Vaccine-induced CD8+ T-cell Responses to MAGE-3 Correlate with Clinical Outcome in Patients with Melanoma[1]

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ABSTRACT

Purpose: Vaccine-induced antitumor CD8+ T-cell responses are believed to play an important role in increasing resistance to melanoma. The following study was conducted to examine whether these responses are associated with improved clinical outcome in melanoma vaccine-treated patients.

Experimental Design: We measured CD8+ T-cell responses to gp100, MART-1, MAGE-3, and tyrosinase by enzyme-linked immunospot assay in peripheral blood of 131 *HLA-A*01*- or *HLA-A*02*-positive melanoma patients before and after immunization to a polyvalent, shed antigen, melanoma vaccine, and correlated the results with clinical outcome.

Results: Fifty-six percent of patients had a vaccine-induced CD8+ T-cell response to at least one of the four antigens. Recurrences were significantly reduced in patients with vaccine-induced responses to MAGE-3 (hazard ratio, 0.42; 95% confidence interval, 0.18–0.99; P = 0.03) by the Cox proportional hazard model but were unrelated to responses to the other three antigens. Patients with a preexisting response to any of the four antigens were significantly more likely to have a further vaccine-boosted response to that same antigen (P < 0.0001–0.036).

Conclusions: There was a correlation between vaccine-induced CD8+ T-cell responses to melanoma-associated antigens and improved clinical outcome, but the correlation depended on the antigen against which the response is di- rected. The only significant correlation was with responses to MAGE-3.

INTRODUCTION

It is generally accepted that CD8+ T-cell responses are an important indicator of the effectiveness of cancer vaccines. However, there is little information demonstrating that the induction of such responses correlates with improved clinical outcome. Two recent reviews of the assays most frequently used to measure vaccine-induced CD8+ T-cell responses (1, 2) concluded that although there are several promising methods, additional studies are needed to evaluate their value due to the small numbers of patients that have been studied.

One of the most promising methods to measure antigen-specific CD8+ T-cell responses is the ELISPOT[3] assay. It has been used to monitor several early-phase cancer vaccine trials (3–8), and several groups, including ours (9, 10), have found that it can detect vaccine-induced CD8+ T-cell responses. In some trials, the induced CD8+ T-cell responses correlated with improved clinical outcome (3, 5, 9), but in all cases, the numbers of patients evaluated were too small to draw statistically reliable conclusions about the value of the assay in predicting clinical outcome.

In this study, we have taken advantage of a large number of patients that have been treated with a polyvalent, shed antigen, melanoma vaccine to examine whether there is a correlation between CD8+ T-cell responses induced by the vaccine and clinical outcome in *HLA-A*01*- and *HLA-A*02*-positive patients.

MATERIALS AND METHODS

Patients. The study was conducted on peripheral blood samples from 139 patients with surgically resected melanoma who were treated with a polyvalent, shed antigen, melanoma vaccine at the New York University Kaplan Comprehensive Cancer Center. All patients signed informed consent approved by the New York University School of Medicine Institutional Board of Research Associates and were *HLA*A*02- and/or *HLA*A*01-positive. HLA typing was performed by complement-mediated cytotoxicity by the Rogosin Institute (New York, NY).

Vaccine Treatment. All patients were immunized to a polyvalent melanoma vaccine prepared from antigens shed into culture medium by a pool of melanoma cell lines as described previously (11, 12). The vaccine contains multiple melanoma-associated antigens, including the antigens used in this study, *i.e.,* tyrosinase, gp100, MART-1, and MAGE-3 (9, 10). For administration, it was combined with either alum or interleu-

[3] The abbreviations used are: ELISPOT, enzyme-linked immunospot assay; AJCC, American Joint Committee on Cancer; CI, confidence interval.

kin-2 liposomes (13) as adjuvants. Immunizations were given intradermally into all four extremities, every 2–3 weeks × 4, monthly × 3, every 3 months × 2, and then every 6 months for 2 years or until disease progression. Some patients were also treated with a low-dose (1–10 million units) of IFN-α-2b administered three times a week for the duration of the trial. Peripheral blood was collected before immunization, and one week after the fourth immunization. Mononuclear cells were separated on Ficoll-Hypaque and frozen in liquid N_2 until used.

ELISPOT Assay. The number of peptide-specific CD8+ T cells in peripheral blood was determined by ELISPOT assay as described previously (9, 10, 14). Briefly, 96-well polyvinylidene difluoride filter plates (Millipore, Bedford, MA) were precoated with monoclonal antibody to human IFN-γ (BioSource, Camarillo, CA). SFM20-A2 targets (9) were used for peptides presented by *HLA-A*02*, and HT-144 targets (American Type Culture Collection, Manassas, VA) were used for those presented by *HLA-A*01*. The target cells were pulsed with 20 nM test peptide before the addition of effectors. Monoclonal antibody IVA12 (anti-HLA-DR, -DP, -DQ from American Type Culture Collection; HB145) was added to all wells to prevent presentation of HLA class II antigens by targets or residual monocytes. Effector peripheral blood mononuclear cells were thawed, depleted of monocytes on plastic, and added to wells. They were incubated for 48 h at 37°C and 5% CO_2, washed with PBS-Tween 20, and incubated overnight with goat anti-IFN-γ (R&D, Minneapolis, MN), followed by alkaline phosphatase-conjugated donkey antigoat (Jackson ImmunoResearch, West Grove, PA). Spots, visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (KPL, Gaithersburg, MD) and representing individual T cells that had been stimulated by peptides to release IFN-γ, were counted on the automated KS ELISPOT system (Zeiss, Thornwood, NY). The criterion for a minimum positive response was an increase of at least 5 peptide-specific CD8+ T cells per 500,000 peripheral blood lymphocytes in the post-fourth vaccination blood sample compared with baseline (preimmunization) level in the same patient. Both baseline and postvaccination measurements were conducted simultaneously on the same ELISPOT plate, and the clinical outcome of the patients was unknown at the time of the assays.

Peptides were prepared as described previously (14). Patients who were *HLA-A*01* positive were tested for CD8+ T-cell responses directed against peptides from MAGE-3$_{(168-176)}$ (15), tyrosinase$_{(240-251)}$ (16), and influenza-NP$_{(44-52)}$ (17). Those who were *HLA-A*02* positive were tested for responses against peptides from gp100$_{(178-186)}$, gp100$_{(280-288)}$, gp100$_{(457-466)}$, gp100$_{(570-578)}$, and gp100$_{(585-593)}$ (10, 18, 19); MART-1$_{(27-35)}$ (20); MAGE-3$_{(159-169)}$, MAGE-3$_{(188-198)}$, and MAGE-3$_{(271-279)}$ (10, 21); tyrosinase$_{(2-10)}$ and tyrosinase$_{(487-495)}$ (10); and influenza-M1$_{(58-66)}$ (22). Influenza-NP$_{(44-52)}$ and influenza-M1$_{(58-66)}$ were used as positive controls.

Statistical Analysis. The Cox proportional hazards model was used to evaluate the association between vaccine-induced CD8+ T-cell responses and other potential prognostic factors with recurrence-free survival. Recurrence-free survival was calculated from the date of assessment of vaccine-induced CD8+ T-cell response (*i.e.,* 1 week after the fourth immunization) to the date of recurrence, death, or last follow-up visit,

Table 1 Patient characteristics ($n = 131$)

Parameter	n (%)
Age[a] (yrs)	
Median (range)	53.2 (18.0–77.3)
<47	40 (30.8%)
47–60	47 (36.2%)
>60	43 (33.1%)
Sex	
Male	83 (63.4%)
Stage	
IIb–IIIa	65 (49.6%)
IIIb	41 (31.3%)
IV	25 (19.1%)
Clinically palpable nodes[a]	
No	89 (70.6%)
Yes	37 (29.4%)
Histologically positive nodes[a]	
0	42 (35.3%)
1	39 (32.8%)
>1	38 (31.9%)
Site of primary[a]	
Limbs	42 (35.6%)
Trunk	38 (32.2%)
Head & neck	38 (32.2%)
Thickness of primary[a] (mm)	
Median (range)	2.9 (0.4–20)
<2	42 (35/6%)
2–4	38 (32.2%)
>4	38 (32.2%)

[a] Missing for some subjects.

whichever occurred first. Date of measurement of CD8+ T-cell response was chosen as the start date rather than date of first immunization because the primary objective of this study was to assess the prognostic value of the vaccine-induced response. The two continuous variables, age and thickness of primary, were assessed both as continuous variables and classified in tertiles. The hazard ratio, obtained from the Cox model, is the ratio of the instantaneous rate of recurrence in patients within a specific level of a prognostic variable to the rate of recurrence in patients in the reference category for this same prognostic variable, adjusted for other variables included in the model. The binomial test was used to assess whether the proportion of vaccine-induced responders differed in patients who recognized the antigens preimmunization from those who did not.

RESULTS

Patient Characteristics. The study was conducted on 139 patients with resected melanoma who were participating in melanoma vaccine trials between May 1995 and December 1999. Two patients who did not have their responses assessed within the 3 months after their first immunization and six patients who had a recurrence within 3 months were excluded, leaving 131 patients for the analysis. The characteristics of the patients and their disease are provided in Table 1. The median lag time between baseline and assessment of vaccine-induced response was 7 weeks (range, 6–12 weeks). Sixty-three percent were male. Fifty percent had AJCC stage IIb (primary melanoma 4 mm or thicker) or stage IIIa melanoma (nodes clinically negative and <2 histologically positive), 31% had stage IIIb disease (nodes clinically positive or 2 or more histologically positive), and 19% had stage IV (distant metastases)

Table 2 Frequency of pre-existing CD8+ T cell responses to specific antigens

| Patient's HLA allele | n | % of patients with preexisting CD8+ T-cell responses at baseline | | | | | |
		gp100	MART-1	MAGE-3	Tyrosinase	Any[a]	Influenza
*A*01*	54			30	30	32	81
*A*02*	87	30	24	21	20	31	74
*A*01/A*02*	131[b]	30	24	24	24	32	73

[a] Excluding influenza.
[b] Ten patients were positive for both *A*01* and *A*02*.

Table 3 Frequency of vaccine-induced, peptide-specific, CD8+ T-cell responses to specific antigens

| Patient's HLA allele | n | % of patients with vaccine-induced CD8+ T-cell response[a] | | | | | |
		gp100	MART-1	MAGE-3	Tyrosinase	Any[b]	Influenza[c]
*A*01*	54			30	28	52	43
*A*02*	87	30	18	15	14	49	63
*A*01/A*02*	131[d]	30	17	19	18	56	75

[a] Vaccine-induced response is defined as >5 peptide-specific IFN-γ-producing cells/500,000 peripheral blood mononuclear cells above baseline recognition.
[b] Excluding influenza.
[c] Positive at post time point.
[d] Ten patients were positive for both *A*01* and *A*02*.

melanoma. All of the patients had resected disease except for nine of the stage IV patients who had measurable disease. Of the 131 patients, 44 were *HLA-A*01* positive, and 77 were *HLA-A*02* positive. There were 10 patients who were positive for both alleles.

CD8+ T-cell Responses. CD8+ T-cell responses were measured at baseline (before initiation of vaccine treatment) and 1 week after the fourth immunization using the panel of peptides described in "Materials and Methods." At the time of the study, there were no *HLA-A*01*-restricted peptides identified for either gp100 or MART-1. Therefore, we tested these patients for responses to only MAGE-3 and tyrosinase. The panel of *HLA-A*02*-restricted peptides we used consisted of the most immunoreactive peptides from gp100, MART-1, MAGE-3, and tyrosinase that we have identified in prior studies (10). We used *HLA-A*02*- and *HLA-A*01*-restricted influenza peptides as positive controls.

The frequency of CD8+ T-cell responses existing before vaccine treatment is shown in Table 2. A response was considered positive to a melanoma antigen if one or more peptides derived from that antigen were recognized. Thirty-two percent of the patients had a preexisting CD8+ T-cell response to at least one peptide from at least one of the four antigens studied. The recognition of individual antigens ranged from 20–30%. Seventy-three percent of the patients recognized an influenza peptide positive control.

The proportion of patients who developed vaccine-induced CD8+ T-cell responses to each antigen is shown in Table 3. Overall, 56% of the 131 patients developed a response to at least one antigen. Responses in *HLA-A*01*-positive patients were directed almost equally to MAGE-3 (30%) and tyrosinase (28%). Responses in *HLA-A*02*-positive patients were most often directed to gp100 (30%) and least frequently directed to tyrosinase (14%). The median and range of vaccine-induced CD8+ T-cell responses to individual antigens (number of cells/500,000 peripheral blood mononuclear cells) were as follows: gp100, 13.1 (5.1–30.8);

MART-1, 12.7 (5.0–45.5); MAGE-3, 14.3 (5.2–89.2); and tyrosinase, 13.4 (6.0–31.7). The influenza control median and range were 15.0 (5.2–82.6) in postimmunization samples. Some patients (<5%) had a decrease in CD8+ T-cell responses after the fourth immunization that was greater than 5 peptide-specific cells/500,000 peripheral blood mononuclear cells. The percentages of such patients were 1.2% to gp100, 4.5% to MART-1, 3.8% to MAGE-3, and 3.0% to tyrosinase. These decreased responses were counted as negative in subsequent analyses.

Correlation of Vaccine-induced CD8+ T-cell Responses with Recurrence-free Survival. We performed an initial analysis of risk factors that are commonly associated with recurrence-free survival: age, stage of disease, sex, presence of histologically positive nodes, number of clinically positive nodes, and thickness and site of the primary lesion. Only one variable, stage of disease, was significantly associated with recurrence-free survival in that stage IV patients were more likely to recur earlier than those in other stages ($P = 0.03$). There was also a trend for older patients to recur sooner ($P = 0.06$). Recurrence-free survival was not associated with whether or not the patient was *HLA-A*01* positive ($P = 0.34$) or *HLA-A*02* positive ($P = 0.46$) or whether the patient was or was not concurrently treated with IFN-α-2b ($P = 0.32$).

Overall, there was a trend for association between a vaccine-induced CD8+ T-cell response to at least one antigen and prolonged recurrence-free survival ($P = 0.08$; see Table 4). The correlation was dependent on the antigen to which the CD8+ T-cell response was directed. When the responses to individual antigens were analyzed, there was a highly significant association between vaccine-induced CD8+ T-cell responses to MAGE-3 and delayed recurrence ($P = 0.009$), and the hazard ratio for recurrence was 0.37 (95% CI, 0.18–0.87). When the response to MAGE-3 was adjusted for age and stage of disease using multivariate analysis, it still remained significant ($P =$

Table 4 Relationship between vaccine-induced CD8+ T-cell responses to individual melanoma-associated antigens and recurrence-free survival by Cox's proportional hazards model

Factor	Hazard ratio (95% CI)	P
Univariate analysis		
Stage[a]	1.47 (1.05–2.05)	0.03
Age[b]	1.37 (0.98–1.92)	0.06
Any vaccine-induced CD8+ T-cell response[c]	0.59 (0.33–1.06)	0.08
" " to gp100[d]	0.58 (0.27–1.22)	0.13
" " to MART-1[e]	0.66 (0.28–1.56)	0.32
" " to MAGE-3	0.37 (0.18–0.87)	0.009
" " to tyrosinase[f]	1.02 (0.51–2.05)	0.96
Multivariate analysis (adjusted for stage and age)		
Any vaccine-induced CD8+ T-cell response[c]	0.68 (0.37–1.22)	0.19
" " to gp100[d]	0.63 (0.23–1.33)	0.21
" " to MART-1[e]	0.63 (0.26–1.50)	0.27
" " to MAGE-3	0.42 (0.18–0.99)	0.03
" " to tyrosinase[f]	1.11 (0.55–2.25)	0.77

[a] Stage was divided into three groups by AJCC staging [low risk (IIb–IIIa), moderate risk (IIIb), and high risk (IV)].

[b] Age was divided into tertiles (<47, 47–60, and >60 years).

[c] Vaccine-induced CD8+ T-cell response to any of the four antigens.

[d] Based on 92 subjects.

[e] Based on 100 subjects.

[f] Based on 119 subjects.

0.03) with a hazard ratio of 0.42 (95% CI, 0.18–0.99). There was also a trend between vaccine-induced responses to gp100 and MART-1 and an improved recurrence-free survival (hazard ratios of 0.63 for both by multivariate analysis), but the differences were not statistically significant. There was no correlation between responses to tyrosinase and clinical outcome (hazard ratio, 1.02).

To see whether there were clinical differences between the patients who responded to MAGE-3 by vaccine immunization and those who did not respond, we compared the two groups for factors associated with early recurrence. There were no differences in age, sex, clinical protocol, stage of disease, or thickness of primary lesion between MAGE-3 responders and nonresponders (See Table 5) other than their MAGE-3 response and prolonged length of time until recurrence.

The importance of a MAGE-3 response was further evidenced by analysis of 17 patients who recurred while receiving vaccine treatment and then continued on vaccine treatment but with the concurrent addition of low doses of IFN-α-2b. All of these patients had advanced but nonbulky disease (*i.e.,* AJCC stage IIIb or IV). Only two of the six patients who had a vaccine-induced CD8+ T-cell response to MAGE-3 after continued immunization relapsed again within 12 months, whereas all 11 patients who were lacking a MAGE-3 response relapsed within this time frame. This difference was significant by Fisher's exact test ($P < 0.01$). However, we cannot formally exclude the possibility that these differences in outcome resulted from differences in extent of disease between CD8+ T-cell responders and nonresponders.

Probability of a Vaccine-induced Response to Previously Recognized Antigens. Because we tested pre- and postimmunization responses simultaneously to each antigen, we were able to examine whether initial recognition of an antigen was associated with subsequent recognition of that antigen after immunization. As shown in Table 6, we found that the patients were significantly more likely to increase their response to an

Table 5 Characteristics of patients with and without vaccine-induced CD8+ T-cell responses to MAGE-3

Factor	MAGE-3 (+) response (n = 26)	MAGE-3 (−) response (n = 105)	P[a]
Age (yrs)			
Mean	50.4	53.0	0.38[b]
Sex			
Male	13 (50%)	70 (66.7%)	0.50[c]
Clinical protocol			
Alum	3 (27.3%)	8 (73.8%)	0.62[c]
Interleukin 2 liposomes	19 (18.1%)	86 (81.9%)	
IFN Rx	4 (26.7%)	11 (73.3%)	
Stage			
IIb–IIIa	15 (57.7%)	50 (47.6%)	0.47[c]
IIIb	8 (30.8%)	33 (31.4%)	
IV	3 (11.5%)	22 (21.0%)	
Thickness of primary (mm)			
Mean	3.27	3.96	0.41[b]
<2	10 (28.6%)	32 (33.0%)	0.43[c]
2–4	6 (47.6%)	32 (33.0%)	
>4	5 (23.8%)	33 (33.0%)	

[a] P for test of no difference in factors in MAGE-3 responders *versus* nonresponders.

[b] One-way ANOVA.

[c] χ^2 test (likelihood ratio).

antigen postimmunization if it was recognized preimmunization. This was true for all four antigens ($P = 0.036$ to <0.0001), and in nearly every case, the vaccine-enhanced recognition was to the same peptide.

DISCUSSION

Our most important finding is that there was a correlation between vaccine-induced CD8+ T-cell responses to melanoma-associated antigens and improved clinical outcome. The strength of the correlation depended on the antigen against which the

Table 6 Correlation between preexisting CD8+ T-cell response to an antigen and vaccine-induced response to that antigen

Preexisting CD8+ T-cell response	Posttreatment vaccine-induced CD8+ T-cell response[a]			
	gp100 (n = 92)	MART-1 (n = 92)	MAGE-3 (n = 131)	Tyrosinase (n = 119)
Present	52%	32%	41%	45%
Absent	22%	13%	13%	10%
P	0.004	0.036	0.0007	<.0001

[a] >5 vaccine-induced CD8+ T cells above the preexisting level of response to that same antigen (usually the same peptide).
[b] Number of patients evaluated.

response was directed, with the strongest correlation being for CD8+ T-cell responses directed against MAGE-3.

Ability to stimulate antigen-specific CD8+ T-cell responses is one of the important parameters used to evaluate the immunological activity of cancer vaccines (2). However, there is little information on whether these responses correlate with the clinical effectiveness of the vaccine (2). Only one small study thus far has reported immune responses that significantly correlated with improved survival in patients after treatment with a cancer vaccine (23). Several other studies have reported the development of immune responses in patients who were treated with vaccines (3, 5, 9), but these observations were based on small numbers of patients and were not subjected to statistical analysis.

In this study, we took advantage of a large number of patients treated with a polyvalent, shed antigen, melanoma vaccine to evaluate the correlation between vaccine-induced CD8+ T-cell responses to several melanoma-associated antigens and clinical outcome. The vaccine appears to be clinically effective, as shown in a small, double blind and placebo-controlled trial in resected AJCC stage IIIb melanoma patients. Vaccine-treated patients had a median recurrence-free survival more than twice as long as that of patients treated with a placebo vaccine. The difference was statistically significant ($P = 0.03$; Ref. 24).

The vaccine contains multiple melanoma-associated antigens including MAGE-3, MART-1, gp100, and tyrosinase, the antigens against which CD8+ T-cell responses were measured in this study. These responses were measured by ELISPOT, first described by Czerkinsy *et al.* (25) to measure antigen-specific B cells. Many investigators have recently adapted the assay to measure small numbers of antigen-specific CD8+ T cells in the circulation (3–10). We selected this assay to measure antigen-specific CD8+ T cells over the two other methods most currently used because the ELISPOT is more sensitive than the tetramer assay, and the results are more reproducible than the cytokine-flow cytometry assay (2).

As observed previously (9, 10, 14), we found that immunization with the vaccine could induce specific CD8+ cells to at least one of the four antigens studied in approximately half of the patients. Responses were induced in patients who were *HLA-A*01* positive as well as in those who were *HLA-A*02* positive, with little difference in the frequency of responses between these two groups. However, in *HLA-A*02*-positive patients, there were differences in the ability of the vaccine to induce CD8+ T-cell responses to the different antigens, with

responses induced most often to gp100 and least often to tyrosinase.

Overall, there was a correlation between the ability of the vaccine to induce CD8+ T-cell responses and improved clinical outcome, but the correlation was entirely dependent on the antigen to which the response was directed. There was a strong and statistically significant correlation between vaccine-induced CD8+ T-cell responses to MAGE-3 and delayed tumor recurrence, with the chance of recurrence in these patients being one-third that of nonresponders. This difference remained statistically significant after Cox multivariate analysis ($P = 0.03$). Patients with vaccine-induced CD8+ T-cell responses to gp100 and MART-1 were also less likely to develop recurrent disease, but the improvement was not statistically significant. However, because we did not test responses to all known CD8+ T cell-reactive gp100 or MART-1 peptides including gp100$_{(209-217)}$ or gp100$_{(154-163)}$ (26, 27), it is possible that the frequency of responses to these antigens would have been higher than we observed and/or that there would have been a stronger correlation with clinical outcome than we observed. There was no correlation between vaccine-induced CD8+ T-cell responses to tyrosinase and clinical outcome. It is interesting that the only significant correlation between vaccine-induced CD8+ T-cell responses and clinical outcome was with MAGE-3, because of the four antigens, this is the one that was the most selectively associated with melanoma. The other three antigens (MART-1, gp100, and tyrosinase) are normal differentiation antigens, which are also expressed in melanocytes. This observation suggests that antigens that are more selectively associated with tumor cells may be more effective for vaccine immunotherapy than antigens that are also expressed by normal cells.

Lastly, we found that vaccine immunization was much more likely to enhance CD8+ T-cell responses to antigens for which a response existed before vaccine treatment than to antigens that were previously not recognized by a patient. This suggests that one mechanism of action of cancer vaccines may be to enhance the magnitude of preexisting antitumor immune responses rather than induce new responses or overcome tolerance. One implication of this observation is that the search for new cancer vaccine antigens should include examination of antigens that are naturally recognized by patients with cancer.

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