The Journal of Immunology

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J Immunol 1998; 160:1750-1758; ;
http://www.jimmunol.org/content/160/4/1750

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Enhanced Generation of Specific Tumor-Reactive CTL In Vitro by Selected Melan-A/MART-1 Immunodominant Peptide Analogues[1]

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The *Melan-A/MART-1* gene, which is expressed by normal melanocytes as well as by most fresh melanoma samples and melanoma cell lines, codes for Ags recognized by tumor-reactive CTL. HLA-A*0201-restricted Melan-A-specific CTL recognize primarily the Melan-A_{27-35} (AAGIGILTV) and the Melan-A_{26-35} (EAAGIGILTV) peptides. The sequences of these two peptides are not necessarily optimal as far as binding to HLA-A*0201 is concerned, since both lack one of the dominant anchor amino acid residues (leucine or methionine) at position 2. In this study we introduced single amino acid substitutions in either one of the two natural peptide sequences with the aim of improving peptide binding to HLA-A*0201 and/or recognition by specific CTL. Surprisingly, analogues of the Melan-A_{27-35} peptide, which bound more efficiently than the natural nonapeptide to HLA-A*0201, were poorly recognized by tumor-reactive CTL. In contrast, among the Melan-A_{26-35} peptide analogues tested, the peptide ELAGIGILTV was not only able to display stable binding to HLA-A2.1 but was also recognized more efficiently than the natural peptide by two short-term cultured tumor-infiltrated lymph node cell cultures as well as by five of five tumor-reactive CTL clones. Moreover, in vitro generation of tumor-reactive CTL by stimulation of PBMC from HLA-A*0201 melanoma patients with this particular peptide analogue was much more efficient than that observed with either one of the two natural peptides. These results suggest that the Melan-A_{26-35} peptide analogue ELAGIGILTV may be more immunogenic than the natural peptides in HLA-A*0201 melanoma patients and should thus be considered as a candidate for future peptide-based vaccine trials. ***The Journal of Immunology*, 1998, 160: 1750–1758.**

Melanoma-associated Ags recognized by CTL from cancer patients can be divided into three broad categories. The first category includes products of normal genes such as the *MAGE* multigene family (1), which are expressed in some melanomas as well as in subsets of different human tumor types, but not in normal tissues except testis and placenta. A second category includes Ags derived from mutant proteins (2–4). The third category, which constitutes the largest group of melanoma Ags identified to date, is represented by melanocyte lineage-specific Ags and includes peptides derived from tyrosinase, gp100, gp75, and Melan-A/MART-1 proteins (5–10).

Melan-A/MART-1 (Melan-A)[3] is expressed by most fresh melanoma samples and by about 60% of melanoma cell lines (9, 10). Melan-A-specific CTL have been identified in both PBMC and tumor-infiltrating lymphocytes (TIL) from HLA-A*0201 melanoma patients (10, 11). Nine of 10 independent HLA-A*0201-restricted Melan-A-specific TIL lines were found to recognize the nonapeptide Melan-A_{27-35} (AAGIGILTV) (11). Melan-A-specific CTL could be induced by stimulation of PBMC from HLA-A*0201 normal donors and melanoma patients with peptide Melan-A_{27-35} (Refs. 12–15; and S. D'Souza, F. Lejeune, D. Rimoldi, D. Liénard, J.-C. Cerottini, and P. Romero, manuscript in preparation). Since this antigenic peptide appeared to be an immunodominant Melan-A epitope (11, 12), it has been proposed as a target for the development of vaccines in HLA-A*0201 patients with melanoma. More recently, we found that the decapeptide Melan-A_{26-35} (EAAGIGILTV) was better recognized than the nonapeptide Melan-A_{27-35} by 4 of 4 tumor-infiltrated lymph nodes (TILN) as well as by 10 of 13 Melan-A-specific CTL clones derived from melanoma patients (16). Whether the Melan-A decapeptide is more immunogenic than the nonapeptide when used in peptide-based vaccines has not yet been determined.

The majority of natural peptides bound to HLA-A*0201 have a restricted size of 9 to 10 amino acids and contain two dominant anchor residues within their sequence: leucine (L) or methionine (M) at position 2, and valine (V) at position 9 (17, 18). Amino acids present at other positions within the peptide may play a role

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Received for publication October 10, 1997. Accepted for publication October 30, 1997.

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[1] J.-F.F. thanks the Ligue Departementale de Loire-Atlantique contre le Cancer, France, for its funding. This work was partly supported by the Federal Office for Education and Science (OFES), Switzerland, and the European Community (Contract BMH4-CT95-1627). C.M.L. thanks the Junta de Andaluera, Spain, for financial support.

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[3] Abbreviations used in this paper: Melan-A, Melan-A/MART-1; TIL, tumor-infiltrating lymphocyte; TILN, tumor-infiltrated lymph node.

0022-1767/98/$02.00

in HLA-A*0201-peptide interactions (19). In particular, the presence of negatively charged residues at position 1 such as glutamic acid (E) or aspartic acid (D) was reported to be associated with poor binding to HLA-A*0201 (19). In this respect, it is noteworthy that both Melan-A antigenic peptides lack the major anchor residue at position 2. Moreover, the Melan-A_{26-35} peptide has glutamic acid at position 1.

In the current study, we introduced single amino acid substitutions in the Melan-A nonapeptide or decapeptide sequences with the aim of improving peptide binding to HLA-A*0201 and/or recognition by tumor-reactive CTL. This approach led to the identification of a Melan-A_{26-35} peptide analogue, which exhibited more stable binding to HLA-A*0201 and improved recognition by Melan-A-specific TILN and CTL clones from HLA-A*0201 melanoma patients. In addition, this particular peptide analogue was more efficient than the natural peptides in inducing Melan-A-specific, melanoma-reactive CTL responses in vitro in PBMC from HLA-A*0201 melanoma patients. These results suggest that the efficacy of Melan-A peptide-based immunization of melanoma patients may be enhanced by using such a selected peptide analogue.

Materials and Methods

Cell lines and clones

Tumor cell lines and EBV-transformed B cell lines were maintained in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS, 0.55 mM Arg, 0.24 mM Asn, and 1.5 mM Gln. Melanoma cell lines Me 256, Me 260, Me 275, and Me 290 were established at the Ludwig Institute for Cancer Research, Lausanne Branch, from surgically excised melanoma metastases from patients LAU84, LAU149, LAU50, and LAU203, respectively. CTL line 198NS, which is specific for peptide MAGE-$3_{271-279}$ presented by HLA-A2, was derived as previously described (20). Melan-A-specific CTL clones M77.80 (Vβ3) and M77.86 (Vβ14) were derived from TIL of melanoma patient M77 as described (21). Briefly, CTL clones were obtained from limiting dilution cultures in the presence of irradiated autologous tumor cells, EBV-transformed B lymphocytes, PHA, and rIL-2. Clones were derived from wells having a probability of clonality higher than 90% according to single hit Poisson distribution. They were subsequently expanded by plating 5×10^3 cells, every 3 to 4 weeks, into microtiter plates together with irradiated feeder cells (5×10^4 allogenic PBMC and 2×10^4 EBV-transformed-B-LAZ cells). CTL clones 1.13 (Vβ14), 7.10 (Vβ17), and Mel 1.33 (Vβ9) were derived from PBMC of normal HLA-A2 donors (N. Gervois, S. Le Guiner, N. Labarriere, J.-F. Fonteneau, A. S. Beignon, E. Diez, and F. Jotereau, manuscript in preparation). All the clones used in this study efficiently recognized HLA-A*0201-positive Melan-A-expressing tumor cell lines.

Synthetic peptides

Peptides were synthesized by standard solid phase chemistry on a multiple peptide synthesizer (Applied Biosystems, Foster City, CA) by using standard F-moc for transient NH_2-terminal protection, and were analyzed by mass spectrometry. All peptides were >90% pure as indicated by analytical HPLC. Lyophilized peptides were diluted in DMSO and stored at −20°C.

*HLA-A*0201 binding assay*

The peptide binding capacity to HLA-A*0201 was assessed in a functional competition assay based on inhibition of recognition of the antigenic peptide MAGE-$3_{271-279}$ by the HLA-A*0201-restricted CTL line 198NS (20). Briefly, various concentrations of competitor peptides (50 μl) were incubated with ^{51}Cr-labeled T2 cells (50 μl) (1000 cells/well) for 15 min at room temperature. A suboptimal dose (1 nM) of the antigenic peptide MAGE-$3_{271-279}$ (50 μl) was then added together with specific CTL (5000 cells/well) (50 μl). Chromium release was measured after a 4-h incubation at 37°C. The concentration of each competitor peptide required to achieve 50% inhibition of target cell lysis was then determined and indicated as [nM] 50%. To facilitate comparison, the relative competitor activity of each peptide was calculated as the [nM] 50% of the unmodified Melan-A nonapeptide AAGIGILTV divided by the [nM] 50% of the competitor peptide.

*Assessment of the stability of Melan-A-derived peptides/HLA-A*0201 complexes*

The stability of peptide/HLA-A*0201 complexes was assayed using the TAP-deficient T2 cells. These cells were loaded with peptide by overnight incubation at room temperature with saturating concentrations (10 μM) of the different peptides and human β2 microglobulin (3 μg/ml) (Sigma, Buchs, Switzerland) in serum-free medium (X-VIVO 10; BioWhittaker, Walkersville, MD). After peptide removal and addition of emetine (10^{-4} M; Sigma) to block protein synthesis, cells were incubated at 37°C for the indicated time periods. For each time point, an aliquot of cells was stained with mAb BB7.2 (HLA-A2 specific) to measure HLA-A2 Ag expression. Influenza $matrix_{58-66}$ peptide, which has been shown to bind to HLA-A*0201 with high affinity and form stable peptide/HLA-A*0201 complexes (22), was used as an internal standard. Results are expressed as:

Relative complex stability = 100
× [(mean fluorescence with Melan-A-derived peptide
− background mean fluorescence)
÷ (mean fluorescence with Flu matrix peptide
− background mean fluorescence)]

Where background mean fluorescence represents the fluorescence value obtained on a sample of T2 cells treated in similar conditions except for the absence of exogenous peptide (23).

Cytokines

Human rIL-2 (Glaxo, Geneva, Switzerland) was kindly provided by Dr. M. Nabholz (ISREC, Epalinges, Switzerland) and human rIL-7 was donated by Dr. N. Vita of Sanofi Recherche (Labège, France). One unit of IL-2 is defined as the concentration that gives 50% maximal proliferation of CTLL-2.

Generation of TILN and Melan-A-specific CTL

TILN were generated from tumor-infiltrated lymph nodes of HLA-A*0201$^+$ melanoma patients obtained by surgery at the Centre Pluridisciplinaire d'Oncologie, CHUV, Lausanne, Switzerland. Tumoral lymph node fragments were minced to single cell suspensions and cultured in 24-well tissue culture plates (Costar corporation, Cambridge, MA) in 2 ml of Iscove's Dulbecco medium supplemented with Asn, Arg, and Gln and 10% pooled human A$^+$ serum in the presence of IL-2 and IL-7 (100 U/ml and 10 ng/ml, respectively). After 2 to 3 wk of cell culture, the TILNs were tested for cytolytic activity and their cell surface phenotype was determined by flow cytometry. The TILN chosen for this study were >90% CD3$^+$CD8$^+$ cells that had cytolytic activity against autologous or HLA-A*0201$^+$ tumor cell lines and recognized the Melan-$A_{(26)27-32}$ peptides presented by HLA-A*0201. Peptide-specific CTL were generated as previously described (20) with minor modifications. Briefly, PBMC from HLA-A*0201$^+$ melanoma patients were isolated by centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden). After enrichment for CD3$^+$ lymphocytes by treatment with Lympho-Kwik T (One Lambda, Canoga Park, CA), CD8$^+$ T lymphocytes were isolated using a miniMACS device (Miltenyi Biotec GmBH, Sunnyvale, CA). The resulting populations routinely contained >75% CD8$^+$ T cells and were used as responder cell populations. Purified CD8$^+$ T cells were plated at 1 to 2×10^6 cells/well together with 2×10^6 stimulator cells/well in 24-well plates in a total volume of 2 ml of Iscove's medium supplemented with 10% human serum, Asn, Arg, and Gln (complete medium) in the presence of IL-7 (10 ng/ml) and IL-2 (10 U/ml). Stimulator cells were prepared as follows: 2×10^6 autologous PBMC were incubated for 2 h at 37°C in serum-free medium (X-VIVO 10; BioWhittaker) with a Melan-A-derived peptide (20 μg/ml) and human β2 microglobulin (3 μg/ml). Peptide-pulsed PBMC were then washed, irradiated (3000 rad), and adjusted to the appropriate volume before addition to the CD8$^+$-enriched responder cell populations. On day 7, cells were restimulated with peptide-pulsed autologous PBMC in complete medium supplemented with IL-2 (10 U/ml). Subsequent restimulations were performed weekly with peptide-pulsed and irradiated autologous PBMC. CTL activity was first tested at the end of the second in vitro restimulation.

Table I. *Identification of the peptide(s) recognized by HLA-A2-restricted, Melan-A specific TILN*

Peptide Sequence	TILN LAU 203 Peptide[a] [nM] 50%	TILN LAU 203 Relative Activity[b]	TILN LAU 132 Peptide [nM] 50%	TILN LAU 132 Relative Activity
$AAGIGILTV_{27–35}$	40	1	15	1
$EAAGIGILTV_{26–35}$	1.5	27	1	15
$AAGIGILTVI_{27–36}$	600	0.06	300	0.05
$ILTVILGVL_{32–40}$	$>10^4$	$<4 \times 10^{-3}$	$>10^4$	$<1.5 \times 10^{-3}$

[a] T2 cells were incubated with various concentrations of the Melan-A peptides $_{27–35}$, $_{26–35}$, $_{27–36}$, and $_{32–40}$. Lysis by TILN LAU 203 and LAU 132 was measured by a 4-h ^{51}Cr release cytotoxicity assay at a lymphocyte to target cell ratio of 30:1. The peptide concentration giving 50% of maximal activity, [nM] 50%, was then determined from the titration curve for each peptide.

[b] The relative antigenic activity for each peptide compared with that of the nonapeptide Melan-$A_{27–35}$ was calculated as described in *Materials and Methods.*

Assessment of Ag recognition by TILN, Melan-A-specific CTL clones, and peptide-induced CTL

Ag recognition was assessed using chromium-release assays. Target cells were labeled with ^{51}Cr for 1 h at 37°C and washed two times. Labeled target cells (1,000 cells in 50 μl) were then added to varying numbers of effector cells (50 μl) in V-bottom microwells in the presence or absence of 1 μg/ml of the antigenic peptide (50 μl). In the peptide titration experiments, target cells (1,000 cells in 50 μl) were incubated in the presence of various concentrations of peptide (50 μl) for 15 min at room temperature before the addition of effector cells (50 μl). In each case the effector cells were preincubated for at least 20 min at 37°C with unlabeled K562 cells (50,000/well) to eliminate nonspecific lysis due to NK-like effectors present in stimulated T cell populations. Chromium release was measured in supernatant (100 μl) harvested after 4 h of incubation at 37°C. The percent specific lysis was calculated as:

$$100 \times [(\text{experimental} - \text{spontaneous release}) \div (\text{total} - \text{spontaneous release})]$$

Analysis of mRNA expression

The analysis of mRNA expression was performed as described (24). Briefly, total cellular RNA was extracted by the guanidine-isothiocyanate/cesium chloride procedure. cDNA synthesis from 2 μg of RNA was accomplished by priming with oligo(dT) and aliquots corresponding to 100 ng of RNA were amplified by 30 cycles of PCR using oligonucleotide primers specific for the gene *Melan-A* (24). A 10-μl aliquot from each reaction was run on a 2% agarose gel and visualized by ethidium bromide fluorescence. To verify RNA integrity, a 21-cycle PCR assay with primers specific for β-actin was conducted in each case.

Results

*Identification of the Melan-A-derived peptide(s) recognized by TILN from HLA-A*0201 melanoma patients*

Several overlapping antigenic peptide sequences have been mapped in the Melan-A protein region containing the immunodominant HLA-A2-restricted CTL epitope (11). To determine which of these peptides were recognized by TILN populations derived from two HLA-A*0201 melanoma patients, we tested the peptides listed in Table I. In an attempt to minimize Ag-specific selection in vitro, we used TILN that were cultured for a short time period in the presence of IL-2 and IL-7 alone. Two melanoma lines (Me 290 and Me 260) were used as a source of Melan-A-expressing target cells. As shown in Figure 1, both TILN populations were able to lyse the HLA-A*0201-positive melanoma line Me 290 equally well in the absence or presence of exogenously added peptide Melan-A_{26-35}. In contrast, the HLA-A*0201-negative melanoma line Me 260 was not recognized by the two TILN populations, either in the absence or in the presence of exogenously added peptide. T2 target cells, which express HLA-A*0201 but not Melan-A, were lysed only in the presence of exogenously added peptide. These results show that the two TILN populations used in this study recognized Melan-A-derived epitopes expressed in HLA-A*0201-positive melanoma cells.

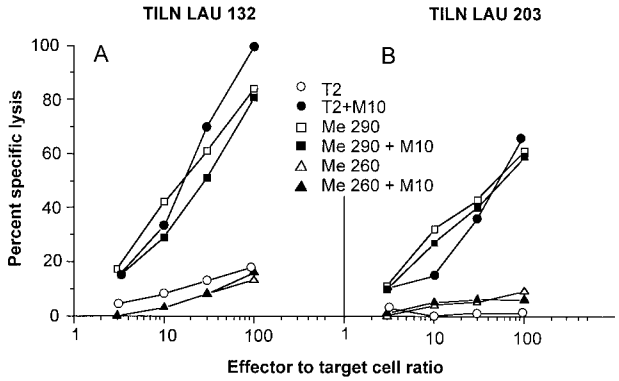

FIGURE 1. Ag specificity and HLA-A2 restriction of TILN LAU 132 and LAU203. Lysis of Me 290 (□, ■), Me260 (△, ▲), and T2 (○, ●) by TILN from HLA-A*0201 melanoma patients was measured in a 4-h ^{51}Cr release assay in the absence (open symbols) or presence (closed symbols) of exogenously added peptide Melan-A_{26-35} (M10, 1 μM).

Peptide Melan-A_{26-35} (EAAGIGILTV) was recognized at least one order of magnitude more efficiently than peptide Melan-A_{27-35} (AAGIGILTV) by both TILN (Table I). In contrast, peptide Melan-A_{27-36} (AAGIGITLVI) was poorly recognized while the partially overlapping peptide Melan-A_{32-40} (ILTVILGVL) was not recognized at all. Similar results have been reported with TILN populations from three other HLA-A*0201 melanoma patients (16). It thus appears that peptide Melan-A_{26-35} is recognized most efficiently by TILN populations from HLA-A*0201 melanoma patients compared with other overlapping peptides. Based on these results, we selected peptides Melan-A_{26-35} and Melan-A_{27-35} for further studies.

*Binding of Melan-A peptide analogues to HLA-A*0201*

In an attempt to identify Melan-A peptides with enhanced binding activity to HLA-A*0201, we synthesized the peptide analogues

Table II. *Binding of modified Melan-A peptides to HLA-A*0201 allele*[a]

Peptide	Sequence	Competitor Activity[b] [μM] 50%	Relative Competitor Activity[c]
Melan-$A_{27–35}$	AAGIGILTV	60	1
	ALGIGILTV	1.5	40
	AMGIGILTV	2	30
	LAGIGILTV	65	1
	MAGIGILTV	55	1
Melan-$A_{26–35}$	EAAGIGILTV	15	4
	ELAGIGILTV	6.5	9
	EMAGIGILTV	20	3
	EALGIGILTV	100	0.6
	EAMGIGILT	100	0.6
	YAAGIGILTV	4	15
	FAAGIGILTV	2	30
Influenza A matrix$_{58–66}$	GILGFVFTL	1	60
MAGE-$3_{168–176}$	EVDPIGHLY	>100	<0.6

[a] Data reported are from one of three independent experiments giving comparable results.

[b] Competitor activity was measured on the basis of the inhibition of recognition of the MAGE-$3_{271–279}$ antigenic peptide in the context of HLA-A*0201 by the peptide-specific CTL line 198NS.

[c] The relative competitor activity was calculated as described in *Materials and Methods* using the Melan-$A_{27–35}$ as the reference peptide with an arbitrary competitor activity of 1.

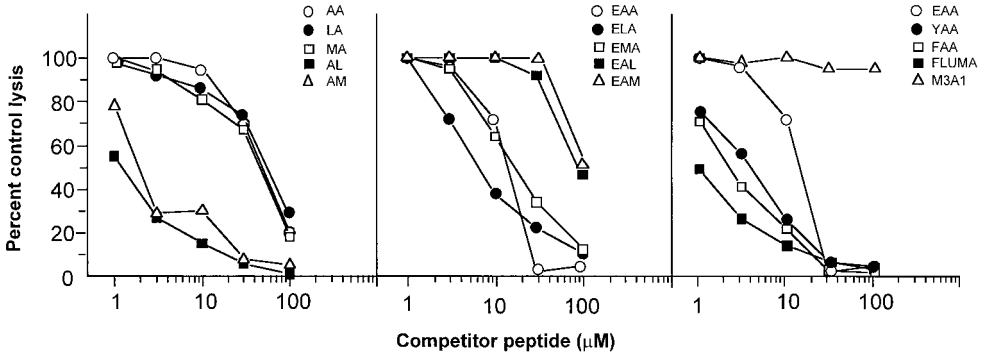

FIGURE 2. Binding of Melan-A Ag peptide analogues to HLA-A*0201. Binding was measured in a functional competition assay by using the MAGE-$3_{271-279}$-specific, HLA-A*0201-restricted CTL line 198NS. Test peptides were incubated at various concentrations (1 μM to 100 μM) with ^{51}Cr-labeled T2 cells (1000/well) for 15 min at room temperature. A suboptimal dose of the antigenic peptide (MAGE-3 $_{271-279}$, 1 nM) was then added together with the effector cells at 5:1 E:T. The assay was then carried on as a conventional chromiun release assay. For details of peptide origin and amino acid sequence, see Table II.

listed in Table II. Peptide analogues Melan-A_{27-35} A28L and A28 M contain single amino acid substitutions corresponding to the HLA-A*0201 dominant anchor residues at position 2 of the nonapeptide. The same substitutions were introduced at position 2 of the decapeptide (peptide analogues Melan-A_{26-35} A27L and Melan-A_{26-35} A27M). For comparison, we also synthesized the analogues Melan-A_{27-35} A27L and A27 M as well as Melan-A_{26-35} A28L and A28M. Two additional peptide analogues were synthesized (peptides Melan-A_{26-35} E26F and E26Y) in which E at position 1 of the decapeptide (described as being associated with less efficient peptide-HLA-A2 binding) was replaced by an aromatic amino acid (Y or F) known to favor decapeptide binding to HLA-A2 (19).

As illustrated in Figure 2, binding of the various peptides to HLA-A*0201 was evaluated in a functional competition assay based on inhibition of specific recognition of peptide MAGE-$3_{271-279}$ by the HLA-A*0201-restricted CTL line 198NS (20). The nonsubstituted nonapeptide was a relatively weak competitor (50% inhibition at 60 μM) compared with the internal positive control peptide (Influenza A matrix $_{58-66}$ peptide, 50% inhibition at 1 μM) (Fig. 2, Table II). As expected, the HLA-A1-restricted MAGE-$3_{168-176}$ peptide did not show competitor activity even at the highest concentration tested. Substitution of A at position 2 of the nonapeptide with either L or M (Melan-A_{27-35} A28L, A28M) resulted in a 30- to 40-fold increase in competitor activity. In contrast, substitution of A at position 1 of the nonapeptide with L or M (Melan-A_{27-35} A27L, A27M) had no apparent effect. Peptide Melan-A_{26-35} was a fourfold better competitor than the peptide Melan-A_{27-35}. Substitution of A at position 2 of the decapeptide with L (Melan-A_{26-35} A27L) resulted in an additional twofold increase in competitor activity, while substitution with M (Melan-A_{26-35} A27M) had no further effect. Substitution of L or M for A at position 3 of the decapeptide (Melan-A_{26-35} A28L, A28M) reduced its competitor activity. In contrast, substitution of Y or F for E at position 1 (Melan-A E26Y, E26F) significantly enhanced the competitor activity of the decapeptide (Table II).

*Assessment of the dissociation rate of Melan-A peptide analogues from HLA-A*0201*

The stability of complexes formed between HLA-A*0201 and the different Melan-A peptides was assayed on T2 cells, which lack TAP function and consequently are defective in properly loading class I molecules with antigenic peptides generated in the cytosol. The association of exogenously added peptides with thermolabile, empty HLA-A2 molecules stabilizes them and results in an increase in the level of surface HLA-A2 recognizable by conformation-dependent α1/α2-specific mAb such as W6/32 or BB7.2 (25). Indeed, overnight incubation of T2 cells with saturating amounts of HLA-A*0201 binding peptides and human β2 microglobulin resulted in increased surface expression of HLA-A*0201 molecules. After peptide removal and addition of emetine to inhibit protein synthesis, T2 cells were incubated at 37°C and the amount of HLA-A*0201 molecules remaining at the cell surface was determined after various incubation times (as illustrated in Figure 3*A* for some peptides). The stability of each peptide/HLA-A*0201 complex was then normalized relative to that observed for the Influenza A matrix $_{58-66}$ peptide/HLA-A*0201 complex (Fig. 3, *B–D*). HLA-A*0201 complexes formed with peptides Melan-A_{27-35} and Melan-A_{26-35} were unstable, reaching background levels in less than 1 h of incubation at 37°C (Fig. 3, *A–C*). Similarly, complexes formed with peptides Melan-A_{27-35} A27L and A27M dissociated rapidly (Fig. 3*B*). In contrast, peptides Melan-A_{26-35} A27L, A27M (Fig. 3*C*), E26Y, and E26F (Fig. 3*D*) formed complexes that were relatively stable over a 6-h period. Complexes of intermediate stability were observed with the remaining peptide analogues tested (Fig. 3, *B* and *C*).

Recognition of Melan-A peptide analogues by Melan-A-specific TILN and CTL clones

The relative antigenic activity of the peptide analogues was quantitated in a standard CTL assay using Melan-A-specific TILN and CTL clones. A dose-response curve was generated for each peptide analogue (Fig. 4). The antigenic activity of each peptide analogue was then calculated relative to that of the Melan-A_{27-35} peptide (which was 60 and 30 nM for 50% maximal lysis by TILN LAU 203 and LAU 132, respectively, Table III). The two Melan-A-specific TILN populations recognized the reference nonapeptide with a similar efficiency (Table III). Substitution of A at position 1 of the nonapeptide with L or M resulted in enhanced peptide recognition of about 10-fold. Surprisingly, substitution of A at position 2 of the nonapeptide with either L or M, which strongly increased peptide binding to HLA-A*0201, resulted in a >50-fold reduction of their antigenic activity.

In agreement with the results mentioned previously, peptide Melan-A_{26-35} was better recognized than peptide Melan-A_{27-35} by both TILN populations. Remarkably, substitution of A at position 2 of the decapeptide with L or M further improved the efficiency of peptide recognition. However, L or M substitution for A at

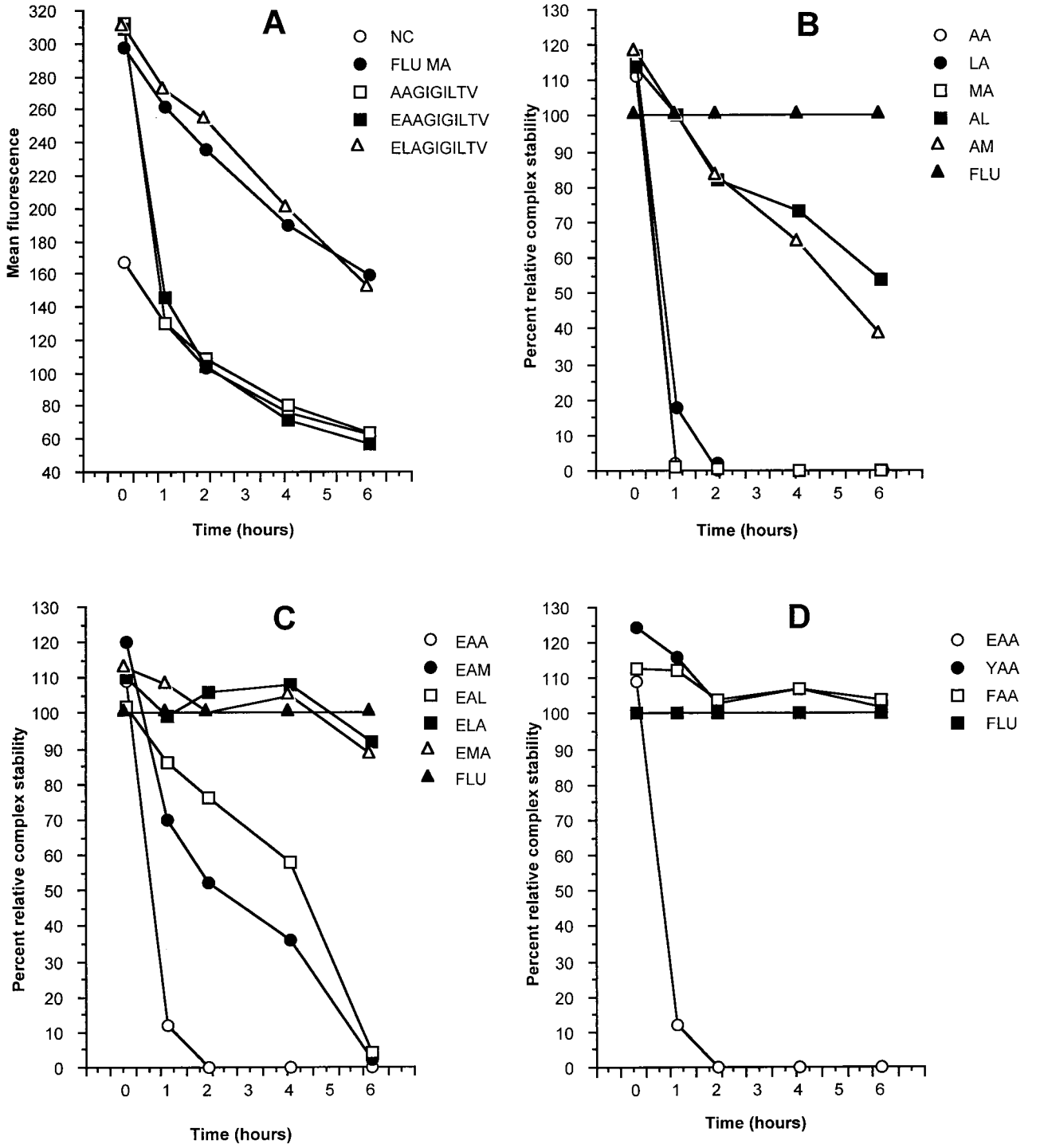

FIGURE 3. Assessment of the dissociation rate of Melan-A peptide analogues from HLA-A*0201. After overnight incubation with saturating amounts of peptide, T2 cells were treated with emetine (to inhibit protein synthesis) and incubated at 37°C. At the indicated time points, cells were stained for HLA-A2 Ag expression. The mean fluorescence intensity was determined for each histogram (*A*), where NC refers to the HLA-A2 Ag expressed by T2 cells treated in the same conditions but in the absence of exogenous peptide. The stability of each peptide/HLA-A*0201 complex was normalized relative to that observed for the Influenza A matrix $_{58-66}$ peptide/HLA-A*0201 complex (*B-D*).

position 3 of the decapeptide resulted in strongly reduced efficiency of recognition, as expected from the relatively low HLA-A*0201 binding activity displayed by these two peptide analogues. Finally, substitution of E at position 1 with Y and, particularly, with F, in accordance with the effect on peptide binding, also resulted in highly improved peptide recognition (Table III).

To further document these findings, we tested the different peptides and their analogues for recognition by five independent, HLA-A*0201-restricted Melan-A-specific CTL clones known to lyse appropriate melanoma target cells. As shown in Table IV, the CTL clones recognized the peptide Melan-A_{27-35} with variable efficiency, as reflected by the concentration of peptide required to achieve 50% of maximal target cell lysis (between 15 and 4000 nM). Substitution of A at position 1 of the nonapeptide with L enhanced peptide recognition by four of the five clones, whereas a similar substitution at position 2 resulted in a general loss of antigenic activity. Three of the

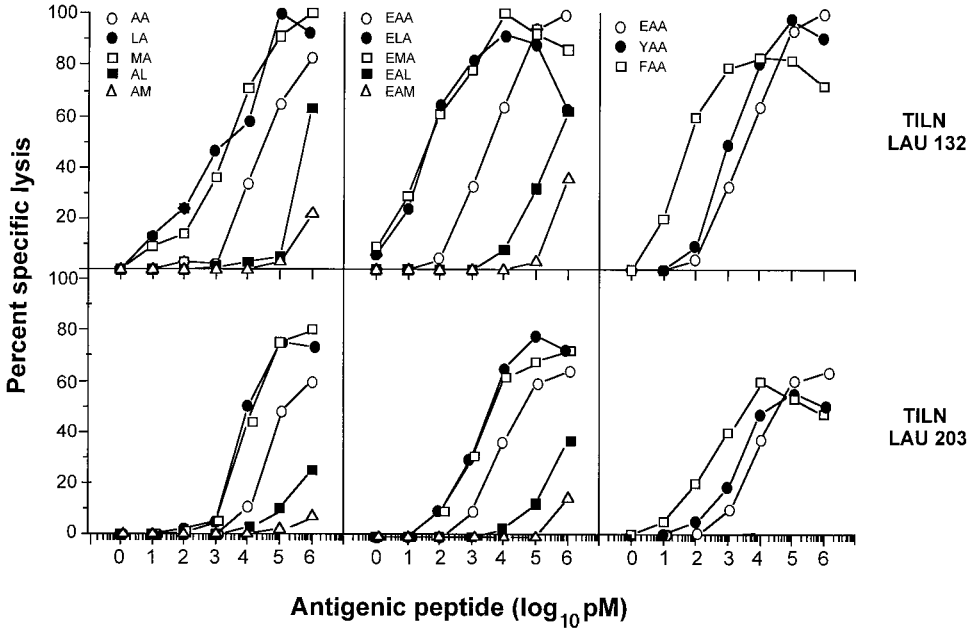

FIGURE 4. Recognition of Melan-A peptide analogues by Melan-A-specific TILN. The relative antigenic activity of Melan-A peptides was measured by assessing lysis of T2 cells incubated with increasing concentrations of the Melan-A peptide analogues by TILN LAU 132 and LAU 203 in a 4-h ^{51}Cr release cytotoxicity assay at a lymphocyte to target cell ratio of 30:1.

five CTL clones recognized peptide Melan-A_{26-35} more efficiently than peptide Melan-A_{27-35}. Nevertheless, all five CTL clones recognized very efficiently the decapeptide analogue containing L at position 2. In contrast, substitution of A at this position with M decreased efficiency of peptide recognition to variable degrees. Moreover, substitution of L for A at position 3 of the decapeptide resulted in greatly reduced efficiency of recognition by four of the five clones. Surprisingly, substitution of A at position 3 with M, in contrast with the findings obtained with TILN, improved peptide recognition by four of the five clones. Finally, substitutions at position 1 of Melan-A_{26-35} resulted in reduced recognition by several of the clones tested.

Taken collectively, the results of this analysis indicated that two Melan-A peptide analogues, namely Melan-A_{26-35} A27L and, to a lesser extent, Melan-A_{27-35} A27L, were better recognized than the corresponding natural peptides by the various CTL lines and clones tested, whereas much more variability was observed with the other peptide analogues tested.

Table III. *Recognition of modified Melan-A derived peptides by Ag-specific TILN from HLA-A2$^+$ melanoma patients.*

Peptide Sequence	TIL LAU 132 Peptide [nM] 50%[a]	TIL LAU 132 Relative Activity[b]	TIL LAU 203 Peptide [nM] 50%	TIL LAU 203 Relative Activity
AAGIGILTV	30	1	60	1
ALGIGILTV	600	0.05	>1000	<0.06
AMGIGILTV	>1000	<0.03	>1000	<0.06
LAGIGILTV	1.5	20	6	10
MAGIGILTV	2.5	12	8	7.5
EAAGIGILTV	3	10	12	5
ELAGIGILTV	0.05	600	2	30
EMAGIGILTV	0.05	600	2	30
EALGIGILTV	400	0.07	1000	0.06
EAMGIGILTV	>1000	<0.03	>1000	<0.06
YAAGIGILTV	1	30	5	20
FAAGIGILTV	0.05	600	1	60

[a] Relative antigenic activity of Melan-A peptides was measured as described in the legend to Figure 4. The peptide concentration required to obtain 50% of maximal activity ([nM] 50%) was then determined for each peptide.

[b] The relative antigenic activity was calculated as the [nM] 50% for the reference peptide Melan-A_{27-35} divided by that of the corresponding analog peptide.

Induction of Melan-A-specific, tumor-reactive CTL by in vitro stimulation with Melan-A peptide analogues

Based on the above-mentioned data, the peptide analogue Melan-A_{26-35} A27L was selected for in vitro CTL induction studies. The ability of this peptide to induce melanoma-reactive CTL specific for Melan-A was evaluated by stimulating CD8$^+$-enriched T lymphocytes derived from PBMC of melanoma patients with autologous irradiated PBMC pulsed with peptide. Stimulation with Melan-A_{27-35} and Melan-A_{26-35} peptides was also performed in each experiment to allow an appropriate comparison between the CTL responses generated with the natural and the modified peptides. The results obtained on day 7 after the second restimulation of CD8$^+$-enriched T cells from melanoma patient LAU 203 are shown in Figure 5. Peptide-specific CTL activity was barely detectable in the cultures stimulated with the natural peptides, whereas the culture stimulated with the Melan-A_{26-35} A27L peptide analogue exhibited strong CTL activity. This activity was directed not only against the peptide analogue used for stimulation but was also cross-reactive with the nonsubstituted Melan-A decapeptide. More importantly, the CTLs induced by stimulation with the Melan-A_{26-35} A27L peptide analogue were able to lyse the Melan-A-expressing autologous melanoma cell line Me 290 (Fig. 5). After three additional rounds of stimulation, peptide-specific activity as well as tumor reactivity was also clearly detectable in those cultures stimulated with the natural peptides (data not shown). Similar results were obtained after stimulation of PBMC CD8$^+$ T cells from patient LAU 132 (data not shown).

These results were extended in an experiment including CD8$^+$-enriched T cells from eight HLA-A*0201 melanoma patients. The T cells were stimulated in parallel with peptides Melan-A_{27-35}, Melan-A_{26-35}, and Melan-A_{26-35} A27L. CTL activity of individual cultures was assayed on day 7 after the third in vitro restimulation using as a source of target cells T2 cells with or without exogenously added

Table IV. *Recognition of peptide analogs by Melan-A-specific CTL clones*[a]

	Recognition by CTL Clone									
	M77.86		7.10		Mel 1.33		M77.80		1.13	
Peptide Sequence	Peptide [nM] 50%	Relative Activity	Peptide [nM] 50%	Relative Activity	Peptide [nM] 50%	Relative Activity	Peptide [nM] 50%	Relative Activity	Peptide [nM] 50%	Relative Activity
AAGIGILTV	15	1	50	1	300	1	300	1	4000	1
ALGIGILTV	90	0.16	>1000	<0.015	>1000	<0.3	>1000	<0.3	>10000	<0.4
AMGIGILTV	>1000	<0.015	>1000	<0.015	>1000	<0.3	>1000	<0.3	>10000	<0.4
LAGIGILTV	0.08	187	1.5	33	150	2	0.03	10000	30	1300
MAGIGILTV	0.6	25	15	3	200	1.5	0.5	600	80	50
EAAGIGILTV	0.15	100	4	12	0.06	5000	600	0.5	2000	2
EALGIGILTV	300	0.05	>1000	<0.015	40	7.5	>1000	<0.3	>10000	<0.4
EAMGIGILTV	0.5	30	1	50	0.02	15000	5	60	50	80
ELAGIGILTV	0.015	1000	0.5	100	0.015	20000	0.5	600	20	200
EMAGIGILTV	550	36	>1000	<0.015	40	7.5	>1000	<0.3	>10000	<0.4
YAAGIGILTV	0.015	1000	35	1.4	>1000	<0.3	1000	0.3	>10000	<0.4
FAAGIGILTV	0.005	3000	7	7	>1000	<0.3	>1000	<0.3	200	20

[a] Relative antigenic activity of Melan-A-derived peptides was measured as described in the legends to Figure 4 and Table III.

peptide as well as Melan-A-expressing melanoma cells described above. As shown in Table V, HLA-A*0201-restricted Melan-A-specific tumor-reactive CTL were detected in PBMC cultures from five of eight patients after stimulation with the Melan-A_{26-35} A27L peptide analogue. In contrast, none of the PBMC cultures stimulated with the nonsubstituted Melan-A nona- or decapeptides exhibited specific cytolytic activity. Together, these data indicate that the Melan-A_{26-35} A27L peptide analogue displays greater immunogenicity than the two parental peptides with regard to induction in vitro of Melan-A-specific and tumor-reactive CTL.

Discussion

The Melan-A (also termed MART-1) gene product encodes Ags that are recognized by HLA-A*0201-restricted CTL (9–12, 26, 27). A synthetic peptide corresponding to residues 27 to 35 from the Melan-A protein was initially identified as the determinant recognized by the majority of HLA-A*0201-restricted TIL derived from patients with metastatic melanoma (11). TIL as well as CTL lines generated by in vitro stimulation of PBMC from HLA-$A*0201^+$ melanoma patients with peptide Melan-A_{27-35} were reported to cross-react with two overlapping peptides, namely Melan-A_{26-35} and Melan-A_{27-36} (11, 12). Another overlapping antigenic peptide, which corresponds to Melan-A residues 32 to 40, was identified by tandem mass spectrometry as the active peptide species in peptides eluted from HLA-A2 molecules purified from a melanoma cell line. This peptide was reported to be frequently recognized by CTL lines and TIL from melanoma patients (27). Yet another Melan-A-derived Ag was characterized as a peptide species associated with HLA-A2 molecules extracted from an autologous melanoma cell line. The identity of this peptide has not yet been established, although it does not appear to correspond to any of the Melan-A-encoded peptides containing the HLA-A2 consensus motif (26).

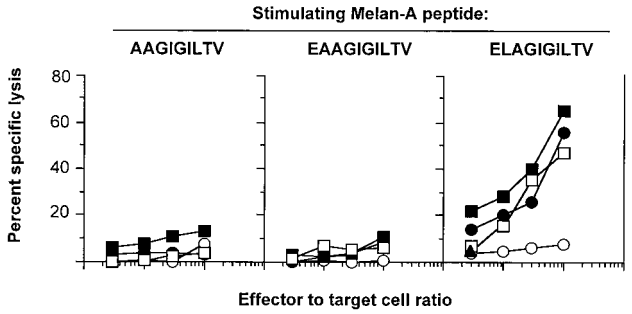

FIGURE 5. Induction of Mela-A-specific, tumor-reactive CTL by in vitro stimulation of PBMC with Melan-A peptide analogues. $CD8^+$-enriched T cells from melanoma patient LAU 203 were restimulated twice with the indicated peptide-pulsed and irradiated autologous PBMC. CTL activity was measured 7 days after the second in vitro restimulation. Lysis on autologous tumor cells, Me 290 (□), or T2 was measured in a 4-h ^{51}Cr release assay in the absence (○) or presence of exogenously added parental peptide Melan-A_{26-35} (■) or peptide analogue Melan-A_{26-35} A27L (●).

In the present study, we used short-term-cultured Melan-A-specific HLA*0201-restricted TILN from two melanoma patients. The TILN had been incubated in the presence of cytokines without addition of exogenous antigenic peptides to further define Melan-A peptide sequence(s) recognized by specific, tumor-reactive CTL. Quantitative assessment of peptide recognition indicated that the most efficiently recognized natural peptide was peptide Melan-A_{26-35}, followed by peptide Melan-A_{27-35}, whereas peptides Melan-A_{27-36} and Melan-A_{32-40} were not recognized. These results confirm and extend our previous study in which four TILN from HLA-A*0201 melanoma patients were tested for recognition of various Melan-A peptides (16). Similar analyses performed with monoclonal populations of Melan-A-specific, tumor-reactive CTL revealed that the majority (10 of 13 CTL clones tested) recognized more efficiently peptide Melan-A_{26-35} than peptide Melan-A_{27-35} (Ref. 16 and this study).

Interestingly, a few Melan-A-specific CTL clones have been shown to recognize peptides Melan-A_{26-35} and Melan-A_{27-35} equally well (Refs. 11 and 16, and this study), or peptide Melan-A_{27-35} better than peptide Melan-A_{26-35} (11). Altogether, these results suggest a diversity in the fine specificity of recognition by CTL directed against the Melan-A immunodominant epitope. Although surprising, this finding is not without precedent. Indeed, an extensive analysis of Ag recognition by a large panel of H-$2K^d$-restricted CTL clones directed against a parasite nonapeptide, PbCS $_{252-260}$, showed not only cross-reactivity with the octapeptide PbCS $_{253-260}$, but also clonal diversity in the efficiency of recognition of the two peptides (28). Additional studies using single Ala-substituted peptide analogues of the Melan-A_{26-35} decapeptide are in progress to determine the extent of clonal diversity in Melan-A Ag recognition by CTL.

In agreement with a recent report (15), the current study indicates that peptides Melan-A_{26-35} and $_{27-35}$ have relatively low

Table V. *Induction of Melan-A-specific and tumor-reactive CTL by in vitro stimulation with Melan-A peptide analog*

		Percentage Specific Lysis from Cultures Stimulated with Peptide[a]											
		Melan-$A_{27–35}$				Melan-$A_{26–35}$				Melan-$A_{25–35}$A27L			
Patient	E/T[b]	T2	T2 +M10	Me260	Me290	T2	T2 +M10	Me 260	Me 290	T2	T2 +M10	Me 260	Me 290
LAU 203	100	38[c]	29	7	17	37	41	15	6	32	**83**	18	**81**
	30	29	11	10	0	17	23	7	1	26	**96**	4	**75**
	10	3	6	2	0	9	17	0	0	17	**73**	1	**62**
LAU 132	100	9	12	1	0	19	19	6	3	34	**50**	6	**31**
	30	3	7	2	0	5	10	1	2	16	**32**	3	**18**
	10	0	0	5	1	0	0	1	0	5	**23**	2	6
LAU 145	100	15	24	4	1	39	40	5	9	29	**50**	6	**30**
	30	9	12	3	1	15	25	2	1	10	**29**	5	**19**
	10	3	6	0	0	4	6	0	0	10	16	3	7
LAU 86	100	36	29	22	5	44	38	14	10	35	45	24	15
	30	17	15	9	5	20	26	6	0	24	23	10	4
	10	16	5	2	0	10	10	1	0	14	9	1	0
LAU 50	100	21	26	7	5	18	20	5	5	19	26	6	20
	30	7	16	4	5	8	13	1	0	10	18	3	8
	10	7	7	0	4	0	4	1	0	3	12	0	0
LAU 148	100	51	39	13	4	46	45	9	0	34	39	9	4
	30	19	8	5	4	20	26	1	2	19	27	9	3
	10	3	6	1	0	14	14	6	0	13	13		0
LAU 161	100	24	22	6	1	33	31	3	1	25	**38**	4	**23**
	30	3	8	6	1	16	12	3	0	18	23	2	**13**
	10	2	0	5	0	9	7	2	0	5	11	3	4
LAU 119	100	31	27	5	12	33	31	1	4	18	**46**	5	**45**
	30	7	13	1	1	17	23	3	4	13	**39**	4	**25**
	10	4	0	0	0	9	12	1	0	7	**17**	2	**16**
Clone 6[d]	10	7	**78**	2	**63**								
	3	3	**74**	0	**61**								
	1	0	**65**	0	**51**								

[a] Lytic activity was assayed 7 days after the third restimulation with the indicated Melan-A peptide.
[b] Lymphocyte to target cell ratio titration was performed for every culture.
[c] Numbers represent the percentage specific lysis obtained for each target. Me 290 is a Melan-A and HLA-A*0201-positive melanoma cell line obtained from patient LAU 203. Me 260 is a HLA-A*0201 negative melanoma cell line obtained from patient LAU 149. Each number represents the geometric mean of duplicate cultures. Numbers in bold face type indicate significant specific CTL activity, when the differences in specific lysis obtained on T2 cells in presence or in absence of Melan-$A_{26–35}$ (1 μM) (abbreviated as M10) and on Me 290 or Me 260 are equal or higher than 10%.
[d] Clone 6 is a Melan-A-specific CTL clone derived from the TILN LAU 132, used as positive control.

binding affinities for HLA-A2. Moreover, complexes between these peptides and cell-associated HLA-A2 molecules were found to be unstable at 37°C, with a half-life less than 1 h (Fig. 3). In the same study mentioned above (15), it was reported that complexes between peptide Melan-A_{26-35} and HLA-A2 molecules were less stable than Melan-A_{27-35} peptide/HLA-A2 complexes, in contrast to our own data.

Although the reason for this discrepancy may be due to technical differences in the procedures used to measure the stability of complexes, it is clear that both peptides lack one of the major anchor residues found in the majority of natural peptides associated to HLA-A*0201.

In an attempt to overcome the relatively poor binding of natural Melan-A peptides to HLA-A*0201, we synthesized a series of single amino acid substituted peptide analogues and tested them for 1) binding to HLA-A*0201, 2) ability to form stable peptide/HLA-A*0201 complexes, and 3) recognition by a panel of Melan-A-specific, HLA-A*0201-restricted TILN and CTL clones.

Among the peptide analogues tested, the decapeptide Melan-A_{26-35} that was substituted with L for A at position 2 (Melan-A_{26-35} A27L) was found to form stable complexes with cell-associated HLA*0201 molecules and, more importantly, to be recognized more efficiently than the natural decapeptide by TILN (6- to 60-fold) as well as by the Melan-A-specific CTL clones tested (4- to 1000-fold) (Tables III and IV).

Surprisingly, the same amino acid substitution at position 2 of the nonapeptide Melan-A_{27-35} resulted in a strong reduction in the efficiency of recognition by Melan-A-specific T cells in spite of the enhanced binding affinity to HLA-A2. Although it has been observed in another class I-restricted T cell recognition system that changes in peptide residues buried in the peptide binding cleft can negatively influence recognition by specific CTL (29), it is striking that introduction of L at position 2 in the nonapeptide is deleterious for Ag recognition whereas it has the opposite effect at position 2 in the decapeptide. It is conceivable that when the nonapeptide is engineered to bind with high affinity to the HLA-A2 molecule, it displays a qualitatively different epitope from that displayed by the corresponding high-affinity bound decapeptide analogue. In support of this interpretation is the crystallographic observation that the orientation of the side chains from the central peptide residues relative to the HLA-A2 binding site is similar for four nonapeptide co-crystals, but is nearly the opposite for one decapeptide co-crystal (30). The change in peptide amino acid side chain orientations would be induced by a zig-zag movement in the decapeptide main chain, allowing the longer peptide to fit within the limited size of the HLA-A2 peptide binding cleft.

Based on these results, the immunogenicity of Melan-A_{26-35} A27L analogue was assessed by its ability to induce CTL from PBMC of HLA-$A2^+$ melanoma patients. Clearly, the Melan-A_{26-35} A27L peptide analogue was more efficient than the two natural peptides in inducing melanoma-reactive CTL. The enhanced CTL stimulatory capacity of the Melan-A_{26-35} A27L peptide analogue may have several applications. First, and most important, this peptide analogue could be used as a vaccine able to elicit potent antitumor CTL responses. It has been shown for a viral peptide that analogues with the ability to form long-lived complexes with HLA-A2

molecules were more immunogenic in vitro than their natural counterparts (23). Moreover, a high correlation has been found between overall peptide affinity for MHC class I molecules and in vivo peptide immunogenicity in HLA-A2K^b transgenic mice (31). An even better correlation with the peptide's ability to form stable HLA-A2 complexes has been reported (22). Improved immunogenicity in HLA-A2K^b transgenic mice has also been reported for analogues of a self-peptide, namely the gp100$_{154-162}$, displaying both higher affinity and more prolonged complex stability than the natural peptide (32). Of note, one of these analogues incorporated an amino acid substitution at a nonanchor peptide residue (32). Second, the Melan-A peptide analogues may be advantageous in the monitoring of CTL responses in melanoma patients against the natural tumor peptide Ags. In this regard, use of analogues of the peptides gp100 $_{209-217}$ and gp100 $_{280-288}$ has been shown to reduce the number of stimulations required to reveal a CTL response in patients immunized with the corresponding natural peptides (33). Finally, analogues with greater immunogenicity than their natural counterparts may be useful in shortening the stimulation time required to obtain the large numbers of peptide-specific effector CTL populations required for adoptive transfer therapy. As a first step toward possible applications of our findings to cancer patients, we are currently assessing the immunogenicity of the Melan-A peptide analogues described here in HLA-A2/K^b transgenic mice (34).

Acknowledgments

We gratefully acknowledge Dr. K. Servis for peptide synthesis and M. van Overloop for assistance in manuscript preparation.

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