of LMP-positive and LMP-negative cells by NY-ESO-1 157–165-specific CTL after infection with NY-ESO-1 vaccinia virus. These results were controlled for by analyzing simultaneously presentation of the LMP7-dependent influenza matrix HLA-A2 epitope (Fig. 2, *A* and *B*).

In the second part of these studies, we analyzed whether antigenicity of the peptide NY-ESO-1 157–165 was enhanced by modifying the peptide's carboxyl-terminal amino acid. As peptide 157–165 has a cysteine at position 9, we reasoned that its sulfhydryl modification could reduce its binding affinity and antigenicity. Our results were consistent with this possibility, as we showed that target cells pulsed with peptide NY-ESO-1 157–165 in the presence of reducing agents were recognized 10 times more efficiently (Fig. 3). These findings are in line with previously published data, demonstrating that modification of cysteine residues affects the immunogenicity of MHC class I viral determinants (9).

We then identified a series of NY-ESO-1 peptide analogues with greater antigenicity than the wild-type peptide. In particular, we showed that peptide analogues with cysteine 165 substituted by either a valine, isoleucine, or leucine were recognized at least 100 times more efficiently than the wild-type peptides (Fig. 4*A*). Binding of peptide NY-ESO-1 157–165 (V), NY-ESO-1 157–165 (I), and NY-ESO-1 157–165 (L) to HLA-A2 molecules was more efficient than the wild-type peptide NY-ESO-1 157–165. However, it is worth noting that HLA-A2 tetramers containing either the wild-type peptide NY-ESO-1 157–165 or peptide NY-ESO-1 157–165 (I) gave similar intensity of staining of the NY-ESO-1-specific CTL clone (data not shown).

Finally, we studied the immunogenicity of NY-ESO-1 peptide analogues using PBL from melanoma patients. The peptide NY-ESO-1 157–165 (V) was capable of inducing a significantly more efficient expansion of NY-ESO-1-specific CTL from patients' PBL, as compared with wild-type peptide NY-ESO-1 157–165 in the presence or absence of reducing agents. The specificity of peptide NY-ESO-1 157–165 (V)-expanded CTL was confirmed by their ability to be stained with HLA-A2 tetramers containing the wild-type peptide NY-ESO-1 157–165. Over a 2-wk restimulation, the frequency of NY-ESO-1-specific CTL was more than 50 times greater in cultures expanded in the presence of the peptide analogue NY-ESO-1 157–165 (V). Increased antigenicity and immunogenicity of NY-ESO-1 peptide analogues may result from a combination of their higher binding affinity to HLA-A2 molecules and better interaction with TCRs of specific CTL clones and lines. The relationship between Ag presentation, peptide-binding affinity, and expansion of CTL in vitro and in vivo is still unclear. Results obtained with different *Listeria monocytogenes* mutants showed that the extent of T cell priming and expansion is not related in a linear fashion to the amount of presented epitope (20). It has been shown, however, that recombinant vaccinia virus encoding HLA-A2/Melan-A epitope 27–35 analogues can prime more efficiently HLA-A2 transgenic mice than vaccinia encoding wild-type Melan-A peptide (21, 22).

In conclusion, we have demonstrated that processing of NY-ESO-1 protein by tumor cells results in the generation of an A2-restricted CTL epitope. Since specific killing of NY-ESO-1-positive tumor cells was shown using tetramer-sorted NY-ESO-1-specific CTL clones, our results definitively prove that the NY-ESO-1 epitope 157–165 is generated by tumor cells, hence emphasizing the importance of this epitope in cancer vaccines. We have defined the minimal overlapping peptide region required for the recognition of this epitope and demonstrated that its presentation is not dependent on the presence of immunoproteasome. Finally, we extended these results by identifying peptide analogues capable of an enhanced stimulation of NY-ESO-1-specific CTL from melanoma patients' PBL. Since NY-ESO-1 is expressed in 20–40% of several common tumor types and HLA-A2 is expressed in 40% of Caucasian population, our findings confirm the importance of vaccines capable of expanding NY-ESO-1-specific CTL in cancer patients. Phase 1 clinical trials using NY-ESO-1 synthetic peptides are already in progress aimed at eliciting a tumor-specific CTL response. The use of peptide analogues could result in a more efficient induction of NY-ESO-1-specific CTL in cancer patients.

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