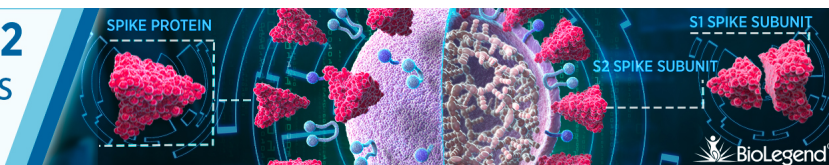


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Tumor Progression Can Occur despite the Induction of Very High Levels of Self/Tumor Antigen-Specific CD8⁺ T Cells in Patients with Melanoma

Steven A. Rosenberg,¹ Richard M. Sherry, Kathleen E. Morton, William J. Scharfman,² James C. Yang, Suzanne L. Topalian, Richard E. Royal, Udai Kammula, Nicholas P. Restifo, Marybeth S. Hughes, Douglas Schwartzentruber, David M. Berman,³ Susan L. Schwarz, Lien T. Ngo, Sharon A. Mavroukakis, Donald E. White, and Seth M. Steinberg⁴

The identification of many tumor-associated epitopes as nonmutated “self” Ags led to the hypothesis that the induction of large numbers of self/tumor Ag-specific T cells would be prevented because of central and peripheral tolerance. We report in this study on vaccination efforts in 95 HLA-A*0201 patients at high risk for recurrence of malignant melanoma who received prolonged immunization with the “anchor-modified” synthetic peptide, gp100_{209–217(210M)}. Vaccination using this altered peptide immunogen was highly effective at inducing large numbers of self/tumor-Ag reactive T cells in virtually every patient tested, with levels as high as 42% of all CD8⁺ T cells assessed by tetramer analysis. From 1 to 10% of all CD8⁺ cells were tumor-Ag reactive in 44% of patients and levels >10% were generated in 17% of patients. These studies were substantiated using the ELISPOT assay and a bulk cytokine release assay. Although our data regarding “tumor escape” were inconclusive, some patients had growing tumors that expressed Ag and HLA-A*0201 in the presence of high levels of antitumor T cells. There was no difference in the levels of antitumor Ag-specific T cells in patients who recurred compared with those that remained disease-free. Thus, the mere presence of profoundly expanded numbers of vaccine-induced, self/tumor Ag-specific T cells cannot by themselves be used as a “surrogate marker” for vaccine efficacy. Further, the induction of even high levels of antitumor T cells may be insufficient to alter tumor progression. *The Journal of Immunology*, 2005, 175: 6169–6176.

Since the identification of tumor-associated Ags more than a decade ago, cancer vaccines aimed at the induction of robust tumor-specific T cell responses have been a focus of translational cancer immunotherapies (1, 2). Multiple immunization strategies have been evaluated including the use of recombinant viruses encoding cancer Ags, immunization with whole tumor cells, Ag-loaded dendritic cells, recombinant proteins, or immunogenic peptides derived from cancer Ags. These efforts have resulted in generally low rates of immunization as assessed using a variety of techniques (3, 4) and objective clinical responses to vaccines used alone are rare and sporadic (reviewed in Refs. 5–7). Immunizations with peptide in saline without adjuvant, used alone or in combination with vaccines encoded by an avian poxvirus, have confirmed the poor T cell responses—even among vaccinated patients showing a detectable CTL response, many have tumor-specific T cell responses ranging between 0.001 and 0.001% of CD8⁺ T cells. Immunization with avipox and vaccinia virus encoding carcinoembryonic Ag (8) or

prostate-specific Ag (9, 10) or peptides from melanoma-melanocyte Ags generally results in antitumor precursor frequencies of <0.01% (11, 12). Despite these wide ranging and ongoing efforts, objective clinical responses are not reliably induced and patient benefit has not been reproducibly demonstrated with current vaccines (7). Although little definitive data exists, some workers have suggested that Ag-specific CD8⁺ T cell levels in the range of 1% will be necessary to mediate tumor regression (13, 14).

The self nature of many of the potentially most useful tumor Ags has led us to question whether antitumor vaccine responses can be generated at high levels, or whether the mechanisms of central and peripheral tolerance would intrinsically limit the levels of antitumor T cells. To test this question, we studied vaccine optimization in 95 patients who had previously been diagnosed with melanoma and were without clinical evidence of disease but were at high risk for recurrence. This patient population enabled us to perform repetitive immunizations over 48 wk. Because altered peptide ligands may be useful in obtaining strong antiself immune response (15), we immunized patients with a heteroclitic version of the gp100 self/tumor Ag. The immunogen was a synthetic peptide comprised of an altered form of the gp100_{209–217} epitope that contains a threonine→methionine substitution (16). This modified epitope has a slower off-rate than the native epitope (17), but its anchor modification does not alter the external structure of the MHC class I/peptide complex as shown in a recently obtained crystal structure of the native and modified epitopes (18).

Materials and Methods

Patients and clinical protocol

Ninety-five HLA-A*0201 patients with a confirmed diagnosis of melanoma were enrolled in this protocol. All patients were clinically free of

Surgery Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

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¹ Address correspondence and reprint requests to Dr. Steven A. Rosenberg, National Cancer Institute, National Institutes of Health, Clinical Research Center, Room 3W/3-3940, 10 Center Drive, Bethesda, MD 20892-1202. E-mail address: sar@nih.gov

² Current address: Johns Hopkins Medical Center, Baltimore, MD 21287.

³ Current address: Laboratory of Pathology, National Cancer Institute, Bethesda, MD 20892.

⁴ Current address: Biostatistics and Data Management Section, National Cancer Institute, Bethesda, MD 20892.

disease and eligible for the protocol if they 1) had primary melanomas ≥ 1.5 mm of thickness or were ulcerated, 2) had one or more resected positive lymph nodes, or 3) had completely resected metastatic melanoma. All patients were entered into the protocol within 6 mo of surgical resection of their melanoma. No patients had previously been immunized with gp100 or tyrosinase.

Patients received 1 mg of gp100_{209–217(210M)} and tyrosinase_{368–376(370D)} emulsified separately in IFA and injected s.c. in different extremities. Patients were prospectively randomized into one of the following three arms of the study. In Arm 1, a course consisted of weekly injections for 10 wk followed by a 3-wk break. In Arm 2, a course consisted of injections every 3 wk for four injections. In Arm 3, a course consisted of injection of the peptides for 4 days in a row every 3 wk for four cycles. Patient characteristics for each arm are shown in Table I. All patients were scheduled to receive four courses over 48 wk. Patients underwent leukapheresis before immunization and 3 wk after each course of immunization. PBMC were cryopreserved at -180°C after Ficoll-Hypaque separation.

Toxicities seen in patients receiving peptide immunization were local induration and erythema at the injection site, generally only at the site of the gp100_{209–217(210M)} peptide. Local discomfort caused 16 patients to stop peptide administration before completing the full four courses (three in Arm 1, five in Arm 2, and eight in Arm 3).

Media and tissue culture

Melanoma cell lines 888mel(A2⁻), 938mel(A2⁻), 624mel(A2⁺), and 526mel(A2⁺) were grown from resected specimens. T2 cells (peptide transporter-associated protein-deficient T-B hybrid) or C1R-A2 (19) cells were pulsed with peptide and used as targets. All cell lines were cultured in either RPMI 1640 supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine (Invitrogen Life Technologies) or DMEM (Invitrogen Life Technologies) containing 10% heat-inactivated FBS, 2 mM L-glutamine, and 10 mM HEPES buffer. Human lymphocytes were cultured in complete medium (CM) consisting of RPMI 1640, 2 mM L-glutamine, 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen Life Technologies), and 10% heat-inactivated human AB serum (Gemini Bio-Product; Valley Biomedical).

Immunologic assays

For all assays, pretreatment and posttreatment cryopreserved PBMC samples from a patient were evaluated simultaneously.

ELISPOT assays. The ELISPOT assay was performed blindly on coded specimens (20). In brief, PBMC were thawed from cryopreservation and cultured overnight in complete medium at a density of 10^5 cells/well. PBMC were then incubated for 24 h with peptide-pulsed C1R-A2 cells in 96-well plates coated with anti-IFN- γ Ab (BD Pharmingen), developed

with avidin-alkaline phosphatase, and stained. The number of spots per experiment were counted using a Immunospot Analyzer (CTL Analyzers) and were corrected by subtracting background spots resulting from PBMC incubated with unpulsed C1R-A2 cells.

Tetramer analysis. PBMC were FcR-blocked with mouse IgG (Caltag Laboratories), stained with fluorochrome-labeled gp100:209–217:HLA-A*0201 tetramers (Beckman Coulter Immunomics) and Ab against CD8 (BD Biosciences) and analyzed by using a FACSCalibur with CellQuest (BD Biosciences).

In vitro sensitization assay. In vitro sensitization assays were performed as previously described (21). Briefly, PBMC were cultured in Iscove's medium with 10% heat-inactivated human AB serum with 1 μM specific or control native peptides and 300 IU/ml IL-2. After 11–13 days, these T cells were harvested and cocultured with peptide-pulsed T2 cells overnight and IFN- γ release in the supernatant measured by ELISA. A positive assay was defined as IFN- γ release by postvaccination PBMC >100 pg/ml during incubation with the native peptide and more than two times the IFN- γ released by prevaccination PBMC with native peptide.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissues were subject to Ag retrieval in 10 mM citrate, pH 6.0, with 0.1% Tween 20. The slides were then immunostained using the Ventana DAB detection system with Abs to HLA-DR (TAL.1B5; DAKO); gp 100(HMB45; ENZO); Tyrosinase (T311; Vision Biosystems); MHC-1(HC-10; kindly provided by Dr. S. Ferrone, Roswell Park Cancer Institute, Buffalo, NY) and MART-1. Both positive and negative controls were included with each reaction and all slides were reviewed by one pathologist (D. M. Berman). Intensity of immunoreactive staining was graded using a four-tier system: 0, 1+ (weakly visible at $\times 400$), 2+ (weakly visible at $\times 40$), and 3+ (strongly visible at $\times 40$). Percent immunoreactivity of viable cells was graded using a four-tier system (0, $<5\%$, 5–50%, $>50\%$).

Peptide preparation

The gp100_{209–217(210M)} peptide, IMDQVPFSV, was produced to GMP grade by solid phase synthesis techniques by Multiple Peptide Systems. The peptide was vialied in 1.5 ml containing 1.5 mg of peptide. To prepare the 1-mg peptide dose, 1.5 ml of montanide ISA-51 were combined with 1.5 ml of the peptide solution and vortexed vigorously for 12 min. Two vials were administered in two 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 above.

Statistical analysis

Analyses were performed to compare log 10 assay values in each of the posttreatment courses to the corresponding pretreatment values to identify

Table I. HLA-A2 adjuvant protocol

Schedule	Arm1 (qw)	Arm2 (q3w)	Arm3 (qdx4 q3w)
	Number of patients (%)		
	31 (100)	31 (100)	33 (100)
Sex			
Male	17 (55)	16 (52)	24 (73)
Female	14 (45)	15 (48)	9 (27)
Age			
21–30	1 (3)	2 (6)	0 (0)
31–40	6 (19)	3 (10)	4 (12)
41–50	11 (35)	8 (26)	10 (30)
51–60	9 (29)	12 (39)	8 (24)
61–70	4 (13)	5 (16)	10 (30)
Over 70	0 (0)	1 (3)	1 (3)
Eligibility criteria			
Resected primary	6 (19)	14 (45)	5 (15)
Positive nodes	19 (61)	13 (42)	23 (70)
Resected mets	6 (19)	4 (13)	5 (15)
Number of positive lymph nodes			
0	12 (39)	18 (5)	10 (30)
1–2	16 (52)	9 (29)	10 (30)
3–4	2 (6)	4 (13)	5 (15)
5–6	0 (0)	0 (0)	4 (12)
7–8	0 (0)	0 (0)	3 (9)
9–10	0 (0)	0 (0)	1 (3)
>10	1 (5)	0 (0)	0 (0)

whether any of the three arms were associated with a stronger tendency than others to produce immunologic changes. The five changes in assay values over time (each course plus the 1 year assay) were compared in a pairwise fashion for all three assay types using the Wilcoxon rank sum test. An exception was made for the analysis of post cycle one data involving the tetramer assay and Arm 3. Because virtually all of these changes were zero, the fractions of outcomes increasing in value were compared between Arm 3 and both Arms 1 and 2 used the χ^2 test. A formal adjustment for multiple hypothesis tests was not made and thus only p values <0.01 should be interpreted as being reasonable evidence for a statistically significant difference between arms. Values of p between 0.01 and 0.05 suggest a strong trend.

To determine whether immunologic parameters changed as the number of courses increased, comparison of course 2 to course 1, course 3 to course 2, and course 4 to course 3 were performed for all three assays types and all three arms of the trial. The differences in the log 10 paired values were assessed using the Wilcoxon signed rank test using a two tailed p value. As above, in view of the multiple comparisons, any results with a p value <0.01 were interpreted as being statistically significant. Correlation between actual immune results from the three assay types were done pairwise by arm, separately at each of the postadministration time points using Spearman nonparametric rank correlation. Only correlations such that $r_s > 0.70$ would be interpreted as being strong correlations, while those such that $0.5 < r_s < 0.7$ would be moderately strong, $0.3 < r_s < 0.5$ would be weak to moderately strong, and those with $r_s < 0.3$ would be considered weak. In addition, because later courses were based only on patients who had not progressed, the results based on earlier courses are likely to be

more representative of the results which could be obtained in all patients on the trial.

Results

Immunization with altered peptide in adjuvant is highly effective at inducing tumor-specific T cells

We sought to study the impact of a highly effective vaccination in the setting of minimal residual disease. Recent papers have described repeated immunization of peptides in saline in the absence of immunological adjuvants (22–24). Tumor-specific T cell precursors in patients vaccinated with peptide alone without adjuvant are reportedly very low ($p < 0.01\%$ in the peripheral blood). We tested pre- vs postimmunization with the optimized anchor-modified gp100_{209-217(210M)} peptide immunogen in saline using either T2 target cells alone, pulsed with the native gp100₂₀₉₋₂₁₇ peptide or an irrelevant epitope gp100:280–288. We found no evidence that the peptide was immunogenic when injected in saline using a total of 16 immunizations in four patients (data not shown). These data are consistent with those in most immunological studies which find that peptide immunization is ineffective at inducing T cells in the absence of adjuvant.

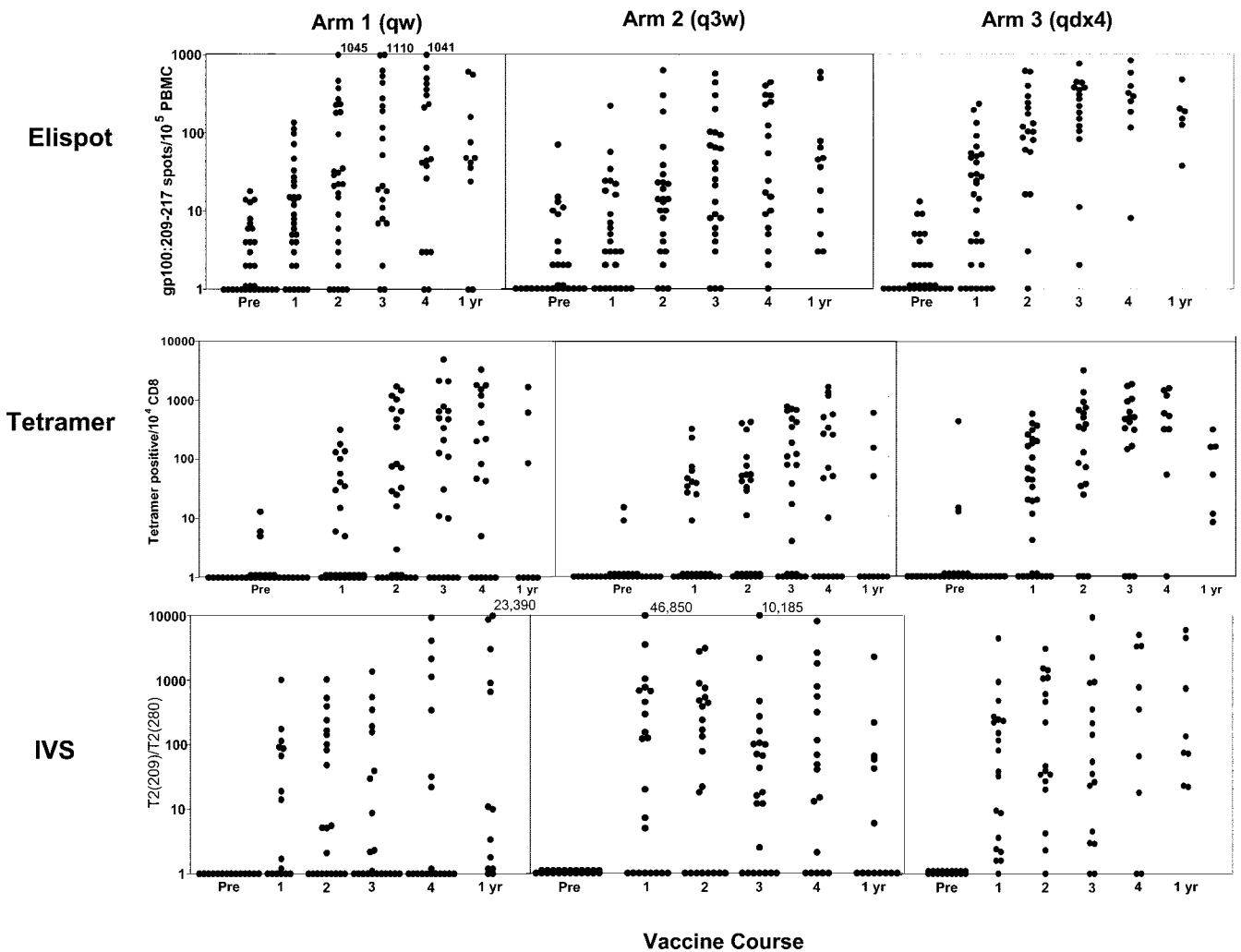


FIGURE 1. Evidence for the generation of high self/tumor Ag-specific T cells using multiple assays. Results of the ELISPOT assay (*upper*), the tetramer assay (*middle*), and the in vitro sensitization boost assay (*lower*) are shown. Each dot represents results from an individual patient. Lymphocytes were obtained by apheresis before all immunizations and 3 wk following each of the first four courses of immunization as well as 1 year after the last administration of peptide. As noted in the text, increasing immunization was seen throughout three courses but little advantage was seen with a fourth course of immunization. In addition, Arm 3 appeared to be more effective in generating precursor T cells compared with Arms 1 and 2.

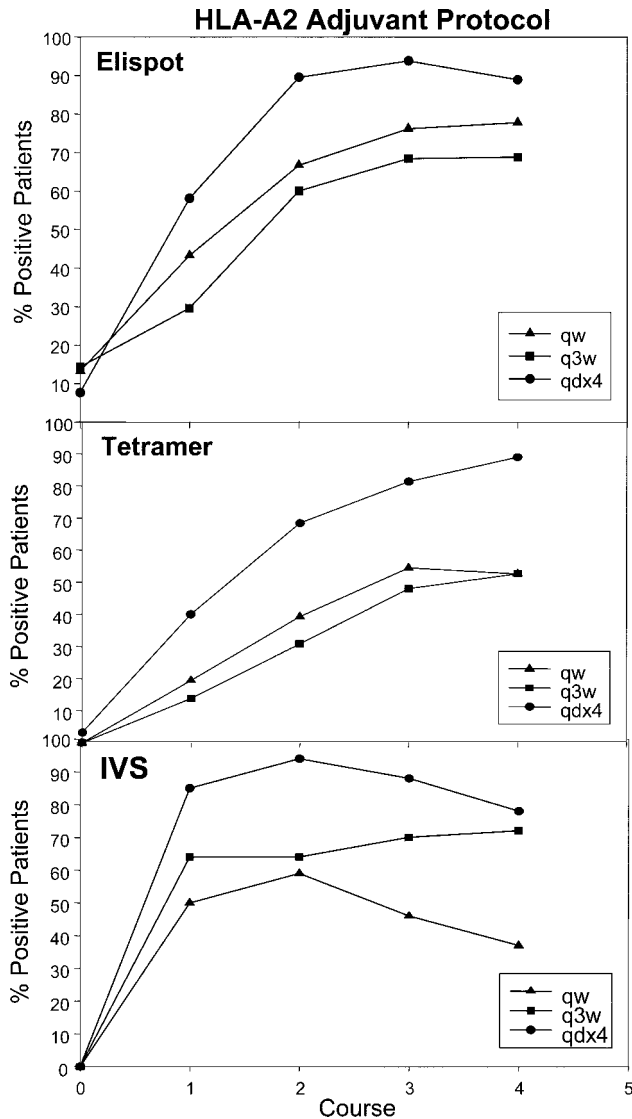


FIGURE 2. The frequency of patients with evidence of positive immunization in each of the three arms of the protocol. Arm 1 (qw), Arm 2 (q3w), Arm 3 (qdx4). Positive in the ELISPOT assay was defined as ≥ 10 spots per 10^5 PBMC and more than or equal to twice background (*upper*). Positive in the tetramer assay was defined as $\geq 0.5\%$ of all $CD8^+$ cells (*middle*). A positive assay was defined as IFN- γ release by postvaccination PBMC >100 pg/ml during incubation with the native peptide and more than two times the IFN- γ released by prevaccination PBMC with native peptide.

We and others have previously observed that immunization using peptide in adjuvant dramatically increases the effectiveness of vaccination. The absence of measurable disease in the current patients made it possible to design immunization schema that were much longer and involved repeated immunization. Pre- and postimmunization samples were thawed and tested simultaneously.

In each of the three arms of the study (see *Materials and Methods*) patients were scheduled to receive four courses over 48 wk. All tests were performed against the native peptides rather than the modified peptides. The results of assays on individual patients are shown using the ELISPOT assay (Fig. 1, *upper*), tetramer assay (Fig. 1, *middle*), and the in vitro sensitization assay (Fig. 1, *lower*). Minimal or no activity was seen in all assays in the pretreatment samples. The ELISPOT and tetramer assays that allowed enumeration of reactive or peptide-binding cells revealed that 53–89% of all patients were eventually successfully immunized to the native gp100 peptide (Fig. 2 and Table II). Surprisingly high levels of immunization were seen after prolonged (three or four courses) immunization. Using the tetramer assays, 44% of patients achieved 1–10% of $CD8^+$ cells that recognized the native peptide and 17% of patients achieved levels $>10\%$ of all $CD8^+$ cells (Table II). The ELISPOT assay that measured IFN- γ release from total PBMC also revealed high levels of immunization with 53% achieving levels greater than 0.1% of PBMC ($CD8^+$ cells were $\sim 10\%$ of all PBMC). Statistical analysis of the effect of increasing courses of immunization on the generation of immune precursors as measured by the ELISPOT assay indicated that two courses of immunization provided increased precursors compared with one course in all three arms ($p = 0.009$, $p = 0.0004$, $p = 0.0006$, respectively) and three courses were better than two courses ($p = 0.009$, $p = 0.06$, $p = 0.002$, respectively). Using the tetramer assay, two courses were an improvement compared with one course in Arms 1 and 3 only ($p = 0.007$ and $p = 0.0002$, respectively) and three courses appeared superior to two courses for Arms 1 and 2 only ($p = 0.01$, $p = 0.02$, respectively). No difference was seen when comparing the first, second and third courses using the in vitro sensitization assay. In none of the assays were four courses of immunization significantly superior to three courses.

Analysis of the ability of each of the three immunization arms to generate precursors against gp100 revealed that Arm 3 often showed a significant improvement or a trend toward improvement in precursor generation compared with Arms 1 and 2 tested by both the ELISPOT and tetramer assay ($P_2 = 0.0005$ to 0.09 for several of the comparisons performed at each of the four courses

Table II. Results of ELISPOT and tetramer assays before immunization and after three or four courses

	Arm1		Arm2		Arm3		Total	
	Pre (n = 31)	Post 3–4 (n = 22)	Pre (n = 28)	Post 3–4 (n = 25)	Pre (n = 31)	Post 3–4 (n = 16)	Pre (n = 90)	Post 3–4 (n = 63)
ELISPOT assay								
Spots/ 10^5 PBMC								
<10	27 (87%)	4 (18%)	23 (82%)	7 (28%)	30 (97%)	1 (6%)	80 (89%)	12 (19%)
10–100	4 (13%)	7 (32%)	5 (18%)	10 (40%)	1 (3%)	1 (6%)	10 (11%)	18 (29%)
10–1,000	0	10 (45%)	0	8 (32%)	0	14 (88%)	0	32 (51%)
>1,000	0	1 (5%)	0	0	0	0	0	1 (2%)
Tetramer assay %								
$CD8^+$ cells								
<0.1%	30 (97%)	6 (27%)	28 (97%)	9 (36%)	28 (90%)	2 (12%)	86 (95%)	17 (27%)
0.1–1%	1 (3%)	3 (14%)	1 (3%)	4 (16%)	2 (7%)	0	4 (4%)	7 (9%)
1.1–10%	0	8 (36%)	0	9 (36%)	1 (3%)	11 (69%)	1 (1%)	28 (44%)
>10%	0	5 (23%)	0	3 (12%)	0	3 (19%)	0	11 (17%)

of immunization). There was never a significant difference between Arms 1 and 2. Fewer than 10% of patients exhibited evidence of immunization against the tyrosinase peptide using ELISPOT or in vitro sensitization assays even after four courses.

Fig. 3 presents combined patient data across all time points to display the overall assay results on large numbers of individual PBMC samples. There was often a strong correlation between the results on large numbers of individual PBMC samples. There was often a strong correlation between the results of the ELISPOT and tetramer assays (Fig. 3, upper), with individual correlations of $r_s = 0.41, 0.75, 0.79,$ and 0.87 postcourses 1 through 4, respectively. Similarly, the in vitro sensitization assay generally tended to be moderately well correlated with both the tetramer assay (Fig. 3, middle; $r_s = 0.16, 0.57, 0.57,$ and 0.62) and with the ELISPOT assay (Fig. 3, lower; $r_s = 0.40, 0.61, 0.55,$ and 0.67). As shown, substantially more scatter was seen in these latter two figures. Each of these comparisons were highly significant when analyzing patients after three or four courses of immunization individually ($p < 0.001$).

PBMC that are highly reactive to peptide can react to tumor cells

As shown above, the majority of patients immunized with the modified gp100_{209–217(210M)} peptide developed reactivity against the native gp100_{209–217} peptide in tetramers or when pulsed onto APCs in the ELISPOT and in vitro sensitization assays as measured by IFN γ secretion. To test the ability of PBMC to react to melanoma tumor cells, 17 patients who developed highly reactive lymphocytes against peptide after four courses were tested in an ELISPOT assay simultaneously against peptide pulsed C1R-A2 APCs as well as two HLA-A2⁺ melanoma cell lines (526mel and 624mel) and two HLA-A2⁻ melanoma lines (888mel and 938mel) (Table III). Values twice both controls are underlined. None of the PBMC obtained before immunization recognized the native peptide or tumor. All 17 patients showed high degrees of specific reactivity against the gp100_{209–217} peptide compared with the control gp100_{280–288} peptide. Six of these 17 patients showed specific reactivity against the specific peptide and against both of the HLA-A2⁺ melanoma cell lines while showing only background levels of reactivity against the HLA-A2⁻ lines. Patients with the highest levels of IFN- γ secretion to peptide tended to recognize tumors. If cytokine secretion to peptide was < 700 pg/ml none of nine PBMC recognized tumor compared with six of eight that recognized tumor when secretion to peptide exceeded 700 pg/ml. It should be emphasized that this tumor recognition was seen in PBMC without any in vitro boost sensitization.

A similar study was conducted using the in vitro sensitization assay. PBMC from 62 patients were tested after three or four courses of immunization. After stimulation with $1 \mu\text{M}$ of the gp100_{209–217(210M)} peptide, 40 (65%) developed reactivity against $1 \mu\text{M}$ of peptide pulsed on T2 cells, 63% were reactive against $0.1 \mu\text{M}$ peptide, 53% against $0.01 \mu\text{M}$ peptide, 40% against $0.001 \mu\text{M}$, and 15% against $0.0001 \mu\text{M}$ peptide. Thirty-three of the 62 patients (53%) developed specific reactivity to melanoma cell lines after 11–13 days of incubation. In a detailed study of five patients in this protocol Powell et al. (25) demonstrated that prolonged immunization led to a phenotypic shift of peptide-specific CD8 T cells from an early effector to an effector memory (CD27⁻, CD28⁻, CD62L⁻, CD45RO⁺) phenotype that correlated with peptide-specific T cell precursor frequencies.

Thus, following in vitro stimulation with peptide in the presence of 300 IU of IL-2/ml, antitumor reactivity was generated in PBMC from about half of immunized patients.

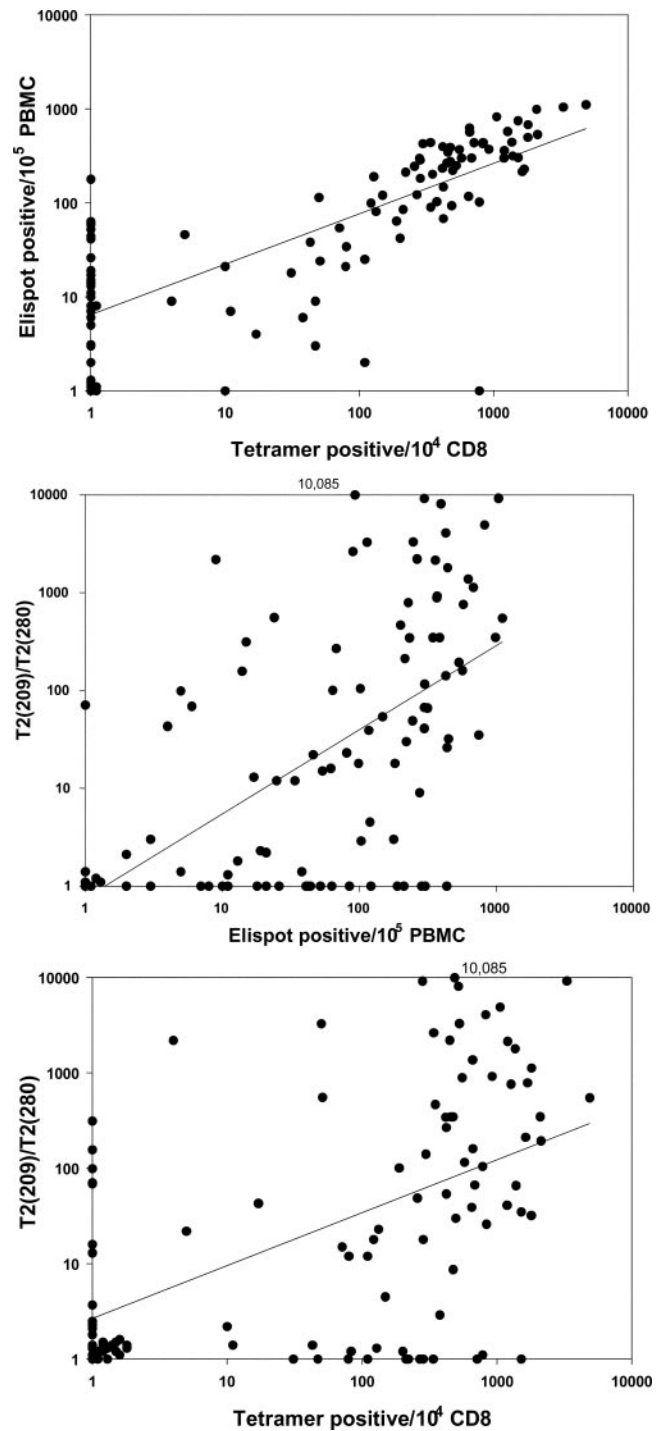


FIGURE 3. Correlation of assays. The correlation of the ELISPOT and tetramer assays (upper), of the in vitro sensitization and tetramer assays (middle), and of the in vitro sensitization and ELISPOT assays (lower) are shown taking all patients after three and four courses. Each dot represents an individual patient tested with each of these assays. In general, correlations were moderately strong (see Results).

Tumor recurrence in patients following immunization

This clinical trial was not designed to test whether peptide immunization could decrease the rate of recurrence in this high risk population because all patients received the peptides. However, despite the generation of high levels of anti-tumor Ag specific T cells, 45 to 65% of patients recurred at 3 years. The actuarial disease free survival curves of patients with resected primaries, with

Table III. Recognition of peptide and tumor before and after immunization: ELISPOT assay

Patient	Pre								After Three or Four Courses							
	CIRA2 + peptide			Melanoma					CIRA2 + peptide			Melanoma				
	0	280	209	526	624	888	938	0	280	209	526	624	888	938		
				(A2 ⁺)	(A2 ⁺)	(A2 ⁻)	(A2 ⁻)				(A2 ⁺)	(A2 ⁺)	(A2 ⁻)	(A2 ⁻)		
(Number spots/10 ⁵ PBMC)																
1	24	24	28	7	10	6	10	10	8	<u>290</u> ^a	41	41	5	7		
2	10	8	11	7	11	5	1	7	3	<u>321</u>	22	29	5	8		
3	26	17	26	8	10	5	1	25	18	<u>1160</u>	<u>432</u>	<u>227</u>	15	93		
4	21	17	25	79	152	75	26	10	10	<u>266</u>	220	300	161	63		
5	12	7	8	9	3	7	3	8	8	<u>509</u>	3	4	0	4		
6	33	30	47	17	5	5	9	52	24	<u>489</u>	89	37	7	44		
7	84	62	79	211	24	18	24	13	15	<u>577</u>	284	117	11	150		
8	24	9	23	14	20	5	8	5	3	<u>507</u>	97	34	0	69		
9	35	22	17	5	30	6	8	2	0	<u>710</u>	42	17	0	24		
10	16	15	11	12	14	16	5	67	48	<u>636</u>	105	36	19	67		
11	5	5	5	9	11	12	4	15	10	<u>782</u>	<u>299</u>	<u>334</u>	30	15		
12	12	11	18	11	14	18	11	11	13	<u>725</u>	88	143	11	7		
13	13	10	13	13	13	18	4	29	20	<u>1486</u>	<u>219</u>	<u>234</u>	50	38		
14	5	5	5	8	17	20	11	2	3	<u>377</u>	56	52	31	16		
15	13	9	9	6	8	20	10	13	4	<u>1474</u>	<u>613</u>	<u>574</u>	25	24		
16	20	16	20	20	21	32	35	33	15	<u>2082</u>	<u>627</u>	<u>773</u>	43	39		
17	8	9	10	11	31	20	18	6	3	<u>831</u>	<u>113</u>	<u>165</u>	28	23		

^a Values twice both controls are underlined.

positive lymph nodes, or with resected metastases are shown in Fig. 4, *left*, and the recurrence rates for patients in each of the three immunization arms are shown in Fig. 4, *right*. There were no significant differences in recurrence rates among the three arms. The apparent lower disease-free survival seen in Fig. 4, *right*, for patients in Arm 3 could be accounted for by the increased number of patients with large numbers of positive lymph nodes that randomized to this arm of the treatment protocol (Table I).

To estimate whether tumor recurrence was influenced by the level of antitumor T cells, we evaluated patients who received at least three full courses of immunization. There was no statistically significant difference in the number of CTL precursors in patients who remained disease free compared with patients who recurred using the ELISPOT assay (215 ± 35 vs 152 ± 44 spots per 10^5 PBMC) or using the tetramer assay (5.0 ± 1.0 vs $3.5 \pm 1.3\%$ of $CD8^+$ cells), respectively. Thus, although surprisingly high levels of anti-peptide T cells were generated as a result of this prolonged

immunization, there was little apparent effect of the T cells on the incidence of recurrent tumors.

Immunohistochemical analysis of Ag expression on recurrent tumors that could be resected revealed that some recurrent tumors expressed both gp100 and HLA class I Ags. These high levels of target Ags were expressed by tumors in patients with high levels of circulating anti-gp100 T cell precursors based on ELISPOT and tetramer assays including one of the most highly immunized patients with 15.2% of all $CD8^+$ cells that were tetramer positive (Table IV).

Discussion

We vaccinated 95 HLA-A*0201⁺ patients at high risk for the recurrence of melanoma with three vaccination schedules in a randomized clinical trial. We found that very high levels of T cells specific for self/tumor peptide gp100₂₀₉₋₂₁₇ can be achieved using repeated immunization schedules that use an altered form of the

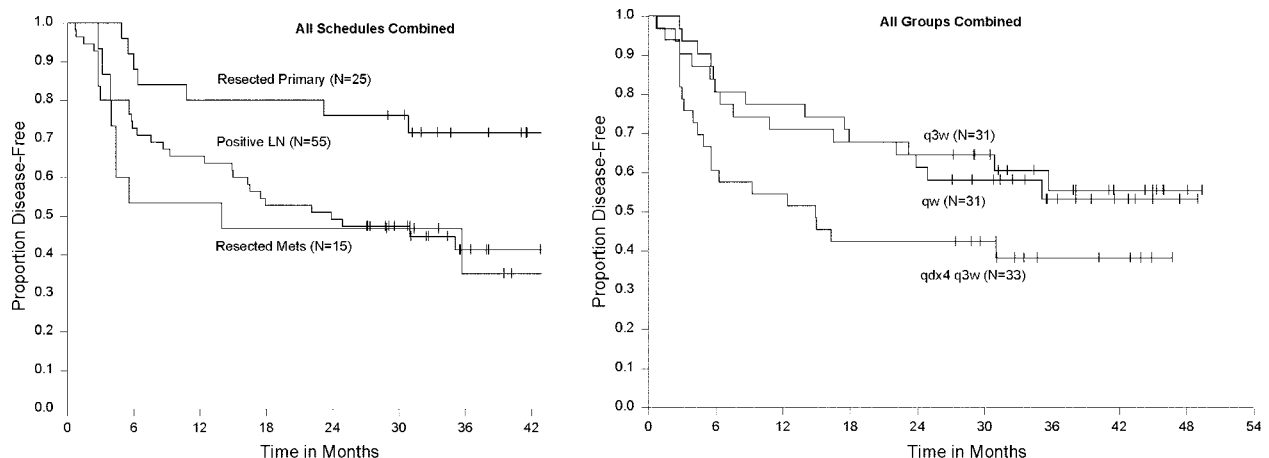


FIGURE 4. Survival curves. Actuarial curves of disease-free survival of patients in the three eligibility groups (*left*) and in the three (*right*) immunization arms of the protocol.

Table IV. Patients with recurrence after ≥ 2 courses of immunization

	Course	Intensity/% Cells Positive				ELISPOT (per 10^5 PBMC)	Tetramer (% of CD8 ⁺)
		gp100	MART1	Tyrosinase	MHC1		
Arm1(qw)	4	3/>50%	0/0	0/0	0/0	42	2.01
	4	2/>50%	2/>50%	0/0	1/<5%	12	2.21
	2	0/0	0/0	2/>50%	3/>50%	4	0.29
	2	3/>50%	1/>50%	3/>50%	1/>50%	0	0
	2	2/>50%	3/>50%	3/>50%	1/<5%	0	0
	4	1/5–50%	3/>50%	3/>50%	0/0	606	16.70
	4	3/>50%	3/>50%	3/>50%	2/>50%	160	0
	4	3/>50%	3/>50%	2/>50%	2/5–50%	303	15.2
Arm2/(q3w)	4	0/0	0/0	0/0	3/>50%	245	2.56
	4	1/5–50%	0/0	3/>50%	2/>50%	24	0.51
	4	0/0	1/>50%	0/0	3/>50%	228	16.85
	4	3/>50%	3/>50%	3/>50%	3/>50%	9	0.47
	4	0/0	3/>50%	3/>50%	3/>50%	0	0
Arm3(qdx4)	4	3/5–50%	1/>50%	3>50%	3>50%	114	0.5
	4	2/>50%	n.d.	0/0	0/0	15	13.89
	2	0/0	0/0	0/0	3/<5%	16	0.01

self/tumor peptide in which methionine is substituted for threonine at position 210.

These self/tumor Ag-specific T cells were revealed using three independent assessments of the immune response. First, we used a highly sensitive assay in which PBMC were stimulated *in vitro* with peptide. We found that the vast majority of patients could be immunized, as assessed by the specific production of γ -IFN. Second, we used a tetramer assay containing the native peptide, which enumerates the number and phenotype of T cells with specificity for the epitope in question. Repeated immunization lead to levels of self/tumor epitope-specific CD8⁺ T cells in excess of 1% in 61% of the patients studied and >10% in 17% of the patients studied—levels far higher than those seen after immunization with other self/tumor peptides. Third, we confirmed similar levels using the ELISPOT assay, which enables the determination of the number of PBMC capable of secreting IFN- γ when stimulated with the native peptide. Levels of reactivity to HLA-A*0201-matched tumors were also high.

Some patients had melanoma recurrences despite the presence of very high levels of tumor-reactive T cells. We did not observe predominance at sites of immune privilege such as the brain. Although some lesions that recurred failed to express the target Ag or the restricting MHC class I, there were at least four examples where recurrent tumors stained with Abs specific for both the gp100 target Ag and MHC class I Ags. It is not possible to determine whether the immunization schedules we performed resulted in any immunoselection of Ag loss variants. Tumor escape mutations can result from a myriad of potential mechanisms (26). Instead, our findings simply make the point that the mere presence of T cells capable of reacting with self/Ag in humans is insufficient to prevent tumor recurrence. Thus, T cell induction cannot be a “surrogate marker” for the success of an anticancer vaccine.

Immune-mediated destruction of tissues such as tumors or allografts is dependent on the generation of T cells capable of recognizing immunogenic determinants on the target tissues. Thus, considerable effort has been devoted to evaluating a variety of immunization approaches directed against growing cancers in humans. Although many early approaches used immunization against whole tumor cells or subcellular components the molecular identification of tumor Ags has led to attempts to more selectively immunize against defined tumor Ags. Immunization strategies have included the use of recombinant viruses encoding cancer Ags,

Ag-loaded dendritic cells, recombinant proteins, heat shock proteins, or immunogenic peptides derived from cancer Ags. Several recent reviews of the administration of these more selective cancer vaccines in humans have been published (7, 7, 27, 28). Immunization with peptides in adjuvant has been most successful in generating measurable levels of antitumor T cells. Although a variety of adjuvants have been evaluated in immunizing with peptides including IFA (12, 29, 30), GM-CSF (11, 23), QS-21 (12), IL-2 (21), IL-12 (31), and others we selected emulsification in IFA because of the known ability of this adjuvant to generate T cells in our pilot studies (21). Because many tumor Ags are generally non-mutated normal proteins, modification of anchor amino acids in immunogenic peptides can significantly enhance the ability to generate T cells reactive with the native peptides (16, 21).

Despite a major effort to evaluate a wide variety of cancer vaccines, none have been reproducibly successful in mediating the regression of metastatic cancer and only rare and sporadic objective responses have been seen. A review of over 1200 patients treated with vaccines in our own and other institutions revealed an overall objective response rate of 3.3% when using standard World Health Organization or Response Evaluation Criteria in Solid Tumors (RECIST) criteria (7). An explanation for the lack of clinical effectiveness despite the ability to generate antitumor T cells *in vivo* has been the subject of considerable immunologic investigation but has thus far been elusive.

A significant problem confronting the administration of molecularly defined vaccines in patients with metastatic cancer is the inability to perform prolonged immunization. We used patients who were disease free but at high risk for recurrence because this enabled the testing of vaccines in the minimal disease setting and made possible prolonged immunization for up to one year. It is possible that immunotherapy approaches using vaccines may be more effective in the adjuvant rather than in the metastatic disease setting although there are few, if any, examples in oncology that demonstrate that treatments ineffective for patients with metastatic disease from solid tumors are effective for the treatment of patients with subclinical disease in the adjuvant setting.

The present study demonstrates that it is possible to administer multiple immunizations over a prolonged time in the adjuvant setting. Prolonged immunization resulted in surprisingly high levels of anti-peptide T cells. Tumor progression restricts the ability to

perform multiple immunizations in patients with widespread metastases. Prospective randomized trials with large numbers of patients will be required to determine whether these prolonged peptide immunization regimens can reduce tumor recurrence.

A variety of new cancer vaccine approaches are under active investigation. Although it is not clear that the simple induction of large numbers of antitumor T cells will result in therapeutic effectiveness, it may be possible that qualitatively different and more effective T cells can be induced. The use of new adjuvants, such as new TLR agonists that activate innate immunity (32), the simultaneous stimulation with agonistic Abs such as anti-4-1BB to stimulate CD8 cells, the use of other adjuvants such as IL-15 (33), or the blockade of immunoregulatory components such as TGF- β , IL-10 or IL-13, or CD4⁺/CD25⁺ regulatory cells may increase the likelihood that vaccines can be effective in the future.

Several recent findings have provided encouragement that immunotherapeutic approaches can be effective in mediating cancer regression. The blockade of a negative costimulatory molecule, CTLA-4, using a mAb has been shown to result in cancer regression in patients with metastatic melanoma (34). The adoptive transfer of tumor-reactive T cells following lymphodepleting chemotherapy can mediate the regression of metastatic cancer in approximately half of heavily pretreated patients with metastatic melanoma (35). Animal models predict that concurrent vaccination can substantially enhance the clinical effectiveness of such immunotherapies based on the adoptive transfer of anti-tumor T cells (36).

Disclosures

The authors have no financial conflict of interest.

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