Cancer Immunol Immunother (2000) 49:504–514

ORIGINAL ARTICLE

Heidi Hörig · David S. Lee · William Conkright
Joe Divito · Henry Hasson · Michelle LaMare
Audrey Rivera · David Park · John Tine · Ken Guito
Kwong Wong-Yok Tsang · Jeffrey Schlom
Howard L. Kaufman

Phase I clinical trial of a recombinant canarypoxvirus (ALVAC) vaccine expressing human carcinoembryonic antigen and the B7.1 co-stimulatory molecule

Received: 6 May 2000 / Accepted: 13 July 2000

Abstract The generation of cytotoxic effector T cells requires delivery of two signals, one derived from a specific antigenic epitope and one from a costimulatory molecule. A phase I clinical trial was conducted with a non-replicating canarypoxvirus (ALVAC) constructed to express both human carcinoembryonic antigen (CEA) and the B7.1 costimulatory molecule. This was the first study in cancer patients to determine if the delivery of costimulation with a tumor vaccine was feasible and improved immune responses. Three cohorts of six patients, each with advanced CEA-expressing adenocarcinomas, were treated with increasing doses of an ALVAC-CEA-B7.1 vaccine (4.5×10^6, 4.5×10^7, and 4.5×10^8 plaque-forming units, PFU). Patients were vaccinated by intramuscular injection every 4 weeks for 3 months and monitored for side-effects, tumor growth and anti-CEA immune responses. ALVAC-CEA-B7.1 at doses up to 4.5×10^8 PFU was given without evidence of significant toxicity or autoimmune reactions. Three patients experienced clinically stable disease that correlated with increasing CEA-specific precursor T cells, as shown by in vitro interferon-γ enzyme-linked immunoassay spot tests (ELISPOT). These three patients underwent repeated vaccination resulting in augmented CEA-specific T cell responses. This study represents the first use of costimulation to enhance antitumor vaccines in cancer patients. This approach resulted in CEA-specific immunity associated with stable diseases in three patients. This study also demonstrated that CEA-specific T cell responses could be sustained by repeated vaccinations. Although the number of patients was small, the addition of B7.1 to virus-based vaccines may improve immunological and stable diseases to vaccination against tumor-associated antigens with tolerable toxicity.

Key words Tumor vaccine · Costimulation · Poxvirus · Carcinoembryonic antigen

H. Hörig · D. S. Lee · W. Conkright · J. Divito · H. Hasson
M. LaMare · A. Rivera · D. Park · H. L. Kaufman (✉)
Albert Einstein Cancer Center,
1300 Morris Park Avenue, Bronx, NY 10461, USA
Tel.: +1-718-430-3517
Fax: +1-718-430-3099

J. Tine
Virogenetics Corporation,
Troy, NY, USA

K. Guito
Pasteur Mérieux Connaught Laboratories,
Swiftwater, Pa., USA

K. W.-Y. Tsang · J. Schlom
Laboratory of Tumor Immunology and Biology,
National Cancer Institute, Bethesda, Md., USA

Introduction

Vaccination has become standard practice for the prevention of various infectious diseases. The possibility of using similar strategies for cancer was suggested nearly a century ago and has been supported by a prolific body of research in animal models. A recent resurgence in cancer vaccine development has been spurred-on by a better understanding of how immune responses are induced in both normal and cancer-bearing individuals. The ability to elicit antigen-specific T cell responses appears to be of central importance in eradicating established tumors in animals and has, hence, been the focus for tumor vaccine development in humans. New generations of vaccines are now being designed to activate T cells more efficiently and specifically in cancer patients.

Tumor-associated antigens have been identified for a wide range of tumors, although none has been entirely tumor-specific. The array of isolated antigens includes viral proteins (i.e. human papillomavirus E6 and E7 proteins), mutated self-proteins (i.e. ras), and non-mutated self-proteins (i.e. carcinoembryonic antigen and prostate-specific antigen). Human T cell epitopes have been identified in representative antigens from each of these groups, suggesting that these antigens may be able to function as targets for cytotoxic T cells under appropriate conditions [39]. The method for delivering

individual T cell epitopes by vaccination has utilized many different strategies. The simplest has been the use of a short peptide containing the epitope sequence, often admixed with a non-specific adjuvant, such as Freund's incomplete adjuvant. Other approaches used whole proteins pulsed onto dendritic cells, tumor cell lysates, or recombinant viruses engineered to express the human tumor antigen. While many viruses may be used for this purpose, the poxviruses are frequently chosen because of their efficient antigen presentation and ability to induce strong T cell responses. The development of neutralizing antibodies against vaccinia virus, the prototype poxvirus, has led to the use of other non-replicating poxviruses. Canarypoxvirus (ALVAC) does not replicate in mammalian cells, is not pathogenic for humans, can be delivered repetitively without neutralization, and generates strong cytotoxic T lymphocyte (CTL) responses [42].

Carcinoembryonic antigen (CEA) is a self-protein expressed by fetal colon epithelium and over-expressed in a large number of adenocarcinomas, including those of the colon, rectum, stomach, pancreas, lung, and breast [11]. Recently, isolation of several class-I-restricted T cell epitopes from CEA has been reported [14, 28, 37]. Vaccine strategies targeting CEA have included recombinant vaccinia viruses and ALVAC expressing the full-length CEA gene, as well as CEA-pulsed dendritic cells and HLA-restricted CEA peptide vaccines [6, 19, 21, 24, 37]. CEA-specific proliferative T cell and antibody responses have also been seen in patients vaccinated with a CEA anti-idiotype monoclonal antibody [9], and with a CEA protein in combination with granulocyte/macrophage-colony-stimulating factor [32]. Although these strategies were generally safe and demonstrated CEA-specific T cell and antibody responses, few patients responded with significant stable disease.

The clonal expansion of antigen-specific effector T cells requires the delivery of costimulatory signals by antigen-presenting cells. When antigen is presented to naïve T cells, the simultaneous ligation of homodimeric B7.1 (CD80) to CD28 on the surface of the T cell results in increased production of interleukin-2 (IL-2), synthesis of high-affinity IL-2 receptors (CD25), and T cell proliferation [4]. Recombinant viral vaccines expressing tumor-associated antigens are thought to generate effector T cells through presentation of epitopes following host immunization. Several tumor-derived antigens have been identified as self-proteins that possess putative specific T cell epitopes [31]. However, these antigens may not ordinarily elicit effector T cell responses because costimulation does not occur at the time of antigen presentation by the tumor cell [23]. In fact, studies have shown that, when B7.1 was introduced into tumor cells, enhanced recognition by $CD8^+$ T cells was observed against specific antigens and tumor cells expressing the antigen [15, 40]. Furthermore, recombinant vaccinia virus co-expressing tumor antigen and B7.1 genes resulted in improved therapeutic responses in murine tumor models [1, 12]. The introduction of B7.1 as an adjuvant to virus-based tumor vaccination has not been previously reported in human patients. We sought to evaluate the effect of costimulation using a recombinant ALVAC virus expressing both CEA and the B7.1 costimulatory molecule.

The objectives of this trial were to determine the dose and toxicity of three monthly intramuscular vaccinations with ALVAC-CEA-B7.1 vaccine. The effects of this vaccine on development of CEA-specific T cell responses and disease progression were also evaluated. Interestingly, we observed 3 patients who developed evidence of CEA-specific T cell responses after vaccination, which correlated with stable diseases. Although the responses were modest, all 3 patients received a second cycle of vaccinations and were able to boost their CEA-specific T cell responses. These results are the first to show a correlation of clinical outcome with in vitro T cell responses after administration of CEA-directed vaccines and suggest that continued boosting may be critical for achieving meaningful stable diseases in patients harboring established tumors. The co-expression of B7.1 in recombinant vaccines appears to be safe in humans and may provide a method for enhancing the therapeutic effectiveness of vaccines targeted against tumor-associated antigens.

Patients and methods

Clinical protocol

Patients with measurable and unresectable metastatic adenocarcinoma expressing CEA were eligible for this study. All patients had CEA expression confirmed by measurement of serum CEA or histological expression by immunohistochemical staining of tumor biopsy specimens as previously described [11]. Patients with cardiac, pulmonary, hepatic, renal, or neurological disorders were excluded. A history of eczema, ulcerative colitis, recent steroid use, or other cancer therapy within 4 weeks was also considered a contraindication to study entry. Patients were required to sign an informed consent form and the clinical protocol was approved by our Institutional Review Board. Prior to starting the trial patients had a complete history and physical examination, complete blood count, electrolyte panel, liver function studies, pregnancy test (if indicated), and computed tomography (CT) scan or magnetic resonance imaging of all sites of disease. Blood was also obtained for HLA typing by a nested-sequence polymerase chain reaction assay and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque separation and cryopreserved at −125 °C with approximately 10^7 cells/vial. Sera were collected from separated samples and stored at −80 °C.

Patients were treated at the General Clinical Research Center at our institution. A dose-escalation study design was employed with 6 patients receiving 4.5×10^6 plaque-forming units (PFU), 6 receiving 4.5×10^7 PFU, and 6 receiving 4.5×10^8 PFU of the ALVAC-CEA-B7.1 vaccine by intramuscular injection in the upper, outer arm. Vaccination was performed every 4 weeks for three total doses. Blood was obtained at each monthly visit for further toxicity analysis and PBMC collected for in vitro immune assays. One month after completion of the vaccination regimen the patients underwent radiological imaging of their disease. If there was no evidence of disease progression, patients were offered another series of three vaccinations at the same dose they had received previously.

Evaluation of treatment response

Treatment response was determined by measuring the volume of radiologically defined tumor lesions. The volume was calculated by

multiplying the maximum length of index lesions by the maximum width. In cases where multiple lesions were present, each was recorded separately and followed for response. Disease progression was defined as an increase of at least 25% in any lesion followed or the appearance of new lesions. Patients without change in the size of any lesion or with less than 25% increase of the lesion were recorded as having stable disease.

Clinical toxicity was determined through history, physical examination, and blood testing for evidence of subclinical abnormalities. Monthly complete blood counts, electrolytes, blood urea nitrogen, serum creatinine, bilirubin, alkaline phosphatase, liver transaminases, albumin, lactic acid dehydrogenase, prothrombin time, partial thromboplastin time, and serum CEA levels were determined in the clinical laboratories of the hospital. Laboratory abnormalities were graded according to the standard NCI common toxicity criteria. Evidence of grade III or greater non-hematological events was used to stop further treatment. The occurrence of the same grade III or greater side-effects in 2 patients at a given dose was used to define the dose-limiting toxicity.

Antibody titers

Antibody responses against ALVAC were tested by standard enzyme-linked immunosorbent assay (ELISA). Briefly, serial dilutions of patient pre-immune and post-vaccination sera (1:200 and 1:800) and positive sera [from 5 patients treated with ALVAC-RG (rabies glycoprotein) vaccine] were applied to plates coated with purified ALVAC virus. Wells were incubated with anti-(human IgG) conjugated to horseradish peroxidase and developed using a horseradish-peroxidase-specific substrate. The absorbance (A) at 450 nm was determined every 3 min for 21 min. After plotting the A_{450} against time in a preliminary assay, it was decided to use calculations based on 3- to 15-min intervals, since this represented the steepest slope of the curve. Post-vaccination titers were considered significant if they were greater than three standard deviations above the mean A_{450}/minute observed in pre-immune sera from each patient.

The immunofluorescent (HE-2000) anti-(nuclear antigen) (ANA) assay (ImmunoConcepts, Sacramento, Calif.) was used to detect auto-antibodies to nuclear antigen in patient sera according to the manufacture's instructions. Briefly, sera were diluted to 1:40 in phosphate-buffered saline (PBS) and incubated with slides covered with nuclear antigen substrate to allow specific binding of autoantibodies to cell nuclei. After washing, a fluorescein anti-(human IgG) antibody was added and cells were observed under light microscopy by a blinded investigator. Positive ANA samples were detected as bright fluorescence with characteristic staining patterns. If a sample was positive at 1:40 dilution, further serial dilutions were prepared (up to 1:1,280), and cells examined in a blinded fashion to determine the ANA titer.

Vaccine

ALVAC-CEA-B7.1 vaccine was derived from an unmodified parental canarypoxvirus (ALVAC) [35] by in vitro homologous recombination. CEA was obtained from the plasmid pGEM-CEA and modified by replacement of the 5′-untranslated sequences with the vaccinia early/late H6 promoter and replacement of the 3′-untranslated region with a T_5AT, vaccinia early transcription-termination signal [5, 29]. The H6-CEA-T_5AT expression cassette was cloned into an ALVAC donor plasmid containing flanking sequences corresponding to the non-essential ALVAC C3 locus. Homologous recombination was used to insert the CEA expression cassette into the ALVAC genome. Owing to the presence of extensive inverted terminal repetitions within the C3 locus, two copies of the CEA gene were present in the recombinant ALVAC-CEA genome, as shown in Fig. 1. The B7.1 gene containing the 867-nucleotide coding sequences and adjacent 5′- and 3′-untranslated regions was derived from the plasmid pBS-HB7–1. The B7.1 was modified in a similar manner to the CEA gene to include the vaccinia H6 promoter in the 5′-untranslated region before the ATG

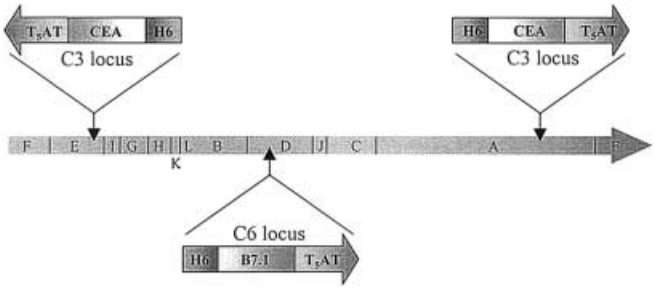

Fig. 1 Schematic diagram showing the construction of ALVAC-CEA-B7.1 virus. The full-length carcinoembryonic antigen (*CEA*) gene with the vaccinia H6 early/late promoter at the 5′-terminus and the vaccinia early-transcription activator at the 3′-terminus was inserted into an ALVAC donor plasmid. The plasmid was used for homologous recombination into the ALVAC C3 locus, a non-essential region generating an ALVAC-CEA virus. Owing to extensive inverted terminal repeats in this region, the CEA is duplicated as shown. The B7.1 gene with similar vaccinia H6 promoter and transcription activator sequences was inserted into a separate ALVAC donor plasmid. This was use to introduce the B7.1 gene into the ALVAC C6 locus to generate the ALVAC-CEA-B7.1 virus. The donor plasmids contain flanking sequences corresponding to the ALVAC insertion sites

start site and the T_5AT sequence in the 3′-untranslated region. An expression cassette, H6-B7.1-T_5AT, was inserted into an ALVAC donor plasmid containing flanking sequences of the ALVAC C6 locus. The ALVAC-CEA-B7.1 virus was generated by homologous recombination of the donor B7.1 plasmid into the ALVAC-CEA virus. The CEA sequence specifies a 180-kDa polypeptide and the B7.1 expresses a 50 to 55-kDa polypeptide (data not shown). Vaccine was produced in accordance with good manufacturing practices by Pasteur-Merieux-Connaught Laboratories (Swiftwater, Pa.) and was provided by the Cancer Therapy Evaluation Program (National Cancer Institute, Bethesda, Md.).

Peptides and cell lines

HLA-A2-restricted CEA peptides included the CEA-associated peptide, CAP-$1_{571–579}$ (YLSGANLNL) [37] and CAP-1-6D (YLSGA*D*LNL) [47]. The HLA-A24-restricted CEA peptide was $CEA_{570–579}$ (SYLSGANLNL). The HLA-A2-binding influenza viral protein was $M1_{58–66}$ (GILGFVFTL) [13]. An HLA-A3-binding CEA epitope, $CEA_{61–69}$ (HLFGYSWYK) and an HLA-A3-binding Flu control, $NP_{265–273}$ (ILRGSVAHK) was kindly provided by A. Sette (Epimmune, San Diego, Calif.) [14]. All peptides were dissolved in dimethylsulfoxide to achieve a final concentration of 5 mg/ml and further diluted in PBS. The T2-A2 cell line (HLA-$A2^+$, transporter in antigen processing (TAP)-deficient T-B cell hybrid) and the C1R-A24 (HLA-$A24^+$, B lymphoblast cell line) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Gaithersburg, Md.). G418 (300 μg/ml) was added to the medium used for culturing the C1R-A24 cell line. The EHM-A3 (HLA-$A3^+$, Epstein-Bair-virus-transformed) cell line was kindly provided by A. Sette (Epimmune, San Diego, Calif.) and cultured in RPMI-1640 medium with 10% FCS. Human TK^-143 cells, a human embryonic kidney cell line susceptible to poxvirus infection, were kindly provided by N. Restifo (Bethesda, Md.). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2% glutamine, 1% penicillin/streptomycin and 0.1% gentamicin (all Gibco, Grand Island, N.Y.).

ALVAC-CEA-B7.1 expression

To verify expression of CEA and B7.1, TK^-143 cells were plated onto 6-well tissue-culture plates and infected at a multiplicity of

infection of approximately 1:10 with ALVAC-CEA-B7.1 virus. Briefly, virus was diluted in DMEM containing 2.5% FCS for 2 h at 37 °C. The medium was replaced with complete DMEM and cells were placed in a CO_2 incubator at 37 °C for 24 h. Surface protein expression was evaluated by FACScan (BectonDickinson, San Jose, Calif.) analysis. Cells were stained with the mouse anti-(human CEA) mAb, COL-1 [26] and fluorescein-isothiocyanate-labeled secondary polyclonal goat anti-(mouse IgG) (PharMingen, San Diego, Calif.) for CEA expression. Cells were stained with a mouse anti-(human CD80) mAb labeled with phycoerythrin for B7.1 expression. Viable cells were gated by forward- and side-scatter with virus-infected cells additionally stained with propidium iodine for further exclusion of dead cells (data not shown). Fluorescence-activated cell sorting (FACS) analyses were conducted using CellQuest software (Becton Dickinson, San Jose, Calif.). CEA and B7.1 expression by normal TK^-143 cells was confirmed by FACS analyses of uninfected cells. Isotype-matched IgG control antibodies (IgG1 and IgG2a) were used to detect non-specific staining.

T cell phenotyping

Patient whole-blood samples were stained with fluorochrome-conjugated monoclonal antibodies (mAb) directed against CD3, CD4, CD8, CD14, CD16, CD45, CD56, CD69, and CD80 (Becton Dickinson, San Jose, Calif.), and HLA-DR (PharMingen, San Diego, Calif.). Whole-blood samples were incubated with appropriate combinations of mAb for 15 min at room temperature, red blood cells were lysed using Becton Dickinson lysis buffer, and cells were washed and fixed prior to analysis on a Becton Dickinson FACScan flow cytometer (San Jose, Calif.).

Interferon-γ (IFN-γ) ELISPOT assay

The precursor frequency of CEA-specific T lymphocytes was determined by enzyme-linked immunoassay spot tests (ELISPOT) for all patients expressing HLA types A-2, A-3, and A-24 (see Table 1). ELISPOT assays were performed on 96-well nitrocellulose plates (Millititer HA, Millipore, Bedford, Mass.) coated overnight with 10 μg/ml mouse anti-(human IFN-γ) monoclonal antibody (PharMingen, San Diego, Calif.). After washing, wells were blocked for 2 h at 37 °C with 200 μl complete medium containing DMEM (Gibco, Grand Island, N.Y.), 10% human AB serum (Bioreclamation Inc., Hicksville, N.Y.), 1% glutamine and penicillin/streptomycin. Cryopreserved PBMC were thawed, cultured overnight in complete medium, and plated onto nitrocellulose-coated 96-well plates at 2×10^5 cells/well with 6 wells/treatment group. Antigen-presenting cells (APC), such as T2-A2, C1R-A24 or EHM-A3 (see Peptides and cell lines), were added at 1×10^5 cells/well together with 50 μg/ml corresponding HLA-type-matched CEA peptides as indicated or, as control, without peptide. After a 24 h incubation at 37 °C and 5% CO_2, plates were washed several times with PBS containing Tween 20 (0.05%) and incubated with 2 μg/ml biotinylated mouse anti-(human IFN-γ) mAb (PharMingen, San Diego, Calif.) at 4 °C overnight. Subsequently, wells were washed with PBS containing Tween 20 (0.05%) and incubated with a 1:4,000 dilution of avidin-linked alkaline phosphatase (Gibco BRL, Grand Island, N.Y.) for 2 h, followed by washing with PBS. After addition of 100 μl 5-bromo-4-chloro-3-indolyl phosphate/nitroblue/tetrazolium substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) for 1 h, plates were washed and allowed to dry in air. IFN-γ spot-forming units (SFU) were counted by an observer in a blinded manner on a stereomicroscope. The precursor frequency of CEA-specific cells was calculated as the number of SFU from PBMC + APC + peptide after subtraction of the background (PBMC + APC) per number of PBMC seeded.

Results

Clinical characteristics of the patients

A recombinant ALVAC virus was engineered to co-express human CEA and B7.1 as shown in Fig. 1. ALVAC virus administration results in abortive replication after intramuscular injection into mammalian cells. Despite this, the virus expresses foreign transgenes and has been shown to function effectively as an immunization vehicle, with the ability to elicit strong CTL responses [29, 35]. The expression of CEA and B7.1 was confirmed following in vitro infection of a human cell line with ALVAC-CEA-B7.1 (Fig. 2).

Eighteen patients with measurable, metastatic CEA-expressing adenocarcinomas were enrolled in a phase I clinical trial after giving informed consent. A dose-escalation study design was employed with 6 patients

Table 1 Patient characteristics of ALVAC-CEA-B7.1 clinical trial. *CEA* carcinoembryonic antigen, *C* chemotherapy, *S* surgical resection, *RT* radiation therapy, *NA* not available

Vaccine dose (PFU)	Patient number	Age/sex	HLA-A status	Primary cancer/metastases	Prior therapy	Serum CEA (ng/dl)	
						Pre-vaccine	Post-vaccine
4.5×10^6	1[a]	70/F	2, 3	Pancreas/liver	None	1[b]	0.5
	2	74/M	1, 2	Gastric/liver	C, S	9	NA
	3[a]	59/M	2, 3	Colon/liver	C, S	297	970
	4	66/M	2, 2	Colon/liver	C, S	5	14
	5[a]	50/M	24, 24	Colon/liver	C, S, RT	62	275
	6	73/F	3, 23	Lung/lung	C	35	62
4.5×10^7	7	57/F	1, 31	Lung/lung	C	12	40
	8	68/F	1, 1	Colon/lung	C, S	15	25
	9	63/M	2, 24	Colon/liver	C, S	154	807
	10	56/F	24, 11	Colon/liver	C, S	637	1960
	11	60/F	1, 2	Rectal/lung	C, S, RT	296	780
	12	45/F	1, 3	Lung/lung	C, S, RT	76	NA
4.5×10^8	13	66/F	21, 28	Colon/liver	C, S	166	223
	14	74/M	29, 66	Colon/liver	C, S	2013	3613
	15	51/M	2, 2	Colon/liver	C, S	41	86
	16	61/M	24, 24	Rectal/lung	C, S, RT	380	643
	17	30/M	2, 24	Rectal/perineum	C, S, RT	3[b]	NA
	18	57/M	2, 34	Colon/liver	S	25	284

[a] Patients were treated with a second cycle of vaccine therapy
[b] Tumor stained for CEA expression

receiving 4.5×10^6 plaque-forming units (PFU) by intramuscular injection every 4 weeks for 3 months. A second cohort of 6 patients were given 4.5×10^7 PFU in a similar manner, and a third group of 6 patients received 4.5×10^8 PFU. The mean age of the patients was 60 years (range, 30–74 years) and all had a good performance status. The characteristics of the patients treated are shown in Table 1 and the cohort includes 10 colon, 3 rectal, 3 non-small-cell lung, 1 pancreatic, and 1 gastric cancer patients. All patients, except 1, had received extensive prior therapy consisting of surgical intervention ($n = 15$), chemotherapy ($n = 16$), or radiation therapy ($n = 5$). None of the patients had received prior immunotherapy.

Clinical evaluation of the patients

Two patients could not be fully evaluated, 1 (patient 2) dying of progressive disease 2 weeks after starting the trial and 1 (patient 17) withdrawing. Of the remaining 16 patients, 3 (19%) met the criteria for stable disease. Patient 1 was a 68-year-old woman who presented with a pancreatic mass and liver metastases. A biopsy of her liver lesions revealed a CEA-expressing pancreatic adenocarcinoma, although her serum CEA was normal. She completed one cycle (three immunizations) of the ALVAC-CEA-B7.1 vaccine and a repeat CT scan showed a growing pancreatic mass with minimal growth of the intrahepatic metastases. She received a course of radiation therapy directed to the pancreatic lesion, successfully limiting tumor growth over a 4-month period. During this time her liver lesions remained stable and she was offered a second cycle of vaccine treatment upon completion of her radiation therapy. Patient 3 was a 59-year-old man with a history of resected colon cancer who presented with a single large hepatic metastasis. He had an elevated serum CEA level and completed the first cycle of vaccination without incident. His CT scan revealed less than 25% increase in the liver lesion and he was offered a second cycle of vaccine treatment. Patient 5 was a 50-year-old man with metastatic colon cancer involving the liver and spleen. He tolerated the first cycle of vaccinations well and had stable disease on repeat radiological imaging. He was also offered a second cycle of vaccine therapy. The remaining patients all experienced progression of their disease after the first cycle of vaccine therapy and were not submitted to a second cycle.

Clinical toxicity was minimal except for mild pain at the site of injection lasting several days. Laboratory analysis revealed an increase in the alkaline phosphatase and hepatic transaminases in 7 patients (Table 2). The increase in liver function parameters was seen predominantly in patients with hepatic metastases and could have been due to progressive disease. We also observed thrombocytopenia in 3 patients, 2 with marginal decreases (104,000/μl and 121,000/μl) and 1 (patient 2) who died of progressive disease had a dramatic decrease to 25,000/μl before death. One patient (patient 6) experienced an exacerbation of pre-existing psoriasis after the first dose of vaccine, which improved without intervention in 2 weeks. Other minor side-effects included mild anemia in 1 patient and low-grade fever in 3 patients. These effects did not seem to correlate with the dose of virus used after patient 2 had been excluded from analysis, since this patient was responsible for all of the grade 3 toxicity observed at the lowest vaccine dose (Table 2). This patient was a 79-year-old man with

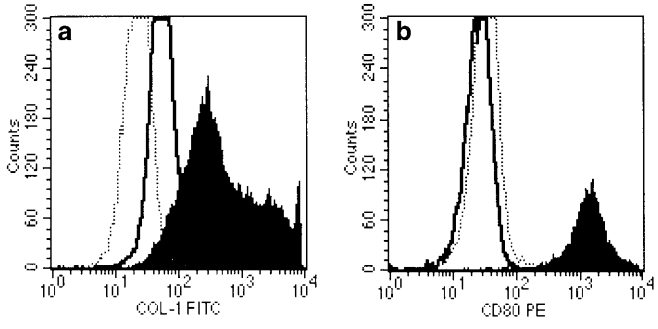

Fig. 2a, b Expression of CEA and B7.1 by the ALVAC-CEA-B7.1 vaccine was confirmed by in vitro infection of human TK^-143 cells. Histograms of gated cell populations are plotted as fluorescein-isothiocyanate-labeled COL-1(*COL-1 FITC*) for CEA expression (**a**) and phycoerythrin-labeled CD80 (*CD80 PE*) for B7.1 expression (**b**) as described in Patients and methods. CEA and B7.1 expression was observed in infected cells (■), whereas uninfected (–) cells were negative. Non-specific staining was excluded by IgG isotype controls (·····)

Table 2 Clinical toxicity of ALVAC-CEA-B7.1 vaccine. Note that three complications listed in the 4.5×10^6 dose (liver function, thrombocytopenia, and anemia) all occurred in one patient who died of progressive disease (see text). *LFT* liver function tests (includes alkaline phosphatase, bilirubin, aspartate aminotransferase, alaninine aminotransferase)

Vaccine dose (PFU)	Toxicity	Incidence (%)	Maximum grade[a]	Treatment
4.5×10^6	Increased LFT	3 (17)	3	None
	Thrombocytopenia	1 (5)	3	None
	Anemia	1 (5)	2	None
	Fever/dehydration	1 (5)	1	Fluids
	Psoriasis activation	1 (5)		None
4.5×10^7	Increased LFT	1 (5)	3	None
	Thrombocytopenia	1 (5)	1	None
4.5×10^8	Increased LFT	3 (17)	2	None
	Thrombocytopenia	1 (5)	1	None
	Fever	2 (11)	2	Acetaminophen

[a] Maximum grade according to NCI common toxicity criteria

advanced gastric carcinoma who expired 2 weeks after receiving the first vaccine. A post-mortem examination concluded that the cause of death was related to extensive gastric cancer.

In order to determine the induction of autoimmune phenomena due to B7.1 expression by the virus, we carefully monitored the patients for clinical evidence of autoimmunity. We were especially concerned about generating an autoimmune colitis since CEA is expressed on the surface of normal colonic epithelial cells. However, we observed no clinical evidence of immune-mediated toxicity, such as diarrhea, hematochezia, myalgias, arthralgias, or other symptoms. Serum CEA levels obtained every 4 weeks during the trial did not show any significant decrease after vaccination (see Table 1). We also determined the presence of ANA titers in all patients. Although several patients had pre-immune ANA titers, none of the patients experienced an increase in ANA antibodies after vaccination (Table 3).

Immunological evaluation of the patients

In order to determine the immune response in vaccinated patients we collected blood and separated PBMC by Ficoll centrifugation before treatment and every 4 weeks during the trial. Antibody titers against ALVAC were evaluated to ensure that patients were successfully immunized with the virus. As observed previously in phase I vaccine trials using ALVAC-based vaccine candidates, we observed an increase in ALVAC titers by ELISA after three immunizations in most patients [35]. The greatest increase was observed in sera from patients given the highest dose of vaccine (Fig. 3).

Table 3 Anti-(nuclear antigen) antibody titers. − The anti-(nuclear antigen) antibody titer is negative when there is no reactivity at 1:40 dilution of sera; + Positive titers at 1:40–1:80, ++ positive titers at 1:160–1:320, +++ positive titers at 1:640–1:1280 dilutions of sera. Patients 2 and 17 did not complete the vaccination schedule and were excluded from analysis

Vaccine dose (PFU)	Patient number	Pre-vaccine	Post-vaccine
4.5×10^6	1	−	−
	3	−	−
	4	−	−
	5	−	−
	6	+	+
	7	+++	+++
4.5×10^7	8	+++	+++
	9	++	++
	10	−	−
	11	+	+
	12	−	−
	13	++	++
4.5×10^8	14	−	−
	15	−	−
	16	−	−
	18	−	−

CEA-specific cellular immunity was evaluated by testing patient-derived PBMC for recognition of class-I-restricted CEA peptides using an in vitro IFNγ ELISPOT assay. Unstimulated PBMC isolated from patients were thawed from cryopreserved samples collected every 4 weeks during the trial. PBMC were

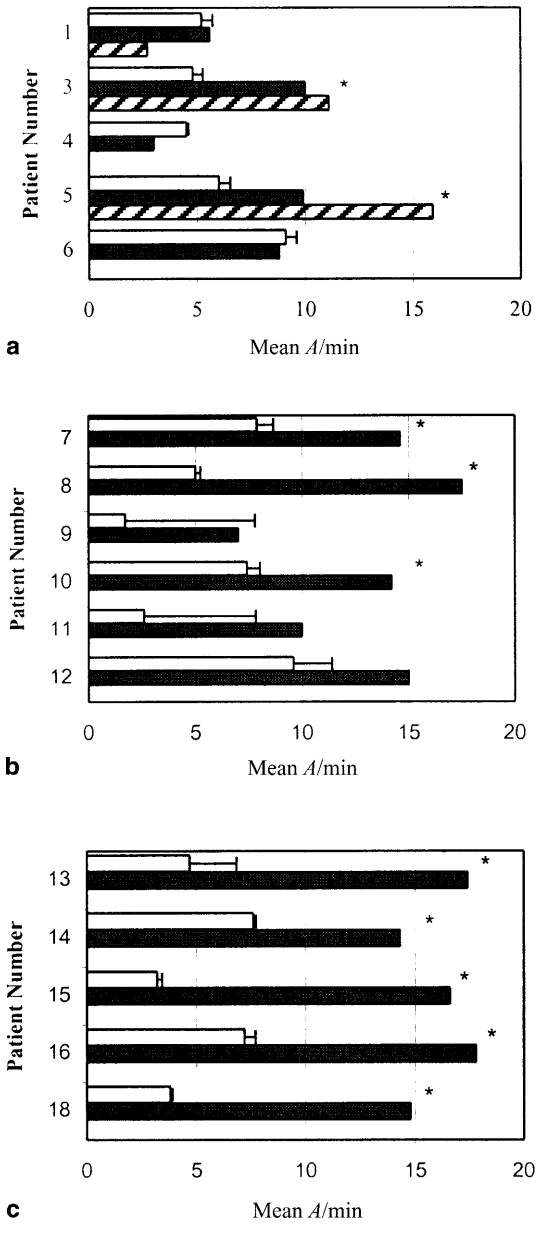

Fig. 3a–c Anti-ALVAC antibody responses showing pre-immune titers (□), titers after one cycle of vaccination (■), and after two cycles of vaccination (▥). The mean A_{450}/minute (±standard deviation) is shown for points corresponding to 3–15 min, representing the steepest slope of the plotted curve. Patients received 4.5×10^6 (**a**), 4.5×10^7 (**b**), or 4.5×10^8 (**c**) PFU ALVAC-CEA-B7.1 vaccine. Significantly increased titers are defined as those greater than three standard deviations above the pre-immune titer for each individual patient (*). Patients receiving the highest dose of vaccine tended to have the highest anti-ALVAC titers. Patients 2 and 17 did not complete the vaccination schedule and were excluded from analysis

co-cultured with appropriate HLA-matched, CEA-peptide-pulsed antigen-presenting cells for 24 h (see Patients and methods). HLA-A2-, -A3-, and -A24-class-I-restricted CEA peptide epitopes have been previously described [14, 28, 37]. Of the 16 evaluable patients, 12 expressed one of these alleles (see Table 1). Eight patients with progressive disease (by clinical evaluation) exhibited no evidence of reactive T cell responses by ELISPOT assay (data not shown). We did see an increase in the CEA-specific T cell precursor frequency after immunization in 4 patients (Figs. 4, 5). While 1 of these patients had progressive disease, the other 3 patients were the those (patients 1, 3, and 5) who experienced stable clinical disease.

These 3 patients were offered and completed a second cycle of vaccine therapy and in vitro IFN-γ ELISPOT data in response to CEA peptide stimulation are shown in Fig. 4. Patient 1 showed a minimal increase in T cell response with a precursor frequency below $1/10^6$, which is at the lower limits of ELISPOT sensitivity. Further, we found that the precursor frequency of PBMC in this patient had declined to undetectable levels by the start of the second cycle, but increased again after another three vaccinations (Fig. 4a). Patients 3 and 5 both had metastatic colon cancer, with a significant increase in CEA-specific precursor frequency responses to about 1/10,000 and 1/5,000 respectively after the first cycle of vaccination. After an interval of 16 weeks for patient 3 and 4

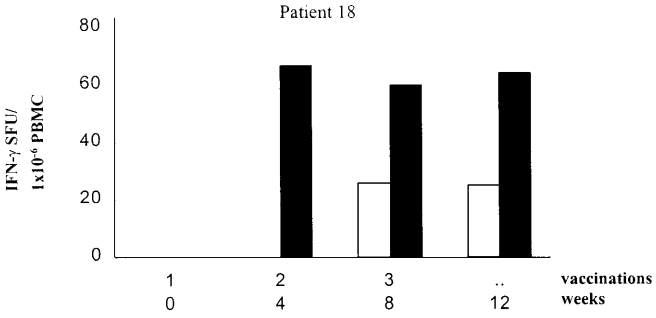

Fig. 5 CEA-specific T cell precursor frequency of in vitro unstimulated PBMC from ALVAC-CEA-B7.1-immunized, HLA-A2-positive patient 18, assessed by ELISPOT. Pre- (*0 week*) and post-vaccination (4–12 weeks) PBMC were tested against the HLA-A2-restricted CAP-1 peptide, $CEA_{571-579}$ (□) and the modified (Asn → Asp) CAP-1-6D peptide (■), modified to display higher T cell receptor affinity. Data represent a single ELISPOT assay and show an increase in precursor frequency after stimulation with the CAP-1-6D peptide. Calculation of precursor frequency is based on the number of IFN-γ spot-forming units (*SPU*) as described in Patients and methods

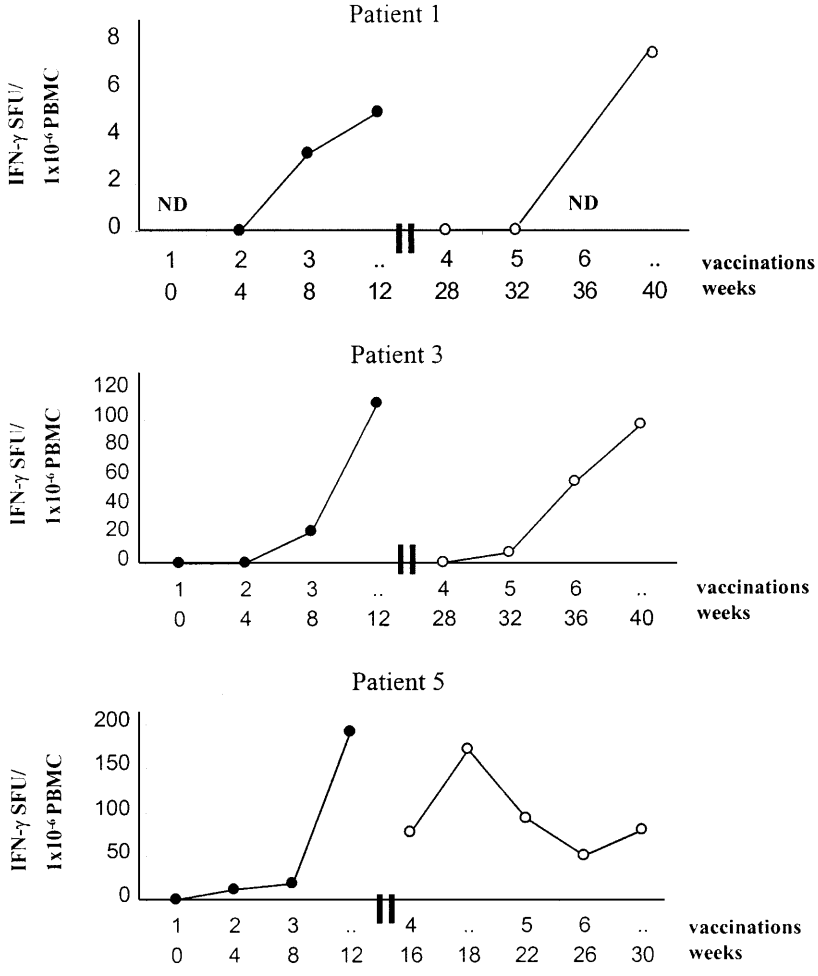

Fig. 4a–c CEA-specific T cell precursor frequency of in vitro unstimulated peripheral blood mononuclear cells (*PBMC*) from ALVAC-CEA-B7.1-immunized cancer patients assessed by enzyme-linked immunoassay spot tests (ELISPOT). Pre- (*0 week*) and post-vaccination (4–40 weeks) PBMC of patients 1 (**a**) and 3 (**b**) were tested against the HLA-A2-restricted CAP-1 peptide ($CEA_{571-579}$) using the TAP-deficient, HLA-A2-expressing T2 cells, and patient 5 (**c**) was tested against the HLA-A24-restricted $CEA_{570-579}$ using the HLA-A24-expressing C1R cells. Data representing the first and second vaccination cycle are displayed discontinuously indicating the intervening weeks. Data represent a single ELISPOT assay showing the interferon-γ (*IFN-γ*) spot-forming units (*SFU*) representing the CEA-specific T cell precursor frequency as calculated in patients and methods; *ND* not determined

weeks for patient 5, vaccine treatment was continued. CEA-specific T cell responses were induced after continuous ALVAC-CEA-B7.1 exposure to levels similar to those seen after the first cycle of vaccination (Fig. 4b, c).

We assessed the phenotypic characteristics of lymphocyte populations from all patients before and after vaccination. There were no obvious differences observed during the course of the trial (data not shown). However, analysis of specific T cell activation markers was performed on the 3 patients with a stable disease (Table 4). Markers of T cell activation were elevated in patients 1 and 3 as shown by expression of the CD69 early T cell activation marker on $CD8^+$ T cells and an increase in the late activation marker HLA-DR on $CD3^+$ T-cells after the second cycle of vaccine therapy. Patient 1 also showed an inverse CD4:CD8 ratio with a threefold increase in $CD8^+$ T cells at the beginning of the second vaccine treatment cycle, which could have been attributed to her interval radiation therapy. This is interesting since radiation induces apoptosis in tumor cells and apoptotic cells may be efficiently processed by APC to induce class-I-specific CTL responses [2]. Patient 5 manifested an increased NK cell population ($CD3^-$/$CD16^+$ $CD56^+$) throughout the study.

Since CEA-specific precursor T cells may be very low, we utilized a modified peptide that displays enhanced T cell receptor recognition to see if we could amplify in vitro immune responses. Cryopreserved T cells were available from patient 18 for further analysis. A modified HLA-A*0201-restricted CEA peptide, designated CAP-1-6D, was generated by substituting aspartic acid (D) for asparagine (N) at position 6, and displayed increased T-cell receptor affinity [41]. This peptide was used to pulse T2-A2 (TAP-deficient APC expressing HLA-A2) cells in an ELISPOT assay with PBMC derived from patient 18. While this patient had a modest ELISPOT response after stimulation with the native HLA-A2-restricted CEA peptide, CAP-1 ($CEA_{571–579}$), a higher precursor frequency was observed when the CAP-1-6D was used to stimulate post-vaccine PBMC (Fig. 5).

Table 4 Phenotype analysis of lymphocyte populations derived from 3 patients receiving two cycles of vaccine. Relative expression of subset markers evaluated at 12 weeks for the first vaccination cycle and at 40 weeks (patients 1 and 3) or 30 weeks (patient 5) for the second vaccination cycle. Data indicated as a percentage of all lymphocytes, defined by $CD45^+$ bright/$CD14^-$. Fraction of cells expressing T cell activation markers: −0–10%, +/−10–20%, +20–40%, ++40–60%, +++60–80%

Vaccination cycle	Patient 1		Patient 3		Patient 5	
	1st	2nd	1st	2nd	1st	2nd
Subset markers (%)						
$CD3^+$	71	70	66	61	50	48
$CD3^-/CD16^+$ 56^+	9.5	9.6	9.0	13.6	34.5	43
CD4:CD8	1:0.3	1:3.5	1:1.6	1:1.9	1:1.0	1:1.1
T cell activation markers						
$CD8^+/CD69^+$	−	+	−	+/−	−	−
$CD3^+$/HLA-DR	+/−	+++	+/−	+	+	+

Discussion

The development of therapeutic vaccines for cancer depends on strategies that induce tumor-antigen-specific effector T cells. Activation of such T cells requires two signals, one delivered by the interaction of peptide-MHC complexes with a T cell receptor, and the second delivered through ligation of co-stimulatory molecules expressed on APC with CD28 on the surface of responding T cells. Failure to elicit T cell responses in cancer patients may occur through a variety of mechanisms, including loss of tumor antigen expression, down-regulation of MHC, lack of β-2 microglobulin, secretion of immunosuppressive cytokines, and the absence of adhesion or costimulatory molecules on the tumor cell [16]. The dependence on costimulation has been shown in experiments where the transduction of tumor cells with costimulatory molecules enhances their recognition by antigen-specific T cell populations [3]. Alternatively, costimulatory molecules can be added to a vaccine vector for systemic immunization with improved therapeutic responses, as shown in several murine tumor models [1, 12, 18, 27]. The most widely studied costimulatory molecules are B7.1 and B7.2, which bind to the T cell surface receptors, CD28 and CTLA-4. CD28 is usually expressed on the surface of recently activated T cells and ligation of CD28 enhances activation in the presence of T cell receptor signaling. Shortly after activation, CTLA-4 appears and binds to B7 molecules, leading to inhibition of T cell responsiveness [36]. While the exact role of B7.1 compared to B7.2 has yielded conflicting results, B7.1-transduced tumor cells induced stronger $CD8^+$ T cell responses than did B7.2-transduced cells in a murine P815 tumor model [10, 17]. B7.1 was also more effective than B7.2 in generating allo-specific T cell populations in a keratinocyte cell line [7]. Interestingly, expression of B7.1 and B7.2 may lead to a distinct pattern of cytokine release, with B7.1 inducing a Th1 profile and B7.2 inducing a Th2 profile [34]. Thus, current evidence supports the hypothesis that B7.1 and B7.2 may have differential effects in the regulation of effector T cell responses and suggests that B7.1 may be more effective as a vaccine adjuvant [30].

We have reported the first use of B7.1 co-expressed with a tumor-associated antigen in human patients. The vaccine appeared to be safe with few major side-effects, even at the highest administered dose of 4.5×10^8 PFU. While we did not observe clinical evidence of autoimmunity, 1 patient did experience an outbreak of psoriasis. This did not require therapy and resolved quickly, but the possibility that vaccination precipitated the exacerbation cannot be completely excluded. Because B7.1 has not been previously injected into patients we sought other evidence of autoimmunity by screening patient sera for ANA titers. Advanced cancer patients have been

known to have elevated ANA titers, but we did not find any increase in pre-existing titers in our patient population [22]. Evaluation of anti-ALVAC antibody titers revealed an increase after vaccination, particularly in patients receiving the highest dose of vaccine. This is consistent with previous human vaccine studies with ALVAC-based vaccine candidates. Interestingly, CEA-specific T cell responses were able to be boosted in patients receiving up to six doses of ALVAC-CEA-B7.1 over a relatively short time interval (7–10 months). This is consistent with results from other studies using ALVAC-CEA vaccine candidates and differs from human studies using vaccinia-based CEA candidates, where prior exposure to vaccinia significantly dampened CEA-specific booster responses [6, 37, 42]. Although we did not observe any decrease in serum CEA levels in any patient, the significance of following tumor markers in such patients is unclear. The initiation of an immune response against tumor cells expressing specific antigens can induce an inflammatory reaction with an increase in tumor marker secretion after vaccination [8].

Vaccination using the ALVAC-CEA-B7.1 vaccine resulted in three significant treatment responses as defined in standard oncology clinical trials over a period of 4 months. Although these responses were modest, it is intriguing that all 3 patients had evidence of CEA-specific T cell responses by ELISPOT assay. Since these patients were all heavily pre-treated and had advanced metastatic disease, one would be unlikely to see significant treatment responses. For this reason, peripheral T cell immune reactivity represented a critical study endpoint of this study. We used cryopreserved PBMC in a 24-h ELISPOT without prior in vitro stimulation, providing a sensitive assay for determination of antigen-specific T cells [33]. This assay also may more accurately represent actual in vivo T cell responses, since they do not receive extensive stimulation before assay. In order to show that these T cells could recognize tumor, we attempted to establish autologous tumor cell lines from the 3 patients with increased ELISPOT reactivity (see Fig. 4). However, tumor cells obtained by percutaneous liver biopsy from these patients did not survive in vitro culture for more than a few days (data not shown).

The induction and monitoring of immune responses may be more difficult in patients with CEA-expressing tumors than for patients with melanoma. The reasons for this discrepancy are not entirely clear. One possibility is that CEA is less immunogenic than most melanoma antigens. Another possibility is that CEA-expressing tumors occur in different sites from melanoma, which could ultimately influence the type and extent of antitumor immunity. Many CEA-bearing cancers arise in the gastrointestinal epithelium, where they are likely to encounter mucosal immune effector cells first. Oral tolerance of peripheral T cell responses has been well described and may be a factor in the poor immune responses observed in colon cancer patients [25]. This is distinct from melanoma, which arises in the skin where more efficient APC may regulate the presentation of tumor antigens through different mechanisms. Furthermore, analysis of vaccine trials is often easier for melanoma patients because of the ease of generating T cell clones and autologous tumor cell lines from these patients.

One possible method for improving the isolation of CEA-specific T cells is to use a modified CEA peptide agonist, such as CAP-1-6D [41]. This peptide contains an asparagine $\rightarrow$ aspartic acid substitution at position 6, resulting in higher affinity for the CEA-specific T cell receptor. CAP-1-6D has been used in vitro to generate stronger cytotoxic T cell activity from patients vaccinated with a CEA-expressing vaccinia virus [41]. When we used this peptide to stimulate PBMC in 1 patient, we also observed an increased frequency of CEA-reactive T cells after ALVAC-CEA-B7.1 vaccination. Although this peptide has only been used for in vitro analyses thus far, it is possible to consider the use of this epitope for clinical study by using the single peptide or incorporating this mutation into a full-length CEA sequence expressed by a viral vector. Evaluation of low-frequency T cell responses in vitro using agonist peptides or other techniques, such as tetramers, awaits further validation.

The vaccination strategy employed in this trial included monthly vaccinations for three total doses of vaccine. This design is arbitrary since the optimal number of boosters and the timing of administration for these vaccines are not known. Interestingly, of the 3 patients who received two cycles of vaccine, 2 had an interval of 16 weeks between cycles and both had lost evidence of CEA-reactive cells at the start of the second cycle of vaccination. After completing the second cycle, however, both patients showed increasing numbers of reactive T cells similar to levels detected after the first cycle. The 3rd patient continued the second cycle after only a 4-week interval and continued to show high levels of CEA-specific precursors. This suggests that repeated immunizations with frequent boosting may be important in maintaining a pool of CEA-specific T cells following vaccination. Repeated exposure to antigen may be one method of breaking tolerance to weakly immunogenic proteins, such as CEA [20]. Future vaccine trials should consider that continued immunization might increase immune responses against tumor-associated self-antigens.

Improved understanding of the mechanisms through which effector T cells are activated has led to the addition of costimulation as an adjunct to antigen-specific vaccine therapy. This study was the first to use B7.1 with the tumor antigen CEA, in an ALVAC vector for patients with advanced CEA-expressing tumors. There was little clinical evidence of autoimmunity in vaccinated patients, except for one mild exacerbation of pre-existing psoriasis. Although the objectives of the study were largely predicated to an analysis of dose-limiting toxicity, we also observed stable disease in 3 patients and evidence of CEA-specific T cell immunity in 4 patients. While the stable diseases occurred in patients

receiving the lowest dose of vaccine (4.5×10^6 PFU), it is difficult to know if this is meaningful given the low numbers of patients treated. T cell responses were maintained when a second cycle of vaccine was given, suggesting that repeated exposure to antigen may be necessary for providing sustained antigen-specific immunity after vaccination. Preliminary results from another phase I clinical trial using ALVAC-CEA-B7.1 had 6 of 28 patients classified as stable after four vaccinations with doses ranging from 2.5×10^7 to 4.5×10^8 PFU vaccine [38]. Although these results are modest, most patients treated in these trials have advanced disease with large tumor burdens. These minor responses, however, provide support for the feasibility of vaccinating against CEA. Recombinant vaccines expressing costimulatory molecules may provide an effective strategy for further enhancing tumor immunotherapy. Additional studies with larger cohorts of patients and use of the vaccine at earlier stages of disease may help to define the full therapeutic potential of tumor vaccine therapy.

Acknowledgements We thank Drs. Betty Diamond, Anne Davidson, James Tartaglia and Neil Berinstein for critical review of the manuscript. We also thank Drs. James Tartaglia and Elizabeth Kauffman for providing reagents for the anti-ALVAC antibody assay and Dave Gebhard Jr. for FACS assistance. This work was supported by the Albert Einstein Cancer Center, NIH grant RO3 80647–01, and Doris Duke Foundation Award T98052.

References

1. Akagi J, Hodge JW, McLaughlin JP, Gritz L, Mazzara G, Kufe D, Schlom J, Kantor JA (1997) Therapeutic antitumor response after immunization with an admixture of recombinant vaccinia virus expressing a modified MUC-1 gene and the murine T cell costimulatory molecule B7. J Immunother 20: 38
2. Albert ML, Sauter B, Bhardwaj N (1998) Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 392: 86
3. Bixby DL, Yanelli JR (1998) CD80 expression in an HLA-A2-positive human non-small cell lung cancer cell line enhances tumor-specific cytotoxicity of HLA-A2-positive T cells derived from a normal donor and a patient with non-small cell lung cancer. Int J Cancer 78: 685
4. Bluestone JA (1995) New perspectives of CD28-B7-mediated T cell costimulation. Immunity 2: 555
5. Broyless SS, Yuen L, Shuman S, Moss B (1988) Purification of a factor required for transcription of vaccinia virus early genes. J Biol Chem 263: 10 754
6. Cole DJ, Wilson MC, Baron PL, O'Brien P, Reed C, Tsang KY, Schlom J (1996) Phase I study of recombinant CEA vaccinia virus vaccine with post vaccination CEA peptide challenge. Hum Gene Ther 7: 1381
7. Dijk AM van, Otten HG, Vercauteren SM, Kessler FL, de Boer M, Verdonck LF, de Gast GC (1996) Human B7-1 is more efficient than B7-2 in providing costimulation for alloantigen-specific T cells. Eur J Immunol 26: 2275
8. Eisenberger MA, and Nelson WG (1996) How much can we rely on the level of prostate-specific antigen as an end point for evaluation of clinical trials? A word of caution! J Natl Cancer Inst 88: 779
9. Foon KA, John WJ, Chakraborty M, Sherratt A, Garrison J, Flett M, Bhattacharya-Chatterjee M (1997) Clinical and immune responses in advanced colorectal cancer patients treated with anti-idiotype monoclonal antibody vaccine that mimics the carcinoembryonic antigen. Clin Cancer Res 3: 1267
10. Gajewski TF (1996): B7-1 but not B7-2 efficiently costimulates $CD8^+$ T lymphocytes in the P815 tumor system in vitro. J Immunol 156: 465
11. Goldenberg DM, Sharkey RM, Primus FJ (1976) Carcinoembryonic antigen in histopathology: immunoperoxidase staining of conventional tissue sections. J Natl Cancer Inst 57: 11
12. Hodge JW, McLaughlin JP, Abrams SI, Shupert WL, Schlom J, Kantor JA (1995) Admixture of a recombinant vaccinia virus containing the gene for the costimulatory molecule B7 and a recombinant vaccinia virus containing a tumor-associated antigen gene results in enhanced specific T cell responses and antitumor immunity. Cancer Res 55: 3598
13. Jameson J, Cruz J, Ennis FA (1998) Human cytotoxic T-lymphocyte repertoire to influenza A viruses. J Virol 72: 8682
14. Kawashima I, Tsai V, Southwood S, Takesako K, Sette A, Celis E (1999) Identification of HLA-A3-restricted cytotoxic T lymphocyte epitopes from carcinoembryonic antigen and HER-2/neu by primary in vitro immunization with peptide-pulsed dendritic cells. Cancer Res 59: 431
15. La Motte RN, Rubin MA, Barr E, Leiden JM, Bluestone JA, Mokyr MB (1996) Therapeutic effectiveness of the immunity elicited by P815 tumor cells engineered to express the B7-2 costimulatory molecule. Cancer Immunol Immunother 42: 161
16. Lee PP, Yee C, Savage PA, Fong L, Brockstedt D, Weber JS, Johnson D, Swetter S, Thompson J, Greenberg PD, Roederer M, Davis MM (1999) Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat Med 5: 677
17. Levine BL, Ueda Y, Craighead N, Huang ML, June CH (1995) CD28 ligands CD80 (B7-1) and CD86 (B7-2) induce long-term autocrine growth of $CD4^+$ T cells and induce similar patterns of cytokine secretion in vitro. Int Immunol 7: 891
18. Liebowitz DN, Lee KP, June CH (1998) Costimulatory approaches to adoptive immunotherapy. Curr Opin Oncol 10: 533
19. Marshall JL, Hawkins MJ, Tsang KY, Richmond E, Pedicano JE, Zhu MZ, Schlom J (1999) Phase I study in cancer patients of a replication-defective avipox recombinant vaccine that expresses human carcinoembryonic antigen. J Clin Oncol 17: 332
20. Matzinger P (1994) Tolerance, danger, and the extended family. Annu Rev Immunol 12: 991
21. McAneny D, Ryan CA, Beazley RM, Kaufman HL (1996) Results of a phase I trial of a recombinant vaccinia virus that expresses carcinoembryonic antigen in patients with advanced colorectal cancer. Ann Surg Oncol 3: 495
22. McCormick NK, McCormick KJ, Trentin JJ (1976) Antinuclear antibodies and elevated anti-Epstein-Barr virus titers in cancer patients. Infect Immun 13: 1382
23. Miller SD, Vanderlugt CL, Lenschow DJ, Pope JG, Karandikar NJ, Dal Canto MC, Bluestone JA (1995) Blockade of CD28/B7-1 interaction prevents epitope spreading and clinical relapses of murine EAE. Immunity 3: 739
24. Morse MA, Deng Y, Coleman D, Hull S, Kitrell-Fisher E, Nair S, Schlom J, Ryback ME, Lyerly HK (1999) A phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. Clin Cancer Res 5: 1331
25. Mowat AM, Viney JL (1997) The anatomical basis of intestinal immunity. Immunol Rev 156: 145
26. Muraro R, Wunderlich D, Thor A, Lundy J, Noguchi P, Cunningham R, Schlom J (1985) Definition by monoclonal antibodies of a repertoire of epitopes on carcinoembryonic antigen differentially expressed in human colon carcinomas versus normal adult tissues. Cancer Res 45: 5769
27. Nawrocki S, Mackiewicz A (1999) Genetically modified tumor vaccines – where we are today. Cancer Treat Rev 25: 29
28. Nukaya I, Yasumoto M, Iwasaki T, Ideno M, Sette A, Celis E, Takesako K, Kato I (1999) Identification of HLA-A24 epitope

peptides of carcinoembryonic antigen which induce tumor-reactive cytotoxic T lymphocyte. Int J Cancer 80: 92
29. Perkus ME, Tartaglia J, Paoletti E (1995) Poxvirus-based vaccine candidates for cancer, AIDS, and other infectious diseases. J Leukoc Biol 58: 1
30. Prabhu Das MR, Zamvil SS, Borriello F, Weiner HL, Sharpe AH, Kuchroo VK (1995) Reciprocal expression of co-stimulatory molecules, B7-1 and B7-2, on murine T cells following activation. Eur J Immunol 25: 207
31. Rosenberg SA, Kawakami Y, Robbins PF, Wang R (1996) Identification of the genes encoding cancer antigens: Implications for cancer immunotherapy. Adv Cancer Res 70: 145
32. Samanci A, Yi Q, Fagerberg J, Strigard K, Smith G, Ruden U, Wahren B, Mellstedt H (1998) Pharmacological administration of granulocyte/macrophage-colony-stimulating factor is of significant importance for the induction of a strong humoral and cellular response in patients immunized with recombinant carcinoembryonic antigen. Cancer Immunol Immunother 47: 131
33. Scheibenbogen C, Lee KH, Stevanovic S, Witzens M, Willhauck M, Waldman V, Naeher H, Rammensee HG, Keilholz U (1997) Analysis of the T cell response to tumor and viral peptide antigens by an IFNgamma-ELISPOT assay. Int J Cancer 71: 932
34. Soos JM, Ashley TA, Morrow J, Patarroyo JC, Szente BE, Zamvil SS (1999) Differential expression of B7 costimulatory molecules by astrocytes correlates with T cell activation and cytokine production. Int Immunol 11: 1169
35. Tartaglia J, Excler JL, El Habib R, Limbach K, Meignier B, Plotkin S, Klein M (1988) Canarypox virus-based vaccines: prime-boost strategies to induce cell-mediated and humoral immunity against HIV. Aids Res Hum Retroviruses 14: 291
36. Townsend SE, Allison JP (1993) Tumor rejection after costimulation of $CD8^+$ T cells by B7-transfected melanoma cells. Science 259: 368
37. Tsang KY, Zaremba S, Nieroda CA, Zhu MZ, Hamilton JM, Schlom J (1995) Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. J Natl Cancer Inst 87: 982
38. Von Mehren M, Davey M, Rivera V, Yeslow G, Gillon T, Alpaugh K, Cheng J, Meropol N, Revold E, Scher R, Cooper H, Schlom J, Weiner LM (1999) Phase I trial with ALVAC-CEA-B7.1 immunization in advanced CEA-expressing adenocarcinomas. Proc ASCO 18: 437a
39. Wang RF (1999) Human tumor antigens: implications for cancer vaccine development. J Mol Med 77: 640
40. Yu X, Abe R, Hodes R (1998) The role of B7-CD28 costimulation in tumor rejection. Int Immunol 10: 791
41. Zaremba S, Barzaga E, Zhu MZ, Soares N, Tsang KY, Schlom J (1997) Identification of an enhancer agonist cytotoxic T lymphocyte peptide from human carcinoembryonic antigen. Cancer Res 57: 4570
42. Zhu MZ, Marshall J, Cole D, Schlom J, Tsang KY (2000) Specific cytolytic T cell responses to human CEA from patients immunized with recombinant avipox-CEA vaccine. Clin Cancer Res 6: 24