Int. J. Cancer: **78,** 518–524 (1998)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

IDENTIFICATION OF GP100-DERIVED, MELANOMA-SPECIFIC CYTOTOXIC T-LYMPHOCYTE EPITOPES RESTRICTED BY HLA-A3 SUPERTYPE MOLECULES BY PRIMARY *IN VITRO* IMMUNIZATION WITH PEPTIDE-PULSED DENDRITIC CELLS

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The human melanocyte lineage-specific antigen gp100 contains several epitopes recognized by cytotoxic T lymphocytes (CTL). However, most of the epitopes reported to date are HLA-A2.1-restricted. Despite the high frequency of HLA-A2.1 in melanoma patients, effective population coverage requires the identification of epitopes restricted by other frequent HLA alleles. Herein, HLA-A3 binding, gp100-derived synthetic peptides were tested for their capacity to elicit anti-melanoma CTL *in vitro* using $CD8^+$ T cells from healthy donors as responders and peptide-pulsed autologous dendritic cells as antigen-presenting cells. Of 7 peptides tested, 2 (gp100[9_{87}] and gp100[10_{86}]) induced CTLs that killed melanoma cell lines expressing HLA-A3 and gp100. Additional MHC-binding studies to various HLA molecules belonging to the HLA-A3 superfamily (HLA-A*1101, -A*3101, -A*3301 and -A*6801) were performed to determine whether these CTL epitopes could further increase potential population coverage. Further experiments indicated that the peptide gp100[9_{87}], which bound to HLA-A11 with high affinity, was capable of inducing specific CTLs that killed melanoma cells expressing gp100 and HLA-A11 molecules. Our results indicate that the gp100[9_{87}] peptide corresponds to a CTL epitope which may be restricted by either the HLA-A3 or HLA-A11 allele, emphasizing its utility for the design and development of epitope-based therapies for melanoma. *Int. J. Cancer,* 78:518–524, 1998.
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Cytotoxic T lymphocytes (CTLs) play a key role in the immune response to viral infections and tumors by directly lysing antigen-expressing target cells or secreting a diverse array of lymphokines which amplify immune reactions (Berke, 1994). CTLs can recognize complexes formed between peptide fragments, length of 8 to 12 amino acids, derived from viral proteins or tumor-associated antigens (TAAs) and major histocompatibility complex (MHC) molecules expressed on the cell surface (Rammensee *et al.,* 1993; York and Rock, 1996; Zinkernagel and Doherty, 1997). Thus, to develop epitope-based immunotherapy for viral infections and tumors, it is essential to identify CTL epitopes that are derived from viral proteins and TAA.

Since the discovery of the *MAGE* family of genes in 1991, which encode for several CTL epitopes (Van der Bruggen *et al.,* 1991), numerous additional TAAs have been identified (Van den Eynde and Boon, 1997). These TAAs can be classified into 3 groups according to pattern of expression: *(i)* tumor-specific shared antigens, *(ii)* antigens specific for individual tumors (point mutations) and *(iii)* differentiation lineage-specific antigens. In the case of malignant melanoma, several melanocyte lineage-specific antigens, such as Melan-A/MART-1, tyrosinase, gp100/pmel 17, gp75/TRP-1, and TRP-2 (Bakker *et al.,* 1994; Brichard *et al.,* 1993; Coulie *et al.,* 1994; Cox *et al.,* 1994; Kawakami *et al.,* 1994*a,b;* Wang *et al.,* 1995, 1996) have been described as TAAs containing epitope peptides for class I MHC-restricted CTLs. Among these, gp100 could be most relevant for the development of antigen-specific immunotherapy because of the reported correlation between the recognition of gp100 by HLA-A2.1-restricted, tumor-infiltrating lymphocytes (TILs) and the clinical efficacy of adoptive immunotherapy of the TILs administered to melanoma patients (Kawakami *et al.,* 1995). In addition, the importance of gp100 is further substantiated by Rosenberg *et al*. (1998), who observed that 42% of patients receiving a peptide vaccine representing an immunodominant, HLA-A2.1-restricted CTL epitope analog from gp100 (which was modified to increase its binding to HLA-A2) in combination with IL-2 had objective tumor responses. These results emphasize the need for epitopes with good HLA-binding affinity in order to develop effective immunotherapy.

Although several CTL epitopes have been identified from gp100 (Cox *et al.,* 1994; Kawakami *et al.,* 1994*b,* 1995; Tsai *et al.,* 1997), most of these epitopes are restricted to HLA-A2.1 and only one epitope has been described for the HLA-A3 allele (Skipper *et al.,* 1996), which is expressed in about 21% of Caucasians, the ethnic group most susceptible to melanoma. However, the studies in HLA-A3 are preliminary and it remains unknown whether this particular epitope is immunodominant and the most appropriate for clinical studies. Herein, we examined whether additional HLA-A3-restricted epitopes from gp100 exist to allow selection of the most appropriate epitope candidate(s) for the development of epitope-based immunotherapy in this population group. Furthermore, the use of more than one CTL epitope per vaccine formulation could increase the efficacy of the therapy (Jaeger *et al.,* 1996*a,b*).

Two gp100-derived epitopes were capable of eliciting tumor-reactive HLA-A3-restricted CTLs using an *in vitro* lymphocyte immunization method developed in our laboratory (Tsai *et al.,* 1998). These 2 peptides and the previously identified HLA-A3-restricted epitope also bound HLA-A11 molecules (expressed in approx. 10% of Caucasians) with high affinity. Indeed, at least one of these newly identified epitopes can also readily induce melanoma-reactive CTLs restricted to this allele. These results demonstrate the feasibility of developing epitope-based CTL immunotherapy for melanoma patients expressing either HLA-A3 or -A11 (approx. 30% of Caucasians).

MATERIAL AND METHODS

Synthetic peptides

Peptides were synthesized according to standard solid phase synthesis methods using an Applied Biosystems (Foster City, CA) synthesizer and purified by HPLC as described (Sidney *et al.,* 1996). The purity (>95%) and identity of peptides were determined by mass spectrometric analysis.

MHC-binding assay

The binding of peptides to HLA-A*0301 molecules was measured based on the inhibition of binding of a radiolabeled standard peptide to purified MHC molecules as described previously (Sidney *et al.,* 1996). Briefly, various doses of the test peptides were incubated with standard peptide (KVFPYALINK) and deter-

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Received 4 May 1998; Revised 4 June 1998

gent-solubilized HLA-A*0301 molecules in the presence of a mixture of protease inhibitors and β_2-microglobulin (Scripps Laboratories, San Diego, CA). The binding of peptides to HLA-A*1101, HLA-A*3101 and HLA-A*6801 molecules was measured by the same protocol used for the HLA-A*0301 assay, with KVFPYALINK as standard peptide. In the HLA-A*3301 assay, STLPETYVVRR was used as standard peptide. The percentage of MHC-bound radioactivity was determined by gel filtration, and the concentration of the tested peptides to inhibit 50% of the binding of the labeled peptide (IC_{50}) was calculated.

Cell lines

The Epstein-Barr virus (EBV)–transformed human B-cell lines EHM (homozygous for A*0301, obtained from the ASHI Repository Collection, Lenexa, KS) and BVR (homozygous for A*1101, obtained from the Human Genetic Mutant Repository, Camden, NJ) were used as targets for peptide-mediated cytotoxicity assays. The HLA-typed melanoma cell lines 624mel (A2.1/A3), 526mel (A2.1/A3), 697mel (A2.1/A11) and A375 (A1/A2.1) were a generous gift from Drs. Y. Kawakami and S. Rosenberg (NCI, Bethesda, MD). The gp100 molecule is expressed in 624mel, 526mel and 697mel cells and not in A375 (Kawakami *et al.*, 1994*b*). All cell lines were cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FCS and antibiotics. Expression of HLA-A3 and A11 on the cell lines was checked by flow-cytometric analysis using monoclonal antibodies (MAbs) 0170HA and 0284HA (both from One Lambda, Canoga Park, CA), respectively.

In vitro *generation of dendritic cells (DCs)*

Peripheral blood mononuclear cells (PBMCs) from normal volunteers were purified by centrifugation in Ficoll-Paque (Pharmacia, Piscataway, NJ) from leukopheresis products. The Institutional Review Board on Human Subjects (Epimmune) approved this research, and informed consent for blood donation was obtained from all volunteers. Monocyte-derived and cytokine-generated DCs were prepared as described (Romani *et al.*, 1994; Sallusto and Lanzavecchia, 1994; Tsai *et al.*, 1998). Briefly, PBMCs were plated in 6-well plates (10×10^6 cells/well), and non-adherent cells were washed off after 2-hr incubation at 37°C. Monocyte-enriched adherent cells were cultured in the presence of 50 ng/ml of rGM-CSF and 1,000 U/ml of rIL-4 (both from Endogen, Cambridge, MA) in complete medium [RPMI-1640 supplemented with 5% human AB serum (Gemini, Calabasas, CA), 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine and 50 μg/ml gentamicin]. After 7 days, the cytokine-treated cells, which display typical cell-surface markers of DC ($CD3^-$, $CD14^-$, $CD86^+$, HLA class I^{high}, $HLA\text{-}DQ^+$; data not shown) were harvested and used as antigen presenting cell (APC) as described below.

Primary CTL induction using MHC-binding peptides and DCs

For CTL induction, cytokine-generated DCs were pulsed with peptide and used as APC to stimulate autologous $CD8^+$ T cells. To increase the density of relevant peptide–MHC complexes on the surface of DCs, one of the following procedures was employed: *(i)* before pulsing with the peptide, DCs were treated with a mild acidic buffer to release MHC-bound endogenous peptides (Storkus *et al.*, 1993; Wentworth *et al.*, 1995). Acid-treated DCs, which contain large amounts of ''empty'' MHC molecules, were then pulsed with peptides as described below in more detail. *(ii)* Peptide was introduced into the DC cytoplasm by osmotic lysis of pinocytic vesicles (Mehta-Damani *et al.*, 1994; Okada and Rechsteiner, 1982).

Specifically, for the first protocol, DCs (approx. 2×10^6 cells) were washed twice with 1% BSA/0.9% NaCl and resuspended in 1 ml of cold citrate-phosphate buffer (0.13 M citric acid, 0.06 M sodium phosphate monobasic, pH 3) containing 1% BSA and 3 μg/ml of β_2-microglobulin. After 2-min incubation on ice, 5 volumes of cold 0.15 M sodium phosphate monobasic (pH 7.5) containing 1% BSA, 3 μg/ml of β_2-microglobulin and 10 μg/ml of peptide were added and cells harvested by centrifugation. For the second procedure, DCs (approx. 2×10^6 cells) were resuspended in 50 μl of 1% BSA/PBS containing 0.4 mg/ml of peptide, then 50 μl of the hypertonic solution (1 M sucrose and 20% polyethylene glycol 1500) were added to the suspension. After 10-min incubation at 37°C, 10 ml of warm 1% BSA/PBS were added and DCs harvested by centrifugation after 3-min incubation at 37°C. After one of these pre-treatments, DCs were pulsed with 40 μg/ml of peptide in the presence of 3 μg/ml of β_2-microglobulin for 4 hr at 20°C in PBS with 1% BSA.

Peptide-loaded DCs were irradiated with 4,200 rads and mixed with $CD8^+$ cells, obtained by positive selection with Dynabeads M-450 CD8 and Detachabead (both from Dynal, Lake Success, NY), at a ratio of 1:20. Cultures were set up in 48-well plates; each well contained 0.25×10^5 APC, 5×10^5 $CD8^+$ cells and 10 ng/ml of rIL-7 (Genzyme, Boston, MA) in 0.5 ml of complete medium. On day 1, a final concentration of 10 ng/ml rIL-10 (Endogen) was added to the culture. On days 7 and 14, the responder cells were restimulated with autologous peptide-pulsed adherent APC. To prepare the APC, 2×10^6 irradiated autologous PBMCs in 0.5 ml of 1% BSA/D-PBS were added to each well of 48-well plates. After incubation at 37°C for 2 hr, non-adherent cells were washed off and adherent cells were incubated for 2 hr with 10 μg/ml of peptide and 3 μg/ml of β_2-microglobulin in a final volume of 0.25 ml 1% BSA/D-PBS per well. The supernatant of the responder cultures was aspirated and fresh complete medium added to the total volume of 0.5 ml per well. Excess peptide was removed from the adherent APC plate, and responder cultures were transferred to the corresponding wells containing peptide-pulsed APC. Each individual well was restimulated separately. The next day of each restimulation, a final concentration of 10 ng/ml rIL-10 was added to the culture. Cultures were fed every 2 to 3 days with fresh medium containing a final concentration of 50 IU/ml rIL-2. Cytotoxicity was first tested after 3 rounds of peptide stimulation (day 21).

After the initial cytotoxicity assays, more detailed analyses on the specificity of the CTLs were performed by expanding the cells in culture with anti-CD3 MAb (OKT-3) and IL-2 in the presence of PBMCs and EBV-transformed cell lines as feeder cells (Riddell *et al.*, 1996; Tsai *et al.*, 1998).

Cytotoxicity assays

Cytolytic activity was determined in a standard 4- to 6-hr ^{51}Cr-release assay, as described previously (Tsai *et al.*, 1997). Peptide-pulsed targets were prepared by incubating cells with 10 μg/ml of peptide overnight at 37°C. Tumor cell lines were treated with 100 U/ml of IFN-γ (Genzyme) for 48 hr before assays to increase the level of MHC class I expression. Adherent target cells were removed from culture flasks with trypsin-EDTA solution. Target cells were labeled with 300 μCi of ^{51}Cr sodium chromate (Dupont, Wilmington, DE) for 1 hr at 37°C. Labeled target cells (10^4/well) and various numbers of effector cells were plated in a final volume of 0.2 ml in 96-well plates. After 4 to 6 hr at 37°C, 100 μl of supernatant were collected from each well and percent specific lysis was determined according to the formula [(cpm of tested sample – cpm of spontaneous ^{51}Cr release) / (cpm of maximal ^{51}Cr release – cpm of spontaneous ^{51}Cr release)] × 100.

Antigen specificity and tumor reactivity were confirmed by cold target inhibition experiments, by determining the capacity of peptide-pulsed (10 μg/ml for 16 hr at 37°C) unlabeled (cold) EBV-transformed cell lines (EHM for HLA-A3 and BVR for HLA-A11) to inhibit the lysis of ^{51}Cr-labeled melanoma cells.

RESULTS

Selection of HLA-A3-binding peptides from gp100

From the melanoma antigen gp100, only one CTL epitope which is restricted for HLA-A3 has been reported (Skipper *et al.*, 1996).

TABLE I – LIST OF HLA-A*0301-BINDING PEPTIDES FROM GP100

Peptide[1]	Sequence	A*0301-binding[2] IC_{50} (nM)	Number of cultures containing CTL reactive with[3]	
			Peptide	Tumor cells
gp100[9_{614}]	LIYRRRLMK	5.5	NT[4]	NT
gp100[9_{460}]	GTATLRLVK	6.9	0	0
gp100[9_{87}]	ALNFPGSQK	8.5	4	3
gp100[10_{608}]	AVVLASLIYR	30.6	0	0
gp100[10_{86}]	IALNFPGSQK	36.7	2	2[5]
gp100[9_{17}]	ALLAVGATK[6]	37.9	7	0
gp100[10_{613}]	SLIYRRRLMK	39.3	0	0
gp100[9_{551}]	QLVLHQILK	52.4	1	0

[1]Numbers in brackets represent peptide size and the position on the protein sequence (subscript).– [2]Measured as concentration of test peptide required for 50% inhibition of binding of the standard peptide (see ''Material and Methods'' for details).–[3]Lymphocytes were stimulated using the ''optimized'' protocol (acid-treated plus hypertonic solution) and considered to contain CTLs when specific cytotoxicity toward antigen-containing target (peptide-pulsed or melanoma cell line) was >10% above cytotoxicity with negative control target. Values represent the number of positive wells out of 48 wells tested.–[4]Not tested.–[5]Values represent the number of positive wells out of 32 wells tested.–[6]This peptide was described as an HLA-A3-restricted epitope (Skipper *et al.*, 1996).

To identify additional HLA-A3-restricted CTL epitopes from gp100 in order to select the most appropriate epitopes for immunotherapy, the protein sequence of gp100 was first analyzed for the presence of peptide sequences containing the HLA-A3-binding motif: a positively charged amino acid (R or K) at the C terminus of peptides consisting of 9 or 10 residues and a hydroxyl-containing (S or T) or hydrophobic (L, V, I or M) residue at position 2 (Sidney *et al.*, 1996). A total of 25 peptides containing the HLA-A3-binding motif were synthesized and tested for the capacity to bind purified HLA-A*0301 molecules *in vitro*. The 8 peptides with the highest binding affinity to HLA-A3 are shown in Table I. Interestingly, the previously described HLA-A3-restricted epitope, gp100[9_{17}] (Skipper *et al.*, 1996), ranked as the 6th highest affinity binder to purified A*0301 molecules (IC_{50} of 37.9 nM, Table I).

Optimization of CTL induction protocol for HLA-A3-binding peptides and identification of CTL epitopes from gp100

Seven of the 8 high-affinity HLA-A3-binding peptides listed in Table I were selected to determine whether they were capable of eliciting CTL responses *in vitro.* Peptide gp100[9_{614}], the highest affinity binder to HLA-A3, could not be tested because we were unable to produce sufficient quantities (approx. 5 mg) with a high degree of purity (>95%) of this peptide, possibly because of its highly positively charged nature. When the 7 selected peptides were first tested using the primary CTL induction protocol developed for the screening of A2.1-binding peptides (Tsai *et al.*, 1997), we were unable to detect any specific killing, even against the peptide-pulsed target cells (data not shown). A possible explanation for these results is that peptide pulsing of DCs under conventional conditions (40 μg/ml peptide for 4 hr at 20°C) did not result in sufficient peptide–MHC complexes to stimulate the naive CTL precursors. Accordingly, we attempted to optimize the induction protocol for HLA-A3-binding peptides by increasing the density of peptide–MHC complexes on the surface of APC. Brief treatment of DCs with the acidic buffer to remove naturally presented peptides and allow more efficient peptide pulsing or the use of a hypertonic solution to allow introduction of the peptides into the cell interior was utilized (see ''Material and Methods'' for details).

The 7 peptides described above were tested again for the capacity to elicit CTLs utilizing this optimized peptide-pulsing protocol. The results showed that 4 of these peptides, gp100[9_{87}], gp100[10_{86}], gp100[9_{17}] and gp100[9_{551}], induced CTLs that killed peptide-sensitized (EBV-transformed) target cells (Table I). Thus, the modified procedure appeared to be effective at inducing CTL responses to the HLA-A3-binding peptides. Furthermore, 2 of the peptides (gp100[9_{87}] and gp100[10_{86}]) elicited CTLs which were capable of specifically recognizing and killing the HLA-A3$^+$/gp100$^+$ 624mel melanoma cell line (Table I).

The CTLs induced by peptide gp100[9_{87}] were further expanded and characterized in more detail. These CTLs displayed high killing activity even at low effector:target (E:T) ratios directed against 2 HLA-A3$^+$/gp100$^+$ melanoma cell lines, 624mel and 526mel, while they did not lyse the A375 melanoma cell line, which does not produce gp100 and expresses a different HLA allele (Fig. 1*a*). The tumor specificity of the gp100[9_{87}]-reactive CTLs was further demonstrated by showing that unlabeled (cold) targets that were pulsed with peptide gp100[9_{87}], but not those pulsed with an irrelevant control peptide, were effective at blocking the lysis of both HLA-A3$^+$/gp100$^+$ melanoma cell lines (Fig. 1*b*). When the gp100[9_{87}] peptide was used to induce CTLs in another HLA-A3$^+$ healthy donor, 2 of 32 cultures produced CTLs that recognized and killed both peptide-loaded targets and gp100$^+$ melanoma cell lines after 2 cycles of restimulation with peptide (data not shown).

Similarly, the CTLs induced by the gp100[10_{86}] peptide effectively recognized the melanoma cell lines 624mel and 526mel (Fig. 2*a*), and the antigen specificity of this CTL was confirmed by cold target inhibition assay (Fig. 2*b*). Significant CTL responses were also induced in a second lymphocyte donor, and the resulting CTLs clearly recognized both the peptide-pulsed cells and the 624mel targets (data not shown).

Peptide gp100[10_{86}] is the 10mer of gp100[9_{87}], which contains an Ile residue at the N terminus of gp100[9_{87}]. Thus, the possibility exists that both peptides represent the same CTL epitope. Indeed, the CTLs induced by peptide gp100[9_{87}] were also capable of recognizing target cells pulsed with gp100[10_{86}], and the CTLs induced by peptide gp100[10_{86}] killed the targets pulsed with peptide gp100[9_{87}] (Fig. 3). These results suggest that peptides gp100[10_{86}] and gp100[9_{87}] activate the largely overlapping sets of CTL specificities. Peptide dose titrations were done to determine whether the affinity of each CTL population for its respective ligand differed significantly. The results presented in Figure 4 indicate that both the CTLs induced by peptide gp100[9_{87}] and the CTLs induced by peptide gp100[10_{86}] had very similar relative affinities toward the peptides originally used to stimulate them. These results also show that although these peptides differ significantly with regard to MHC-binding affinity (Table I), they are quite similar with respect to antigenicity.

Interestingly, peptide gp100[9_{17}], which was reported to represent a CTL epitope in an HLA-A3$^+$ melanoma patient, was very effective at inducing peptide-reactive CTLs in a total of 3 lymphocyte donors, but none of these T-cell cultures was capable, in our hands, of lysing the gp100$^+$ tumor cells (an example obtained with one of the donors is shown in Table I).

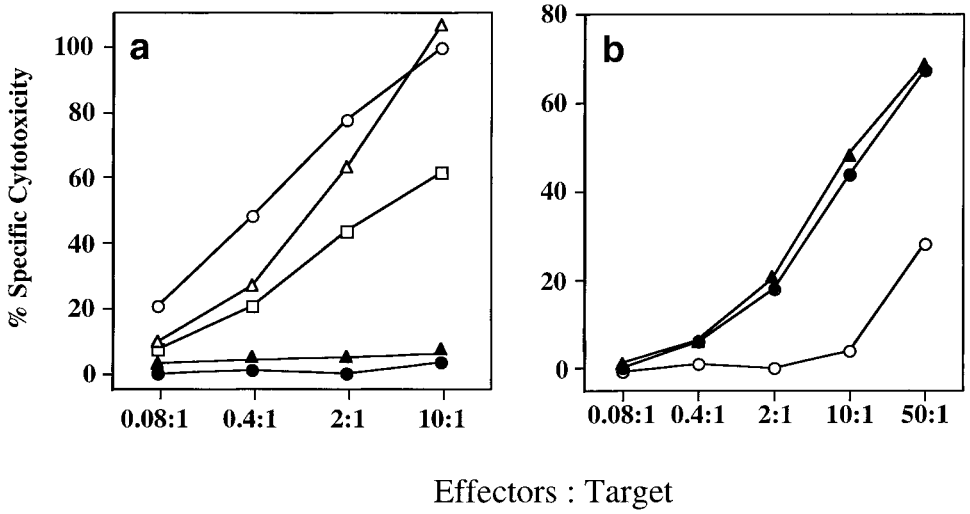

FIGURE 1 – Recognition of melanoma cell lines by HLA-A3-restricted, gp100[9_{87}]-specific CTLs. *(a)* The gp100[9_{87}]-specific CTLs, expanded after 4 cycles of restimulation with peptide, were tested for lytic activity against the following target cell lines: ○, EHM pulsed with gp100[9_{87}]; ●, EHM without peptide; △, 624mel (melanoma, A3$^+$, gp100$^+$); □, 526mel (melanoma, A3$^+$, gp100$^+$); ▲, A375 (melanoma, A3$^-$, gp100$^-$). *(b)* Cold target inhibition assays to demonstrate the antigen specificity of gp100[9_{87}]-specific CTLs. The melanoma cell line 526mel was ^{51}Cr-labeled and mixed at a ratio of 1:30 with the following cold targets: ○, EHM pulsed with gp100[9_{87}]; ▲, EHM pulsed with irrelevant A3 binding peptide [Flu, NP_{265}, ILRGSVAHK, A*0301 binding (IC_{50}) = 7.3 nM]; ●, EHM without peptide.

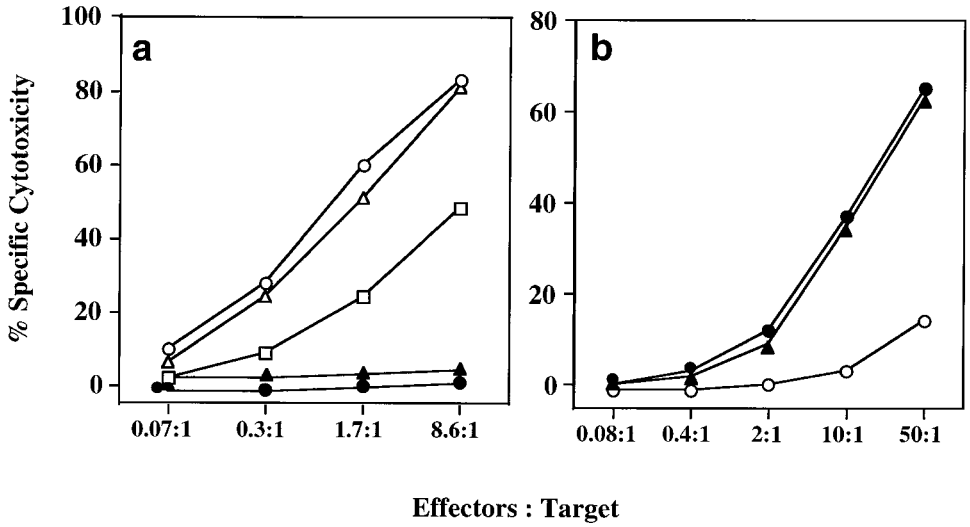

FIGURE 2 – Recognition of melanoma cell lines by HLA-A3-restricted, gp100[10_{86}]-specific CTLs. *(a)* The gp100[10_{86}]-specific CTLs after 3 cycles of restimulation with peptide were tested for lytic activity against the following target cell lines: ○, EHM pulsed with gp100[10_{86}]; ●, EHM without peptide; △, 624mel (melanoma, A3$^+$, gp100$^+$); □, 526mel (melanoma, A3$^+$, gp100$^+$); ▲, A375 (melanoma, A3$^-$, gp100$^-$). *(b)* Cold target inhibition assays to demonstrate the antigen specificity of gp100[10_{86}]-specific CTLs. The melanoma cell line 526mel was ^{51}Cr-labeled and mixed at a ratio of 1:60 with the following cold targets: ○, EHM pulsed with gp100[10_{86}]; ▲, EHM pulsed with irrelevant A3-binding peptide (Flu, NP_{265}); ●, EHM without peptide. The expanded CTLs after 2 cycles of restimulation with peptide were used as effectors.

Cross-reactive binding of known gp100 CTL epitopes to HLA-A3 supertype family

HLA-A*0301 is the prototype allele of the HLA-A3 supertype family, which is formed of several MHC class I molecules that share overlapping peptide-binding specificities (Sidney *et al.*, 1996). Next, we wished to determine whether the peptides representing the newly identified HLA-A3-restricted CTL epitopes from gp100 and/or the previously reported CTL epitope (Skipper *et al.*, 1996) corresponding to peptide gp100[9_{17}] could also bind to other members of the HLA-A3 supertype. To this end, we performed quantitative peptide/MHC-binding assays against 4 of the most frequent members of the HLA-A3 supertype (HLA-A*1101, -A*3101, -A*3301 and A*6801). The results presented in Table II show that all 3 peptides also bound well to the HLA-A*1101 allele but poorly to the other 3 molecules (A*3101, A*3301 and A*6801).

Immunogenicity of gp100[9_{87}] in HLA-A11 allele

Although the peptide gp100[9_{87}] binds well to HLA-A11, the gp100[9_{87}]-specific CTLs induced using lymphocytes derived from HLA-A3$^+$ donors did not recognize HLA-A11$^+$ target cells that were pulsed with the gp100[9_{87}] peptide nor the A11$^+$/gp100$^+$ melanoma cell line 697mel (data not shown). These results indicate

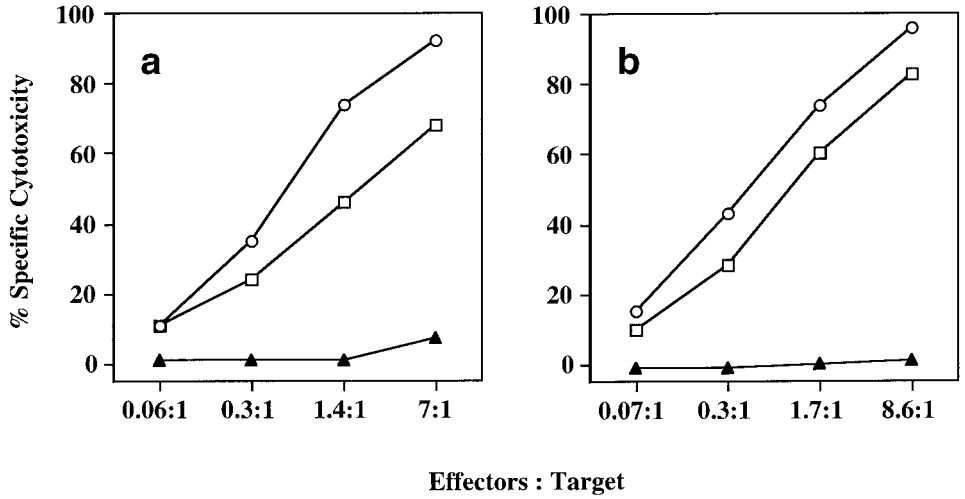

FIGURE 3 – Cross-reactivity of anti-gp100[9_{87}] CTLs and anti-gp100[10_{86}] CTLs. Anti-gp100[9_{87}] CTLs *(a)* and anti-gp100[10_{86}] CTLs *(b)* after 3 cycles of restimulation with each peptide were tested for lytic activity against the following target cell lines: ○, EHM pulsed with gp100[9_{87}]; □, EHM pulsed with gp100[10_{86}]; ▲, EHM without peptide.

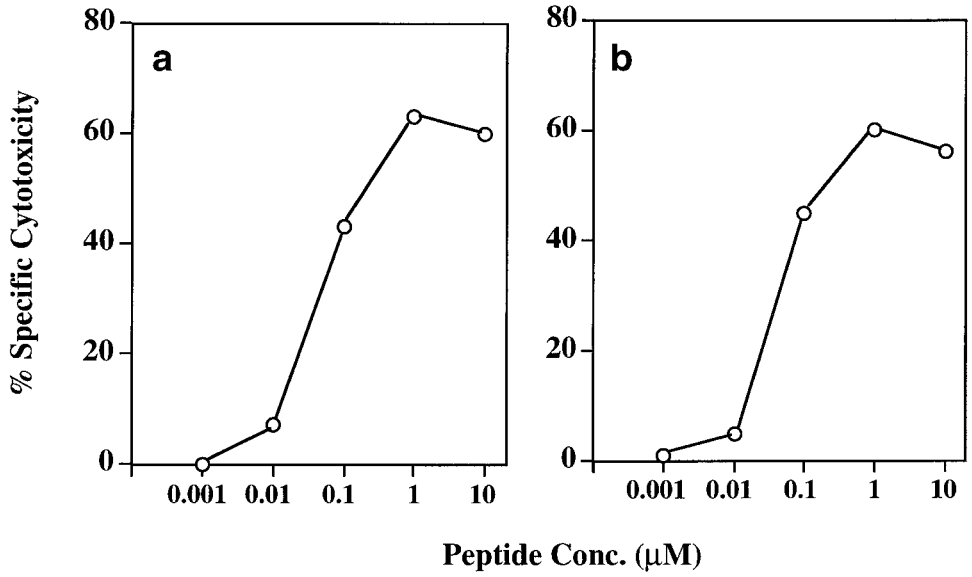

FIGURE 4 – Peptide dose responses of gp100-reactive CTLs. T-cell lines induced with peptide gp100[9_{87}] *(a)* or peptide gp100[9_{86}] *(b)* were tested against various concentrations of peptide-pulsed (1 hr at 4°C) EHM target cells (○) at an E:T ratio of 10:1. Cytotoxicity was measured after 4-hr incubation at 37°C.

TABLE II – A3 SUPERTYPE-BINDING DATA

Peptide	Sequence	A*0301 IC_{50} (nM)	A*1101 IC_{50} (nM)	A*3101 IC_{50} (nM)	A*3301 IC_{50} (nM)	A*6801 IC_{50} (nM)
gp100[9_{87}]	ALNFPGSQK	8.5	4.6	>10,000	>10,000	>10,000
gp100[10_{86}]	IALNFPGSQK	36.7	316	>10,000	>10,000	4,705
gp100[9_{17}]	ALLAVGATK	37.9	40.0	3,333	>10,000	3,077

that the T-cell receptors from these HLA-A3-restricted CTL lines do not recognize HLA-A11–gp100[9_{87}] complexes. Next, the immunogenicity of this peptide in HLA-A11 individuals was examined. Peptide-pulsed DCs from an HLA-A11 normal volunteer were used to stimulate autologous $CD8^+$ T cells following the procedure described above. One CTL culture recognized both the peptide-loaded target cells and the $A11^+/gp100^+$ melanoma 697mel (Fig. 5*a*). The recognition of 697mel was antigen-specific, as demonstrated by cold target inhibition assay (Fig. 5*b*). Similar to the description above, when gp100[9_{87}] was presented on the HLA-A3 molecules, this HLA-A11-restricted CTL could not kill the peptide-pulsed $HLA\text{-}A3^+$ EBV-transformed cells nor the cells of the $HLA\text{-}A3^+/gp100^+$ melanoma line 624mel (data not shown). These results demonstrate that the gp100[9_{87}] peptide binds and is

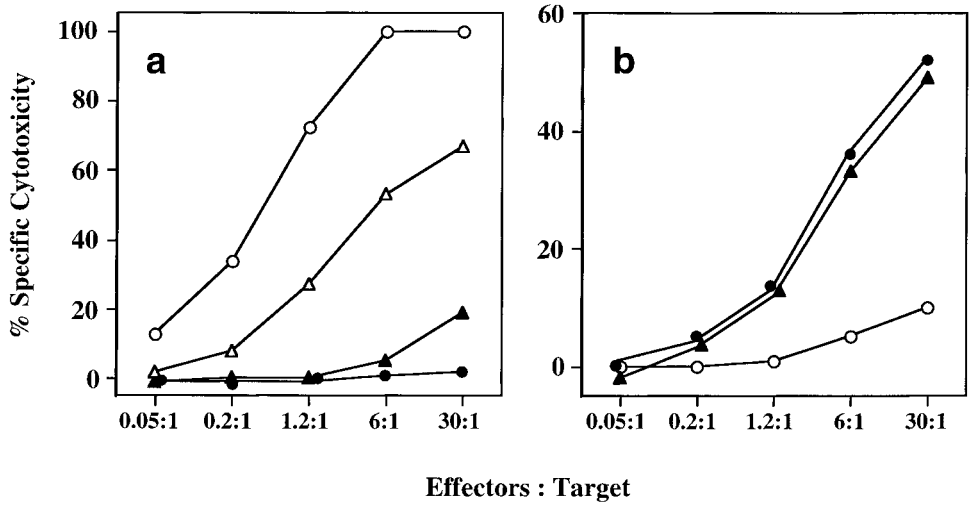

FIGURE 5 – Tumor reactivity and antigen specificity of HLA-A11-restricted, gp100[9_{87}]-specific CTLs. *(a)* The gp100[9_{87}]-specific CTLs expanded after 3 cycles of restimulation with peptide were used as effectors to test the lysis of the following target cell lines: ○, BVR pulsed with gp100[9_{87}]; ●, BVR without peptide; △, 697mel (melanoma, A11$^+$, gp100$^+$); ▲, 624mel (melanoma, A3$^+$, gp100$^+$). *(b)* Antigen specificity demonstrated by a cold target inhibition experiment. The melanoma cell line 697mel was ^{51}Cr-labeled and mixed at a ratio of 1:40 with the following cold targets: ○, BVR pulsed with gp100[9_{87}]; ▲, BVR pulsed with irrelevant A11-binding peptide [gp100[9_{460}], A*1101 binding (IC_{50}) = 5.5 nM]; ●, BVR without peptide. The same CTLs as used in *(a)* were used as effectors.

immunogenic in the context of both HLA-A3 and HLA-A11 molecules but that the CTLs recognizing this epitope are specific and restricted by the allele utilized to induce the antigenic response.

DISCUSSION

In the present study, we have identified 2 immunogenic peptides from the melanoma-associated antigen gp100 (gp100[9_{87}] and gp100[10_{86}]). When these peptides were pulsed onto DCs, HLA-A3-restricted CTL responses were induced *in vitro* using lymphocytes from normal human volunteers. Furthermore, the present results demonstrate that the gp100[9_{87}] peptide is capable of binding to both HLA-A3 and HLA-A11 and is accordingly immunogenic in individuals expressing these MHC class I alleles. Because the HLA-A3-restricted, gp100[9_{87}]-reactive CTLs could not recognize peptide-pulsed HLA-A11 targets (though these cells bind the peptide well), it is possible that the peptide binds with different conformations to HLA-A3 and -A11. Alternatively, it is also possible that the corresponding T-cell receptors from both the A3- and A11-restricted CTLs need to interact directly with polymorphic residues on the MHC molecules, which differ in these alleles. In contrast to our results, Threlkeld *et al.* (1997) have observed that HLA-A3- and HLA-A11-restricted CTLs both induced by HIV-1 RT/325–333 peptide (AIFQSSMTK) exhibited MHC cross-reactivity (*i.e.*, recognized both peptide-pulsed syngeneic and allogeneic targets). Thus, recognition by the same T-cell receptor of a peptide presented by different MHC molecules on heterologous (allogeneic) cells probably depends on the specificity of the T-cell receptor, the difference of peptide conformation in the binding groove and the variation of amino acids outside the peptide-binding pockets of these MHC molecules.

Since the gp100[9_{87}] and gp100[10_{86}] peptides significantly overlap and the CTLs induced with either peptide were capable of recognizing both peptides, it is possible that there is in fact only one HLA-A3-restricted CTL epitope (represented by peptide gp100[9_{87}]) in this region of gp100. In contrast, in the HLA-A2.1 system, we have reported that the overlapping peptides gp100[9_{178}] (MLGTHTMEV) and gp100[10_{177}] (AMLGTHTMEV) were both capable of inducing CTLs recognizing distinct epitopes (Tsai *et al.*, 1997). In these studies, in contrast to the present report, the CTLs induced with the 10mer (gp100[10_{177}]) were not able to recognize the 9mer (gp100[9_{178}]), indicating that these peptides form different epitopes when complexed to HLA-A2.1 molecules.

It was somewhat puzzling that peptide gp100[9_{17}], which represents the previously reported HLA-A3 CTL epitope present on melanoma patients, did not, in our hands, elicit tumor-reactive CTLs in any of the 3 healthy HLA-A3$^+$ donors tested. However, this peptide induced CTLs capable of recognizing exogenously peptide-pulsed target cells (one example is shown in Table I). Nevertheless, Castelli *et al.* (1998) have corroborated that peptide gp100[9_{17}] can indeed be recognized as a tumor-reactive CTL epitope by some melanoma patients (2 of 7), but this peptide was not capable of stimulating CTL responses in lymphocytes isolated from 7 healthy HLA-A3$^+$ donors. Thus, it is possible that the majority of the CTLs induced by peptide gp100[9_{17}] under our experimental conditions are of low affinity, rendering detection of naturally processed antigen more difficult (Wentworth *et al.*, 1995). Thus, these low-affinity CTLs require higher amounts of peptide–MHC complexes per cell as compared with high-affinity CTLs in order to exert their lytic function. Alternatively, it is also possible that the overall affinities of the CTLs induced by peptide gp100[9_{87}] or gp100[9_{17}] are similar (Fig. 4) but that the number of gp100[9_{87}]–HLA-A3 complexes on the surface of melanoma cells is greater than the number of gp100[9_{17}]–HLA-A3 complexes. Further experiments are required to determine which of these possibilities is correct since currently there are no reliable methods to quantitate specific peptide–MHC complexes on cell surfaces.

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