

MAGE-A1-, MAGE-A10-, and gp100-Derived Peptides Are Immunogenic When Combined with Granulocyte-Macrophage Colony-Stimulating Factor and Montanide ISA-51 Adjuvant and Administered as Part of a Multi-peptide Vaccine for Melanoma¹

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Twelve peptides derived from melanocyte differentiation proteins and cancer-testis Ags were combined and administered in a single mixture to patients with resected stage IIB, III, or IV melanoma. Five of the 12 peptides included in this mixture had not previously been evaluated for their immunogenicity in vivo following vaccination. We report in this study that at least three of these five peptides (MAGE-A1_{96–104}, MAGE-A10_{254–262}, and gp100_{614–622}) are immunogenic when administered with GM-CSF in Montanide ISA-51 adjuvant. T cells secreting IFN- γ in response to peptide-pulsed target cells were detected in peripheral blood and in the sentinel immunized node, the node draining a vaccine site, after three weekly injections. The magnitude of response typically reached a maximum after two vaccines, and though sometimes diminished thereafter, those responses typically were still detectable 6 wks after the last vaccines. Most importantly, tumor cell lines expressing the appropriate HLA-A restriction element and MAGE-A1, MAGE-A10, or gp100 proteins were lysed by corresponding CTL. This report supports the continued use of the MAGE-A1_{96–104}, MAGE-A10_{254–262}, and gp100_{614–622} epitopes in peptide-based melanoma vaccines and thus expands the list of immunogenic peptide Ags available for human use. Cancer-testis Ags are expressed in multiple types of cancer; thus the MAGE-A1_{96–104} and MAGE-A10_{254–262} peptides may be considered for inclusion in vaccines against cancers of other histologic types, in addition to melanoma. *The Journal of Immunology*, 2005, 174: 3080–3086.

More than 50 melanoma-associated peptides have been identified, yet only a small portion of these have been evaluated for their immunogenicity as part of experimental vaccine therapies (1). The identification of peptide epitopes for melanoma-specific CTL provides an opportunity to create novel vaccines that can be evaluated directly in terms of the CTL response to the purified immunizing Ag. In this regard, peptide-specific T cell responses have been reported for several clinical studies of peptide-based vaccines (2–5).

The majority of shared melanoma Ags identified thus far can be divided into two groups: melanocyte differentiation proteins

(MDP)³ and cancer-testis Ags (CTA). Generally, both types of proteins are encoded by genes lacking tumor-specific mutations and are differentially expressed at various stages of melanoma (6–8). Therefore, a polyvalent vaccine incorporating epitopes derived from both groups of Ags may be appropriate for the treatment of the majority of patients with melanoma.

Two criteria are important to consider when selecting peptides for a vaccine to be tested in the majority of the melanoma patient population. The first is the frequency of expression of the parent protein from which the epitope is derived, and the second is the frequency of expression of the HLA alleles to which the epitopes are restricted. Both gp100 and members of the MAGE gene family are expressed in a large percentage of melanomas (9, 10). The epitopes described in this study are restricted by HLA-A2 and -A3 molecules, and the percentage of the melanoma patient population expressing at least one of these HLA alleles is ~60%. Thus, each of these epitopes may have a broad application in melanoma vaccines.

gp100 is classified as a MDP and multiple gp100-derived epitopes restricted by HLA-A2, -A3, and -A24 molecules have been identified, some of which have been shown to be immunogenic when administered in peptide-based vaccines (3, 4). Importantly, vaccination with gp100-derived epitopes administered in

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³ Abbreviations used in this paper: MDP, melanocyte differentiation proteins; CTA, cancer-testis Ags; SIN, sentinel immunized node.

combination with low- or high-dose IL-2 therapy has been associated with tumor regression in patients with advanced disease (3, 4). One of the more recently described gp100-derived epitopes, gp100₆₁₄₋₆₂₂ (LIYRRRLMK), was defined as an HLA-A3-restricted epitope using tumor infiltrating lymphocytes from a patient with melanoma (11). However, until this study, this epitope had yet to be evaluated for its immunogenic potential when administered as part of a peptide-based vaccine regimen.

MAGE-A1 and MAGE-A10 are members of the MAGE gene family and are classified as CTA. This family of proteins is expressed in male germline cells and placenta, as well as several different types of tumors including melanoma, bladder, breast, prostate, and nonsmall cell lung cancers (9). The HLA-A3-restricted MAGE-A1₉₆₋₁₀₄ epitope (SLFRAVITK) and the HLA-A2-restricted MAGE-A10₂₅₄₋₂₆₂ epitope (GLYDGM EHL) were recently identified using CTL isolated from melanoma patients (12-14); however, at the time this study was initiated, neither epitope had been evaluated for its ability to stimulate T cell responses following vaccination.

In order for a peptide restricted by class I MHC molecules to have value in cancer vaccines, the peptide must induce a specific immune response, and the responding T cells must be able to recognize cancer cells naturally expressing the peptide-MHC complex. We have evaluated the immunogenicity of the gp100₆₁₄₋₆₂₂, MAGE-A1₉₆₋₁₀₄, and MAGE-A10₂₅₄₋₂₆₂ peptides following vaccination with a multi-peptide vaccine, and have characterized the ability of T cells expanded from the lymph node draining a vaccination site (sentinel immunized node (SIN)) to recognize and to lyse tumor cells expressing the gp100, MAGE-A1, and MAGE-A10 proteins, respectively. These peptides are immunogenic when combined with GM-CSF and Montanide ISA-51 adjuvant and administered as part of a multi-peptide vaccine. This expands the number of epitopes with therapeutic potential for the treatment of cancer.

Materials and Methods

Patients

Patients with resected stage IIB, III, or IV melanoma were eligible to receive the 12 melanoma peptide vaccine. Entry criteria included age ≥ 18 years, expression of HLA-A1, -A2, or -A3, and expression of gp100 (for patients HLA-A2⁺ or HLA-A3⁺) or tyrosinase (for patients HLA-A1⁺ or HLA-A2⁺) by immunohistochemistry. Patients with ocular melanoma were excluded. Patients were not eligible if they were pregnant, had received cytotoxic chemotherapy, IFN therapy, radiation therapy, or steroids within the preceding 4 wks, or were vaccinated previously with any of the peptides in the vaccine. Patients were studied following informed consent, and with Institutional Review Board (HIC no. 8878) and FDA (BB-IND no. 9847) approval.

Peptides

Peptides incorporated in the vaccine and those used for in vitro assays were synthesized by the University of Virginia Biomolecular Core Facility or by Multiple Peptide Systems. Each was synthesized with a free amide NH₂ terminus and free acid COOH terminus and provided as a lyophilized powder. The peptides were solubilized and sterile-filtered. In the case of the vaccines, the concentration of peptide was assessed by amino acid analysis. Peptide solutions were vialled in borosilicate glass vials and stored $\leq -80^{\circ}\text{C}$ protected from light. The peptides include those in the 12 melanoma peptide vaccine (see Table I for sequences) and a modified tetanus toxoid protein-derived class II MHC-restricted helper peptide (AQYIKANSKFIGITEL) (2, 3, 15). Two peptides used as negative controls in chromium release and ELISPOT assays were YLKKIKNSL (malaria CSP₃₃₄₋₃₄₂, derived from malaria circumsporozoite protein (16)) and SLYNTVATL (GAG₇₇₋₈₅, derived from the HIV-1 gag protein (17)).

Cell lines

C1RA2 and C1RA3 are human EBV-transformed B cell lines transfected with the gene for HLA-A2 and HLA-A3 class I molecules, respectively. No other class I molecules are present on the transfectants. T2 is a mutant

Table I. 12 melanoma peptide vaccine

Allele	Sequence	Epitope
HLA-A1	DAEKSDICTDEY	Tyrosinase ₂₄₀₋₂₅₁ ^a
	SSDYVPIPIGT	Tyrosinase ₁₄₆₋₁₅₆
	EADPTGHSY	MAGE-A1 ₁₆₁₋₁₆₉
	EVDPIGHLY	MAGE-A3 ₁₆₈₋₁₇₆ ^b
HLA-A2	YMDGTMSQV	Tyrosinase ₃₆₉₋₃₇₇ ^b
	IMDQVPFSV	gp100 _{209-217M} ^c
	YLEPGPVTA	gp100 ₂₈₀₋₂₈₈
	GLYDGM EHL ^d	MAGE-A10 ₂₅₄₋₂₆₂
	ALLAVGATK	gp100 ₁₇₋₂₅
HLA-A3	LIYRRRLMK	gp100 ₆₁₄₋₆₂₂
	SLFRAVITK	MAGE-A1 ₉₆₋₁₀₄
	ASGPGGGAPR	NY-ESO-1 ₅₃₋₆₂

^a Substitution of S for C at residue 244.

^b Posttranslational change of N to D at residue 371.

^c Substitution of M for T at position 210.

^d Bolded text are epitopes evaluated in this manuscript.

human T/B cell hybrid lacking TAP but expressing HLA-A*0201 (18). C1RA3 and T2 were kindly provided by Dr. P. Cresswell (Yale University School of Medicine, New Haven, CT). K562 is a human myelogenous leukemia cell line and a NK cell target. Melanoma cell lines are shown below in Table II. Those prefixed with "VMM" were established at the University of Virginia and those prefixed with "DM" were established at Duke University and were kindly provided by Drs. T. Darrow and H. Seigler (Duke University, Durham, NC). LB1751, LB373, and AVL3 are melanoma cell lines kindly provided by Dr. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). SkMel2 is a melanoma cell line available from American Type Culture Collection. Ag expression was determined by RT-PCR.

Immunization protocol

Patients received a vaccine containing 12 melanoma peptides, 4 each restricted by HLA-A1, -A2, and -A3, and a modified HLA-DR-restricted helper peptide (p2) derived from tetanus toxoid protein, weekly over a period of 7 wks, with a break at wk 3. For the first 3 wks, vaccines were administered at two sites, the primary and replicate vaccination sites. At each vaccination site, patients received the 12 melanoma peptide mixture containing 100 μg of each individual peptide and 190 μg of tetanus peptide, administered with 110 μg of GM-CSF (Leukine (Sargramostim); Berlex) in 1 ml Montanide ISA-51 adjuvant. The total injection volume at each site was 2 ml. At each site, one-half of the dose was administered intradermally and one-half of the dose was administered s.c. One week after the third vaccine, the node draining the replicate vaccination, the SIN, was harvested and evaluated for an immune response to the vaccine as described (19). For the last 3 wks, vaccines were administered at the primary vaccination site only.

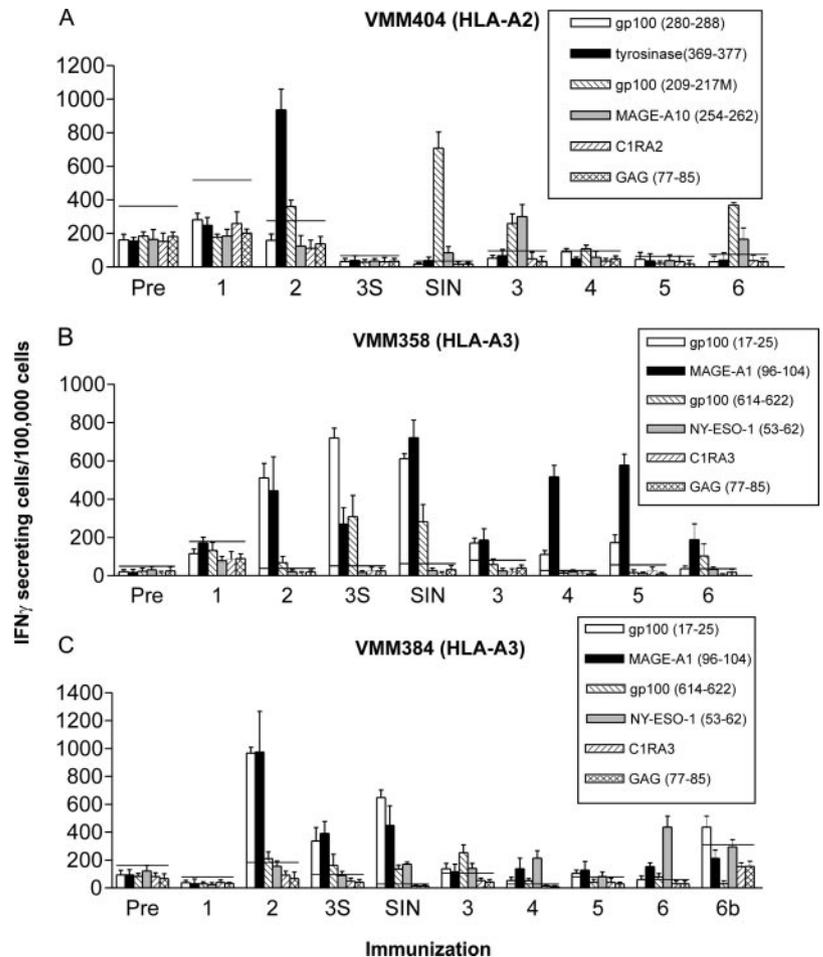
For immune monitoring, PBL were collected before and after each vaccination. For vaccines 1-5, PBL samples were collected 1 wk after each vaccine was administered. The first PBL sample after vaccine 6 was collected an average of 45 days after that vaccine. The second PBL sample collected after vaccine 6 (Fig. 1C; 6b) was collected ~ 6 mo after that vaccine.

Table II. Melanoma cell lines

Cell Line	HLA-Type	Ag Expression
VMM12	-A1, -A3	gp100 ⁺ , MAGE-A1 ⁺ , MAGE-A10 ⁺
VMM18	-A3	gp100 ⁺ , MAGE-A1 ⁺
VMM68	-A11, -A25	gp100 ⁻ , MAGE-A1 ⁺
VMM273	-A3, -A26	gp100 ⁺ , MAGE-A1 ⁺
DM6	-A2	gp100 ⁺ , MAGE-A1 ⁺ , MAGE-A10 ⁺
DM122	-A3	gp100 ⁺ , MAGE-A1 ⁻
DM331 ^a	-A1, -A2	gp100 ⁻
SkMel2	-A3, -A26	gp100 ⁺ , MAGE-A1 ⁺ , MAGE-A10 ⁺
AVL3	-A1, -A2	gp100 ⁻ , MAGE-A1 ⁺ , MAGE-A10 ⁺
LB373	-A2, -A11	gp100 ⁻ , MAGE-A1 ⁺ , MAGE-A10 ⁺
LB 1751	-A2, -A3	gp100 ⁻ , MAGE-A1 ⁺ , MAGE-A10 ⁺

^a In one of two PCR reactions recently performed, gp100 was very weakly positive, but Northern and Western analyses and immunohistochemistry revealed no detectable gp100 expression (36).

FIGURE 1. IFN- γ release in response to the 12 peptide vaccine. ELISPOT assays were performed on PBL and SIN samples and the number of responding lymphocytes, per 100,000 plated, is shown prevaccination and following each immunization (x-axis). Responses shown are for patients VMM404 (HLA-A2⁺) (A), VMM358 (HLA-A3⁺) (B), and VMM384 (HLA-A3⁺) (C). Negative controls include target cells alone, C1RA2 (A) or C1RA3 (B and C), and target cells pulsed with an irrelevant peptide GAG₇₇₋₈₅ (A–C). Horizontal lines represent the threshold of positivity of two times the highest background. PBL collected at the same week in which the SIN biopsy was performed are labeled 3S. PBL drawn after the sixth vaccine were obtained ~6 wk after that vaccine. For patient VMM384, PBL were collected twice after the sixth vaccination and are labeled 6 and 6b.



Lymphocytes were isolated from peripheral blood by Ficoll gradient centrifugation and were cryopreserved. Samples from prevaccination and representative samples after one or more vaccinations were evaluated simultaneously, in parallel with lymphocytes derived from the SIN.

ELISPOT assays

Stimulation of lymphocytes. Lymphocytes (2×10^6 cells/ml) in complete medium (RPMI 1640 (Life Technologies) plus 10% human AB serum (Gemini) plus 1% PenStrep (Invitrogen Life Technologies) and 1% 200 mM L-Glutamine (Invitrogen Life Technologies)) were incubated with the 12 melanoma peptide mixture (40 μ g/ml each) for 2 h at 37°C with 5% CO₂. The lymphocytes were pelleted and resuspended in complete medium with 20 U/ml IL-2 (IL-2; Chiron) and cultured for 14 days. Complete medium was replaced as needed.

Assay procedures. Multiscreen IP sterile plates HTS (Millipore) were prewet with 70% methanol and were coated with anti-IFN- γ mAb (Endogen). Effector cells were plated at 25,000 and 75,000 cells/well. Equal numbers of APCs alone or APC pulsed with peptide (40 μ g/ml) were added to each well. Plates were incubated at 37°C and 5% CO₂ for 18–20 h. After extensive washing with 0.01% Tween, the plates were incubated with a biotin-labeled anti-IFN- γ Ab (Endogen). Then, the plates were washed again and incubated with streptavidin conjugated to alkaline phosphatase (BD Pharmingen). After washing, the plates were developed with NBT/5-bromo-4-chloro-3-indolyl phosphatesubstrate-Toluidine salt (Pierce). Plates were read using an automated plate reader (Bioreader; Biosys).

Each sample is tested in quadruplicate. The number of T cells responding to each of the peptides in the vaccine was calculated as the difference between the number of cells secreting IFN- γ in response to APCs loaded with an immunizing peptide and the highest negative control results (APC alone or loaded with irrelevant peptide).

Assessment of response. Evaluation of T cell responses was based on the following definitions:

$$N_{\text{vax}} = \text{number of T cells responding to peptide in the vaccine}$$

$$N_{\text{neg}} = \text{number of T cells responding to negative control (maximum of}$$

two negative controls: C1RA2 or C1RA3 alone; or C1RA2 or C1RA3 pulsed with an irrelevant peptide)

$$R_{\text{vax}} = \text{ratio of } N_{\text{vax}}/N_{\text{neg}}$$

For evaluations of PBL, a patient was considered to have a T-cell response to vaccination only if all of the following criteria had been met:

N_{vax} exceeded N_{neg} by at least 30 cells per 100,000 (corresponds to ~0.15% of CD8⁺ cells); $R_{\text{vax}} > 2$; ($N_{\text{vax}} - 1 \text{ SD}$) \geq ($N_{\text{neg}} + 1 \text{ SD}$); and R_{vax} after vaccination $\geq 2 \times R_{\text{vax}}$ prevaccination.

The peak CTL response to any peptide after the first vaccine is reported as a fold increase over the negative control, and the increase resulting from vaccination is reported as a ratio of the postvaccine measure to the prevaccine measure. For evaluations of the SIN, the first three listed criteria are required for a T cell response to vaccination. Because prevaccination lymph node samples were not routinely evaluated in this study, the last criterion was not applied to the samples derived from the SIN.

Chromium release assays

Lymphocyte stimulation. Lymphocytes were stimulated in vitro by culturing cells in complete medium (RPMI 1640 plus 10% human AB serum (Gemini) or 10% FCS (Gemini) plus 100 U/ml penicillin plus 100 μ g/ml streptomycin plus 2 mM L-glutamine) with 20 U/ml IL-2 and 10 μ g/ml peptide for 12–15 days. During restimulation, irradiated APC pulsed with 1–5 μ g/ml peptide were cocultured with the lymphocytes in complete medium with 20 U/ml IL-2 at a 10:1 lymphocyte:target ratio for 7–15 days. The lymphocytes were then expanded by specific or nonspecific methods.

Nonspecific lymphocyte expansion. For T cell expansion, we used a protocol modified from Crossland et al. (20). T cells were expanded in vitro without additional Ag stimulation by culturing with 10 ng/ml anti-CD3 Ab (OKT3; BD Pharmingen) in the presence of several groups of feeder cells. T cells ($n = 50,000$) were cocultured at 37°C in a T-25 flask with 5×10^6 irradiated allogeneic EBV-B cells and 25×10^6 irradiated allogeneic PBL for 14 days in complete medium with 25 U/ml IL-2. This usually yields $10\text{--}50 \times 10^6$ T cells with specificity comparable to that of the original T cells.

Specific lymphocyte expansion. A specific lymphocyte expansion served as the third stimulation. Lymphocytes (1×10^6) are cocultured with 5×10^6 irradiated allogeneic EBV-B cells, 5×10^6 irradiated allogeneic PBL, and 5×10^6 irradiated APC pulsed with 0.1–1 $\mu\text{g/ml}$ peptide. Cells are incubated in complete medium containing 15% serum and 20 U/ml IL-2 in a T-75 flask for at least 7 days.

Assay procedures. The ability of peptide-reactive T cells to lyse target cells after vaccination was determined using a standard 4-h chromium release assay. ^{51}Cr -labeled target cells were plated at 1×10^3 cells/well in triplicate in 96-well V-bottom plates (Nunc International) with the indicated ratio of target to effector cells in a final volume of 200 μl . Wells containing either culture medium or 3% Nonidet P40 detergent (Sigma-Aldrich) in place of the effector cells served as spontaneous and maximum ^{51}Cr -release controls, respectively. Percent specific lysis was calculated as $[(\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{background}})/(\text{cpm}_{\text{maximum}} - \text{cpm}_{\text{background}})] \times 100$.

Results

CD8⁺ T cell responses against MAGE-A1_{96–104}

MAGE-A10_{254–262}, and gp100_{614–622} in the sentinel immunized node and the peripheral blood following vaccination

Twenty-five patients were vaccinated with the 12 peptide mixture described in Table I and the number of T cells responding to each peptide in the SIN and the PBL, as measured by release of IFN- γ , was determined using an IFN- γ ELISPOT assay. T cell data from the SIN have identified at least four patients who responded to each of the MAGE-A1_{96–104}, MAGE-A10_{254–262}, and gp100_{614–622} peptides and representative data are shown in Fig. 1. VMM404 (Fig. 1A) responded to the MAGE-A10_{254–262} peptide (GLYDGM EHL), and VMM358 (Fig. 1B) and VMM384 (Fig. 1C) responded to both the MAGE-A1_{96–104} (SLFRVITK) and gp100_{614–622} (LIYRRRLMK) peptides.

In evaluating the data from the SIN, all three patients shown in Fig. 1 responded to additional peptides incorporated in the vaccine. VMM404 (Fig. 1A) responded to the gp100_{209–217M} (IMDQVPFSV) peptide, a peptide that has been shown to be immunogenic in prior peptide-based vaccines (4). VMM358 (Fig. 1B) and VMM384 (Fig. 1C) responded to gp100_{17–25} (ALLAVGATK), which has also been shown to be immunogenic in prior studies (3, 19). In addition, VMM384 responded to the NY-ESO-1_{53–62} peptide (ASGPGGGAPR), which was originally described in association with HLA-A31 (21), but is also presented in the context of HLA-A3 (22).

T cell responses were evaluated in the peripheral blood both before and during the course of vaccination (Fig. 1). Responses against each of the MAGE-A1_{96–104}, MAGE-A10_{254–262}, and gp100_{614–622} peptides were detected at least once in the blood following vaccination. In some cases (Fig. 1, B and C) responses against the MAGE-A1_{96–104} and gp100_{614–622} peptides in the blood drawn at the same time as the SIN biopsy (3S) correlated with those measured in the SIN.

CD8⁺ T cells in the SIN recognize and lyse peptide-pulsed target cells and tumor cells naturally expressing MAGE-A1, MAGE-A10, and gp100 proteins

To evaluate whether Ag-reactive T cells induced by vaccination have adequate avidity to recognize and to lyse tumor cells naturally expressing their cognate peptide Ags, we performed chromium release assays using T cells expanded from the SIN from patients VMM404, VMM358, and VMM384. T cells derived from the SIN were chosen for this analysis because this is the location where immune responses to the vaccine are expected to be initiated. Ideally, the SIN is the site of the primary immune response to vaccination and we have been able to reliably expand vaccine-specific T cells from this site in previous studies (3, 19, 23).

MAGE-A1_{96–104}

Lymphocytes derived from the SIN of patient VMM358 (HLA-A3⁺) and expanded on the MAGE-A1_{96–104} peptide (SLFRVITK) were specific for that peptide. Lymphocytes lysed C1RA3 cells pulsed with this peptide, but failed to lyse C1RA3 cells pulsed with any of the remaining three HLA-A3-restricted peptides incorporated in the 12 peptide vaccine mixture (Fig. 2A). Importantly, CTL specific for the MAGE-A1_{96–104} peptide lysed two of three tumor cell lines, which are HLA-A3⁺ and express the MAGE-A1 protein (SkMel2 and VMM18; Fig. 2B).

gp100_{614–622}

Similarly, lymphocytes derived from patient VMM384 (HLA-A3⁺) SIN cultures expanded on the gp100_{614–622} (LIYRRRLMK) peptide demonstrated specific recognition of this peptide (Fig. 3A). CTL lysed gp100_{614–622}-pulsed C1RA3 cells and failed to lyse C1RA3 cells pulsed with an irrelevant peptide. Three of five tumor cell lines that are HLA-A3⁺ and gp100⁺ (DM122, SkMel2, and VMM18; Fig. 3B) were lysed by the gp100_{614–622}-specific CTL.

MAGE-A10_{254–262}

CTL derived from the SIN of an HLA-A2⁺ patient, VMM404, were expanded in vitro on the MAGE-A10_{254–262} peptide (GLYDGM EHL) and lysed T2 cells pulsed with the GLYDGM EHL peptide, but failed to lyse T2 cells pulsed with the remaining 3 HLA-A2-restricted peptides incorporated in the 12 peptide vaccine mixture (Fig. 4A). In addition, two of four tumor samples that are

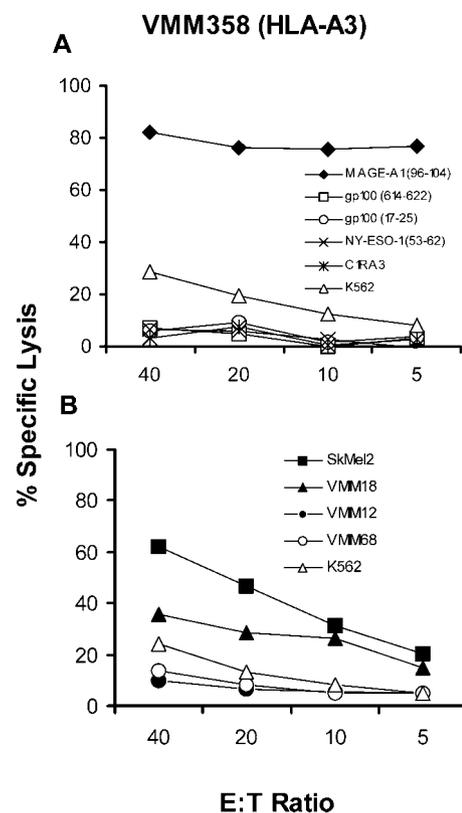


FIGURE 2. CTL responses to the MAGE-A1_{96–104} peptide. T cells cultured from the SIN of patient VMM358 (HLA-A3⁺) were assayed for cytotoxicity at several E:T ratios against peptide-pulsed C1RA3 target cells (A) and melanoma tumors (B), in 4 h ^{51}Cr -release assays. A, C1RA3 represents target cells alone. B, MAGE-A1⁺, HLA-A3⁺ tumor cells are represented by closed symbols. K562 is a NK cell target and is used to assess the level of NK activity in the cultures (A and B).

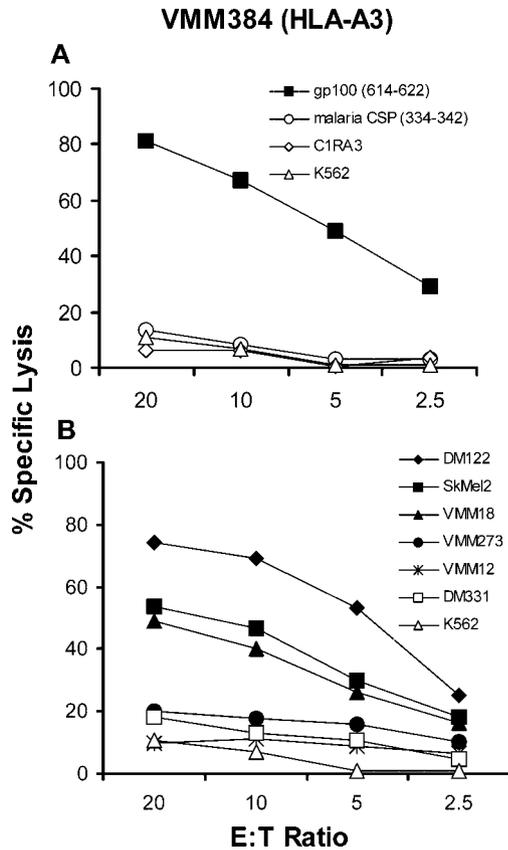


FIGURE 3. CTL responses to the gp100₆₁₄₋₆₂₂ peptide. T cells cultured from the SIN of patient VMM384 (HLA-A3⁺) were assayed for cytotoxicity at several E:T ratios against peptide-pulsed C1RA3 target cells (A) and melanoma tumors (B), in 4 h ⁵¹Cr-release assays. A, C1RA3 represents target cells alone. B, gp100⁺, HLA-A3⁺ tumor cells are represented by closed symbols. K562 is a NK cell target and was used to assess the level of NK activity in the cultures (A and B).

HLA-A2⁺ and express MAGE-A10 (LB1751, LB373; Fig. 4B) were lysed by the MAGE-A10₂₅₄₋₂₆₂-specific CTL.

In summary, the MAGE-A1₉₆₋₁₀₄, MAGE-A10₂₅₄₋₂₆₂, and gp100₆₁₄₋₆₂₂ peptides are immunogenic when incorporated as part of a multipptide vaccine administered in GM-CSF adjuvant, and the Ag-reactive T cells have an avidity for their target Ags that is adequate for recognition and lysis of melanoma cells naturally expressing MAGE-A1, MAGE-A10, and gp100 respectively.

VMM404-derived CTL have a high avidity for the MAGE-A10₂₅₄₋₂₆₂ peptide

Only half of the HLA-A2⁺, MAGE-A10⁺ tumor targets incorporated in the chromium release assay were lysed by the MAGE-A10₂₅₄₋₂₆₂-specific T cells cultured from the SIN of patient VMM404 (Fig. 4B). To further assess the avidity of the MAGE-A10₂₅₄₋₂₆₂-specific CTL, defined as the concentration of peptide required to elicit 50% lysis of target cells, a peptide dose-response curve was generated using the MAGE-A10₂₅₄₋₂₆₂ peptide. Half-maximal lysis of T2 target cells was achieved using ~1 nM of peptide (Fig. 5). This concentration of peptide is similar to the amount of peptide needed to elicit half-maximal lysis of target cells pulsed with other gp100, MART-1, and MAGE-A1 derived peptides (12, 24) and lower than the quantity previously reported using a clone specific for the MAGE-A10₂₅₄₋₂₆₂ peptide (13).

Discussion

Metastatic melanomas contain a heterogeneous population of cells with respect to protein expression (6-8). Thus, vaccines incorpo-

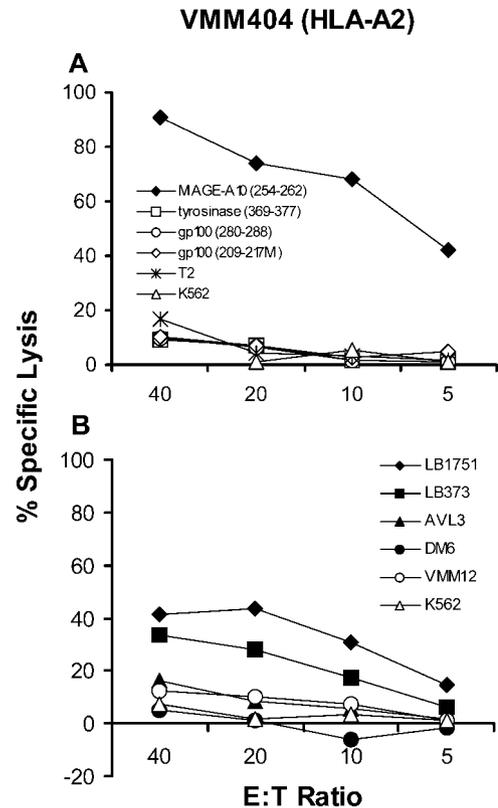


FIGURE 4. Cytotoxic T cell responses to the MAGE-A10₂₅₄₋₂₆₂ peptide. T cells cultured from the SIN of patient VMM404 (HLA-A2⁺) were assayed for cytotoxicity at several E:T ratios against peptide-pulsed T2 target cells (A) and melanoma tumors (B), in 4 h ⁵¹Cr-release assays. A, T2 represents target cells alone. B, MAGE-A10⁺, HLA-A2⁺ tumor cells are represented by closed symbols. K562 is a NK cell target and was used to assess the level of NK activity in the cultures (A and B).

rating single melanoma-derived epitopes may be inadequate in generating a complete immune response against the tumor (25, 26). Instead, a polyvalent vaccine incorporating epitopes derived from MDP and CTA may be necessary to eliminate most tumors. Providing evidence for the immunogenicity of epitopes derived from these proteins in a vaccine regimen is useful for the development of cancer vaccines.

In this study, we report on the immunogenicity of three previously identified epitopes derived from the MAGE-A1, MAGE-A10, and gp100 proteins (11-13) that had not been evaluated for immunogenicity in human peptide vaccines. We determined that the MAGE-A1₉₆₋₁₀₄, MAGE-A10₂₅₄₋₂₆₂, and gp100₆₁₄₋₆₂₂ epitopes are immunogenic and elicit T cell responses that are detectable in the SIN and in the peripheral blood following vaccination (Fig. 1). These responses typically reached a maximum after two vaccines and most responses were still detectable 6 wks after the sixth vaccine. When observed, decreases in response over time may have been due to any of several factors, including exhaustion of the CTL pool, activation-induced cell death, changes in T cell trafficking, or induction of regulatory T cells. Studies have been initiated to characterize these cells functionally ex vivo at varied time points. Initial data show CD8 responses that are detectable by ex vivo tetramer and ELISPOT assays (C. L. Slingluff, Jr., manuscript in preparation) and the data presented in this study support evaluation of the immunogenicity of individual epitopes incorporated in multipptide mixtures.

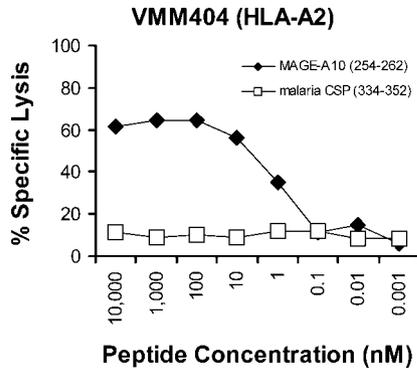


FIGURE 5. Peptide-dose-response curve for MAGE-A10_{254–262}-specific T cells. T cells derived from the SIN of patient VMM404 (HLA-A2⁺) were assayed for cytotoxicity in a 4-h ⁵¹Cr-release assay using dilutions of the MAGE-A10_{254–262} peptide (GLYDGM EHL) or an irrelevant peptide, malaria CSP_{334–352}, pulsed on T2 target cells. The E:T ratio in this experiment was 15:1.

The lytic capacity of MAGE-A1_{96–104}⁺, MAGE-A10_{254–262}⁺, and gp100_{614–622}-specific T lymphocytes was confirmed in chromium release assays (Figs. 2–4). The results of these assays provide evidence that the responding T cell populations are specific for each of the immunizing peptides following in vitro culture. Most importantly, the resulting CTL populations lyse tumor cell targets naturally expressing MAGE-A1, MAGE-A10, or gp100, and the corresponding HLA-A restriction element. These results demonstrate that functional CTL populations can be elicited following vaccination with a multipeptide vaccine administered in GM-CSF adjuvant, and provide evidence that vaccination in this manner elicits T cells with a high enough avidity to recognize and lyse tumor.

However, not all of the MAGE-A1⁺, MAGE-A10⁺, and gp100⁺ tumors tested were lysed by the MAGE-A1_{96–104}⁺, MAGE-A10_{254–262}⁺, and gp100_{614–622}-specific T cell populations. There are several possible explanations for this finding. First, melanoma cells may have processing defects that interfere with the processing and loading of class I-restricted epitopes (27–29). Second, melanomas often lose or down-regulate expression of MHC molecules (30). Third, the melanoma-associated proteins may be expressed at low levels. Fourth, tumor cells may express inhibitory ligands that interfere with the effector function of cytolytic T cells (31). Failure of T cells to lyse tumor targets may also be attributed to the T cells having a low avidity for the corresponding MHC/peptide complex. We questioned whether low avidity could explain failure to lyse two of four MAGE-A10⁺ tumors by VMM404-derived T cells. In a prior study, 100 nM of peptide was required to elicit 50% lysis of peptide-pulsed target cells by a MAGE-A10_{254–262}-specific T cell clone (13). However, the data presented in this study indicate that postvaccination, ~1 nM MAGE-A10_{254–262} peptide is required to elicit 50% lysis of target cells by the responding T cell culture (Fig. 5). Thus, these T cells have a much higher avidity. Our results are comparable to avidities of T cells responding to gp100-, MART-1-, and MAGE-A1-derived peptides (12, 24) and demonstrate that T cells with a sufficient avidity for tumor are induced by our vaccination regimen. Because expression of gp100, MAGE-A1, and MAGE-A10 were assessed by RT-PCR, we cannot state whether the corresponding proteins are present at higher levels in the target cells that are lysed by the corresponding T cell populations. However, we suspect down-regulation of melanoma Ags and/or MHC molecules explains the lack of susceptibility of some tumor cell targets to T cell lysis.

Ideally, we would like to be able to directly assess the cytolytic properties of our responding T cell populations using fresh PBMC, following vaccination with the melanoma-associated peptides. However, it is difficult to make assessments of cytolytic activity using unstimulated cultures because of the low frequency of the responding T cell populations. In our study, in vitro expansions using peptide-pulsed target cells as stimulator cells were performed to obtain adequate cell numbers to assess cytotoxicity. In vitro expansions could, theoretically, induce T cell responses that do not reflect the immune response occurring in vivo. However, our experience is that prevaccine samples from stage III or IV melanoma patients submitted to in vitro sensitization rarely (<5%) have evidence of reactivity to these Ags when evaluated by the ELISPOT assay (C. L. Slingluff, Jr., unpublished observation), which is a more sensitive measure of T cell response than the chromium release assay. Thus, it appears the responses observed in chromium release assays reflect the immunogenicity of the vaccines, and T cells with a sufficient avidity to recognize tumor cells, although present in low proportions, can be generated by our vaccination regimen.

In addition to gp100_{614–622}, VMM384 (Fig. 1C) responded to the NY-ESO-1_{53–62} peptide (ASGPGGGAPR). The NY-ESO-1_{53–62} epitope was identified by Wang et al. (21) and was shown to be restricted by HLA-A31, a member of the A3 supertype family. Using mass spectrometry, we found that this NY-ESO-1-derived peptide is also associated with HLA-A3 from both the VMM12 and VMM18 human melanoma cells (22); therefore, it was included in the 12 peptide mixture. Further studies evaluating the frequency and magnitude of T cells specific for NY-ESO-1_{53–62} presented in the context of HLA-A3, as well as their lytic function, are currently underway.

Other members of the A3 supertype family with similar binding motifs include HLA-A11, -A33, and -A68. Based on the similar binding motifs, the HLA-A3-restricted MAGE-A1_{96–104} and gp100_{614–622} epitopes described in this study may be presented by additional HLA alleles, which would broaden their usefulness in the clinical setting. Experiments are currently underway to determine whether the existing MAGE-A1_{96–104}⁺ and gp100_{614–622}-specific CTL, which recognize these epitopes in the context of HLA-A3, also recognize these epitopes on tumor cells expressing other members of the A3 supertype family, as has been shown previously for the TRP_{197–205} epitope (32).

Prior reports have suggested the immunogenicity of MAGE Ags is low compared with the immunogenicity of MDP (33, 34). In a preliminary assessment of immune response data, we have detected T cell responses to the MAGE-A1_{96–104} and MAGE-A10_{254–262} peptides in at least 67% and 78%, respectively, of patients immunized with the 12 peptide vaccine as reported previously (35). Combined, these results support the continued use of the MAGE-A1_{96–104} and MAGE-A10_{254–262} peptides along with gp100_{614–622} in immunotherapies for melanoma and suggest that CTA peptides may be reliably immunogenic. CTA are expressed in multiple cancers including melanoma, nonsmall cell lung cancer, bladder, breast, and prostate cancers (9). Thus, the immunogenicity data provided in this study in patients with melanoma support the use of the MAGE-A1_{96–104} and MAGE-A10_{254–262} peptides in vaccines for a wide range of cancers of other histologies expressing the MAGE-A1 and MAGE-A10 proteins.

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