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ORIGINAL REPORT

Phase I Study of Sequential Vaccinations With Fowlpox-CEA(6D)-TRICOM Alone and Sequentially With Vaccinia-CEA(6D)-TRICOM, With and Without Granulocyte-Macrophage Colony-Stimulating Factor, in Patients With Carcinoembryonic Antigen–Expressing Carcinomas

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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ABSTRACT

Purpose

Our previous clinical experience with vaccinia and replication-defective avipox recombinant carcinoembryonic antigen (CEA) vaccines has demonstrated safety and clinical activity with a correlation between CEA-specific immune response and survival. Preclinical evidence demonstrated that the addition of the transgenes for three T-cell costimulatory molecules (B7-1, ICAM-1, LFA-3, designated TRICOM) results in a significant improvement in antigen-specific T-cell responses and antitumor activity. We describe here the first trial in humans of the CEA-TRICOM vaccines (also including an enhancer agonist epitope within the *CEA* gene).

Patients and Methods

Fifty-eight patients with advanced CEA-expressing cancers were accrued to eight cohorts that involved vaccinations with the following: replication-defective fowlpox recombinant (rF)-CEA(6D)-TRICOM; primary vaccination with recombinant vaccinia (rV)-CEA(6D)-TRICOM plus rF-CEA(6D)-TRICOM booster vaccinations; and rV-CEA(6D)-TRICOM and then rF-CEA(6D)-TRICOM, plus granulocyte-macrophage colony-stimulating factor (GM-CSF) with vaccines, or with divided doses of vaccine with GM-CSF. Vaccines were administered every 28 days for six doses and then once every 3 months. Reverting to treatments every 28 days was allowed if patients progressed on the 3-month schedule.

Results

In this phase I study, no significant toxicity was observed. Twenty-three patients (40%) had stable disease for at least 4 months, with 14 of these patients having prolonged stable disease (> 6 months). Eleven patients had decreasing or stable serum CEA, and one patient had a pathologic complete response. Enhanced CEA-specific T-cell responses were observed in the majority of patients tested.

Conclusion

We demonstrated that the CEA-TRICOM vaccines are safe and can generate significant CEA-specific immune responses, and they seem to have clinical benefit in some patients with advanced cancer.

J Clin Oncol 23.

INTRODUCTION

The therapy of metastatic carcinoma remains a major concern. Vaccines represent an alternative approach to therapy either as a single modality or in combination with existing therapies. By definition, however, vaccine-targeted tumor-associated antigens

(TAAs) are either weakly immunogenic or functionally nonimmunogenic in the cancer-bearing host. Therefore, vaccine strategies must be developed in which the presentation of a given tumor antigen to the immune system results in far greater activation of T cells than what is being achieved naturally in the host. We and others have developed five such strategies. These include the following: (1) the use of viral vector–based vaccines to enhance presentation of the TAA to the immune system; (2) diversified prime and boost vaccination strategy using two different types of vaccines; (3) the use of T-cell costimulation to enhance T-cell responses; (4) altering the amino acid sequence of the tumor antigen to enhance its immunogenicity; and (5) the use of granulocyte-macrophage colony-stimulating factor (GM-CSF) to enhance recruitment of dendritic cells to the vaccination site. The trial reported here represents the first human clinical trial in which all of these strategies are combined.

The target antigen used in this study is carcinoembryonic antigen (CEA), which has been implicated in the metastatic process.[1] Although CEA is expressed in fetal gastrointestinal tissues and normal adult colonic tissue, it is overexpressed in the vast majority of human colorectal, gastric, and pancreatic cancers, in approximately 70% of non–small-cell lung cancers and 50% of breast carcinomas, and in other cancers such as cervical, ovarian, prostate, and head and neck cancer.[2-6]

Previous phase I trials using recombinant vaccinia (rV)-CEA, avipox-CEA, or avipox-CEA-B7-1 vaccines showed that patients were able to mount a CEA-specific immune response after vaccination.[7-13] Although patients in these trials all had been previously immunized with the smallpox vaccine, they were able to mount a vigorous immune response to the rV-CEA vaccine when given at the higher dose levels; however, the boost in antivaccinia immunity inhibited the continued use of this vector.[7,14] An alternative booster vaccine is the use of avipox vectors (recombinant fowlpox [rF] or canarypox [ALVAC]), which were previously shown to efficiently infect but were incapable of replication in mammalian cells.[15] Preclinical and clinical studies have demonstrated that one could continually boost with a recombinant avipox vector without the induction of host-neutralizing immunity.[16-20]

A phase I to II randomized clinical study was then conducted in patients with advanced CEA-expressing carcinomas in which the following two diversified vaccination strategies were analyzed: priming with rV-CEA (V) followed by boosting with avipox (ALVAC)-CEA (A) (VAAA regimen) versus a regimen using AAAV.[19] Although the two cohorts in this pilot study were small (n = 9 patients per cohort), analysis of this study showed that patients in the VAAA cohort had a statistically significant ($P < .01$) increase in CEA-specific T cells (postvaccination *v* prevaccination) compared with patients in the AAAV cohort. Treatment with VAAA resulted in longer survival than treatment with AAAV ($P = .05$). CEA-specific T-cell responses were also associated with increased survival ($P = .03$) after accounting for disease status. This study also demonstrated that one can give multiple vaccinations of avipox vector that correlate with increases in CEA-specific T-cell responses.[19,20]

Alterations in the amino acids structure of a given cytotoxic T lymphocyte epitope have been shown to enhance its immunogenicity. A modification of the HLA-A2 CEA CAP-1 epitope was shown to result in an agonist epitope, designated CAP1-6D.[21,22] In a clinical trial using CAP1-6D–pulsed dendritic cells, two of 12 patients experienced dramatic tumor regression, one patient had a mixed response, and two patients had stable disease.[23] Clinical response correlated with the expansion of CD8 CEA-specific T cells. Consequently, the vectors used in this study contain the CEA genome with the 6D modification in the CAP-1 epitope.

T-cell costimulation has previously been shown to be essential for efficient T-cell activation, especially when a weak antigen such as a TAA is involved. Preclinical studies have shown that the addition of the transgenes for a triad of costimulatory molecules to vaccinia and avipox vectors along with the CEA transgene enhances both CEA-specific T-cell immunity and antitumor immunity.[9-11,16,17,24-28] This triad of costimulatory molecules (TRICOM) consists of human B7-1, ICAM-1, and LFA-3. The clinical trial reported here is the first to use a TRICOM vector coupled with a TAA.

GM-CSF has been used by multiple investigators in both preclinical and clinical studies to enhance vaccine efficacy via a recruitment of dendritic cells to regional nodes of the vaccination site.[17,28-32] The clinical trial described here also uses GM-CSF at the injection site.

PATIENTS AND METHODS

Patient Eligibility

To be eligible for this phase I trial, patients had to meet the following criteria: histologically confirmed cancer with evidence of metastatic disease; serum CEA level of at least 10 ng/mL at some point in the past or tumor that stained positively for CEA by immunohistochemical techniques; age of at least 18 years; anticipated survival of 6 months; Eastern Cooperative Oncology Group performance status of 0 to 2; adequate organ function as defined by normal hematopoietic, renal, and hepatic function; HIV seronegativity; and no concurrent use of corticosteroids. There were no HLA phenotype restrictions. Contraindications to enrollment included history of radiation to more than 50% of nodal groups, recent major surgery, pregnancy or breast feeding, serious intercurrent illness, and clinically active brain metastasis.

Patients who had received avipox-CEA and/or rV-CEA vaccines in previous trials and had subsequently progressed were able to participate in this trial, provided they still met the eligibility criteria. Four such patients were enrolled. They did not count

toward the determination of primary immunologic end points or the determination of the maximum-tolerated dose, and rV-CEA(6D)-TRICOM vaccinations were not given. This study was approved by the Institutional Review Board of Georgetown University Medical Center and by the Department of Health and Human Services, and written informed consent was obtained from all patients.

Study Design and Treatment

The treatment schema is listed in Table 1. Patients were vaccinated every 28 days. Patients were monitored before each injection by physical examination, performance of CBC, chemistry and liver profiles, urinalysis, and serum CEA level; and peripheral-blood mononuclear cells (PBMC) were collected for T-cell immunologic monitoring. Tumor responses, which were measured according to the standard Response Evaluation Criteria in Solid Tumors Group guidelines, were evaluated after every two treatment cycles. rV-CEA(6D)-TRICOM was administered intradermally into the thigh. rF-CEA(6D)-TRICOM was administered subcutaneously and intradermally using the Biojector 2000 (Bioject Inc., Portland, OR) needle-free system. In cohort 8, rF-CEA(6D)-TRICOM (4×10^8 plaque-forming units [pfu]) was administered subcutaneously in two divided doses given in each thigh.

Patients had restaging studies performed at 2 months after initial vaccination. These studies were used for a new baseline and for subsequent decisions regarding response using standard Response Evaluation Criteria in Solid Tumors Group criteria. Patients who had rapid progression within the first 2 months after initial vaccination were taken off study at that point; however, those patients who had no clinical decline in performance status, despite having radiographic evidence of progression, were allowed to continue vaccinations. Thus, patients who had rapid progression of their disease were classified as having primary progression of disease, and those who continued on study and subsequently reverted to stable disease were classified as having secondary stable disease. Patients who seemed to be benefiting (ie, had stable disease and tolerated treatment) after the fourth vaccination were allowed to continue with rF-CEA(6D)-TRICOM vaccinations at the same dose and frequency of vaccine as per the treatment arm onto which they were enrolled up through the sixth vaccination, and then they were administered the vaccine every 3 months thereafter. The purpose of such continued vaccination was to study the safety of continually boosting the anti-CEA response with T-cell costimulation. If progression of disease on the every 3-month vaccination schedule occurred, patients were allowed to revert to monthly vaccination.

Vaccine Preparation

rV-CEA(6D)-TRICOM and rF-CEA(6D)-TRICOM were manufactured by Therion Biologics Corp (Cambridge, MA) and supplied by the Pharmaceutical Management Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). rV-CEA(6D)-TRICOM was prepared from virus derived from the Wyeth New York City Board of Health (New York, NY) strain of vaccine. Virus for both vaccines was grown in primary chicken embryo dermal cells. rV-CEA(6D)-TRICOM was vialed at 1.75×10^8 pfu/0.3 mL; 200 μL was withdrawn into a syringe for intradermal injection. rF-CEA(6D)-TRICOM was vialed at 4.2×10^8 pfu/0.3 mL; the sample in the vial was diluted with normal saline to a total of 750 μL, and 500 μL was withdrawn into two syringes for the Bioject system. The routes for both CEA-TRICOM–containing vectors were the same as used in a previous trial using the same CEA vectors devoid of TRICOM.[19] Both vaccine vials were kept at −70°C until the day of administration. They were then thawed at room temperature. Vaccine preparations were performed in sterile hoods.

Immunologic Monitoring Methods: ELISPOT

The primary immunologic end point for comparing immune responses in HLA-A2–positive patients was determined by an overnight enzyme-linked immunosorbent spot (ELISPOT) assay using C1R-A2 as antigen-presenting cells as previously described.[33] This assay measures the frequency of interferon gamma (IFN-γ)-releasing T cells specific to CAP1-6D, an HLA-A2–restricted agonist epitope of CEA.[19,33] Immune parameters were measured before treatment and 1 month after the fourth vaccination. The ELISPOT

Table 1. Trial Design: Dose Escalation Schema

Stage of Study	Cohort No.	rV-CEA(6D)-TRICOM (pfu)	rF-CEA(6D)-TRICOM (pfu)	No. of Patients	No. of HLA-A2–Positive Patients
1	1	—	4×10^6	3	—
1	2	—	4×10^7	3	—
1	3	—	4×10^8	10	7
2	4	1.2×10^6	4×10^8	3	—
2	5	1.2×10^7	4×10^8	3	—
2	6	1.2×10^8	4×10^8	11	7
3	7	1.2×10^8 + GM-CSF	4×10^8 + GM-CSF	12	8
4	8	1.2×10^8 + GM-CSF	4×10^8 (split dose) + GM-CSF	13	9

NOTE. The study was conducted in four stages. All patients in stage 1 received rF-CEA(6D)-TRICOM, which was dose escalated between cohorts. Dose escalation was carried out in the standard manner. If no patients on a given cohort developed dose-limiting toxicity after all three patients received at least one immunization, then the next three patients were started at the next higher dose level in the subsequent cohort. All patients in stage 2 were treated with rV-CEA(6D)-TRICOM, which was dose escalated between cohorts followed by fixed doses of rF-CEA(6D)-TRICOM at the MTD. Patients in cohort 7 were treated with a fixed dose of rV-CEA(6D)-TRICOM (MTD) and rF-CEA(6D)-TRICOM (MTD), plus the addition of GM-CSF at a dose of 100 μg administered subcutaneously into the vaccine injection sites on days 1 (day of first vaccination) through 4. Patients in cohort 8 were treated as in cohort 7, except rF-CEA(6D)-TRICOM was divided equally into two doses and administered into each thigh. GM-CSF at a fixed dose of 100 μg was administered subcutaneously into both rF-CEA(6D)-TRICOM vaccine injection sites on days 1 through 4.

Abbreviations: rV, recombinant vaccinia; rF, recombinant fowlpox; CEA, carcinoembryonic antigen; TRICOM, triad of costimulatory molecules, ie, B7-1, ICAM-1, LFA-3; GM-CSF, granulocyte-macrophage colony-stimulating factor; MTD, maximum-tolerated dose; pfu, plaque-forming units.

assay for the monitoring of specific T-cell responses has been validated previously by Arlen et al.[33] For validation of interassay and intra-assay variability, the peripheral-blood mononuclear cells from the same healthy donor were used as an internal control. The flu matrix peptide precursor frequency for the healthy donor was 1/14,283 ± 1/3,742 (mean ± standard deviation). Negative controls for patient samples included wells with no peptide and HIV peptide. Control for the ELISPOT assay using the flu matrix peptide was performed simultaneously. Other studies have shown that flu CD8 responses do not appreciably change after patients receive a flu vaccine. Immunoglobulin (Ig) G, IgM, and IgA responses specific for human B7-1, ICAM-1, or LFA-3 were analyzed in patient serum by fluorescence-activated cell sorter capture assay.[27] The limit of detection was 4 ng/mL. Anti–GM-CSF IgG was detected by enzyme-linked immunosorbent assay (ELISA)[18]; anti-CEA IgG , antifowlpox IgG, and antivaccinia virus IgG were detected by ELISA.[24] Value expressed as reciprocal serum dilution more than 0.5 optical density. Anti–GM-CSF serum antibodies were confirmed by Western blot analysis.

RESULTS

Patient Cohorts

Patient cohorts are listed in Table 1. Because this is the first trial involving a prime and boost regimen using two different recombinant vaccines containing transgenes for a tumor antigen and three T-cell costimulatory molecules, with or without GM-CSF, several different cohorts were used. Cohorts 1 to 3 involved dose escalation of rF-CEA(6D)-TRICOM. Cohorts 4 to 6 involved dose escalation of rV-CEA(6D)-TRICOM and the maximum-tolerated dose of rF-CEA(6D)-TRICOM. Cohort 7 used the same doses of vaccines as cohort 6, except that recombinant GM-CSF protein (100 μg) was administered at the injection site at the time of vaccination and for 3 consecutive days after each vaccination (see legend to Table 1). Cohort 8 involved the same final doses of vaccines as cohort 7, except that the rF-CEA(6D)-TRICOM boost doses were divided equally and vaccinations were given in each thigh (each with the same 100-μg dose of GM-CSF). This was done in an attempt to establish a more robust systemic response to booster vaccinations. Patient characteristics are listed in Table 2. Most patients had gastrointestinal cancers and were heavily pretreated. Forty-eight of 58 patients had received at least two prior treatment regimens, and 36 of these patients received more than two prior treatment regimens. Four patients had received an rV-CEA and/or avipox-CEA vaccine previously.[19,20] These patients are identified and described separately in the "Patients Previously Treated With CEA-Based Vaccines" section. No other patients had prior immunotherapy. Patients with stable disease after their initial four monthly vaccinations went on to receive two additional monthly vaccinations of rF-CEA(6D)-TRICOM and then rF-CEA(6D)-TRICOM vaccinations every 3 months.

Table 2. Patient Characteristics

Characteristic		No. of Patients (N = 58)
Age, years		
Range	38-85	
Median	58	
Performance status		
0		29
1		29
Sex		
Male		29
Female		29
Prior therapy		
Chemotherapy		
No regimen		3
1 prior regimen		7
2 prior regimens		12
> 2 prior regimens		36
Radiation		16
Primary site		
Colorectal		35
Lung		9
Breast		3
Thyroid		1
Unknown primary		2
Ovary		1
Other gastrointestinal		7
HLA-A2		
Positive		31
Negative		27

Toxicity

Extensive monitoring for safety revealed no evidence of cardiac, renal, or any dose-limiting toxicity. Toxicities were limited to grade 1 local skin reactions at vaccine site, regional adenopathy, fatigue, and mild flu-like symptoms lasting a few days after vaccination.

Evaluation of CEA-Specific T-Cell Responses

PBMCs were obtained at time points before and after four vaccinations from 13 patients who were class I HLA-A2 positive. PBMCs were not analyzed from patients who did not get all four vaccines. PBMCs were analyzed for CEA-specific T-cell responses using a previously described ELISPOT assay that measures the amount of IFN-γ released by PBMCs in response to stimulation with a 9-mer CEA agonist peptide (CAP1-6D). Flu peptide–specific immune responses from patients were also evaluated. As can be seen in Table 3, Flu-specific immune responses did not vary appreciably before versus after vaccination with the CEA-TRICOM vaccines. Although the Flu peptide was not in the vaccine, patients may have been exposed to influenza or other immunostimulatory or immunoregulatory factors during or immediately before the vaccinated period, which might account for the minor differences observed.

As seen in Table 3, 10 of 13 of the HLA-A2–positive patients in the different cohorts mounted CEA-specific

Table 3. ELISPOT Results From HLA-A2 Patients From CEA(6D)-TRICOM Trial

Cohort	Patient No.	Sample	Flu Peptide	Post/Pre Ratio	CEA Peptide	Post/Pre Ratio
3	11	Pre	1/24,000		1/200,000	
		Post-4	1/19,354	1.24	1/60,000	3.33
	12	Pre	1/33,333		1/66,666	
		Post-4	1/24,000	1.39	1/31,578	2.11
	14	Pre	1/37,500		1/100,000	
		Post-4	1/37,500	1.00	1/60,000	1.67
6	24	Pre	1/35,294		1/120,000	
		Post-4	1/40,000	0.88	1/26,086	4.60
	27	Pre	1/66,667		1/100,000	
		Post-4	1/71,429	0.93	1/13,514	7.40
	29	Pre	1/40,000		1/100,000	
		Post-4	1/54,545	0.73	1/85,714	1.17
7	36	Pre	1/35,294		1/60,000	
		Post-4	1/31,578	1.12	1/20,000	3.00
	39	Pre	1/15,789		1/50,000	
		Post-4	1/17,647	0.89	1/19,385	2.58
	41	Pre	1/31,578		1/75,000	
		Post-4	1/31,578	1.00	1/25,000	3.00
8	47	Pre	1/13,043		$<$ 1/200,000	
		Post-4	1/20,690	0.63	1/27,273	$\geq$ 7.33
	49	Pre	1/27,273		$<$ 1/200,000	
		Post-4	1/19,355	1.41	1/50,000	$\geq$ 4.00
	53	Pre	1/20,000		$<$ 1/200,000	
		Post-4	1/18,182	1.10	1/30,000	$\geq$ 6.67
	58	Pre	1/27,273		$<$ 1/200,000	
		Post-4	1/13,044	2.10	$<$ 1/200,000	$\leq$ 1.0
Patients who received prior rV-CEA and avipox-CEA vaccines						
6	26	Pre	1/11,765		$<$ 1/200,000	
		Post-4	1/18,750	0.63	1/46,154	$\geq$ 4.33
7	35	Pre	1/18,181		$<$ 1/200,000	
		Post-4	1/27,272	0.67	1/25,000	$\geq$ 8.00
8	55	Pre	1/31,578		1/75,000	
		Post-4	1/31,578	1.00	1/25,000	3.00

Abbreviations: ELISPOT, enzyme-linked immunosorbent assay; CEA, carcinoembryonic antigen; TRICOM, triad of costimulatory molecules, ie, B7-1, ICAM-1, LFA-3; Post, after vaccination; Pre, before vaccination; Post-4, after four vaccinations; rV, recombinant vaccinia.

T-cell responses with a greater than two-fold increase after four vaccinations versus before vaccination. In some patients, those responses were four- to seven-fold greater after versus before vaccination. In all but one of these patients, the Flu-specific T-cell responses were no greater than 1.4-fold greater after versus before vaccination.

It is interesting to note that in several patients, there were pre-existing prevaccination levels of T cells (Table 3, patients 12, 36, 39, and 41). This phenomenon has been reported previously and will be discussed in the Discussion section.

There were four additional patients on this trial who received rV-CEA and/or avipox-CEA vaccine from a previous trial[19] and subsequently progressed. PBMCs before and after four vaccinations of CEA-TRICOM were obtained from three of these patients who were HLA-A2 positive. ELISPOT results are listed in Table 3. Those patients all had CEA precursors decrease from their levels after four vaccinations in the first trial compared with prevaccination levels in this trial (an interval of 36 to 44 months between trials). However, all these patients' precursors to CEA again increased (three- to $>$ eight-fold) after receiving four vaccinations on this trial. None of these patients had increases in their Flu peptide–specific responses.

There were six HLA-A2 patients who went on to receive vaccines at the 3-month interval regimen. No absolute trends were observed in CEA-specific precursor levels. However, in two of six patients, CEA precursors decreased immediately after vaccination at the 3-month interval and then increased after monthly vaccinations were resumed. In a third patient, the CEA precursors dropped immediately before the vaccination, stayed down during the 3-month vaccine interval, and then increased after the monthly vaccinations were resumed.

Serologic Analyses

Sera from patients who completed four vaccination cycles from cohorts 1 to 8 were analyzed for Ig responses to

CEA, vaccinia, fowlpox, GM-CSF, or the three T-cell costimulatory molecules (B7-1, ICAM-1, and LFA-3) expressed by the TRICOM vectors. Six of 33 patients had modest (> three-fold) increases in CEA-specific IgG after four vaccinations (Table 4). Seven patients had pre-existing anti-CEA IgG before vaccination (≥ 1:100 serum dilution, Table 4), which did not increase after vaccination. None of the seven patients from cohort 7 who received primary vaccination with rV-CEA(6D)-TRICOM with GM-CSF and then rF-CEA(6D)-TRICOM booster vaccinations with GM-CSF mounted anti–GM-CSF IgG after four vaccinations (data not shown). Two of seven patients from cohort 8 who received rV-CEA(6D)-TRICOM and then rF-CEA(6D)-TRICOM booster vaccinations plus GM-CSF (the fowlpox boosts given as a split dose at two sites) administered as a split dose mounted a low level of anti–GM-CSF IgG (1:1,250 and 1:3,750). More importantly, none of the 33 patients analyzed from cohorts 1 through 8 mounted any detectable IgG, IgM, or IgA immune responses to B7-1, ICAM-1, or LFA-3 expressed in the TRICOM vectors (data not shown).

Table 4. Serologic Analysis of Patients Receiving CEA(6D)-TRICOM Vaccine Regimen

		Anti-CEA*		Antivaccinia†	
Cohort No.	Patient No.	Pre	Post-4	Pre	Post-4
1	1	1,250	1,250	ND	ND
1	3	50	50	ND	ND
2	4	< 50	< 50	ND	ND
3	7	< 50	300	ND	ND
3	9	< 50	1,250	ND	ND
3	11	250	600	ND	ND
3	12	50	50	ND	ND
3	14	50	50	ND	ND
4	18	50	150	1,250	1,250
4	20	< 50	< 50	6,250	8,000
5	21	4,000	4,000	1,250	1,250
5	23	< 50	< 50	1,000	1,000
6	24	250	250	50	1,000
6	25	50	50	250	6,250
6	27	< 50	< 50	150	1,250
6	28	150	150	250	1,100
6	29	250	250	150	1,250
6	33	< 50	< 50	50	5,000
6	34	50	50	800	3,000
7	36	3,000	3,000	1,250	6,250
7	37	< 50	< 50	250	6,250
7	38	800	800	800	> 8,000
7	39	< 50	400	5,000	> 8,000
7	40	< 50	80	1,000	> 8,000
7	41	250	1,100	200	4,000
7	42	< 50	800	50	6,250
8	47	< 50	< 50	ND	ND
8	48	< 50	< 50	ND	ND
8	49	< 50	< 50	ND	ND
8	53	< 50	< 50	ND	ND
8	55	< 50	< 50	ND	ND
8	57	< 50	150	ND	ND
8	58	< 50	160	ND	ND

Abbreviations: CEA, carcinoembryonic antigen; TRICOM, triad of costimulatory molecules, ie, B7-1, ICAM-1, LFA-3; Pre, before vaccination; Post-4, after four vaccinations; ND, not done; IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; OD, optical density.

*Anti-CEA IgG was detected by ELISA.[24] Values expressed as reciprocal serum dilution > 0.5 OD. Limit of detection was 0.064 ng/ml.

†Antivaccinia IgG was detected by ELISA. Values expressed as reciprocal serum dilution > 1.0 OD. Antivaccinia serum antibodies were confirmed by plaque neutralization assay.

All patients enrolled onto the trial had a previous smallpox vaccine, and all had detectable antivaccinia virus IgG before vaccination as measured by ELISA (Table 4). The majority of the patients (15 of 18 patients) showed increases in antivaccinia IgG after four vaccinations. Antifowlpox IgG titers increased in the majority of patients after four vaccinations (Fig 1). As described previously,[18] however, these antifowlpox IgG are nonneutralizing because the fowlpox recombinants are replication defective in mammalian cells; the CEA and TRICOM transgenes are expressed on early pox promoters, whereas the late pox virus genes encoding for coat proteins are not transcribed in mammalian cells.[15]

Clinical Response

To best demonstrate clinical response, patients were grouped into four categories as shown in Table 5. Primary stable disease was defined as patients who had no disease progression during the first 4 months of therapy; secondary stable disease was defined as patients who had progressed after 2 months (two vaccinations) but achieved stable disease at 4 months (after four vaccinations); primary progression of disease was defined as patients who progressed at 2 months and were removed from the study; and secondary progression of disease was defined as patients who progressed between 2 and 4 months and were removed from

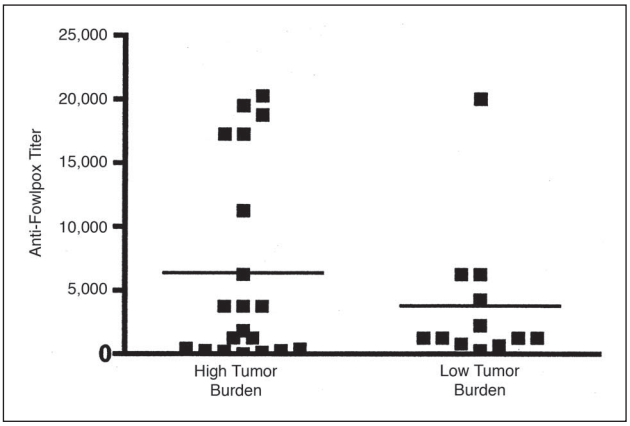

Fig 1. Lack of correlation between tumor burden and the ability of vaccinated patients to mount antifowlpox immunoglobulin G responses by enzyme-linked immunosorbent assay.[24] Patients were classified as high or low tumor burden (see Results). Antifowlpox immunoglobulin G was measured in sera after four vaccinations. The bar in each data set represents the mean titer of the group.

Table 5. Characteristics of Patients With Stable Disease Versus Progressive Disease

Characteristic	Primary Stable Disease	Secondary Stable Disease	Primary Progressive Disease	Secondary Progressive Disease
Total No. of patients	11	12	21	14
Prior therapies, No. of patients				
None	1	1	0	0
1 prior therapy	3	2	1	1
2 prior therapies	4	4	3	2
> 2 prior therapies	3	5	17	11
Baseline serum CEA, ng/mL	9.18	66.8	449.5	137
Time to progression, months				
Median	11.1	8.6	2	4
Range	5-16	4-23	—	—
Mean immune response* (precursor frequency)	1/23,000	1/37,000	NA	1/70,000

NOTE. Primary stable disease was defined as patients who had no disease progression during the first 4 months of therapy; secondary stable disease was defined as patients who had progressed after 2 months (two vaccinations) but achieved stable disease at 4 months (after four vaccinations); primary progression of disease was defined as patients who progressed at 2 months and were removed from the study; and secondary progression of disease was defined as patients who progressed between 2 and 4 months and were removed from the study.

Abbreviations: CEA, carcinoembryonic antigen; NA, not applicable; ELISPOT, enzyme-linked immunosorbent assay.

*Immune response was determined by ELISPOT assay (see Patients and Methods).

the study. Twenty-three patients (40%) achieved stable disease (primary stable disease or secondary stable disease) at 4 months (Table 5). Twelve (52%) of these 23 patients were progressing at 2 months but then achieved stable disease at 4 months (ie, secondary stable disease; Table 5). Nine of the patients with primary stable disease, after six monthly vaccinations, went on to receive vaccinations of rF-CEA(6D)-TRICOM every 3 months. Eight of these patients then progressed and were reverted to monthly vaccinations. Five patients with secondary stable disease, after six monthly vaccinations, went on to receive vaccinations every 3 months, and four of these five patients also developed progressive disease; these patients also were then reverted to monthly vaccination. Six of the 12 patients who were converted to vaccinations every 3 months and who then progressed had disease restabilization after converting back to monthly vaccinations with rF-CEA(6D)-TRICOM. It should be pointed out that the majority of patients with either primary progressive disease or secondary progressive disease had two or more prior therapies. It should also be pointed out that 11 of 14 patients with stable disease more than 6 months after initiation of vaccine had progressive disease at study entry.

Individual Case Reports

One patient (patient 9) had a complete pathologic response to vaccine. This patient was originally diagnosed with limited-stage small-cell lung cancer. She achieved a clinical response after six cycles of concurrent chest radiation with carboplatin and etoposide followed by whole-brain radiation for metastases. A computed tomography scan of the chest was performed before vaccine, demonstrating progression of a lesion in the right upper lung field. A positron emission tomography (PET) scan also performed before initiation of vaccine confirmed a metabolically active lesion in the right lung field (Fig 2A and 2B). After two monthly vaccinations, there was no activity on a subsequent PET scan (Fig 2C and 2D). After six monthly vaccinations, this patient, per the protocol, went on to receive rF-CEA(6D)-TRICOM at 3-month intervals. Evidence of progression via PET scan was then observed, and the patient went on to receive monthly vaccinations, after which the PET scan reverted to normal. The patient died from accidental causes 15 months after initiating vaccine. Autopsy results, including those of lung sections, reported no pathologic evidence of cancer.

Another patient of note is patient 53, a 65-year-old male originally diagnosed with appendiceal adenocarcinoma with peritoneal metastasis. He underwent a debulking surgery followed by postoperative intraperitoneal fluorouracil. Seven months later, he was diagnosed with significant recurrent intra-abdominal disease, bone metastasis, and abdominal wall metastasis. The largest mass measured 18.7 cm in greatest dimension. Although this was a slow-growing tumor, the patient was experiencing significant abdominal distention, requiring frequent paracenteses. His baseline CEA was 20.0 ng/mL. Because he was seeking to avoid debulking operations, he began vaccine treatments 5 months after diagnosis of recurrent disease; he has remained on study for more than 13.5 months. He has undergone only an occasional paracentesis for comfort, his CEA has decreased to 12, and he maintains an excellent performance status. His CEA decreased to a low of 7.6, but upon changing to the 3-month vaccine schedule, his CEA increased to 22. Since reverting back to monthly vaccines, his CEA has decreased to the current mark of 12. In addition to patient 53, 10 patients had either

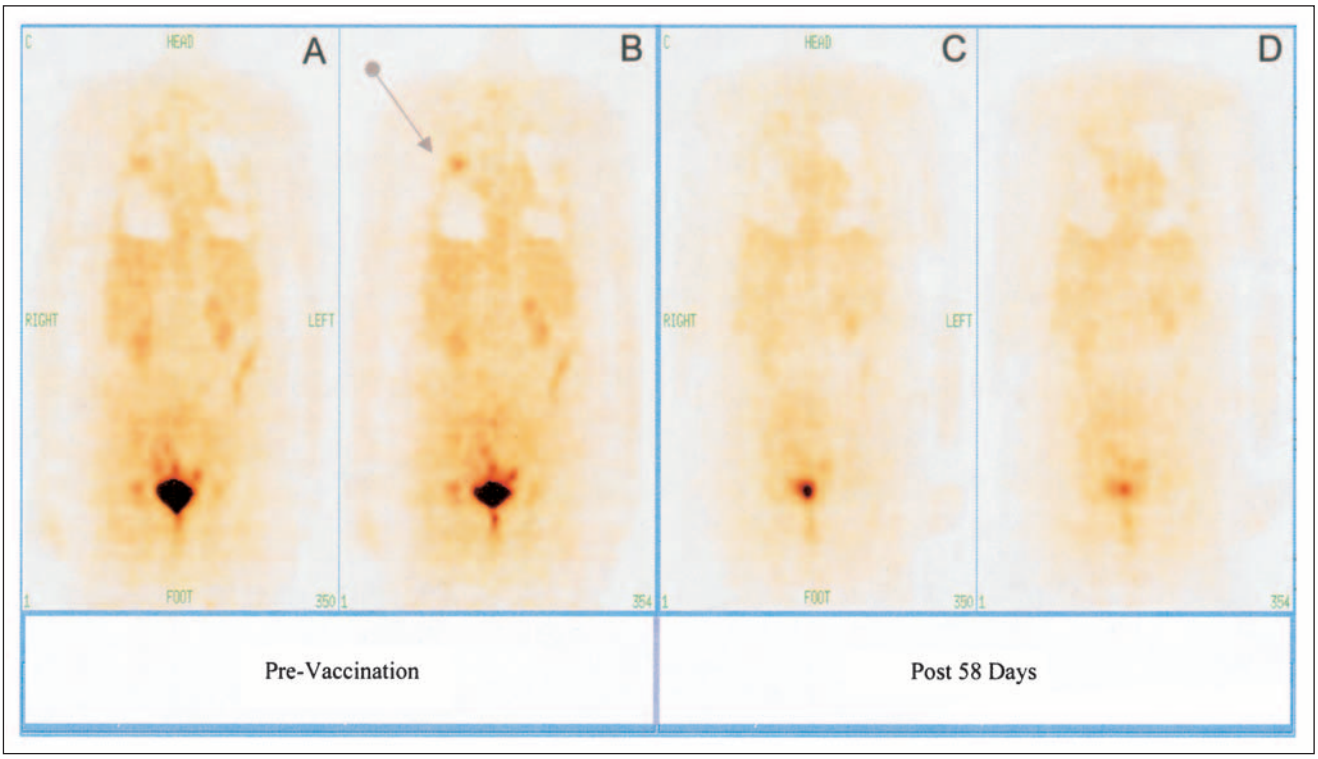

Fig 2. Positron emission tomography (PET) images of patient 9 before (A and B) and after (C and D) two vaccinations with recombinant fowlpox-carcinoembryonic antigen (6D)-TRICOM. A computed tomography scan before vaccine demonstrated progression of a lesion in the right upper lung field. The PET scan also performed before initiation of vaccine confirmed a metabolically active lesion. This patient was eventually defined as having a pathologic complete response. TRICOM, triad of costimulatory molecules, ie, B7-1, ICAM-1, LFA-3.

decreases or stable serum CEA levels after multiple vaccinations versus before vaccination.

Overall and Progression-Free Survival

In view of the phase I nature of this trial, the varied carcinoma types of patients, and especially the limited cohort sizes, no claims can be made concerning overall and progression-free survival. However, there were interesting trends that emerged. Patients in cohorts 3, 6, 7, and 8 ($n = 10$ to 13 patients per cohort) were analyzed for survival because patients in these cohorts received the full dose of vaccine being evaluated. Patients who received only rF-CEA(6D)-TRICOM (cohort 3) did not do as well as patients in the other cohorts who received a primary vaccination with rV-CEA(6D)-TRICOM and then booster vaccinations with rF-CEA(6D)-TRICOM (cohorts 6, 7, and 8). As seen in Figure 3, patients who received GM-CSF with vaccines (cohorts 7 and 8) seemed to do better than those who did not receive GM-CSF (cohorts 3 and 6). Because of the phase I nature of this trial and its diverse patient population and rather small cohorts, the survival differences observed between cohorts 3 and 6 versus 7 and 8 should only be considered trends that merit further study in subsequent trials. Patients who received a split dose of rF-CEA(6D)-TRICOM and GM-CSF (100 μg) into each vaccine injection site (cohort 8) also tended to do better than patients who did not receive the split dose of vaccine but who did receive the same dose of GM-CSF (100 μg) into the injection site (cohort 7).

As mentioned earlier, all patients who were HLA-A2 positive were assayed for the development of CEA-specific T-cell responses to CEA in PBMCs before versus after vaccination. When one analyzes these patients for overall survival versus cohort, there is a trend that shows increased overall survival for those patients who received a primary vaccination with rV-CEA(6D)-TRICOM and then boosters with rF-CEA(6D)-TRICOM (cohorts 6, 7, and 8) versus those patients who received only rF-CEA(6D)-TRICOM vaccinations (cohort 3).

When one analyzes all class I HLA-A2–positive patients for overall survival versus the magnitude of their CEA-specific T-cell responses after four vaccinations, one also sees a trend towards greater survival in those patients who had a CEA-specific T-cell frequency of less than 1/30,000 versus more than 1/30,000; 1/30,000 was the median response observed for all HLA-A2 patients. At 1 year, 83% of patients who had a CEA-specific T-cell response of less than 1/30,000 after four monthly vaccinations were alive versus 41% of patients alive after 1 year if their CEA-specific T-cell responses were more than 1/30,000 after four vaccinations.

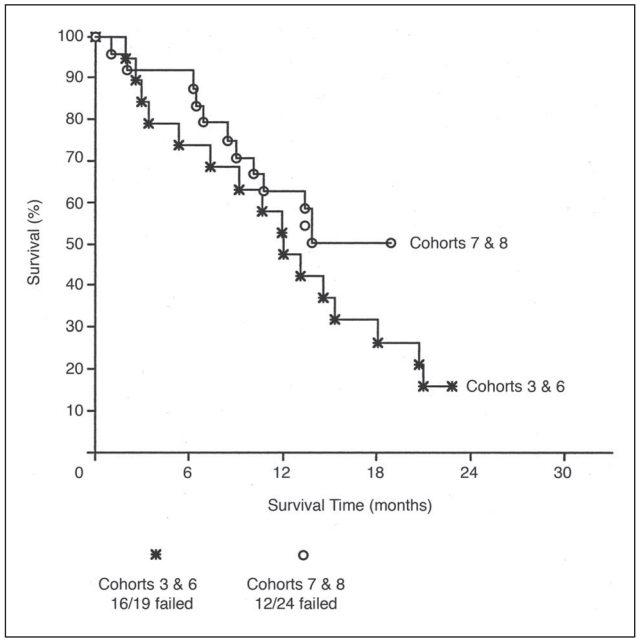

Fig 3. Overall survival by cohorts with or without granulocyte-macrophage colony-stimulating factor (GM-CSF). Cohorts 3 and 6 (16 of 19 patients experienced treatment failure) received no GM-CSF with vaccines; and cohorts 7 and 8 (12 of 24 patients experienced treatment failure) received vaccines plus GM-CSF.

When one analyzes progression-free survival of HLA-A2–positive patients versus CEA-specific T-cell response, there is also a trend ($P = .04$, albeit meaningless because of cohort size, etc) in progression-free survival for those patients whose CEA-specific T-cell responses were less than 1/30,000 versus more than 1/30,000 after four vaccinations. When one analyzes HLA-A2 patients by cohort versus progression-free survival, one also sees a trend in patients who received GM-CSF with vaccine (cohorts 7 and 8) having a longer progression-free survival versus those who did not receive GM-CSF with vaccine (cohorts 3 and 6).

One could hypothesize that any relationship of survival to the generation of CEA-specific T-cell responses could simply be because of the fact that the healthier patients with lower tumor burden would be more likely to mount an immune response to an antigen in a vaccine such as CEA. To evaluate this hypothesis, all patients in the trial were also evaluated for the generation of IgG responses to the fowlpox vector itself (ie, after three monthly vaccinations of rF-CEA-TRICOM). Unlike vaccinia, which patients have seen immunologically in the smallpox vaccine, virtually all individuals are immunologically naive to fowlpox. Using the criteria of high tumor burden as the sum of the long axis of baseline radiographic lesions > 3 cm versus ≤ 3 cm for low tumor burden, the results revealed no significant difference between the high tumor burden group and the low tumor burden group with respect to the generation of antifowlpox antibodies after four vaccinations (Fig 1). Moreover, there was no significant correlation between survival and antifowlpox responses in either the high tumor burden group or the low tumor burden group. Finally, there was no significant difference with regard to the generation of antifowlpox responses in patients with a survival time of more than 12 months versus less than 12 months. Taken together, these results argue that the trend observed that correlates greater anti-CEA–specific T-cell responses with survival is not simply because of the fact that those patients have a more robust immune system.

Patients Previously Treated With CEA-Based Vaccines

Four patients in this study were previously treated (36 to 44 months before current study treatment) with rV-CEA and/or avipox-CEA vaccine,[19,20] subsequently progressed, and went on to receive vaccination with rF-CEA(6D)-TRICOM. Patients 26 and 50 had progression of disease after the second vaccination of rF-CEA(6D)-TRICOM (primary progression) and were taken off study. Patient 55, who was diagnosed with pancreatic cancer, had progressed on prior CEA vaccination with increasing CA 19-9 and pain. After two vaccinations with rF-CEA(6D)-TRICOM, CA 19-9 decreased from 419 to 276 U/mL and pain decreased (now for over 1 year). Patient 35, who was diagnosed with rectal cancer, had increasing serum CEA after prior avipox-CEA vaccinations every 3 months and subsequent monthly avipox-CEA vaccinations on the previous trial. After two monthly vaccinations of rF-CEA(6D)-TRICOM, serum CEA decreased from 35 to 24 ng/mL with disease stabilization. Both these patients went on to receive vaccinations of rF-CEA(6D)-TRICOM every 3 months (Table 5). They then progressed by tumor markers, symptoms, or radiographs and were then reverted to monthly vaccines with rF-CEA(6D)-TRICOM; they continue to have stable disease.

DISCUSSION

This phase I trial demonstrates that rV-CEA(6D)-TRICOM and rF-CEA(6D)-TRICOM vaccines used alone or in combination in patients with advanced cancers are safe. No end organ toxicity or autoimmune toxicity was identified. Treatment was also well tolerated in patients receiving prolonged vaccination. The vaccine in combination with GM-CSF was well tolerated. In contrast to patients receiving vaccine alone, there was an increase in constitutional symptoms such as fatigue and musculoskeletal complaints, although severity was no greater than grade 1. More patients in the vaccine with GM-CSF groups experienced injection site reactions. Patients were monitored for their induction of Ig responses to GM-CSF as well as to the three costimulatory molecules (B7-1, ICAM-1, and LFA-3) expressed by the TRICOM vectors. No such responses were observed

to any of the costimulatory molecules, further supporting the lack of induction of autoimmunity by the vaccination schema. Generation of anti–GM-CSF antibodies was seen in two patients; this has been previously described in other trials.[34]

CEA-specific immune responses were measured in HLA-A2–positive patients using an ELISPOT assay, which measured the production of IFN-γ in response to stimulation with a 9-mer CEA agonist peptide. We used the agonist peptide because we have previously shown[21,22] that T cells activated with CAP-1 recognize CAP1-6D targets, and more importantly, T cells activated with the CAP1-6D peptide will recognize target cells, including human tumor cells, expressing the native CAP-1 epitope. We have also previously shown that T cells activated with CAP1-6D express more IFN-γ than when activated with CAP-1.[21,22,35] Ten of 13 patients analyzed showed the induction of a T-cell response for CEA after vaccination. It is interesting to note, however, that several of the patients had pre-existing CEA-specific T-cell responses before vaccination. It has previously been shown[33,36] that there are differences in the constitutive CEA-specific T-cell levels in PBMCs for patients with CEA-expressing tumors versus normal individuals. The precise reason for this is unknown at this time, but it may be that these patients are mounting an endogenous, albeit insufficient, immune response to the CEA in their tumor. We have analyzed whether there was any difference in increases in CEA-specific T cells in patients who had a prevaccination precursor frequency of CEA-specific T cells of less than 1/100,000 versus those who had prevaccination precursor frequencies of greater or equal to 1/100,000. There was no statistical difference. Thus, at this point, the significance of pre-existing precursors to CEA remains unknown. The purpose of this clinical trial was to use a vaccine and vaccine strategies to enhance the presentation of a weak antigen, such as CEA, to the immune system to render it more immunogenic.

There was a trend towards enhanced CEA-specific immune response to vaccination and an increase in progression-free survival, which was enhanced with the prime-and-boost strategy, the addition of GM-CSF, and the split-dose of rF-CEA(6D)-TRICOM (GM-CSF given at 100 μg/dose for 4 days at each site of vaccination); trends were also observed with these factors and overall survival. The fact that the induction of greater CEA-specific immune responses in patients correlated more favorably with clinical responses could be a result of the fact that these patients simply have a better immune system. It should be pointed out, however, that Flu-specific immune responses were not correlated with any trend towards clinical response. There was no obvious trend relating CEA-specific T-cell responses with number of prior chemotherapies or time since last chemotherapy; however, PBMCs from only 16 HLA-A2–positive patients were available for evaluation.

It is interesting to note that correlations between clinical response and CEA-specific T-cell responses have been observed in two previous trials.[19,37] In a randomized trial using V and A, the cohort receiving the diversified VAAA regimen demonstrated greater survival than the cohort receiving AAAV.[19,37] Survival benefit correlated with the generation of CEA-specific T-cell responses.[19,37] Thus, although these studies are phase I and contain small and diversified cohorts, they do provide some evidence of correlation between CEA-specific immunity and clinical benefit. Larger randomized studies, however, will be needed before more definitive conclusions can be drawn.

Some meaningful anticancer effects were observed in this trial. Twenty-three (40%) of 58 patients achieved stable disease after four monthly vaccinations. There was one pathologic complete response (at autopsy). Of interest, 12 patients who were progressing after their second monthly vaccine went on to have stable disease after their fourth vaccination. The rationale for the use of the categories of primary and secondary stable disease (Table 5) merits discussion. Unlike the use of conventional drugs, the continued use of a vaccine (for example, given at weekly, biweekly, or monthly intervals) has previously been shown in several preclinical studies to amplify the antigen-specific immune response and antitumor response. For example, preclinical studies have shown[16] that, with increasing numbers of rF-CEA-TRICOM vaccinations, the magnitude of the T-cell response specific for CEA increases. Similar findings have been observed in patients in a previous clinical trial using repeated avipox-CEA vaccinations.[19] Thus, it is quite possible for tumor to be progressing during the first two vaccinations of a trial and, after the third or fourth vaccination of the same dose of vaccine, for T-cell responses to the tumor antigen to be amplified and, thus, for tumor growth to stabilize. This phenomenon was indeed observed in several patients in this trial (Table 5). Fourteen (24%) of 58 patients received prolonged vaccination after the six monthly vaccinations defined in the protocol. These patients then went on to receive vaccinations of rF-CEA-TRICOM every 3 months. However, in the majority of cases, after the second dose of vaccine given every third month, patients progressed and were reverted back to the monthly vaccination schedule. Six of 12 of these patients then had restabilized disease.

In the study reported here, it is not surprising that, of the assessable patients, 10 (29%) of 35 patients with high-volume disease were alive at 12 months versus 12 (86%) of 14 patients with low-volume disease. Noting the trend in survival advantage in the cohorts that received the highest dose of vaccine with GM-CSF (cohorts 7 and 8) versus the highest dose of vaccine without GM-CSF (cohorts 3 and 6), it should be pointed out that there was no obvious difference in tumor burden in patients of cohorts 3 and 6 (16 [73%] of 22 patients with high tumor burden) versus patients in cohorts 7 and 8 (20 [80%] of 25 patients with high tumor burden). Similar observations were also

obtained if one evaluates only the colorectal cancer patients (n = 34) in this trial. It should also be pointed out that there was no obvious difference in the level of T-cell response to CEA peptide in high versus low tumor volume patients (12 of 16 HLA-A2 patients evaluated for CEA-specific responses had high volume disease; patients 24, 41, 35, and 55 had low volume disease; Table 3).

The increased trend in survival seen in patients receiving GM-CSF (Fig 3) is potentially important in light of two studies published[38,39] after submission of this article. One study[38] used an autologous whole tumor cell vaccine in patients with non–small-cell lung cancer and demonstrated that vaccine-associated GM-CSF secretion was statistically significantly associated with survival. The second study, involving patients with resected colorectal cancer, used recombinant CEA protein, with half the patients receiving GM-CSF (80 μg/d for 4 consecutive days). In that study,[39] GM-CSF significantly augmented the amplitude of proliferative T-cell responses to CEA protein, and the development of anti-CEA IgG titers were associated with increased survival, whereas standard prognostic factors had no relationship, with the exception of serum CEA values.

It should be pointed out that 12 (86%) of 14 patients with disease stabilization on this trial who went on to receive prolonged vaccines had smaller tumor burden and were not as heavily pretreated as some of the other patients on this trial. To date, no patient with rapidly progressing tumor has responded clinically to the vaccine. These observations underscore the need to further explore these vaccine strategies in patients with minimal disease burden and who are not heavily pretreated. The results reported here also underscore the need to further explore dose scheduling issues and the apparent need for continued vaccination of cancer patients.

Our results also support the concept that the evaluation of cancer vaccines in the clinical setting may require a different paradigm than the evaluation of traditional therapeutics. Traditionally, patients who progress on a cytotoxic agent are immediately taken off trial. The concept of vaccination, however, is based on the induction of an immune response with a primary vaccination and the enhancement of that immune response with subsequent booster vaccinations. One of the confounding factors, however, in the dose scheduling of cancer vaccines is the growth of tumors during the early vaccination phase, especially in phase I trials. Thus, it is quite conceivable that patients will progress after receiving their initial set of primary and booster vaccinations, but they may stabilize after receiving still more vaccinations. Indeed, evidence of this is presented in this trial. As shown in Table 5, 12 of 23 patients who progressed after two vaccinations achieved stable disease at 4 months. It is for this reason that two categories of disease stabilization were used in this study. Primary stable disease was defined as patients who had no progression during the first 4 months (four vaccinations) of therapy; secondary stable disease was defined as patients who had progressed after two monthly vaccinations but who achieved stable disease after four vaccinations.

Although it is clear that the CEA-TRICOM vaccines can induce immune responses specific for CEA in advanced cancer patients, the trends observed in correlating survival or progression-free survival with immune response or with a particular vaccine regimen need to be evaluated with caution. In essence, this was a phase I trial with a primary end point of evaluation of toxicity. Cohorts were small and nonrandomized, and a diverse group of carcinoma patients was administered vaccine. Moreover, the majority of these patients were heavily pretreated with prior therapies. Thus, larger randomized studies are needed to further define the trends seen in this phase I trial.

However, this study supports previous studies that CEA-based vaccines can induce CEA-specific immune responses and antitumor activities in patients with advanced carcinomas.[7-11,19,20,23,39,40] The vaccines and vaccine schema used in this study have, for the first time, used the following five strategies to enhance the immunogenicity of a tumor-associated self antigen: (1) the use of recombinant poxvirus vaccines to enhance tumor antigen presentation and the level of expression of the tumor antigen in virus-infected antigen-presenting cells; (2) the use of an rV virus as a priming vaccination to break tolerance or better present danger signals, followed by multiple vaccinations with recombinant avipox vaccines that can be administered repeatedly with no evidence of host-neutralizing antiviral activity; (3) the insertion of an agonist epitope within the CEA genome to enhance CD8-positive T-cell responses; (4) the use of GM-CSF at the vaccination site to enhance recruitment of dendritic cells to the regional nodes; and (5) the use of recombinant viral vaccines containing the transgenes for a triad of T-cell costimulatory molecules along with the CEA antigen transgene to further enhance T-cell activation.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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