Pilot Study of a Dual Gene Recombinant Avipox Vaccine Containing Both Carcinoembryonic Antigen (CEA) and B7.1 Transgenes in Patients with Recurrent CEA-expressing Adenocarcinomas[1]

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ABSTRACT

Coordinated presentation of antigen and costimulatory molecules has been shown to result in the induction of an antigen-specific T-cell response rather than the development of anergy. This study evaluated the vaccine ALVAC-CEA B7.1, a canary pox virus that has been engineered to encode the gene for the tumor-associated antigen carcinoembryonic antigen (CEA) and B7.1, a T-cell costimulatory molecule. Patients with CEA-expressing tumors were immunized with 2.5×10^7 ($n = 3$), 1.0×10^8 ($n = 6$), and 4.5×10^8 ($n = 30$) plaque-forming units intradermally every other week for 8 weeks. Patients with stable or responding disease received monthly boost injections. Biopsies of vaccine sites were obtained 48 h after vaccination to evaluate leukocytic infiltration and CEA expression. Induction of CEA-specific T-cell precursors was assessed by an ELISPOT assay looking for the production of IFN-γ. Therapy was well tolerated, without significant toxicity attributable to vaccine. All patients had evidence of leukocytic infiltration and CEA expression in vaccine biopsy sites. Six patients with elevated serum CEA values at baseline had declines in their levels lasting 4–12 weeks. These patients all had stable disease after four vaccinations. After four vaccinations, patients who were HLA-A-2-positive demonstrated increases in their CEA-specific T-cell precursor frequencies to a CEA-A2-binding peptide from baseline. The number of prior chemotherapy regimens was inversely correlated with the ability to generate a T-cell response. ALVAC-CEA B7.1 is safe in patients with advanced, recurrent adenocarcinomas that express CEA, and it is associated with the induction of a CEA-specific T-cell response.

INTRODUCTION

Vaccines have been used to successfully prevent disease for many years; however, their value in preventing or treating cancer has not been demonstrated. The majority of preclinical data suggests that the induction of cytotoxic T cells against a tumor-specific antigen can mediate an effective antitumor response and prevent recurrent malignancy. One such tumor-associated antigen is CEA,[3] a 180-kDa glycoprotein present on endodermally derived neoplasms and in the digestive organs of the human fetus (1, 2). CEA is a member of the immunoglobulin gene superfamily located on chromosome 19 (3). This glycoprotein is also classified as an adhesion molecule and may allow tumor cells to attach to normal cells in the metastatic process. Patients with breast, lung, gastric, colon, and ovarian cancers have elevated serum levels of CEA (4). Using immunohistochemistry, CEA is detected in 50% of breast adenocarcinomas (5), in >90% of colorectal (6) and gastric (7) adenocarcinomas, and up to 80% of lung cancers (8); CEA does not appear to be present in normal colonic mucosa, although there are several CEA-like antigens in normal tissues (9).

Adenocarcinomas expressing this antigen do not stimulate a significant immune response against CEA, probably because of its expression during fetal development. Despite this, CEA has been evaluated as an immunogen for antitumor vaccines because there is minimal expression of CEA in adult tissues, limiting the possibility of immunization leading to a potentially harmful autoimmune response. Various vaccines that immunize against CEA have not caused toxicity to normal organs. Approaches have included vaccination with autologous tumor cells (10), rCEA protein (11), anti-idiotypic antibodies (12), various viral constructs encoding the gene for CEA (13–15), naked DNA (16), and mRNA (17).

When an antigen is presented to a naive T cell, two signals are required to stimulate the T cell to divide and differentiate to

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[3] The abbreviations used are: CEA, carcinoembryonic antigen; APC, antigen-presenting cell; TCR, T-cell receptor; IL, interleukin; MAb, monoclonal antibody; GM-CSF, granulocyte macrophage colony-stimulating factor; pfu, plaque-forming unit(s); nCEA, native CEA; rCEA, recombinant CEA; PBMC, peripheral blood mononuclear cell; rV, recombinant vaccinia.

produce an immune response (18). First, an APC presents the protein antigen as peptide fragments in the groove of the MHC class I or MHC class II receptor to the TCR on $CD8^+$ and $CD4^+$ T cells, respectively. The peptide is recognized in a manner restricted by the MHC receptor. A second costimulatory signal, which is antigen-independent and does not bind the TCR, is also required (19). Without such a signal, the T cell develops an anergic response to the antigen. One such signal can be provided by B7 molecules. The two known B7 molecules, B7.1 and B7.2, are also known as CD80 and CD86. Binding of these costimulatory molecules to CD28 results in the production of multiple cytokines, including IL-2, and IFN-γ by $CD4^+$ and $CD8^+$ T cells.

The importance of B7 as a costimulatory molecule for the development of an effective antitumor immune response has been demonstrated by transfecting B7 into nonimmunogenic tumor cells. Tumor cells expressing B7.1 are rejected and also stimulate lasting immunity against wild-type tumor cells in murine models (20–22). Analysis of response at the cellular level reveals production of various cytokines, but not IL-4. This suggests that a subclass of $CD4^+$ cells, known as TH_1, which are important in the generation of cytotoxic T-cell responses, have been stimulated. IL-4 production is characteristic of a TH_2 response, which is responsible for the stimulation of humoral responses (23). B7.1 has been reported by some groups to preferentially stimulate a TH_1 response, whereas B7.2 stimulation induces a TH_2 response (24, 25).

Vaccine strategies that result in the coordinated presentation of antigen with a costimulatory molecule may result in improved immunity. Hodge *et al.* (26) vaccinated tumor-bearing mice with varying ratios of vaccinia vectors containing CEA (rV-CEA) and B7.1 (rV-B7). They examined T-cell responses and found a dose-dependent T-cell response when mice were vaccinated with rV-CEA and control vector, and no response when the animals were vaccinated with rV-B7 and control vector. However, animals receiving rV-CEA and rV-B7 had increased T-cell lymphoproliferative responses, especially at a 3:1 ratio of rV-CEA:rV-B7. There were also similar trends with *in vitro* cytotoxicity assays using a murine adenocarcinoma cell line transfected with CEA. Splenocytes from animals vaccinated once with rV-CEA:rV-B7.1 had a 2.8-fold increase in cytotoxicity against the CEA-expressing tumor cells compared to splenocytes from animals vaccinated with rV-CEA alone. There was no cytotoxicity seen with the parent adenocarcinoma cell line, which did not express CEA irrespective of which vaccine the mice were immunized with.

ALVAC-CEA B7.1 (designated Avipox-CEA/B7.1) is a canary pox vector encoding the gene for CEA and for B7.1. Canary pox is a member of the Avipox genus, along with vaccinia, both of which have been used for vaccine therapy. ALVAC has inherent characteristics that make it more attractive than vaccinia. Vaccinia is very immunogenic, limiting its serial use. Unlike vaccinia, canary pox can only replicate productively in avian species, eliminating the rare risk of a vaccinia infection (27, 28). Although ALVAC does not replicate in nonavian species, inoculations of recombinant Avipox into nonavian cells result in the expression of the transgene(s). ALVAC vectors expressing foreign antigens can elicit protective immune responses to viral pathogens in nonavian species (29–32). An ALVAC-CEA vaccine has undergone Phase I testing in cancer patients and induced CEA-specific CTL responses (15). In these studies (15) and others (14, 17, 33), CEA-specific cytolytic T-cell responses were demonstrated against a 9-mer CEA peptide designated CAP-1. These T cells were shown to be CEA-positive and inhibited by anti-class I MAb. Recently, an enhancer agonist epitope to CAP-1 has been identified (34) and designated CAP-1–6D (YLSGADLNL). This 9-mer peptide has now been shown to (*a*) bind MHC class I, (*b*) induce $CD8^+$ cytolytic T cells, (*c*) induce CTLs that are capable of lysing human carcinoma cells expressing CEA and the MHC-A2 allele, and (*d*) induce $CD8^+$ T cells that produce enhanced levels of TC1-type cytokines, INF-γ, and GM-CSF, but not IL-10 and IL-4 (34, 35).

Here we report on the results of a pilot study treating 39 patients with advanced CEA-producing adenocarcinomas with the recombinant vaccine, ALVAC-CEA B7.1. Toxicity as well as clinical, serological, and immunological responses are presented.

MATERIALS AND METHODS

Patient Eligibility

Patients with advanced or metastatic CEA-expressing adenocarcinoma who had failed standard therapy were eligible for participation after signing informed consent. Patients were required to have an elevated serum CEA, or immunohistochemical evidence of CEA expression in archival tumor samples. Patients were required to be ≥18 years of age, have an Eastern Cooperative Oncology Group performance status of 0–1, and adequate hematological, renal, and hepatic function. In patients with known liver metastases, transaminase elevations up to 3 times the upper limit of normal and total bilirubin up to 1.5 times the upper limit of normal were permitted. All patients had anergy panels performed to assess delayed-type hypersensitivity responses but were not excluded from participation in the study if they were anergic. All patients were HLA-typed to identify HLA-A2-positive patients, but HLA-A2-negative patients were not excluded. A lapse of at least 4–6 weeks from all prior anticancer therapy was required. Measurable and evaluable disease were allowed. Patients were excluded if they had evidence of immunocompromise, such as a known history of HIV infection, active eczema, atopic dermatitis, or any autoimmune disease, prior radiation to >50% of all nodal groups, prior splenectomy, or concurrent use of systemic steroids. Patients were also excluded if there was a prior history of another malignant process active in the last 2 years.

Treatment Schema

An initial dose escalation phase using 2.5×10^7 pfu ($n = 3$) and 1.0×10^8 pfu ($n = 6$)/injection was followed by vaccination with the working dose of 4.5×10^8 pfu/injection ($n = 30$). The vaccine was manufactured by Pasteur-Merieux Sera et Vaccins (Marcy, France/Troy, New York). The vaccine was given as an intradermal injection every 2 weeks for four injections. Patients were evaluated for toxicity using National Cancer Institute Common Toxicity Criteria, and for clinical response using standard response criteria (36). Patients with evidence of objective response or stable disease at 8 weeks were

allowed to continue on study receiving boost injections every 4 weeks, with re-evaluation every 8 weeks. Patients were removed from study for disease progression. While on study, patients were followed with routine laboratory testing as well as serum CEA evaluations.

Correlative Studies

Biopsies. Patients receiving the working dose of 4.5×10^8 pfu had 3-mm punch biopsies of the vaccine site performed 48 h after vaccination. H&E-stained slides were evaluated for evidence of necrosis, dermal leukocytic infiltration, and perivascular inflammation and estimated using a scale of none, minimal (1+), moderate (2+), or significant (3+). Sections were stained for CEA with a 1:4000 dilution of a rabbit polyclonal antibody (DAKO Corp., Carpinteria, CA) using the Techmate 1000 automated stainer with its protocol. Slides were pretreated with heat-induced epitope retrieval in steamer with citrate buffer. Areas of CEA-positive staining were determined by light microscopy. Negative control sections were stained in the same manner with normal rabbit IgG, diluted to the same protein concentration as the antirabbit CEA.

Anti-CEA Antibody ELISA Assay. The presence of anti-CEA antibodies in patient serum before and after vaccination was analyzed using an ELISA. Polyvinyl chloride 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA) were incubated overnight at 4°C with purified preparations of nCEA and rCEA (both from Vitro Diagnostics, Littleton, CO), as well as with BSA or human serum albumin at 100 ng/well in 50 μl of PBS (pH 7.2). nCEA was derived from biopsies of human colon adenocarcinoma from metastases to the liver. rCEA was derived from human MCF-7 carcinoma cells transfected with and expressing a glycosylated CEA. All CEA preparations were negative for the presence of nonspecific cross-reacting antigen (NCA), as determined using the anti-NCA MAb B6.23 (37) and an ELISA (38). The wells were blocked for 1 h at 37°C with PBS containing 5% BSA and then washed once with 1% BSA (assay buffer). Patient serum and control pooled human serum (Gemini Bio Products, Calabasas, CA) were diluted in assay buffer and added to wells in triplicate in a volume of 50 μl/well. Purified IgG of humanized COL-1, a complimentary determining region-grafted antibody derived from the murine COL-1 anti-CEA MAb (39), was used as a positive control antibody for CEA binding. After incubation overnight at room temperature, the wells were washed four times with assay buffer, and 50 μl of a 1:4000 dilution of peroxidase-conjugated goat antihuman IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well. After incubation at 37°C for 1 h, wells were washed four times with assay buffer, and 100 μl each of the chromogen *O*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO) and hydrogen peroxide were added to each well. After a 10-min incubation in the dark, the reaction was stopped with 25 μl of $4NH_2SO_4$. The absorbance of each well was measured at 490 nm using an ELISA microplate autoreader (Bio-Tek Instruments, Winooski, VT).

ELISPOT Assay. Assays were only done on samples from patients positive for the HLA-A2 allele. Samples were analyzed using a modification of the method described by Scheibenbogen *et al.* (40). Ficoll-purified mononuclear cells (PBMCs) were washed three times with PBS, viably frozen at $\sim 1 \times 10^7$ cells/ml in 10% DMSO in heat-inactivated AB serum, and thawed just before use (33). PBMCs obtained before vaccination and 2 weeks after the fourth vaccination were evaluated. Ninety-six-well MultiScreen-HA plates (Millipore Corporation, Bedford, MA) were coated with 100 μl of capture antibody against IFN-γ at a concentration of 10 μg/ml. After a 24-h incubation at room temperature, plates were blocked for 30 min with RPMI 1640 containing 10% human pool AB serum. Added to each well were 1×10^5 cells to be assayed. For each patient, between 5×10^5 and 5×10^6 total cells were analyzed, and the results were expressed as number of spots per 5×10^5. CIR-A2 cells pulsed with 25 μg/ml 9-mer CEA agonist peptide CAP1–6D (YLSGADLNL; Ref. 34) were added to each well as APCs at an effector:APC ratio of 1:3. Unpulsed CIR-A2 cells were used as a negative control. HLA-A2-binding flu matrix peptide 58–66 (GILGFVFTL) was added to identical wells at 5 μg/ml and was used as a peptide control. The responding cells were determined by the use of a Domino Image Analyzer (Optomax, Hollis, NH).

Table 1 Baseline patient characteristics[a]

	n
Sex	
Male	21
Female	18
Age	
Median	57
Range	23–74
Diagnoses	
Colon	22
Rectal	6
Breast	3
Pancreas	3
Appendix	1
Esophagus	1
Gallbladder	1
Lung	1
Thyroid	1
Prior therapy	
Chemotherapy	37
Median	3
Range	1–6
Radiation therapy	16
Immunotherapy	7
Hormonal therapy	2
Positive skin test	16
HLAA-2	26

[a] Frequency of characteristic is listed as *n*, with the exception of age, which is listed in years.

Statistical Methods. Univariate paired *t* test was used to compare baseline outcome measurements taken in the same patient at different time intervals. Multivariate Hotelling's T^2 test was used to compare differences between the treatment group (CEA) and the control (flu). The critical significance level was set to 5%.

RESULTS

Patient Characteristics. Thirty-nine patients were enrolled in this Phase I study. Patient characteristics are outlined in Table 1. The majority of the patients treated had metastatic colorectal cancer, with a minority of patients having breast,

Table 2 Toxicity using standard NCI toxicity scores[a]

Toxicity	Grade (*n*)			
	1	2	3	4
Local site	38	1	0	0
Constitutional				
Fatigue	9	2	0	0
Flu-like symptoms	8	0	0	0
Fever	5	4	2[b]	0
Gastrointestinal symptoms				
Abdominal discomfort	3	0	0	0
Nausea/Vomiting	1	2	2[c]	0
Anorexia	3	0	0	0
Hematologic				
Anemia	1	0	0	0
Leukopenia/thrombocytopenia	0			
Hepatic function				
Transaminases	4	2	3	0
Alkaline phosphatase	2	0	1	0
Total bilirubin	0	0	1	1

[a] Table lists most severe toxicity experienced during the first four vaccine injections.
[b] One patient with presumed pneumonia.
[c] One patient with bowel obstruction related to progressive disease.

lung, thyroid, gall bladder, esophageal, pancreas, and appendix cancers. The majority of patients had received prior therapy. All patients but two had received prior chemotherapy, with a median number of three prior regimens, and a range of one to six. Other prior therapies included radiation therapy, immunotherapies, and hormonal therapy. All patients except for one had measurable disease. The patient with nonmeasurable disease had colon cancer with an elevated CEA with documented intra-abdominal disease at laparotomy that was not visible by computed tomography scan. All patients had metastatic disease. Sixteen of the 39 patients developed a >2-cm reaction to one or more of the antigens of the CMI Multi-test; all others were anergic. Twenty-six of the 39 patients were HLA-A2-positive.

Toxicity. Vaccination with ALVAC-CEA B7.1 was well tolerated by most patients. Three additional patients were added to the 1.0×10^8 pfu dose level because of a grade 3 fever within 24 h of vaccination in one patient, which subsequently proved to be related to pneumonia. The toxicities observed are outlined in Table 2. The most frequent toxicity was local erythema and swelling at the vaccine site, associated with myalgias and flu-like symptoms after vaccination; symptoms were worse with the first vaccination and minimal with subsequent immunizations. There were no hematological toxicities except for anemia in one patient, which was deemed to be related to the underlying malignancy rather than to vaccine therapy. Gastrointestinal side effects such as nausea and vomiting were reported in eight patients. However, the patients with these symptoms had colorectal cancer ($n = 6$), recurrent appendiceal carcinoma ($n = 1$), and metastatic esophageal carcinoma to liver with celiac and retrogastric adenopathy ($n = 1$). It was therefore difficult to determine if these symptoms were attributable to the underlying disease *versus* the therapy. Twenty-six % of patients had elevations in transaminases as well as alkaline phosphatase. Again, the majority of the patients (80%) had known metastatic liver disease; the other two patients had bone ($n = 1$) and lung ($n = 1$) metastases. Seven of these patients exhibited increased liver function tests in association with disease progression, whereas two patients had baseline elevations, which remained unchanged throughout treatment, and another patient had transient elevation of transaminases after the first injection only.

Clinical Response. To be evaluable for response, patients had to receive four injections of vaccine. Of the first 39 patients, 9 patients were not evaluable. Two patients refused further therapy after the first and third injections, respectively. Six patients receiving vaccine at 4.5×10^8 pfu had progressive disease before receiving four vaccines. A final patient was removed from study for noncompliance. The remaining 30 patients received four vaccines, with 8 patients demonstrating stable disease at re-evaluation. These patients, seven with colorectal cancer and one with pancreatic cancer, received one to seven boost injections with continued stabilization of disease. There were no partial or complete responses.

Biopsy Data. All but one patient treated with 4.5×10^8 pfu of ALVAC-CEA B7.1 had a punch biopsy of the vaccine site 48 h after vaccination. All biopsies have shown leukocytic infiltrates in the epidermis and perivascular regions, with 17 demonstrating an infiltrate in the dermis (Fig. 1*A*). Fewer than 10% of patient biopsies demonstrated necrosis within the dermis. All samples stained by immunohistochemistry evaluating CEA expression demonstrated CEA staining in the region of the inflammatory response in leukocytes, in spindle-shaped cells suggestive of dendritic cells, and in fibroblasts (Fig. 1*B*). Negative control sections using an antirabbit IgG did not reveal any positive staining (data not shown).

CEA Responses. Thirty-one patients had serum CEA values >5.0 ng/ml; the remaining patients had evidence of CEA expression in their tumors as shown by immunohistochemistry. Serum CEA levels from baseline, week 4, and week 8 were available for 17 patients. Samples from the remaining patient were unavailable as patients were removed from study ($n = 9$), or samples were not drawn (week 4, $n = 3$; week 8, $n = 2$). Means with 95% confidence intervals of the difference between serum CEA values taken at week 4 to baseline and week 8 to baseline are shown in Table 3. There was an increase in serum CEA between baseline and weeks 4 and 8, and an increase between weeks 4 and 8. Six patients had declines in their CEA levels while receiving vaccine therapy, as outlined in Table 3, lasting a minimum of 4 weeks and as long as 12 weeks. The differences between baseline and the lowest measurement were normalized for each patient by the baseline. A mean decrease of 30.25% (SD = 16.59) was found significantly different than a mean decrease of 0% at $P = 0.0066$ by a one-tailed t test. Patients 11 and 28 were HLA-A2-positive, and each had an increase in CEA-specific T-cell precursor frequencies, as described below. All patients with declines in their CEA had stable disease at initial response evaluation and received vaccine boost injections after the initial four immunizations. Patients 8, 9, 12, and 14 had negative anergy panels at initiation of vaccine therapy.

Anti-CEA Antibody Response. An evaluation of antibody responses specific for CEA was also performed using sera before and after vaccination from 31 patients regardless of whether they were HLA-A2-positive or not. The assay used was a quantitative ELSIA for anti-CEA IgG. Only two patients

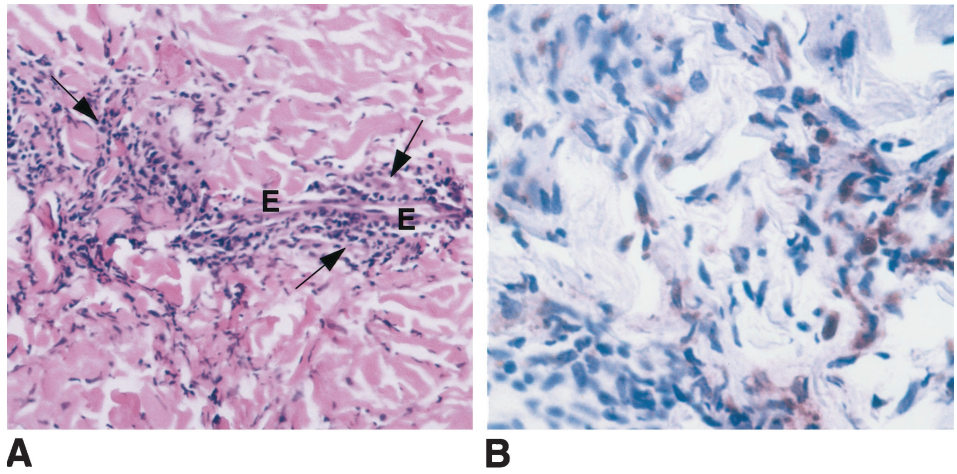

Fig. 1 Photomicrographs of biopsy sites demonstrating leukocytic infiltration and immunohistochemistry showing CEA expression. *A*, region of dermis; *arrows*, perivascular leukocytic infiltration; *E*, the endothelium of the blood vessel (×20). *B*, dermis stained for CEA expression demonstrating staining within leukocytes as well as fibroblasts.

Table 3 Values of serum CEA levels patients with a decline in CEA

Six patients with elevated serum CEA at the time of study entry had declines in their CEA levels. Patients continued to have monthly CEA levels drawn until radiographic evidence of disease progression. The decline in serum CEA is statistically significant by one-tailed *t* test ($P = 0.0066$).

Patient	Baseline	wk 4	wk 8	wk 12	wk 16	wk 20	wk 24	wk 28
8	681	327	289	604	700[a]			
9	204	147	ND[b]	163[a]				
11	273	162	258	331	398	398[a]		
12	42	ND	32	29	37	38	39	52[a]
14	147	116	169	462	524	934		
28	86	83	77	81				

[a] Value at the time of radiographic progression and removed from study.
[b] ND, not done.

showed the induction of CEA-specific IgG; the results of three other patients were marginal. As seen in Fig. 2, prevaccination sera from patient 16 (Fig. 2, ○) showed no reactivity to either native nCEA (Fig. 2*A*) or recombinant rCEA (Fig. 2*B*). Little or no reactivity was also seen in sera obtained after one vaccination. Sera obtained 2 weeks after the third vaccination with ALVAC-CEA-B7.1, however, showed reactivity to both nCEA and rCEA (Fig. 2, *A* and *B*) but did not react to the control BSA protein (Fig. 2*C*).

Results of pre- and postvaccination sera obtained from patient 20 are shown in Fig. 2, *D*, *E*, and *F*). Again, no anti-CEA-specific IgG is observed before vaccination and after one vaccination. However, anti-CEA-specific is seen after vaccination 2 (Fig. 2, ■). This is potentiated after the third vaccination (Fig. 2, ▲). Again, none of the sera reacted with the control BSA protein.

T-Cell Assays. Nineteen of the 26 patients who were HLA-A2-positive received four vaccinations and had baseline and post-vaccine 4 samples available for T-cell assays. These samples were obtained from 1 patient receiving 2.5×10^7 pfu, 2 patients receiving 1×10^8 pfu, and 12 patients receiving 4.5×10^8 pfu. Lymphocytes from four patients were not viable when thawed for evaluation; therefore, data are available for 15 of the HLA-A2-positive patients. T-cell assays using the HLA-A2 class I allele 9-mer CEA peptide CAP1–6D and the HLA-A2 9-mer Flu matrix peptide were used to investigate T-cell responses in patients positive for the HLA-A2 allele. PBMCs were assayed after only 24 h in culture in the presence of peptide to rule out effects of *in vitro* selection of T-cell populations. The ELISPOT assay using CEA and Flu peptides and PBMCs from each patient before and after vaccination was always done simultaneously and coded to minimize interpretive bias. Results are expressed as a precursor frequency of IFN-γ-secreting cells in response to the given peptide. A smaller number in the denominator of the precursor frequency expresses a higher number of precursors.

As seen in Table 4, PBMCs from all 15 HLA-A2-positive patients showed a <2-fold difference in precursor frequency to the Flu 9-mer peptide at baseline and after four vaccinations with ALVAC-CEA-B7.1; this difference was not statistically significant by a univariate paired *t* test ($P = 0.123$). In the same assays, however, these PBMCs showed a statistically significant increase in the CEA-specific T-cell precursor frequency after vaccination as compared to before vaccination ($P = 0.001$). Twelve of 15 patients had at least a 2-fold increase in T-cell precursors specific to CEA peptide. Three of these patients (4, 17, and 20) showed increases of at least 4-fold in postvaccination precursor frequency, whereas patients 30 and 34 showed increases in CEA-specific precursors of 9-fold and 14-fold, respectively, after four vaccinations with ALVAC-CEA-B7.1. Patient 30 had only received concurrent chemoradiotherapy for rectal cancer, and patient 34 received no prior chemotherapy for

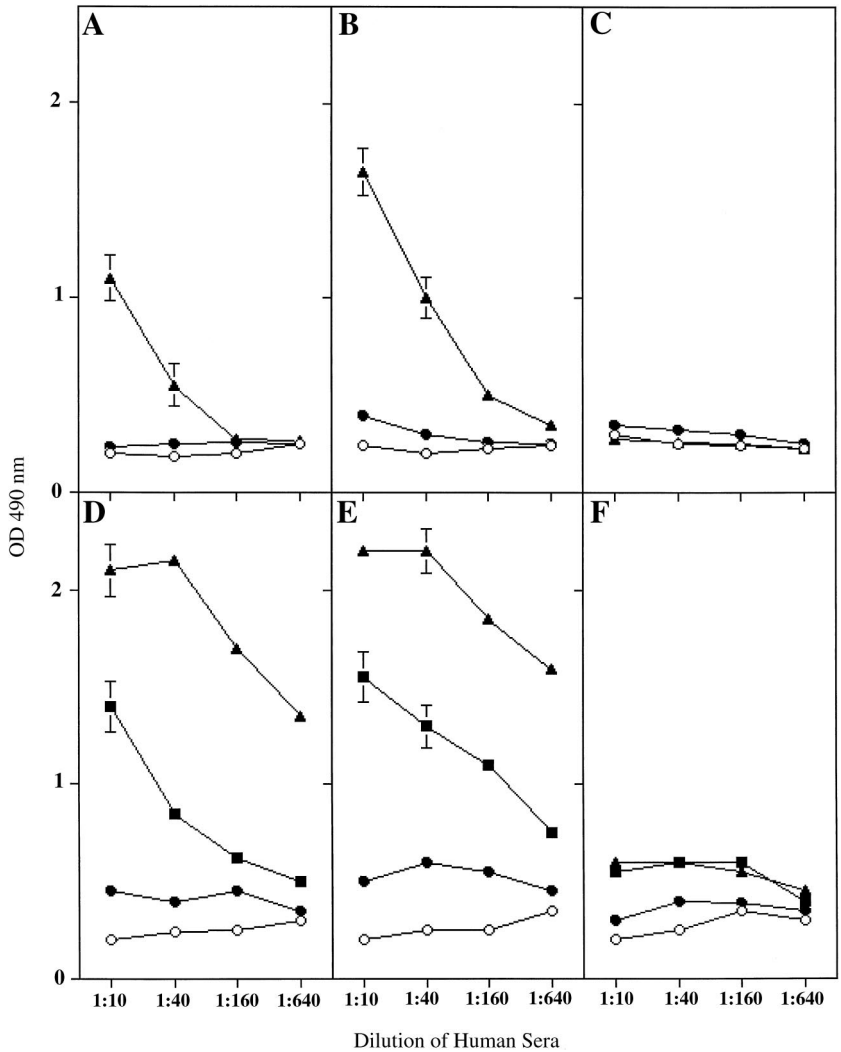

Fig. 2 Generation of CEA-specific IgG after vaccination using a quantitative ELISA. *A*, *B*, and *C*, results from patient 16; *D*, *E*, and *F*, results from patient 20. IgG reactivity was defined *versus* nCEA (*A* and *D*) and BSA control protein (*C* and *F*). Sera were obtained 2 weeks after one vaccination (●), two vaccinations (■), and three vaccinations (▲) with ALVAC-CEA-B7.1.

gallbladder cancer. Patients 4, 10, and 11 had clinically stable disease for 8, 4, and 4 months, respectively; the other patients in this group progressed after the initial four vaccinations. Patients 2, 6, 17, 18, and 30 had negative anergy panels at baseline, whereas all other patients had evidence of a delayed type hypersensitivity response. The hypothesis of equality of mean differences between pre- and postvaccination T-cell precursors for CEA and flu, respectively, was rejected in the multivariate analysis (Hotelling's T^2 test, $P = 0.0035$). Multiple regression analysis evaluated whether the number of prior chemotherapy regimens, length of time with cancer diagnosis, and prevaccine anergy status were associated with the difference between initial and final CEA-specific T-cell responses in HLA-A2-positive patients. The only significant predictor was the number of prior chemotherapy regimens ($P = 0.017$), as shown in Fig. 3.

DISCUSSION

This is the first study to use a dual-gene vector in clinical trials using the tumor-associated and T-cell costimulatory transgenes, CEA and B7.1, respectively. Vaccination with ALVAC-CEA B7.1 is safe for patients with metastatic CEA-expressing adenocarcinomas. No significant toxicity was observed in 39 patients treated with intradermal injections of 2.5×10^7 pfu to 4.5×10^8 pfu of ALVAC-CEA B7.1. Epithelium of the gallbladder and biliary ducts as well as neutrophils express proteins that share some homology to CEA, termed biliary glycoprotein and nonspecific cross-reactive antigen, respectively. There was no clinically evident induction of an autoimmune reaction against CEA or homologous proteins because no evidence of colitis, cholangitis, or neutropenia was observed.

All biopsies of vaccine sites in patients treated with 4.5×10^8 pfu ALVAC-CEA B7.1 showed evidence of CEA expression. This demonstrates that the ALVAC virus can infect cells of the dermis and epidermis and lead to the production of CEA protein that persists for at least 48 h. B7.1 expression was not evaluated because of the normal expression of B7.1 on APCs, which would be expected in the population of infiltrating leukocytes. Biopsies were performed only after the first vaccination. Clinically, patients had less erythema and swelling with subsequent vaccinations. It is possible that this decrease in local

Table 4 Induction of CEA-specific T cells in HLA-A2-positive patients immunized with ALVAC-CEA-B7.1

ALVAC-CEA-B7.1 was injected at three dose levels (2.5×10^7 pfu, 1.0×10^8 pfu, and 4.5×10^8 pfu). Patients were vaccinated on days 1, 14, 28, and 42. Results are expressed as a precursor frequency of IFN-γ-secreting cells. PBMCs from HLA-A2-positive patients from baseline and after four vaccinations (post) were used as effector cells.

Patient no.	Treatment (pfu)	Sample	Flu spots/ 5×10^5	Flu precursor frequency	Ratio of post:pre (Flu)	CEA spots/ 5×10^5	CEA precursor frequency	Ratio of post: pre (CEA)
2	2.5×10^7	Baseline	3	1/166,666	1.3	≤2	<1/200,000	≥1.6
		Post	4	1/125,000		4	1/125,000	
4	1.0×10^8	Baseline	5	1/100,000	0.8	≤2	<1/200,000	≥4.0
		Post	4	1/125,000		8	1/62,500	
6	1.0×10^8	Baseline	12	1/45,454	0.9	6	1/83,333	3.5
		Post	10	1/50,000		21	1/23,809	
10	4.5×10^8	Baseline	3.6	1/140,000	1.0	4.3	1/116,000	1.3
		Post	3.6	1/140,000		5.7	1/87,000	
11	4.5×10^8	Baseline	7.9	1/63,636	0.9	3.6	1/140,000	2.1
		Post	9.3	1/68,333		8.6	1/68,000	
17	4.5×10^8	Baseline	7.9	1/63,636	1.2	≤2	<1/200,000	≤4.3
		Post	9.3	1/53,846		10.7	1/46,666	
18	4.5×10^8	Baseline	5.2	1/19,230	1.1	3.2	1/31,250	2.6
		Post	5.8	1/17,241		8.4	1/11,904	
20	4.5×10^8	Baseline	7	1/71,000	1.2	9	1/55,556	4.2
		Post	8	1/62,000		38	1/13,157	
26	4.5×10^8	Baseline	10.5	1/31,250	0.5	15.5	1/32,258	0.7
		Post	7.5	1/66,666		11.5	1/43,478	
27	4.5×10^8	Baseline	3	1/166,666	1.2	1.5	<1/200,000	≥3.6
		Post	3.5	1/142,857		9	1/55/555	
28	4.5×10^8	Baseline	51.5	1/9,708	1.3	13.5	1/34,483	2.3
		Post	65.5	1/7,633		33	1/15,151	
30	4.5×10^8	Baseline	38.3	1/33,333	1.5	4	1/125,000	9.1
		Post	43.3	1/22,222		36.5	1/13,698	
34	4.5×10^8	Baseline	38.3	1/13,043	1.0	≤2	<1/200,000	≥14.3
		Post	43.3	1/12,500		30.8	1/13,953	
36	4.5×10^8	Baseline	23.4	1/21,429	1.7	6.7	1/75,000	3.8
		Post	39.2	1/12,766		25	1/20,000	
37	4.5×10^8	Baseline	10.9	1/46,153	1.3	4.2	1/120,000	3.2
		Post	14.2	1/35,294		13.4	1/37,500	

reaction was attributable to the development of an immune response against the viral vector, thus limiting infection by the virus, and subsequent production of CEA and B7.1. In other studies using ALVAC-CEA, multiple vaccinations lead to increasing numbers of CEA-specific T-cell precursors.[4] Antibody assays against ALVAC using samples from this study are under way.

This Phase I study also shows that a dual-gene avipox vector containing the CEA gene and the B7.1 gene can generate increases in CEA-specific precursors from PBMCs before and after vaccination using an ELISPOT assay in which cells were cultured less than 24 h. This short-term assay was used to minimize potential artifacts that can be obtained by culturing PBMCs several times *in vitro* in the presence of peptide and IL-2. The use of another 9-mer peptide (Flu matrix) in each assay, which was run simultaneously and coded, helped decrease bias and assay artifacts. Moreover, PBMC ELISPOT assays from pre- and postvaccination samples from the same patient were always thawed and run simultaneously. The ratio of anti-Flu matrix T-cell precursors in HLA-A2-positive patients ranged from 0.5–1.7, with a median of 1.2, and did not statistically significantly differ from baseline to after the vaccine ($P = 0.123$). However, against the CEA peptide, the ratios ranged from 0.7 to ≥14.3, with a median of 3.2, and were statistically different ($P = 0.001$). Patients 4, 10, and 11 had stable disease after the initial four vaccines, with CEA ratios of pre- to postvaccine ratios of ≥4, 1.3, and 2.1.

The incorporation of the whole CEA gene within the vaccine has potential benefits over the use of peptides. Expression of the whole CEA protein *in vivo* allows for processing of the entire protein by each individual patient, leading to presentation of multiple CEA epitopes, including helper epitopes. This approach does not limit the patient population that can be treated, unlike approaches using HLA-restricted peptides. The immunological assessment of this clinical trial was limited to HLA-A2-expressing patients by the use of known HLA-restricted peptides in the ELISPOT assays. The development of an assay using whole CEA protein, which is under way in our laboratory, will allow for the evaluation of all immunological responses irrespective of HLA haplotype, and thus allow for testing of samples for which HLA specific peptides are not known.

There were no clinical responses in this Phase I study, and the majority of patients had rises in the serum CEA values (Fig. 2). However, 20% percent of all study participants and 27% of all evaluable patients had stabilization of disease after four

[4] J. Schlom and J. Marshall, personal communication.

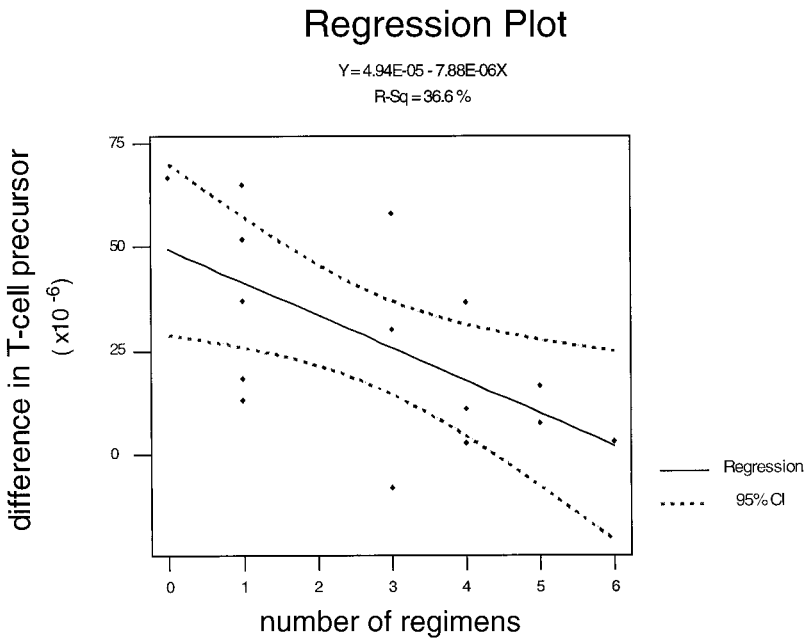

Fig. 3 Regression analysis showing the association between number of prior chemotherapy regimens (*X* axis) and the difference between initial and final CEA-specific T-cell responses in HLA-A2-positive patients (*Y* axis).

immunizations. Eight patients continued therapy for 1 ($n = 1$), 2 ($n = 3$), 3 ($n = 1$), 4 ($n = 1$), and 6 ($n = 2$) months after the initial four immunizations. Six patients also had declines in their serum CEA levels as outlined in Table 3. All patients with an initial decrease in serum CEA had stable disease after four vaccines. Initial decreases in serum CEA may result from the induction of an anti-CEA humoral response, resulting in antibody-antigen complexes. Of the patients with CEA responses, two also were HLA-A2-positive and had ratios of baseline to postvaccination CEA-specific T cells of 2.1 and 2.3, respectively.

The difficulty in evaluating this and other anti-CEA vaccine clinical trials lies in the interpretation of the demonstrated immunological responses. As seen in this study, patients can develop anti-CEA peptide-specific T-cell responses as measured from PBMCs, but these responses are not clearly correlated with clinical response. T-cell responses in the regional lymph nodes and at tumor site(s) were not evaluated. The ELISPOT assay used in this study examines the response to one HLA-A2 peptide (CAP1–6D) and not to autologous tumor cells; it may be an inaccurate predictor of meaningful immunological responses. CAP1–6D, a modification of the CEA immunodominant epitope CAP1, has enhanced binding to CEA-specific TCRs (33). The *in vitro* response thus may overestimate the *in vivo* ability of the T-cell precursor population to be stimulated; however, as discussed above, this method does diminish the risk of stimulating T-cell responses *in vitro* with rounds of antigen-pulsing and IL-2 exposure. In addition, the ELISPOT assay does not measure the binding avidity of the TCR for the CAP1–6D peptide presented in the groove of the HLA-A2 receptor. Binding avidity may be at least as important as precursor frequency for responses against CEA-expressing tumor cells. It has been demonstrated that T-cells stimulated with the CAP1–6D peptide have the ability to lyse human tumor cell expressing CEA in a MHC-restricted manner (33). However, the T-cell precursor frequency alone may not be the appropriate laboratory correlate. A recent study in strains of T-cell transgenic mice whose $CD8^+$ or $CD4^+$ T cells had specificity for an influenza antigen demonstrated, even in the presence of 100% $CD8^+$ T cells, that rejection of a murine tumor cell line transfected with influenza occurred only 60% of the time. Animals with both antigen-specific $CD4^+$ and $CD8^+$ T cells were completely protected from tumor growth (41). Evidence of T-cell stimulation against autologous tumor cells may potentially be a more specific and valuable test; however, such information is difficult to obtain in the patient care setting.

It is unknown if the lack of clinical responses was attributable to an insufficient stimulation of T-cell precursor numbers or to other factors. Phase I studies traditionally include patients with significant prior treatment and with metastatic disease, both of which may impact on the ability of patients to mount effective antitumor immunological responses. In the HLA-A2 patients studied, there was an inverse correlation between their ability to increase their T-cell precursor frequency and the number of prior chemotherapy regimens (Fig. 3). Others have demonstrated defective function of T cells (42) and dendritic cells (43) in cancer patients. Therefore, the lack of clinical response may reflect a relatively immunocompromised patient population attributable to prior therapy and advanced cancer, rather than inefficacy of an immunological approach.

The copresentation of B7.1 with CEA was used to attempt to enhance the anti-CEA T-cell response. There have been no randomized trials comparing the two directly, but in studies using ALVAC-CEA alone using an i.m. injection given every 4 weeks, the increase in T-cell precursor frequency after three vaccinations is less than was observed in this study.[4] Evaluation of adjuvants to enhance immunological response may allow the generation of a higher T-cell precursor frequency and suggest

that a minimum threshold number of antitumor T-cell precursors is required for a clinical response. ALVAC-CEA B7.1 is presently being used with one such adjuvant, GM-CSF. GM-CSF has been shown to up-regulate MHC class II expression on macrophages, enhance the maturation of dendritic cells and stimulate their migration, produce a localized inflammatory response at the site of injection, as well as a systemic response in the bone marrow (44). It stimulates the growth of APCs such as dendritic cells and macrophages. Irradiated tumor cells transfected with GM-CSF have been used as vaccines in murine models and have elicited enhanced immune responses against tumors, even upon rechallenge with nontransfected tumor cells (45). Evidence of an increase in anti-CEA antibodies would indicate $CD4^+$ T cells were also induced against CEA.

The clinical impact of a vaccine approach may not be observable until it is assessed in a patient population without immune compromise. Those patients with the most extensive chemotherapy pretreatment were least likely to have immunological response to vaccination. This supports testing vaccine strategies such as this one in the adjuvant setting with minimal disease. Future studies should prospectively assess the value of costimulation by comparing ALVAC-CEA with ALVAC-CEA B7.1-based strategies and should also formally assess the impact of chemotherapy on the induction of immune responses by vaccination.

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Clinical Cancer Research

Pilot Study of a Dual Gene Recombinant Avipox Vaccine Containing Both Carcinoembryonic Antigen (CEA) and B7.1 Transgenes in Patients with Recurrent CEA-expressing Adenocarcinomas

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