Phase I Trial of a Recombinant Vaccinia Virus Encoding Carcinoembryonic Antigen in Metastatic Adenocarcinoma: Comparison of Intradermal *versus* Subcutaneous Administration

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ABSTRACT

The principal objectives of this trial were twofold: (*a*) to examine the safety and relative efficacy of intradermal needle injection *versus* s.c. jet administration of a carcinoembryonic antigen (CEA)-encoding recombinant vaccinia virus (rV-CEA) over a limited dose range and (*b*) to evaluate CEA-specific immune responses or antitumor effects induced by rV-CEA vaccination. Patients were randomly assigned to one of two groups, depending upon the technique of vaccine administration. All 20 patients received two doses of 10^7 or 10^8 pfu of rV-CEA at a 4-week interval. Toxicity was limited to modest local inflammation at the inoculation site as well as low-grade fever and increased fatigue, each affecting fewer than 20% of the patients. No evidence of CEA-specific lymphoproliferation, interleukin 2 release, delayed-type hypersensitivity, or antibody response was observed. Thus, the efficacy comparison between the two administration techniques was based upon the induction of immune responses to the vaccinia virus vector. Both techniques induced vaccinia-specific lymphoproliferation, interleukin 2 release, and antibody responses of comparable magnitude and frequency as well as protected 80% of patients against pustule formation following vaccinia scarification. Thus, there is no compelling reason to recommend one administration technique over the other based upon toxicity or efficacy. We have selected s.c. jet injection for subsequent trials of rV-CEA based on the ability to accommodate larger injection volumes, enhanced standardization between clinicians, and avoidance of needles that could transmit the vaccine or blood-borne pathogens to health care workers. We recommend use of 10^8 pfu doses for subsequent trials of recombinant vaccinia virus vaccines based upon the favorable toxicity profile and more consistent local pustule formation indicative of an adequate inoculation of live virus. No objective clinical responses to the rV-CEA vaccine were observed among this population of patients with widely metastatic adenocarcinoma.

INTRODUCTION

CEA[2] is a M_r 180,000 membrane-anchored glycoprotein that is expressed on the great majority of colorectal, gastric, and pancreatic carcinomas as well as on ~50% of breast cancers and 70% of non-small cell lung cancers (1). CEA is also expressed, to some extent, in normal colonic mucosa and fetal digestive organs (1). The cDNA for full-length CEA has been cloned from a human colon carcinoma cell library and inserted into the genome of a New York City attenuated strain of vaccinia virus (2). The recombinant virus (rV-CEA) is replication competent and directs cell surface expression of CEA. Immunization with rV-CEA has induced CEA-specific humoral and cellular immune responses in mice and nonhuman primates as well as protection against challenge with syngeneic CEA-expressing colon carcinoma cells in mice (3, 4). However, it is important to emphasize that human CEA is a foreign gene in both mice and nonhuman primates, whereas humans appear to be immunologically tolerant to CEA: it is expressed in fetal and normal adult human tissues.

The first clinical trial of rV-CEA was conducted in 1993 by the NCI-Navy Oncology Branch in 26 patients with metastatic adenocarcinoma (5). The vaccine was administered by dermal scarification monthly for three exposures at doses up to 10^7 pfu. No severe toxicity occurred and no primary T-cell lymphoproliferative responses to soluble CEA protein were observed (5). However, human class I HLA-A2-restricted cytolytic T-cell lines that are responsive to specific epitopes of CEA could be derived by prolonged *in vitro* culture of peripheral blood lymphocytes from patients after vaccination (6).

An abbreviated trial of the same vaccine was conducted at our institution during 1994. Five patients who had surgically

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[2] The abbreviations used are: CEA, carcinoembryonic antigen; rV-CEA, recombinant vaccinia virus encoding carcinoembryonic antigen; NCI, National Cancer Institute; pfu, plaque-forming units; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; SI, stimulation index; IL-2, interleukin 2.

resected Dukes' C colorectal carcinoma and who had completed adjuvant chemotherapy received two vaccinations with 10^7 pfu of rV-CEA 8 weeks apart by scarification (7). The trial was stopped prematurely due to identification of bovine viral diarrhea virus contamination within the rV-CEA vaccine. A new lot of rV-CEA was prepared for use in this study from a different producer cell line, which yielded a lower viral titer of 10^6 pfu/μl. The modest viral titer made it impossible to deliver the desired dose of 10^7–10^8 pfu by the traditional means of scarification, which uses a volume of only 1–2 μl suspended between the prongs of a bifurcated needle. Additional shortcomings of scarification include considerable variation in technique between clinicians and uncertainty regarding the actual dose that penetrates the cornified layer of the epidermis to contribute to productive infection, with resultant expression of the tumor-associated antigen. These problems are relevant to the administration of recombinant vaccinia virus vaccines for a variety of disorders, including cancer and HIV disease. A recognized advantage of scarification is that it delivers the virus to the skin, a highly immunogenic site that is rich in epidermal Langerhans cells, which function as potent antigen-presenting cells. Therefore, we elected to compare two alternative administration techniques that deliver some or all of the dose to the skin in a reproducible manner using injection volumes of ≥100 μl. The techniques selected were intradermal needle injection *versus* s.c. jet injection using the Biojector. A limited dose escalation format was selected because little information was available regarding the toxicity of vaccinia virus when doses of 10^7–10^8 pfu are fully delivered beneath the cornified layer of the epidermis. We hypothesized that more effective delivery of the vaccine to dermal antigen-presenting cells by the administration techniques under investigation would elicit CEA-specific immune responses that are superior to those observed in the NCI Phase I trial of rV-CEA delivered by scarification.

Thus, the goals of this study were twofold: (*a*) to examine the safety and relative efficacy of intradermal needle injection *versus* Biojector administration of rV-CEA over a limited dose range and (*b*) to evaluate any CEA-specific immune responses or antitumor effects induced by rV-CEA immunization.

MATERIALS AND METHODS

Treatment Regimen. Twenty patients with metastatic adenocarcinoma who were demonstrated to express CEA by immunoperoxidase staining or by an elevated serum CEA level were selected. Selected patients had an Eastern Cooperative Oncology Group performance status of 0–2 and a history of prior inoculation with vaccinia virus (*i.e.*, smallpox immunization). Therapy was initiated at least 4 weeks after prior chemotherapy or radiotherapy. A recombinant vaccinia virus encoding full-length human CEA, designated rV-CEA (M_r 180,000), was generated by Therion Biologicals Corporation (Cambridge, MA), using methods described previously (8). The CEA gene was isolated as a cDNA clone from a human colon carcinoma cell cDNA library. The CEA cDNA was inserted, under the control of the vaccinia 40K promoter (8), into the *Hin*dIII M region of the genome of the Wyeth attenuated strain of vaccinia virus. The recombinant virus has been well characterized (2, 4). The rV-CEA (M_r 180,000) vaccine was formulated at 1×10^8 pfu per vial in 0.1 ml of PBS with 10% glycerol and was distributed by the Cancer Therapy Evaluation Program of the NCI (Investigational New Drug No. 6960). All patients received rV-CEA (M_r 180,000) on days 1 and 29, with an additional 4 weeks of clinical and immunological monitoring, such that the off-study evaluation was performed on day 57. Initially, 10 patients were randomly assigned to either group 1 ($n = 5$) or group 2 ($n = 5$). Patients in group 1 received 1×10^7 pfu doses of rV-CEA (M_r 180,000) by intradermal needle injection, whereas those in group 2 received 1×10^7 pfu doses by s.c. jet injection. This starting dose was selected based upon previous observations that 1×10^7 pfu of rV-CEA (M_r 180,000) is well tolerated in humans when administered by scarification (5, 7). Subsequently, 10 additional patients were randomly assigned to either group 3 ($n = 5$) or group 4 ($n = 5$). Because no serious toxicity (grade 3 or 4) was observed among patients in the first two groups, patients in group 3 received 1×10^8 pfu doses of rV-CEA (M_r 180,000) by intradermal needle injection, whereas those in group 4 received 1×10^8 pfu doses by s.c. jet injection.

Patients were examined 3 and 7 days after immunization to assess the degree of inflammation at the inoculation site, regional adenopathy, or other signs of toxicity. A complete blood count, serum CEA level, and serum chemistries were obtained before each immunization as well as 7 and 28 days following each immunization. All patients were evaluated for evidence of objective antitumor response 8 weeks following the primary immunization.

Immunization Techniques. The vaccine was stored in single-use vials at −70°C. On the day of dosing, the vaccine was thawed, vortexed to disperse viral clumps, and diluted as needed with sterile normal saline for injection. All patients received the rV-CEA (M_r 180,000) vaccine over the proximal lateral upper extremity. Patients in groups 1 and 3 received the vaccine by standard intradermal injection using a 0.1-ml volume of appropriately diluted viral stock in an insulin syringe. Those in groups 2 and 4 received the vaccine by s.c. jet injection using the Biojector 2000 needle-free injection device (Bioject, Inc., Raleigh, NC). Each dose consisted of a 0.5-ml volume of appropriately diluted viral stock drawn into a Biojector No. 2 syringe. The tip of the syringe was held firmly at the injection site at a 90° angle to the skin. Pressing the actuator lever results in release of compressed carbon dioxide (CO_2) gas from a cartridge, which drives the syringe plunger to expel the vaccine through a micro-orifice at high velocity in a fraction of a second. Nothing pierces the skin except the fluid stream, and the CO_2 never comes in contact with the vaccine. The Biojector delivers the vaccine in a conical distribution with the apex at the skin surface. Thus, the great majority is delivered to the s.c. tissue, although traces remain in the dermis.

Skin Test Technique and Scarification Challenge with Vaccinia Virus. All patients received 10 μg of baculovirus recombinant CEA protein (MicroGeneSys, Meriden, CT) by intradermal injection on the ventral forearm as a test for delayed-type hypersensitivity to CEA 8 weeks after primary immunization. At the same time, they were challenged with 4×10^7 pfu of a different recombinant vaccinia virus encoding a truncated version of human CEA, rV-CEA (M_r 70,000), by scarification. The local inoculation site was examined for evidence of inflammation and/or pustule formation 3 and 7 days

postscarification to determine the degree of immune-mediated attenuation of the skin reaction induced by the virus.

In a previous trial at our institution, the same dose of rV-CEA (M_r 70,000) administered by scarification induced local pustule formation in 10 of 12 patients, whose only prior exposure to the virus was childhood smallpox immunization. Repeat scarification with rV-CEA (M_r 70,000) 8 weeks later induced a local pustule in only 4 of 12 patients. These observations indicate that scarification boosts the immune response to vaccinia virus, thus attenuating the local skin reaction to subsequent viral challenge in the majority of patients. In this study, patients were vaccinia-primed by childhood smallpox vaccination and boosted with two doses of rV-CEA (M_r 180,000) 4 weeks apart by intradermal needle injection or s.c. jet injection. Viral challenge by scarification was undertaken 4 weeks later to determine whether these administration techniques provide sufficient viral delivery to antigen-presenting cells to boost the antivaccinia immune response with resultant attenuation of the local skin reaction to subsequent viral exposure.

Lymphoproliferative Assay. PBMCs obtained by Ficoll-Hypaque density gradient centrifugation were resuspended in complete medium consisting of RPMI 1640 supplemented with 10% pooled normal human AB serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and antibiotics. Cells were added at 1.5×10^5 per well to 96-well flat-bottomed plates. Stimulated cells were incubated in quadruplicate wells with UV light-inactivated vaccinia virus over a range of concentrations (2×10^6–2×10^7 pfu/ml), baculovirus recombinant human CEA (MicroGeneSys) over a range of concentrations (3–30 μg/ml), BSA (30 μg/ml) as a negative control antigen, tetanus toxoid (Wyeth-Ayerst Laboratories, Paoli, PA) as a positive control antigen, and PHA and pokeweed lectin as positive control mitogens. Control cells were cultured in complete medium alone. All cells were incubated in a humidified atmosphere of 5% CO_2 in air for 6 days, followed by an overnight pulse with 1 μCi/well tritiated thymidine. Cells were harvested, and incorporated radioactivity was quantitated using a solid-phase β-scintillation counter. The SI was calculated as the mean cpm of the stimulated cells divided by the mean cpm of the control cells. A positive response was defined as a postimmunization SI of >3 and at least 2-fold greater than the preimmunization SI for a given antigen.

IL-2 Release. Mononuclear cells were cultured exactly as above with the same panel of antigens and mitogens over the same range of concentrations, with the exception that, after 3 days in culture, cell-free supernatants were harvested and assayed immediately or stored at −70°C. Supernatant IL-2 activity was quantitated based on the ability to support proliferation of CTLL-2 cells (9, 10). CTLL-2 cells were washed to remove exogenous cytokines and added at 5000 cells/well in 100 μl of complete RPMI-10% FCS. Each well also received 100 μl of cell culture supernatant to be assayed for IL-2 activity in triplicate. Following 24 h in culture, cells were pulsed with tritiated thymidine (1 μCi/well) for 8 h and harvested, and incorporated radioactivity was determined using a direct β-scintillation counter. The mean cpm for triplicate cell cultures was converted to mIU/ml IL-2 in the culture supernatant by reading from a standard curve generated for each assay using recombinant human IL-2. A positive response was defined as ≥1 mIU/ml of IL-2 in the culture supernatant postimmunization and at least a 2-fold increase compared with the preimmunization sample.

Antibody Assays. Anti-CEA and antivaccinia antibodies were quantitated by ELISA. To detect anti-CEA antibodies, we coated microtiter plates overnight with 200 ng/well native human CEA purified from human colon cancer liver metastases (Vitro Diagnostics, Littleton, CO). To detect antivaccinia antibodies, we coated microtiter plates overnight with 5×10^6 pfu per well of active vaccinia virus (Therion Biologicals Corporation) in PBS with 0.1 g/liter calcium and 0.1 g/liter of magnesium. The plates were blocked with 1% pig skin gelatin and 3% nonfat dry milk in PBS for 90 min at 37°C, followed by washing. The plates were then incubated with various dilutions of patient or normal donor sera in blocking buffer for 1 h at 37°C. Plates were washed and antibody binding was detected with horseradish peroxidase-conjugated goat antihuman IgG (heavy and light chain) antiserum (1:5000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). This antiserum binds human IgA, IgG, and IgM by virtue of light chain recognition. A positive anti-CEA antibody response was defined as an absorbance greater than the mean plus 2 SDs of 10 normal donor sera at the same dilution. Similarly, serum titers of antivaccinia antibody were calculated as the limiting dilution of serum producing an absorbance of >0.150 and greater than the mean plus 3 SDs of 10 vaccinia-naive normal donor sera at the same dilution.

RESULTS

Patient Characteristics. The clinical aspects of the trial are summarized in Table 1. The patients included 14 men and 6 women with a median age of 65 years (range, 42–77 years). All patients had metastatic disease, and 17 of 20 had elevated serum CEA levels at the time of study entry. Four of 20 patients had stable disease at 8 weeks of follow-up, whereas the remaining 16 patients demonstrated disease progression. None of the patients demonstrated a sustained decrease in serum CEA levels following vaccination, except for patient 15, who has medullary carcinoma of the thyroid, which often follows an indolent course.

Delayed-type hypersensitivity skin testing with baculovirus recombinant CEA protein performed 8 weeks following primary immunization was entirely negative in all 20 patients, with no evidence of erythema or induration. Patients were also challenged 8 weeks following primary immunization with rV-CEA (M_r 70,000) by scarification to determine the degree of immune-mediated attenuation of the skin reaction induced by the virus. Only 4 of 20 patients developed a pustule following scarification with rV-CEA (M_r 70,000), indicating preceding immunity to the vaccinia virus induced by intradermal injection or Biojector delivery of rV-CEA (M_r 180,000) in the majority of patients. Pustule formation following scarification occurred in 3 of 10 patients previously immunized at the 10^7 pfu dose level and only 1 of 10 patients previously immunized with 10^8 pfu doses of rV-CEA (M_r 180,000). Pustules following scarification were observed in two patients from each of the intradermal and Biojector groups.

Toxicology. Both techniques of inoculation produced local skin erythema, induration, and pustule formation similar to

Table 1 Patient characteristics

Patient no.	Age (yr)/sex	Dosage (pfu)	Performance status[a]	Primary site	Metastatic disease	Serum CEA (ng/ml)		Response[b]
						Prestudy	Week 8	
1	55 /M	10^7	1	Colon	Liver	29	109	P
2	66/M	10^7	1	Rectal	Liver, lung	154	288	P
3	59/M	10^7	0	Rectal	Liver, lung	7	25	P
4	63/M	10^7	1	Colon	Lymph nodes	5	2	P
5	65/M	10^7	1	Cholangiocarcinoma	Peripancreatic	2	1	P
6	70/F	10^7	0	Colon	Lymph nodes	188	166	S
7	73/M	10^7	1	Rectal	Liver	41	91	P
8	64/F	10^7	1	Colon	Lymph nodes	180	227	P
9	44/M	10^7	2	Cholangiocarcinoma	Liver, lymph nodes	77	90	P
10	77/M	10^7	2	Colon	Liver	3358	6820	P
11	77/F	10^8	2	Colon	Liver, abdomen wall	45	45	P
12	49/F	10^8	1	Colon	Liver, lung	103	168	P
13	75/M	10^8	0	Colon	Liver, adrenal	1776	2933	P
14	62/M	10^8	1	Rectal	Liver, peritoneal	554	919	P
15	42/M	10^8	1	Thyroid	Mediastinum, bone	667	969	S
16	66/F	10^8	1	Rectal	Liver, lung, nodes	39	44	S
17	53/F	10^8	0	Breast	Peritoneum	33	42	P
18	71/M	10^8	1	Colon	Liver	18	139	P
19	62/M	10^8	1	Gastroesophageal	Lymph nodes	1	1	S
20	67/M	10^8	1	Colon	Liver, lung	306	392	P

[a] Performance status based on the Eastern Cooperative Oncology Group scale (0–4).
[b] Response based on off-study evaluation on day 57. P, progressive disease; S, stable disease.

Table 2 Local inflammation at the inoculation site

	Maximum diameter of erythema and/or induration (cm)[a]									
	A. Patients receiving 10^7 pfu of rV-CEA per dose									
	Group 1 (intradermal injection)					Group 2 (Biojector)				
Patient no.	2	4	5	6	9	1	3	7	8	10
Postprime	3[a]	2P	1P	3	2P	5	6	4P	2P	1P
Postboost	2	1	1	2	2	1	3	2	2	1
	B. Patients receiving 10^8 pfu of rV-CEA per dose									
	Group 3 (intradermal injection)					Group 4 (Biojector)				
Patient no.	11	13	17	18	20	12	14	15	16	19
Postprime	4P	3P	4P	4P	3P	5	7P	6P	6P	3P
Postboost	2	2	5	1	0	0	2	0	1	1

[a] Values are the maximum diameter (cm) of erythema and/or induration at the inoculation site 3–7 days following the dose. P, pustule formation.

that reported following administration of vaccinia virus by scarification for smallpox immunization (11). Local inflammation and pustule formation have traditionally been regarded as desirable consequences of scarification with vaccinia virus, indicating an adequate inoculation with live virus. As shown in Table 2, some degree of erythema and or induration was observed at the site of all initial inoculations with rV-CEA (M_r 180,000). Less local erythema and induration was seen after the second dose. Each technique of inoculation produced a pustule after the first dose in three of five patients at the initial dose level of 10^7 pfu. When the dose was increased to 10^8 pfu of rV-CEA, all five patients in the intradermal group and four of five patients in the Biojector group developed a pustule. Pustules were not observed following the second dose of rV-CEA in either group. Local pruritis at the inoculation site beginning 24 h after administration was noted in 14 of 20 patients, most commonly after the primary immunization. The incidence and severity were not dose related, but pruritis was more frequent and more intense following intradermal administration. Pruritis required no medications and resolved spontaneously within 3–5 days. A few patients reported mild inoculation site discomfort, fatigue, and/or fever, which were not dose related. No regional adenopathy or acute allergic reactions were observed after administration of rV-CEA.

Grade 1–2 anemia developed in a non-dose-related fashion in seven patients and was consistent with anemia of chronic disease in this population of patients with metastatic adenocarcinoma.

Granulocytes have been shown to express nonspecific cross-reacting antigen, raising the possibility that immunization against CEA could lead to autoimmune neutropenia (1, 12). Despite this theoretical concern, no consistent changes in WBC counts or neutrophil counts were observed. No deterioration in renal or hepatic function that could be attributable to immunization was observed.

Table 3 Lymphoproliferative responses to vaccinia virus

	SI[a]									
A. Patients inoculated by intradermal needle injection										
Patient no.	2	4	5	6	9	11	13	17	18	20
Preimmunization	44	1	1	3	2	2	1	5	2	5
Week 4[b]	233	1	2	10	21	89	1	17	1	14
Week 8	753	1	33	729	488	478	27	668	1	23
B. Patients inoculated by Biojector										
Patient no.	1	3	7	8	10	12	14	15	16	19
Preimmunization	431	1	1	9	2	1	4	201	1	1
Week 4	582	670	2	37	16	40	51	1284	50	79
Week 8	8	331	271	41	158	114	1	4	2	7
C. Patients inoculated by scarification										
Patient no.	A	B	C	D	E					
Preimmunization	1	1	4	5	2					
Week 4	159	38	54	148	178					
Week 12	137	10	1	49	51					

[a] Values are SIs in response to UV-inactivated vaccinia virus.
[b] Weeks after primary immunization.

Lymphoproliferative Responses. The lymphoproliferative responses to vaccinia virus are provided in Table 3. Table 3, A and B, refers to patients from this study receiving rV-CEA (M_r 180,000) by intradermal needle injection and s.c. jet injection using the Biojector, respectively. Table 3C provides data from five additional patients with a history of colorectal carcinoma who received 10^7 pfu doses of rV-CEA (M_r 180,000) by scarification on weeks 0 and 8 as participants in a previous trial at our institution. Nine of 25 patients demonstrated lymphoproliferative responses to vaccinia virus prestudy, defined as a SI of >3. These patients were evenly distributed between the three groups. These responses are thought to represent persistent memory T cells induced by previous smallpox immunization (*i.e.*, vaccinia priming), which was an inclusion criterion for each study. Patients 1, 2, and 15, who had robust lymphoproliferative responses to vaccinia virus prestudy, had received smallpox booster immunizations during adulthood as the result of military service. A positive lymphoproliferative response to vaccinia virus as the result of immunization with rV-CEA was defined as a postimmunization SI of >3 and at least 2-fold greater than the prestudy SI. By these criteria, 8 of 10 patients in the intradermal group, 9 of 10 patients in the Biojector group, and 5 of 5 patients immunized by scarification demonstrated a positive lymphoproliferative response to vaccinia virus following immunization with rV-CEA. The only patient in the Biojector group who did not achieve at least a 2-fold increase in SI was patient 1, who was strongly positive prestudy with a SI of 431. The booster immunization with rV-CEA increased the lymphoproliferative response to vaccinia virus in 8 of 10 patients in the intradermal group, 4 of 10 patients in the Biojector group, and 0 of 5 patients immunized by scarification. This is perhaps due to inability of the virus to generate a productive infection upon second exposure in the face of significant antivaccinia immune responses.

For the purpose of comparing vaccinia-specific lymphoproliferative responses to rV-CEA immunization between groups, patients with a prestudy SI of >10 were excluded, and calculations were based upon the maximum postimmunization SI. The prestudy SIs were comparable between the three groups with the mean ± SE being 2.4 ± 0.5, 2.5 ± 1.0, and 2.6 ± 0.8 for the intradermal, Biojector, and scarification groups, respectively. Postimmunization SIs (mean ± SE) were likewise comparable: 272 ± 104, 179 ± 75, and 115 ± 29 for intradermal, Biojector, and scarification groups, respectively. The modes for postimmunization SI to vaccinia virus were 33, 78, and 49 for intradermal, Biojector, and scarification groups, respectively. Thus, each method of administration of rV-CEA induces lymphoproliferation to vaccinia virus with comparable efficacy.

No CEA-specific lymphoproliferative responses were observed in any of the 20 patients studied. Lymphoproliferative responses to tetanus toxoid as well as PHA and pokeweed mitogens were analyzed to determine the effect of progressive metastatic adenocarcinoma upon lymphocyte function *in vitro*. Proliferative responses to PHA and pokeweed mitogen did not change significantly over 8 weeks of follow-up, despite disease progression in 16 of 20 patients. The SIs (mean ± SE) in response to PHA prestudy and 8 weeks after primary immunization were 790 ± 120 and 940 ± 150, respectively. With regard to pokeweed responses, the SIs (mean ± SE) prestudy and 8 weeks after primary immunization were 420 ± 80 and 550 ± 80, respectively.

Ten of 20 patients demonstrated lymphoproliferative responses to tetanus toxoid prestudy, and 8 of these experienced disease progression during study participation. The SI in response to tetanus toxoid 8 weeks following primary immunization was lower than the prestudy SI in all eight of these patients. The tetanus toxoid-specific SI (mean ± SE) decreased by 54 ± 10% over 8 weeks of follow-up. The nonparametric Wilcoxon signed rank test used to perform a paired analysis of prestudy

Table 4 Mononuclear cell release of IL-2 in response to vaccinia virus

	IL-2 (mIU/ml)[a]									
A. Patients inoculated by intradermal needle injection										
Patient no.	2	4	5	6	9	11	13	17	18	20
Preimmunization	35	0	1	9	0	0	3	11	0	0
Week 4[b]	40	0	0	11	80	135	1	4	0	22
Week 8	>300	0	4	>300	22	15	44	13	0	10
B. Patients inoculated by Biojector										
Patient no.	1	3	7	8	10	12	14	15	16	19
Preimmunization	63	1	0	0	0	0	13	43	0	0
Week 4	32	36	4	1	153	148	34	139	28	0
Week 8	>300	39	47	28	51	30	0	0	0	10

[a] Values are mIU/ml IL-2 in cell culture supernatant.
[b] Weeks after immunization.

Table 5 Antibody response to vaccinia virus

	Limiting dilution titer									
A. Patients inoculated by intradermal needle injection										
Patient no.	2	4	5	6	9	11	13	17	18	20
Preimmunization	10,000	–[a]	1,000	–	–	–	1,000	–	–	3,000
Week 4[b]	10,000	30,000	10,000	3,000	10,000	10,000	10,000	10,000	10,000	10,000
Week 8	10,000	10,000	30,000	3,000	3,000	30,000	10,000	3,000	10,000	30,000
B. Patients inoculated by Biojector										
Patient no.	1	3	7	8	10	12	14	15	16	19
Preimmunization	–	–	–	–	–	3,000	10,000	–	–	1,000
Week 4	10,000	10,000	30,000	10,000	100,000	30,000	30,000	10,000	30,000	10,000
Week 8	10,000	3,000	30,000	3,000	100,000	100,000	30,000	3,000	30,000	10,000

[a] –, a negative result, referring to a limiting dilution titer of less than 1:300.
[b] Weeks after immunization.

and week 8 SIs demonstrated that the decrease in SI was significant ($P = 0.03$).

IL-2 Release. The mononuclear cell release of IL-2 in response to vaccinia virus is provided in Table 4. Nine of 20 patients demonstrated IL-2 release in response to vaccinia virus prestudy, thought to represent persistent memory T cells induced by previous smallpox immunization. Patients 1, 2, and 15, who had robust IL-2 release in response to vaccinia virus prestudy, had received recent smallpox booster immunizations as a result of military service.

A positive vaccinia virus-specific IL-2 response as the result of immunization with rV-CEA was defined as a postimmunization level of ≥1 mIU/ml and at least twice the patient's prestudy level. By these criteria, 7 of 10 patients in the intradermal group and 10 of 10 patients in the Biojector group demonstrated a positive IL-2 response to vaccinia virus following immunization with rV-CEA. The booster immunization with rV-CEA increased the vaccinia virus-specific IL-2 response in only 4 of 10 patients in each group, perhaps due to inability of the virus to generate a productive infection upon second exposure. For the purpose of comparing vaccinia virus-specific IL-2 responses, patients with a prestudy IL-2 release of >20 mIU/ml were excluded, and calculations were based upon the maximum postimmunization IL-2 release for each patient. Prestudy vaccinia-specific IL-2 release (mean ± SE) was comparable between the two groups: 2.7 ± 1.4 and 1.8 ± 1.6 mIU/ml for the intradermal and Biojector groups, respectively. Postimmunization IL-2 release (mean ± SE) was likewise comparable: 66 ± 33 and 61 ± 20 mIU/ml for the intradermal and Biojector groups, respectively. Thus, each administration technique induced vaccinia-specific IL-2 release of comparable magnitude, although results were more consistently positive following Biojector delivery. No evidence of CEA-specific IL-2 release was observed among any of the 20 patients studied.

Antibody Responses to Vaccinia Virus and CEA. The limiting dilution titers of antivaccinia antibody are provided in Table 5. Seven of 20 patients demonstrated antivaccinia antibody titers ranging from 1:1000 to 1:10,000 prestudy. Nineteen of 20 patients demonstrated an increase in antivaccinia antibody titer within 4 weeks following primary immunization with rV-CEA. Booster immunization with rV-CEA did not have a consistent effect on the antivaccinia antibody titers. Analysis conducted with a one-sample *t* test confirmed that the antibody titers were significantly greater postimmunization compared with preimmunization ($P = 0.002$ and 0.018 for the intradermal and Biojector groups, respectively). Analysis was also carried

out with a two-sample *t* test and Wilcoxon rank-sum test to examine the equality of prestudy *versus* postimmunization differences in antibody titer between the intradermal injection and Biojector groups. Results show that there is no significant difference between the two groups ($P = 0.17$ and $P = 0.28$ for the *t* test and Wilcoxon rank-sum test, respectively). Thus, both techniques of administering rV-CEA induce antivaccinia antibody responses of comparable magnitude. None of the patients demonstrated an antibody response to CEA as analyzed by ELISA with the native human protein.

DISCUSSION

A major aim of this study was to compare the safety and efficacy of the two techniques for administering the vaccine. Immunization with rV-CEA by intradermal needle injection or s.c. jet injection was associated with modest toxicity. All patients demonstrated some degree of local inflammation at the site of primary inoculation, which was comparable between the two groups. Most patients experienced pruritis at the inoculation site, which was more frequent and more intense with intradermal administration. Pruritis was self-limited and required no therapy. Systemic toxicity was limited to low-grade fever and increased fatigue, each affecting fewer than 20% of the patients. Thus, both techniques of administration were well tolerated, with no compelling reason to recommend one technique over the other, based upon toxicity profiles. Because no evidence of immune response to CEA was observed, the efficacy comparison between the two groups was based upon the induction of immune response to the vaccinia virus vector. Both administration techniques induced vaccinia-specific lymphoproliferative, IL-2 release, and antibody responses of comparable magnitude and frequency. Furthermore, both techniques protected 80% of patients against pustule formation following scarification with rV-CEA (M_r 70,000) on day 57, indicative of immunity to viral challenge. Therefore, there is also no compelling reason to recommend one administration technique over the other, based upon the available efficacy data. Either technique appears reasonable for use in future clinical trials of recombinant vaccinia virus vaccines. We have selected s.c. jet injection using the Biojector for our subsequent trial of rV-CEA, based upon the lower incidence of pruritis at the inoculation site and avoidance of needles, which could transmit the vaccine or blood-borne pathogens to health care workers. This technique also accommodates larger injection volumes up to 1 ml and is more rigorously standardized between clinicians.

With regard to dose, we recommend the use of 10^8 pfu for subsequent clinical trials of recombinant vaccinia virus vaccines administered by intradermal needle injection or s.c. jet injection. This conclusion is based upon the favorable toxicity profile and the observation that this dose induced local pustule formation, which is indicative of an adequate inoculation of live virus, in 90% of patients, compared with 60% of patients receiving doses of 10^7 pfu. Furthermore, 10^8 pfu doses of rV-CEA (M_r 180,000) protected 90% of patients against pustule formation following scarification challenge with rV-CEA (M_r 70,000) on day 57 compared with 70% protection achieved with doses of 10^7 pfu. Vaccinia-specific lymphoproliferation, IL-2 release, and antibody response were not related to patient dose over the limited range studied.

No evidence of CEA-specific lymphoproliferation, IL-2 release, or delayed-type hypersensitivity was observed. The absence of demonstrable cellular immune response to CEA using fresh PBMCs is consistent with the observations of Hamilton *et al.* (5) in the initial trial of rV-CEA administered by scarification in the same patient population. However, Schlom and colleagues (6) have demonstrated that HLA-A2 restricted cytolytic T-cell lines responsive to specific epitopes of CEA could be derived by prolonged *in vitro* culture of postimmunization PBMCs from patients in the initial trial of rV-CEA. These data suggest that immunization with rV-CEA can evoke a cytolytic T-cell response to a specific epitope of the human CEA molecule but that it does so at a precursor frequency that is undetectable by current technology using PBMCs without prolonged *in vitro* culture in the presence of peptide antigen and IL-2. Such studies involving prolonged culture of PBMCs were not systematically undertaken in conjunction with this trial.

None of the patients demonstrated an antibody response to CEA, as determined by an ELISA assay with native human CEA protein. However, 85% of the patients had elevated circulating levels of CEA protein at the time of study entry. Therefore, anti-CEA antibodies in these patients would be expected to exist predominantly in the form of circulating immune complexes with CEA protein. The ELISA assay described herein may be inadequate to detect anti-CEA antibodies in the form of immune complexes. Therefore, we are continuing to analyze these sera by Western blot and modified ELISA techniques in an effort to detect CEA immune complexes. Detection of circulating immune complexes is a challenge shared by a variety of vaccine trials seeking to immunize against tumor associated antigens such as CEA and prostate-specific antigen, which are shed into the bloodstream (13).

Evidence that vaccination is capable of breaking tolerance to CEA in humans has been provided by two sources: (*a*) Schlom and colleagues (6) have described the derivation of CEA-specific cytolytic T cell lines following rV-CEA immunization, as described above; and (*b*) Foon *et al.* (14) have reported CEA-specific antibody and lymphoproliferative responses following vaccination with an anti-idiotype monoclonal antibody to an anti-CEA monoclonal antibody in patients with colorectal carcinoma. Recent murine studies have demonstrated enhanced T-cell responses and antitumor effects when recombinant vaccinia viruses encoding tumor associated antigens were admixed with a recombinant vaccinia virus encoding the murine B7.1 costimulatory molecule (15, 16). Furthermore, priming with rV-CEA followed by multiple boosts with a nonreplicating canarypox recombinant encoding CEA (ALVAC-CEA) has been shown to enhance CEA-specific T cell immune responses and antitumor effects compared with use of either vector alone (17). The use of diversified prime and boost strategies is thought to be particularly critical in vaccinating human populations who have previously received smallpox immunizations. Because vaccinia virus proteins are highly immunogenic, vaccinia recombinants can only be effectively administered once or twice due to the induction of high titers of antivaccinia antibody, which will neutralize subsequent doses of the virus, reducing recombinant gene expression (18, 19). This study confirms this

shortcoming of recombinant vaccinia immunization. Pustules at the inoculation sites that are indicative of productive viral infection were observed after primary immunization in 90% of patients receiving 10^8 pfu of rV-CEA but were completely absent following booster immunization. Furthermore, the second inoculation with rV-CEA did not consistently boost vaccinia-specific lymphoproliferation, IL-2 release, or antibody responses. Therefore, the absence of demonstrable CEA-specific immune responses may be a consequence of inadequate booster immunizations. On the basis of these observations, we plan to carry out an additional clinical trial of vaccination against CEA using recombinant pox viruses. In this trial, patients will receive an admixture of rV-CEA and rV-human-B7.1 followed by multiple boosts with a canarypox recombinant encoding both CEA and human B7.1 to capitalize on the diversified prime and boost strategy as well as the costimulatory activity of B7.1. In this trial, we will use 10^8 pfu doses of the vaccinia recombinants administered by s.c. jet injection with the Biojector.

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