Published in final edited form as:

J Immunother. 2006 ; 29(2): 224–231.

Altered CD8⁺ T-Cell Responses When Immunizing With Multiepitope Peptide Vaccines

Steven A. Rosenberg, Richard M. Sherry, Kathleen E. Morton, James C. Yang, Suzanne L. Topalian, Richard E. Royal, Udai S. Kammula, Nicholas P. Restifo, Marybeth S. Hughes, Susan L. Schwarz, Lien T. Ngo, Sharon A. Mavroukakis, and Donald E. White

From the Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

Summary

Efforts to develop effective cancer vaccines often use combinations of immunogenic peptides to increase the applicability and effectiveness of the immunizations. The immunologic consequences of combining more than 1 self/tumor antigen in a single vaccine emulsion remain unclear, however. We performed 2 sequential clinical trials in patients at high risk for melanoma recurrence. Patients were given the highly immunogenic gp100:209–217(210M) peptide and the less immunogenic tyrosinase:368–376(370D) peptide once every 3 weeks for 4 weeks. This vaccination course was 12 weeks long, and patients were vaccinated for up to 4 courses (16 total vaccinations). In the first trial in 31 patients, the peptides were emulsified separately in incomplete Freund adjuvant and injected at 2 different sites. In the second trial in 33 patients, the peptides were emulsified together and injected at the same site. Cryopreserved lymphocytes were obtained by apheresis after each course and were evaluated for anti-peptide activity using tetramer, enzyme-linked immunospot, and *in vitro* sensitization boost assays. When the peptides were injected at separate sites, robust specific reactivity to the native gp100:209–217 peptide was measured by each of the assays, whereas immunization with the tyrosinase:368–376(370D) peptide was far less effective. When the peptides were emulsified and injected together at the same site, immunization to the gp100:209–217(210M) epitope dropped precipitously, whereas reactivity to the tyrosinase: 368–376(370D) peptide was enhanced. These cautionary data indicate that mixing peptides in the same emulsion can alter reactivity compared with peptides injected separately by mechanisms that may include the induction of localized nonspecific inflammation or competitive binding of peptides to major histocompatibility complex molecules.

Keywords

vaccines; cancer; peptides; melanoma

Attempts to develop therapeutic cancer vaccines are based on stimulating antitumor T cells capable of recognizing cancer antigens.^{1,2} A variety of immunizing vectors have been evaluated in humans, including peptides, proteins, antigen-presenting cells, DNA, and recombinant viruses. Immunization with peptides emulsified in adjuvant is currently the most effective means for the *in vivo* generation of antitumor T cells in humans. The introduction of amino acid substitutions to increase binding to human leukocyte antigens (HLAs) has been used to increase the immunogenicity of peptides.^{3,4} Although no cancer vaccines have yet been demonstrated to mediate cancer regression reproducibly or reduce the rate of tumor recurrence in patients at high risk, the development of cancer vaccines remains a vigorous area of current research.^{5,6}

Clinical trials of multiepitope vaccines have been conducted in humans without evaluating the possible impact of combining peptides on the immunogenicity of the individual peptides in the mixture.^{7–9} The mixing of peptides in multiepitope vaccines has a number of theoretic advantages, including prevention of the escape of tumors after loss of a single epitope or enhancement of the immunogenicity of less immunogenic epitopes by increasing inflammation at the injection site because of highly immunogenic epitopes.

We have now performed 2 sequential trials in patients at high risk for recurrence of melanoma to compare the relative efficacy of immunization with 2 peptides emulsified and injected separately versus immunization with the same 2 peptides emulsified in the same mixture. Surprisingly, the ability of each of the 2 peptides to immunize was substantially altered when they were mixed in the same emulsion.

MATERIALS AND METHODS

Patients and Clinical Protocol

Sixty-four patients with a confirmed diagnosis of melanoma signed an informed consent form and were enrolled in 1 of 2 protocols approved by the Institutional Review Board of the National Cancer Institute. All patients were clinically free of disease based on radiologic studies, including computerized axial tomography of the chest, abdomen, and pelvis and magnetic resonance imaging scans of the brain performed within 6 weeks of protocol entry. All patients expressed HLA-A*0201 as assessed by high-resolution nested-sequence polymerase chain reaction (PCR) subtyping. Patients were eligible for the protocol if they had primary melanomas that were greater than or equal to 1.5 mm in thickness or were ulcerated, if they had 1 or more resected positive lymph nodes, or if they had completely resected metastatic melanoma. All patients were entered into the protocol within 6 months of surgical resection of their melanoma. Other eligibility criteria included serum creatinine of 2 mg/dL or less, total bilirubin of 1.6 mg/dL or less, white blood cell count of 3000 cells/cm or greater, and platelet count of 90,000 cells/cm or greater. Patients with active systemic infections, autoimmune disease, or any known immunodeficiency disease or those who required steroid therapy were not eligible for the trial. No patients had previously been immunized against gp100 or tyrosinase.

In the first protocol, 31 patients received 2 peptides (1 mg each), gp100:209–217(210M) and tyrosinase: 368–376(370D), separately emulsified in incomplete Freund adjuvant and injected subcutaneously in different extremities every 3 weeks for 4 cycles.

In the second protocol, performed sequentially, 33 patients received the 2 peptides (1 mg each) emulsified together in incomplete Freund adjuvant every 3 weeks for 4 cycles. Four cycles comprised 1 treatment course. Patients in both trials were scheduled to receive 4 courses over 48 weeks (16 injections). The patients in protocol 1 were part of a randomized study exploring different schedules of immunization reported elsewhere.¹⁰

In both trials, patients underwent leukapheresis before immunization and 3 weeks after each course of immunization. Peripheral blood mononuclear cells (PBMCs) were cryopreserved at – 180°C after Ficoll-Hypaque separation.

Media and Tissue Culture

T2 cells (peptide transporter–associated protein-deficient T-B hybrid) or C1R-A2 cells (HLA-A/B–deficient B-lymphoblastoid cell line transfected with HLA-A*0201) were pulsed with peptide and used as targets. All cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine (Invitrogen, Carlsbad, CA) or Dulbecco modified Eagle medium (DMEM; Invitrogen) containing 10% heat-inactivated fetal

bovine serum, 2 mM L-glutamine, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer. Human lymphocytes were cultured in complete medium (CM) consisting of RPMI 1640, 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin (Invitrogen), and 10% heat-inactivated human antibody serum (Gemini Bio-Products, Woodland, CA; Valley Biomedical, Winchester, VA).

The CK3H6 lymphocyte clone reactive with the gp100:209–217 peptide and the 1383i lymphocyte line reactive with the tyrosinase 368–376(370D) peptide were grown as previously described.^{11,12}

Immunologic Assays

For all assays, pretreatment and posttreatment cryopreserved PBMC samples from a patient were evaluated simultaneously. All tests were conducted using the native gp100:209–217 peptide and the native tyrosinase: 368–376(370D) peptides as targets.

Enzyme-Linked Immunospot Assay

The enzyme-linked immunospot (ELISpot) assay was performed blindly on coded specimens. In brief, PBMCs were thawed from cryopreservation and cultured overnight in CM. PBMCs were then incubated for 24 hours with peptide-pulsed C1R-A2 cells in 96-well plates coated with anti-interferon- γ (IFN γ) antibody (PharMin-gen), developed with avidin-alkaline phosphatase, and stained. The number of spots per experiment was counted using an Immunospot Analyzer (CTL Analyzers, Cleveland, OH) and was corrected by subtracting background spots resulting from PBMCs incubated with unpulsed C1R-A2 cells.

Tetramer Analysis

PBMCs were Fc receptor–blocked with mouse IgG (Caltag, Burlingame, CA), stained with fluorochrome-labeled gp100:209–217:HLA-A*0201 tetramers (Beckman Coulter Immunomics, San Diego, CA) and antibody against CD8 (BD Biosciences, San Diego, CA), and analyzed by using a FACSCalibur with CELLQUEST (BD Biosciences).

In Vitro Sensitization Assay

As previously described,¹³ PBMCs were cultured in Iscove medium with 10% heat-inactivated human antibody serum with 1 µM specific or control native peptides and 300 IU/mL interleukin (IL)-2. After 11 to 13 days, these T cells were harvested and coincubated with peptide-pulsed T2 cells overnight, and IFN γ release in the supernatant was measured by enzyme-linked immunosorbent assay (ELISA). A positive assay was defined as IFN γ release by postvaccination PBMCs >100 pg/mL during incubation with the native peptide and >2 times the IFN γ released by prevaccination PBMCs with native peptide as well as postvaccination PBMCs plus control peptide.

Peptide Preparation

The gp100:209–217(210M) peptide, IMDQVPFSV, was produced to good manufacturing practice (GMP) grade using solid-phase synthesis techniques by Multiple Peptide Systems (San Diego, CA). The peptide was vialled in 1.5 mL containing 1.5 mg peptide. To prepare the 1-mg peptide dose, 1.5 mL Montanide ISA-51 was combined with 1.5 mL peptide solution and vortexed vigorously for 12 minutes. This procedure produced stable emulsions. Two milliliters was administered in 2 equal volumes of 1 mL each within 2 cm of each other in the same extremity. The tyrosinase:368–376(370D) peptide, YMDGTMSQV, was manufactured by Ben Venue Laboratories, and 1-mg doses were prepared as described previously.

In protocol 2, each of the peptides (1 mg each) was mixed in the same emulsion and administered in 2 equal volumes of 1 mL each within 2 cm of one another in the same extremity.

RESULTS

Patient Characteristics

Characteristics of the 31 patients in protocol 1 who received the peptides in separate emulsions and the 33 patients in protocol 2 who received the peptides combined into the same emulsion are shown in Table 1. All patients had undergone complete resection of all known melanoma and were clinically free of disease at the time of entrance into the immunization protocols.

Assessment of Immunization Using the In Vitro Sensitization Assay

The in vitro sensitization assay was used to assess the ability of the tyrosinase:368–376(370D) peptide and the gp100:209–217(210M) peptide to generate immune precursors (Table 2). When peptides were administered in separate emulsions (protocol 1), the gp100 peptide was more effective than the tyrosinase peptide in successfully immunizing patients as assessed after each immunization course. After 3 and 4 courses, successful immunization against the tyrosinase peptide occurred in 4% and 21% of patients, respectively, compared with 70% and 72% of patients immunized against the gp100 peptide ($P < 0.01$ for both). Because some patients developed disease recurrence during the course of immunization and pheresis samples were not always available after each course, different numbers of patients were assessed after each course, although all available specimens were tested and are included in Table 2.

When the peptides were emulsified together (protocol 2) and lymphocytes were tested after 3 and 4 courses using the in vitro sensitization assay, the incidence of successful immunization against the tyrosinase peptide increased, whereas immunization against the gp100 peptide decreased. After 3 courses of immunization, only 4% of patients were immunized against the tyrosinase peptide in protocol 1 compared with 52% of patients successfully immunized against tyrosinase in protocol 2 ($p = 0.0003$). Similarly after 4 courses, immunization against the tyrosinase peptide increased from 21% in protocol 1 to 54% in protocol 2 ($p = 0.03$). After 3 courses of immunization, reactivity to the gp100 peptide decreased from 70% in protocol 1 to 20% in protocol 2 ($p = 0.001$), and after 4 courses, reactivity of the gp100 peptide decreased from 72% to 42% ($p = 0.07$).

Because these assays had been performed at separate times, samples from patients after 4 immunizations with individual peptides (protocol 1) and with the combined peptides (protocol 2) were simultaneously thawed and tested in the same in vitro sensitization assay. Similar results were obtained. Reactivity against the gp100 peptide was seen in 72% of patients when peptides were administered separately compared with 36% of patients in the combined immunization arm ($p = 0.062$). Conversely tyrosinase reactivity was seen in 17% of patients receiving separate peptides compared with 50% of patients who received the combined peptides ($p = 0.046$). The variability seen is characteristic of the in vitro sensitization boost assay. Although cryopre-served aliquots from the same pheresis are tested, the 12-day culture period can introduce variability between experiments.

Assessment of Immunization Using the Tetramer and ELISpot Assays

The profound decrease in the ability to immunize against the gp100:209–217 peptide when peptides were mixed in the same emulsion was also seen using the tetramer and ELISpot assays. Figures 1 and 2 present the data for each patient tested using the tetramer and ELISpot assays, respectively. The incidences of successful immunization against the gp100 peptide in protocols 1 (separate) and 2 (combined) using the tetramer and ELISpot assay are shown in Figure 3 and Table 3. Using the tetramer assay, rates of successful immunization after 3 and 4 courses of

immunization with the gp100 peptide were 56.0% and 63.2%, respectively, in protocol 1 compared with 13.0% and 17.4% when the peptides were administered in the same emulsion in protocol 2 (see Table 3). Similarly, using the ELISpot assay, separate immunization with the gp100 and tyrosinase peptides resulted in 56.0% and 52.6% rates of successful immunization against the gp100 peptide after 3 and 4 courses compared with 4.2% and 13.0% rates, respectively, when the peptides were combined (see Table 3).

There was an excellent correlation between the results of the tetramer and ELISpot assays after 3 and 4 courses of immunization (Fig. 4; both $P < 0.001$).

In addition to the proportion of patients showing immunization, the intensity of their immunization to the gp100 peptide was also different when comparing protocols 1 and 2 (Table 4). Using the ELISpot assay, after 4 courses of immunization in protocol 1, a mean ($\pm$ standard error of the mean [SEM]) of 118 ± 34 spots/ 10^5 PBMCs was seen compared with 19 ± 11 spots/ 10^5 PBMCs in protocol 2. Similarly, using the tetramer assay after 4 courses, $3.36 \pm 1.19\%$ of tetramer-positive cells was seen in protocol 1 compared with $0.17 \pm 0.16\%$ in protocol 2.

Using the ELISpot assay, none of 25 patients receiving peptides separately developed ≥ 10 spots when tested after 3 or 4 courses against the tyrosinase peptide compared with 8 of 25 patients receiving the mixed emulsion who developed ≥ 10 spots/ 10^5 cells ($P = 0.004$). Reactivity in patients receiving the mixed emulsion was low, however. With the exception of 1 patient with >300 spots/ 10^5 cells, all other patients demonstrated only 10 to 25 spots/ 10^5 cells. Tetramer assays were not conducted to test immunization against the tyrosinase peptide.

Thus, in the in vitro sensitization, ELISpot, and tetramer assays, a substantial decrease in the ability to immunize against the gp100:209–217 peptide was seen when the peptides were combined in the same emulsion with the tyrosinase peptide. Conversely, in the in vitro sensitization and ELISpot assays, immunization against the tyrosinase peptide was increased when it was combined in the same emulsion with the gp100 peptide.

Competition for Binding and Recognition of the gp100 and Tyrosinase Peptides

Experiments were performed to test whether the gp100 and tyrosinase peptides could compete with each other for binding and subsequent recognition of antigen-presenting cells (Fig. 5). The CK3H6 lymphocyte clone that recognized the gp100:209–217 peptide was tested for IFN γ release when incubated for 24 hours with T2 cells pulsed with 0.001 μ M gp100:209–217 peptide in the presence of varying concentrations of the tyrosinase:368–376(370D) peptide. Similar experiments were performed with the 1383i antityrosinase lymphoid line for recognition of T2 cells pulsed with 0.01 μ M tyrosinase peptide in the presence of varying concentrations of the gp100:209–217(210M) peptide. At high concentrations of the competing peptide but not at equimolar concentrations, recognition of each of the cognate peptides was inhibited, providing suggestive evidence that, in vivo, the presence of the 2 peptides emulsified together might well compete with each other for binding to antigen-presenting cells.

Clinical Results

Local inflammatory changes at the site of injection occurred in nearly all the patients at the site of injection of the gp100:209–217(210M) peptides in protocol 1 and at the site of injection of mixed peptides in protocol 2. Only approximately 20% of patients exhibited inflammatory changes at the site of the tyrosinase peptide injection in protocol 1. There was no difference in the length of disease-free survival of patients between protocols 1 and 2, although the sequential nonrandomized nature of this analysis limits the validity of this conclusion (Fig. 6).

DISCUSSION

The characterization of the molecular nature of human cancer antigens stimulated a large number of clinical trials aimed at generating T cells capable of recognizing and destroying tumor in cancer patients.^{1,5} Despite the ability of a variety of immunizing vectors to generate CD8⁺ T cells capable of recognizing immunogenic epitopes on cancer cells, there is currently no cancer vaccine approach that can reliably induce regression of established cancer or reduce the rate of cancer recurrence. Only rare and sporadic objective clinical responses have been seen using cancer vaccines in hundreds of clinical trials.⁵ The successful ability to generate antitumor T cells in cancer patients has continued to spur vigorous attempts to improve on these results, however.

Multiple peptides derived from human cancer antigens have been identified, and immunization with these peptides, often in immune adjuvants, is a particularly effective way to generate CD8⁺ and CD4⁺ antitumor T cells in patients. The optimal methods for immunizing against these peptides have not been clearly determined. Multiple adjuvants have been studied, including incomplete Freund adjuvant, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-12, QS-21, and others.^{13–18} In addition, many workers have mixed peptides and administered them together because of the theoretic advantage gained by preventing the escape of tumors after loss of a single epitope.^{7–9} Thompson et al¹⁹ suggested that mixing peptides did not affect the ability of the peptides to immunize successfully.

In a prior study, we evaluated the ability to immunize against 2 immunogenic epitopes present on the gp100 and tyrosinase human melanoma antigens, respectively.¹⁰ The heteroclitic gp100:209–217(210M) peptide, modified from the naturally presented gp100:209–217 peptide by substitution of a methionine for a threonine in the 210 amino acid position,³ and the naturally posttranslationally modified tyrosinase: 368–376(370D) peptide²⁰ were evaluated in a randomized trial comparing 3 different schedules of immunization. Results from that trial demonstrated that the gp100 peptide was far more immunogenic than the tyrosinase peptide. In addition, local inflammation was often seen at the site of the gp100 peptide and rarely at the site of the tyrosinase peptide injected in a different extremity.

Because of the intense inflammation often seen at the site of the immunization with the gp100 peptide, we hypothesized that this inflammatory environment might promote immunization to the tyrosinase peptide if injected in the same emulsion at the same site. We thus performed a sequential study in 33 melanoma patients using a mixture of the gp100 and tyrosinase peptides injected in the same emulsion and compared the results of the use of these same 2 peptides immunized in separate emulsions. Three different assays were used to assess the immune reactivity to the peptides, including an in vitro sensitization boost assay as well as ELISpot and tetramer assays. Each assay measures a different aspect of the antitumor immune response.

Surprisingly, using all 3 assays, mixing the 2 peptides together in the same emulsion substantially reduced the degree of immunization tested against the native immunogenic gp100:209–217 peptide. A decrease was seen when assessing the number of patients immunized as well as the intensity of the immunization. Protocols 1 and 2 were conducted sequentially, and a prospective randomized trial evaluating peptides injected separately or together would be required to evaluate this phenomenon definitively. The in vitro assays were performed on cryopreserved specimens at the same time to minimize the effects of this nonrandomized comparison, however.

Interestingly, using the in vitro sensitization assay, the mixture of peptides in the same emulsion resulted in an increase in the ability to immunize against the tyrosinase peptide as hypothesized. Quite unpredictably, however, immunization of the gp100 peptide was substantially decreased at the same time.

The mechanism to explain why immunization with mixed peptides presented by the same HLA molecule seemed to interfere with each other is unknown. Competition of peptides for binding to major histocompatibility complex (MHC) molecules on an antigen-presenting cell has been described and can lead to immunodominance of some peptides compared with others.²¹ In our in vitro studies, the gp100 and tyrosinase peptides competed with each other for recognition when pulsed onto HLA-A*0201-positive T cells. It is thus possible that the presence of the tyrosinase peptide in the same emulsion as the gp100 peptide led to decreased presentation of the gp100 peptide in vivo, resulting in decreased immunization to this peptide. Simultaneously, the inflammatory reaction to the gp100 peptide seen in both protocols could have resulted in an increase in immunization against the tyrosinase peptide.

These findings have significance for multiple ongoing efforts using multiepitope vaccines in cancer patients. Many published and ongoing studies use mixtures of peptides injected together without carefully analyzing the impact of each of the peptides on the ability to immunize against the others. Careful attention should be paid to the possible interactions of peptides in these widely used multiepitope peptide vaccines.

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FIGURE 1.

Evaluation using the tetramer assay for the gp100:209–217 peptide of patients receiving immunization with the gp100:209–217(210M) and tyrosinase:368–376(370D) peptides separately (protocol 1) or combined in the same emulsion (protocol 2). Each dot represents the measurement of an individual patient. Analysis after 3 courses (top) and after 4 courses (bottom) is shown.

FIGURE 2.

Evaluation using the ELISpot assay for gp100:209–217-specific T cells of patients receiving immunization separately (protocol 1) or combined in the same emulsion (protocol 2). Each dot represents the measurement of an individual patient. Analysis after 3 courses (top) and after 4 courses (bottom) is shown.

FIGURE 3.

Incidence of positive immunization against the gp100:209–217 peptide in patients receiving the gp100 and tyrosinase peptides injected at separate sites (protocol 1) or combined in the same emulsion (protocol 2). Top, Assessment of patients using the tetramer assay. Positive immunization was defined as $>0.05\%$ of $CD8^+$ cells capable of binding the native gp100:209–217 tetramer. Bottom, Results using the ELISpot assay. Positive immunization was defined as greater than 20 spots/ 10^5 cells against the specific immunizing peptide.

FIGURE 4.

A high correlation was seen between the results obtained in both protocols using the ELISpot and tetramer assays to measure anti-gp100:209–217 reactivity after 3 courses (top) and after 4 courses (bottom) (both $P = 0.001$). Each dot represents simultaneous measurements on the same patient.

FIGURE 5.

Top, CK3H anti-gp100:209–217 clone was tested for IFN γ release against T2 cells pulsed with 0.001 μ M gp100:209–217(210M) peptide in the presence of varying concentrations of the tyrosinase:368–376(370D) peptide. Bottom, 1383i anti-tyrosinase line was tested against T2 cells pulsed with the tyrosinase peptide at 0.01 μ M in the presence of varying concentrations of the gp100 peptide. Each peptide competed with the others for recognition.

FIGURE 6. Disease-free survival of patients in protocol 1 (separate) and protocol 2 (combined). No significant difference was seen.

TABLE 1**Patient Characteristics**

	Protocol 1 Separate Peptides	Protocol 2 Combined Peptides
No. patients (%)	31 (100%)	33 (100%)
Sex		
Male	16 (52%)	23 (70%)
Female	15 (48%)	10 (30%)
Age (y)		
21–30	2 (6%)	0 (0%)
31–40	3 (10%)	10 (30%)
41–50	8 (26%)	11 (33%)
51–60	12 (39%)	7 (21%)
61–70	5 (16%)	5 (15%)
>70	1 (3%)	0 (0%)
Eligibility criteria		
Resected primary	14 (45%)	11 (33%)
Positive nodes	13 (42%)	22 (67%)
Resected metastases	4 (13%)	0 (0%)
No. positive lymph nodes		
0	18 (5%)	11 (33%)
1–2	9 (29%)	18 (55%)
3–4	4 (13%)	3 (9%)
5–6	0 (0%)	0 (0%)
7–8	0 (0%)	0 (0%)
9–10	0 (0%)	0 (0%)
>10	0 (0%)	1 (3%)

TABLE 2

In Vitro Sensitization Assay Comparing Separate Immunization With Combined Immunization of the Gp100 and Tyrosinase Peptides

Course	Protocol 1 Separate Peptides	Protocol 2 Combined Peptides
	No. Patients	Positive/Total
Reactivity to Tyrosinase 368–376 (370D) Peptide		
Pre	0/18 (0%)	1/30 (1%)
1	6/21 (29%)	7/20 (35%)
2	6/19 (32%)	11/29 (38%)
3	1/23 (4%)	13/25 (52%) $p_2 = 0.0003$
4	4/19 (21%)	13/24 (54%) $p_2 = 0.03$
Reactivity to gp 100:209–217 Peptide		
Pre	0/23 (0%)	0/30 (0%)
1	14/22 (64%)	4/20 (20%) $p_2 = 0.006$
2	14/22 (64%)	7/29 (24%) $p_2 = 0.009$
3	16/23 (70%)	5/25 (20%) $p_2 = 0.001$
4	13/18 (72%)	10/24 (42%) $p_2 = 0.07$

TABLE 3

Incidence of Immunization Against the Gp100:209–217 Peptide (% positive patients)

Course	ELISpot [*]		Tetramer [†]	
	Separate	Combined	Separate	Combined
0	3.6%	7.7%	6.9%	0%
1	22.2%	0%	37.9%	0%
2	36.0%	6.3%	50.0%	0%
3	56.0%	4.2%	56.0%	13.0%
4	52.6%	13.0%	63.2%	17.4%

^{*} Positive defined as >20 spots/10⁵ PBMCs.

[†] Positive defined as >0.05% CD8⁺ cells.

TABLE 4

Immunization Against the gp100:209–217 Peptide

Course	ELISpot		Tetramer	
	Separate (no. spots/10 ⁵ , mean ± SEM)	Combined	Separate (% CD8 ⁺ cells, mean ± SEM)	Combined
0	5 ± 3	4 ± 2	0	0
1	17 ± 8	4 ± 2	0.32 ± 0.14	0
2	57 ± 27	3 ± 2	0.64 ± 0.24	0
3	87 ± 29	11 ± 6	1.90 ± 0.54	0.13 ± 0.11
4	118 ± 34	19 ± 11	3.36 ± 1.19	0.17 ± 0.16