

# Vector-based Vaccine/Cytokine Combination Therapy to Enhance Induction of Immune Responses to a Self-Antigen and Antitumor Activity

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## ABSTRACT

Many antigens associated with human tumors are overexpressed in tumor cells as compared with normal tissues; these “self” tumor-associated antigens are also expressed during fetal development, and it is, thus, not surprising that they are either weakly immunogenic or functionally nonimmunogenic in the tumor-bearing host. In the studies reported here, we have used different vaccines and vaccine strategies in an attempt to develop antitumor immunity in a stringent animal model. The tumor antigen used was human carcinoembryonic antigen (CEA). The model used was CEA transgenic mice, in which the human CEA transgene is under the control of the endogenous CEA promoter; CEA is expressed in fetal tissues and normal gastrointestinal tissues, and CEA protein is found in sera. Previous studies have shown these CEA transgenic mice to be tolerant to the induction of CEA immunity using CEA protein in adjuvant as an immunogen. CEA-expressing tumor cells were implanted 14 days before vaccine therapy. The vaccines used were recombinant vaccinia virus containing the transgenes for CEA and three T-cell costimulatory molecules [*B7-1*, *ICAM-1*, and *LFA-3*, designated recombinant vaccinia (rV)-CEA/TRICOM], with each transgene under the control of individual poxvirus promoters, and a replication-defective avipox virus (fowlpox; rF) containing the same four transgenes (designated rF-CEA/TRICOM). The results demonstrate that (a) continued boosting with vaccine is required to maintain CEA-specific T-cell responses, and boosting with rF-CEA/TRICOM is superior to boosting with rF-CEA; (b) a diversified vaccination protocol consisting of primary vaccination with rV-CEA/TRICOM followed by boosting with rF-CEA/TRICOM is more efficacious than homogeneous vaccination with rF-CEA/TRICOM in the induction of both CEA-specific T-cell responses and antitumor activity; and (c) the use of cytokines, local granulocyte macrophage colony-stimulating factor (GM-CSF) and low-dose systemic interleukin 2, in combination with vaccine is essential in inducing antitumor activity, as compared with the use of cytokines alone, or the use of vaccines without cytokine. Both GM-CSF and interleukin 2 were shown to contribute to the induction of CEA-specific T-cell responses. These studies thus provide a “proof of concept” that potent vaccines and vaccine strategies, in combination with cytokines, may be essential to obtain the level of T-cell responses directed against a self-antigen that is necessary to achieve antitumor responses.

## INTRODUCTION

The vast majority of TAAs<sup>2</sup> that are now being targeted by a vaccine therapy have been shown to be “self” antigens that are either overexpressed in tumor as compared with normal tissue or are expressed in tumor tissue and a nonvital organ. Many of these TAAs have also been shown to be expressed during fetal development. Thus, one would expect that a host immune response to vaccination with

such a TAA would be weak at most and would be exacerbated in the presence of tumor. To evaluate different vaccines and vaccine strategies directed against the self TAA, we have used human CEA as a self target antigen in CEA Tg mice. These mice contain the human CEA transgene under the control of the endogenous human CEA promoter and express CEA in normal gastrointestinal tissue and in fetal tissue in a manner similar to that expressed in humans (1, 2). Moreover, these CEA Tg mice contain serum CEA protein in levels (5–100 ng/ml) similar to those found in patients with CEA-expressing carcinomas (1). Previous studies have demonstrated that these mice are tolerant to CEA by their inability to mount either CEA-specific T-cell responses or CEA-specific antibody responses after multiple vaccinations with CEA protein in adjuvant (2). These mice thus provide a model for peripheral tolerance to a self TAA. In these studies, we have used a carcinoma cell (MC38) expressing the human CEA gene as a result of retroviral transfection. Tumor therapy was initiated 14 days after tumor transplant.

The vaccine vectors used in the studies reported here are rV-, which is replication competent, and recombinant avipox (fowlpox; rF-), which is replication defective in mammalian cells. The vaccines contain the CEA transgene as well as the transgenes for a triad of T-cell costimulatory molecules (*B7-1*, *ICAM-1*, and *LFA-3*, designated TRICOM; 3). Each of the four transgenes in the rV-CEA/TRICOM and the rF-CEA/TRICOM vectors is under the control of individual poxvirus promoters. Previous studies in experimental models (4, 5) and in clinical trials (6–9) have shown that vectors can be used only once, or at most twice, to express transgenes because of host-limiting immune responses directed against the vector. On the other hand, preclinical studies (4, 5, 10), and now clinical studies (8, 11), have demonstrated that rF-vectors can be used multiple times without inhibition of transgene expression, because of the lack of induction of host-neutralizing anti-vector immune responses. Previous studies in non-CEA Tg, *i.e.*, conventional C57BL/6 mice, have demonstrated that rV-CEA/TRICOM and rF-CEA/TRICOM are more effective than rV-CEA and rF-CEA in inducing CEA-specific T-cell responses (3, 5). Previous studies in conventional mice have also shown that rGM-CSF protein can enhance CEA-specific T-cell responses when given at the site of vaccination with rV-CEA vectors, and that rF-GM-CSF can enhance T-cell responses when given in combination (admixed) with rF-CEA vectors (10). In the studies reported here, we have used a stringent animal model in which CEA-expressing tumors are implanted 14 days before the initiation of vaccine therapy in an attempt to understand which vaccines and vaccine strategies are most efficient in enhancing antigen-specific T-cell responses as well as antitumor responses. The results demonstrate that (a) continued boosting with vaccine is required to maintain CEA-specific T-cell responses, and boosting with rF-CEA/TRICOM was superior to boosting with rF-CEA; (b) a diversified vaccination protocol (primary vaccination with rV-CEA/TRICOM) followed by boosting with rF-CEA/TRICOM is more efficacious than homogeneous vaccination with rF-CEA/TRICOM in the induction of both CEA-specific T-cell responses and antitumor activity; and (c) the use of cytokines, local GM-CSF, and low-dose IL-2 in combination with vaccine is essential in inducing antitumor activity, as compared with the use of both cytokines without vaccine, or the use of vaccines

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<sup>2</sup> The abbreviations used are: TAA, tumor-associated antigen; CEA, carcinoembryonic antigen; Tg, transgenic; rV-, recombinant vaccinia; rF-, recombinant avipox (fowlpox); pfu, plaque forming unit(s); APC, antigen-presenting cell; PCC, pigeon cytochrome C; GM-CSF, granulocyte macrophage colony-stimulating factor; rGM-CSF, recombinant GM-CSF; IL, interleukin; WT, wild-type; VSV, vesicular stomatitis virus; ConA, concanavalin A.

without cytokine. Both cytokines were shown to contribute to the induction of CEA-specific T-cell responses. These studies, thus, provide a "proof of concept" for the use of vaccines in patients with advanced cancers that potent vaccines (CEA/TRICOM) and vaccine strategies (diversified prime and boost) in combination with cytokines (both GM-CSF and low-dose IL-2) may be essential to achieving the level of T-cell responses directed against the self-antigen that is necessary to achieve antitumor responses.

## MATERIALS AND METHODS

**Recombinant Poxviruses.** The rF-virus designated rF-CEA contains the human *CEA* gene under the control of the poxvirus 40k promoter (12). The rV- and fowlpox viruses containing the human *CEA* gene and the murine *B7-1*, *ICAM-1*, and *LFA-3* genes (designated rV-CEA/TRICOM and rF-CEA/TRICOM, respectively) have been described (3). The recombinant fowlpox virus designated rF-GM-CSF contains the gene for murine GM-CSF under control of the poxvirus 40k promoter (10). Nonrecombinant WT vaccinia virus (Wyeth strain) was designated V-WT, whereas nonrecombinant WT fowlpox virus was designated WT-FP. Therion Biologics Corp. (Cambridge, MA) provided all of the Orthopox viruses. Per vaccination,  $10^8$  pfu of virus were given s.c. in 100  $\mu$ l, except for rF-GM-CSF, which was administered at  $10^7$  pfu/injection in 100  $\mu$ l.

**Animals/Cells.** C57BL/6 mice Tg for human CEA were obtained from a breeding pair provided by Dr. John Thompson (Institute of Immunobiology, University of Freiburg, Freiburg, Germany). The generation and characterization of the CEA Tg mouse has been described previously (1). PCR of DNA from whole blood to detect the *CEA* gene was used to screen for CEA-positive mice as described previously (2). Mice were housed and maintained under pathogen-free conditions in microisolator cages. For experiment 1 (efficacy of TRICOM boosting, see Table 1), 6- to 30-week-old male CEA Tg mice were used. For all of the survival experiments, 8-week-old female CEA Tg mice were used. For *in vitro* readouts of experiments 2 and 3 (see Table 1), 8-week-old male CEA Tg mice were used.

Murine colon adenocarcinoma cells expressing human CEA (MC-38-CEA) were generated by retroviral transduction of MC-38 cells with CEA cDNA (13). Before transplantation to mice, the cells were trypsinized, dispersed

through a 70  $\mu$ m cell strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ), and washed twice in HBSS before final suspension in HBSS.

**Cytokines.** In addition to prime vaccinations, mice of indicated groups (see Table 1 and figure legends) received 20  $\mu$ g of rGM-CSF s.c. at the vaccination site (once a day for 4 days), and/or 16,000 IU recombinant IL-2 i.p. (every 12 h for 4 days, designated low-dose IL-2; Ref. 14). GM-CSF was obtained from PeprTech, Inc. (Rocky Hill, NJ); IL-2 was obtained from Hoffmann-La Roche Inc. (Nutley, NJ). For boost vaccinations, vaccine vector was admixed with  $10^7$  pfu of rF-GM-CSF, and/or low-dose IL-2 was given i.p.

**Lymphoproliferative Assay.** CEA-specific T-cell responses of vaccinated mice were analyzed 21 days after the final vaccinations, as described previously (15). Briefly, CEA-specific lymphoproliferation was evaluated by adding splenic T cells from immunized mice at  $1.5 \times 10^5$ /well in 96-well flat-bottomed plates (Costar, Cambridge, MA). APCs consisted of irradiated (20 Gy) naive syngeneic splenocytes and were added at  $5 \times 10^5$ /well. Cells were stimulated with 80  $\mu$ g/ml purified human CEA or less (Aspen Bio, Littleton, CO) as indicated in individual figures (see Table 2, Figs. 1A and 3A). The endotoxin levels within the purified CEA were reported to be very low (<400 EU/mg). As a negative control, either mouse albumin or ovalbumin (Sigma Chemical Co., St. Louis, MO) was used at 80 or 50  $\mu$ g/ml, as indicated in individual figures (see Table 2, Figs. 1A and 3A). As a positive control for T-cell proliferation, cells were stimulated with the T-cell mitogen ConA (2.5  $\mu$ g/ml; Sigma). Control wells received T cells, APCs and medium only. Cells were labeled for the final 12–18 h of the incubation with 1  $\mu$ Ci/well [ $^3$ H]thymidine (New England Nuclear, Wilmington, DE) and harvested with a Tomtec cell harvester (Wallac Incorporated, Gaithersburg, MD). Incorporated radioactivity was measured by liquid scintillation counting (Wallac 1205 Betaplate; Wallac, Inc.). Results from triplicate wells were averaged and reported either as stimulation index [ $SI = (\text{cpm stimulated wells})/(\text{cpm control wells})$ ] or as mean cpm  $\pm$  1 SD.

**Cytokine Release Assay.** To evaluate CD8<sup>+</sup> T-cell responses, spleens from three animals/group were removed 3 weeks after the last immunization and were dispersed into single-cell suspensions, pooled, and coincubated with 10  $\mu$ g/ml of the H-2D<sup>b</sup>-restricted 8-mer peptide CEA<sub>526–533</sub> (EAQNTTYL, further referred to as CEA peptide; Refs. 2 and 16) for 6 days. Bulk lymphocytes were recovered by centrifugation through a Ficoll-Hypaque gradient. T cells were restimulated with fresh irradiated naive splenocytes and either 10  $\mu$ g/ml CEA peptide or 10  $\mu$ g/ml VSV-N<sub>52–59</sub> (RGVYVQGL; further referred

Table 1 Outline of experiments

Experiment	Group	Vaccinations					
		1	2	3	4	5	6
1 <sup>a</sup>	A	HBSS	HBSS	HBSS	HBSS		
	B	V-WT + rF-GM-CSF + IL-2	FP-WT + rF-GM-CSF + IL-2	FP-WT + rF-GM-CSF + IL-2	FP-WT + rF-GM-CSF + IL-2		
	C	rV-TRICOM + rGM-CSF + IL-2	rF-TRICOM + rF-GM-CSF + IL-2	rF-TRICOM + rF-GM-CSF + IL-2	rF-TRICOM + rF-GM-CSF + IL-2		
	D	rV-CEA/TRICOM + rGM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2		
	E	rV-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA/TRICOM		
2	A	rV-CEA/TRICOM	rF-CEA/TRICOM	HBSS	HBSS	HBSS	HBSS
	B	rV-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA	rF-CEA	rF-CEA	rF-CEA
	C	rV-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA/TRICOM
3	A	rV-CEA/TRICOM + rGM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2		
	B	rV-CEA/TRICOM + rGM-CSF	rF-CEA/TRICOM + rF-GM-CSF	rF-CEA/TRICOM + rF-GM-CSF	rF-CEA/TRICOM + rF-GM-CSF		
	C	rV-CEA/TRICOM + IL-2	rF-CEA/TRICOM + IL-2	rF-CEA/TRICOM + IL-2	rF-CEA/TRICOM + IL-2		
	D	rV-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA/TRICOM		
4	A	rV-CEA/TRICOM + rGM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2	rF-CEA/TRICOM + rGM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2		
	B	rF-CEA/TRICOM + rF-GM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2	rF-CEA/TRICOM + rF-GM-CSF + IL-2		
	C	rF-GM-CSF + IL-2	rF-GM-CSF + IL-2	rF-GM-CSF + IL-2	rF-GM-CSF + IL-2		

<sup>a</sup> For certain experiments, it was necessary to compress the vaccination schedule to every 7 days when treating mice bearing 14-day established tumors in order to complete the vaccine regimen. For experiments 1, 3, and 4: CEA-Tg mice were immunized weekly. For experiment 2, CEA-Tg mice were immunized biweekly.

Table 2 Cellular immune responses to CEA after multiple vaccinations with CEA/TRICOM vectors

Vaccination <sup>a</sup>			Antigen <sup>b</sup>							Peptide <sup>c</sup>		Anti-CEA IgG <sup>d</sup>
			CEA ( $\mu\text{g/ml}$ )							CEA <sub>526-533</sub>	VSV-N <sub>52-59</sub>	
1 (prime)	2	3-6	ConA	Albumin	80	40	20	10	5			
rV-CEA/TRICOM	rF-CEA/TRICOM	HBSS	478	0.2	8.5	3.6	2.7	2.8	1.9	ND <sup>e</sup>	ND	<50
rV-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA	496	0.4	10.3	7.2	4.5	6.2	3.6	4131 $\pm$ 125	310 $\pm$ 80	270
rV-CEA/TRICOM	rF-CEA/TRICOM	rF-CEA/TRICOM	521	0.7	<b>15.5<sup>f</sup></b>	<b>13.5<sup>f</sup></b>	5.5	9.5	5.6	<b>8357 <math>\pm</math> 225<sup>g</sup></b>	2571 $\pm$ 60	500

<sup>a</sup> Five CEA-Tg mice/group were vaccinated once with  $1 \times 10^8$  pfu of rV-CEA/TRICOM. Fourteen days later, mice were boosted with  $1 \times 10^8$  pfu of rF-CEA/TRICOM. Mice then received four biweekly vaccinations with the indicated boosting reagent. Responses from pooled splenic T cells were analyzed 21 days after the final vaccination for CEA protein-specific proliferation and CEA peptide-specific IFN- $\gamma$  production.

<sup>b</sup> For CEA-specific proliferation, antigen concentrations were: ConA (2.5  $\mu\text{g/ml}$ ); mouse albumin (80  $\mu\text{g/ml}$ ); and CEA (80  $\mu\text{g/ml}$  to 5  $\mu\text{g/ml}$ ). Each value represents the stimulation index of the mean cpm of triplicate samples *versus* medium. SD never exceeded 10%.

<sup>c</sup> For CEA-specific IFN- $\gamma$  production, concentrations of peptides CEA<sub>526-533</sub> and VSV-N<sub>52-59</sub> were 10  $\mu\text{g/ml}$ . Each value represents the mean IFN- $\gamma$  (pg/ml) of triplicate samples.

<sup>d</sup> IgG specific for CEA. Antibody titer defined as reciprocal dilution of sera yielding a one-half-maximal absorbance at 490 nm.

<sup>e</sup> ND, none detected.

<sup>f</sup> Values in bold are significant when compared with either rF-CEA- or HBSS-boosted animals ( $P = 0.034$  and  $< 0.001$ , respectively).

<sup>g</sup> Values in bold are significant when compared with rF-CEA-boosted animals ( $P = 0.001$ ).

to as VSV peptide) peptide (17) for 24 h. Supernatant was collected and analyzed for murine IFN- $\gamma$  by capture ELISA as described previously (18). For the TRICOM boosting study, CD8<sup>+</sup> T cells were purified by negative selection using anti-MHC class II, anti-natural killer and anti-CD4 magnetic beads (Miltenyi Biotec, Auburn, CA) and were used in this assay.

**Titration of Serum Antibodies.** Anti-CEA antibody (IgG) was quantified in the serum of each animal by ELISA. Microtiter plates were coated with either purified CEA (100 ng/well) or ovalbumin (100 ng/well). The plates were blocked for 1 h at 37°C with 5% BSA in PBS. The plates were incubated with serum serially diluted from 1:50 to 1:6250, with the CEA-specific monoclonal antibody COL-1 (19) as control, or with the isotype control UPC-10 (IgG2a), for 1 h at 37°C. Plates were washed several times with PBS containing 1% BSA and were incubated at 37°C for 45 min with horseradish peroxidase-conjugated goat antimouse IgG specific antiserum (1:4000) and antibody was detected by horseradish peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer's instructions. The absorbance of each well was read at 490 nm using a Bio-Tek ELx808 microplate ELISA reader (Winooski, VT).

**Tumor Therapy Studies.** CEA Tg mice were transplanted with 50,000 MC38-CEA cells as experimental metastases, as described previously (20). Briefly, the spleens of anesthetized mice were exteriorized by a small subcostal incision. Cells were directly injected into 100  $\mu\text{l}$  of HBSS using 1-ml syringes with 26-gauge, 5/8-inch needles. Splenectomy was performed ~2 min after tumor cell injection by cauterization using a high-temperature cautery (Roboz, Rockville, MD). The abdominal cavity was closed in one layer using 9-mm wound autoclips. This dose of tumor cells is lethal to >80% of mice within 12 weeks, with the primary tumor arising in the peripancreatic area. In previous studies (5) and in some of the studies reported here, it has been demonstrated that increased abdominal circumference correlated with massive abdominal tumor. Mice were killed when they became moribund. This tumor model has been described previously (20).

**Statistical Analysis of the Data.** Where indicated (Figs. 1 and 3, Table 1), the results of tests of significance are reported as  $P$ s and are derived from Student's  $t$  test using a two-tailed distribution. Evaluation of survival patterns in mice bearing peripancreatic tumors was performed by the Kaplan-Meier method and ranked according to the Mantel-Cox log-rank test using Statview 4.1 (Abacus Concepts Inc., Berkeley, CA) software package. For graphical representation of data,  $Y$ -axis error bars indicate the SD of the data for each point on the graph (Figs. 1 and 3).

## RESULTS

**Efficacy of Different Boosting Regimens.** We have previously shown, using conventional mice, that the use of CEA/TRICOM vectors is superior to the use of vectors containing CEA/B7-1 or CEA alone in the induction of CEA-specific immune responses. Studies in CEA Tg mice were first conducted to determine whether (a) continued boosting is necessary, and (b) boosting with rF-CEA/TRICOM vectors is superior to boosting with rF-CEA in the absence of the TRICOM costimulatory molecules. Mice were primed with rV-CEA/

TRICOM and boosted 2 weeks later with rF-CEA/TRICOM. Fourteen days later, mice were divided into three groups and received four biweekly boosts of HBSS, rF-CEA, or rF-CEA/TRICOM. The vaccination schedule of this study (study 1) and all of the subsequent studies is shown in Table 1. Three weeks after the final vaccination, splenic T cells were analyzed for proliferative responses to CEA protein and mouse albumin as a negative control protein (Table 2). ConA was used as a positive control. T cells from mice continuously boosted with rF-CEA/TRICOM (vaccinations 3-6) proliferated significantly more in response to CEA than did T cells from mice that received HBSS for vaccinations 3-6 ( $P < 0.001$ ; both at 80 and 40  $\mu\text{g/ml}$  CEA). T cells from all of the groups failed to react with the negative control protein, mouse albumin. In addition, T cells from rF-CEA/TRICOM-boosted mice showed a higher proliferation in response to CEA at all of the CEA concentrations than did T cells from rF-CEA-boosted mice ( $P = 0.034$  at 40  $\mu\text{g/ml}$  CEA). Activation of CEA-specific CD8<sup>+</sup> T cells was also investigated by an analysis of IFN- $\gamma$  production on stimulation with APCs and either CEA peptide or negative control peptide VSV. Greater levels of IFN- $\gamma$  were produced by CD8<sup>+</sup> T cells from mice boosted with rF-CEA/TRICOM for vaccinations 3-6 than from rF-CEA-boosted mice in response to the CEA peptide ( $P = 0.001$ ; Table 2). Previous studies (21) have shown correlations between IFN- $\gamma$  production and CTL activity. Anti-CEA IgG antibody responses showed no significant differences between mice boosted with rF-CEA/TRICOM or mice boosted with rF-CEA for vaccinations 3-6 (Table 2).

**Vaccine/Cytokine Combination Therapies.** The above study indicated that continued boosting with rF-CEA/TRICOM was more effective in generating CEA-specific T-cell responses than continued boosting with rF-CEA. No cytokines were used in those studies. To determine whether the addition of GM-CSF and/or IL-2 would further enhance antigen-specific immune responses, CEA Tg mice were vaccinated with rV-CEA/TRICOM as a prime and were boosted three times weekly with rF-CEA/TRICOM. Group A received only CEA/TRICOM vectors and no additional cytokines, as described in Table 1 (study 2). Group B received rGM-CSF GM-CSF with the prime and rF-GM-CSF with the three weekly boosts, as described in "Materials and Methods." Group C received low-dose IL-2, as described in "Materials and Methods," in addition to the CEA/TRICOM vectors. Group D received both low-dose IL-2 and GM-CSF in addition to the CEA/TRICOM vectors. As seen in Fig. 1A, proliferation of CEA-specific T cells was slightly enhanced by the addition of low-dose IL-2 to the CEA/TRICOM vectors. Significantly greater proliferation of T cells was seen with the addition of GM-CSF only (Fig. 1A, ■), as compared with CEA/TRICOM vectors alone ( $P = 0.003$  at 80  $\mu\text{g/ml}$  CEA). The addition of both GM-CSF and IL-2 (Fig. 1A, ●) to



the vaccinations enhanced T-cell proliferation to a greater level than did GM-CSF only or IL-2 only ( $P = 0.021$  and  $P < 0.001$ , respectively, at  $80 \mu\text{g/ml}$  CEA). T cells from all of the groups failed to react with the negative control protein, ovalbumin (Fig. 1A,  $\Delta$ ). CD8<sup>+</sup>-specific T-cell responses were investigated by a cytokine release assay using CD8<sup>+</sup> T cells from vaccinated mice and defining IFN production in response to CEA protein or the control VSV-N protein. IFN- $\gamma$  production was enhanced by the addition of cytokines to the CEA/TRICOM vectors (Fig. 1B). The addition of only GM-CSF was more efficient than the addition of only low-dose IL-2 ( $P = 0.002$ ), whereas the addition of GM-CSF and low-dose IL-2 was more efficient than the use of either cytokine alone ( $P < 0.001$ ; Fig. 1B). CEA-specific antibody titers changed slightly with the addition of cytokines (Fig. 1C). At one-half maximum absorbance, the titers were 98 for group A (Fig. 1C,  $\blacklozenge$ ), which received no additional cytokines; 190 for group B (Fig. 1C,  $\blacksquare$ ), which received only GM-CSF in addition to the CEA/TRICOM vectors; 130 for group C (Fig. 1C,  $\blacktriangle$ ), which received only IL-2 in addition to the CEA/TRICOM vectors; and 410 for group D (Fig. 1C,  $\bullet$ ), which received both GM-CSF and IL-2 in addition to the CEA/TRICOM vectors. Titers in groups B and D were significantly different from those in groups A and C ( $P < 0.05$ ).

A tumor therapy study was then conducted to determine the effect of cytokine addition on the survival of tumor-bearing CEA Tg mice. Mice were vaccinated in a manner identical to that described above (Fig. 2), but they received CEA-expressing tumor cells 14 days before vaccination, as described in "Materials and Methods." As can be seen in Fig. 2, only 14% of the mice from group A, which received only CEA/TRICOM vectors and no additional cytokines, survived for the duration of the study (22 weeks). Fifty-seven % of the mice from group B, which received only GM-CSF in addition to the CEA/TRICOM vectors, survived during the 22-week observation period. Fifty % of the mice from group C, which received only IL-2 in addition to the CEA/TRICOM vaccines, survived during the 22-week observation period. However, 83% of mice that received CEA/TRICOM and both GM-CSF and IL-2 (group D) survived during the 22-week observation period. The addition of GM-CSF to the CEA/TRICOM vaccine regimen (group B) resulted in a significant increase in survival when compared with the mice that received only CEA/TRICOM vaccines ( $P = 0.04$ ). No significant differences were found between groups A and C ( $P > 0.05$ ), and groups B and C ( $P > 0.05$ ). In contrast, mice in group D, which received both GM-CSF and IL-2 in addition to CEA/TRICOM viral vectors, differed significantly in their survival from mice that received only CEA/TRICOM vaccines ( $P = 0.016$ ; group A).

**Diversified Prime and Boost Vaccination versus Homogeneous Prime and Boost.** The above experiments used rV-CEA/TRICOM to prime immune responses; rF-CEA/TRICOM was used for continued boosting. However, unlike fowlpox virus, vaccinia virus can replicate in mammalian cells and can have adverse effects (22). The data in the above experiments clearly showed that the addition of GM-CSF and low-dose IL-2 to CEA/TRICOM viral vectors markedly and significantly enhanced the survival of CEA Tg mice bearing CEA-positive tumors. Studies were then undertaken to determine whether the use of rF-CEA/TRICOM, in combination with both GM-CSF and low-dose IL-2, would enhance immune responses to such a level that a diversified prime and boost would not be necessary. CEA Tg mice either were vaccinated with rV-CEA/TRICOM and after 7 days received three weekly boosts with rF-CEA/TRICOM (Fig. 3, group A,  $\bullet$ ), or were vaccinated weekly four times with rF-CEA/TRICOM, *i.e.*, without a rV-CEA/TRICOM primary vaccination (Fig. 3, group B,  $\blacktriangle$ ). All of the mice received GM-CSF and low-dose IL-2. Vaccine regimen details are described in Table 1, study 3. Control mice were given HBSS as a vaccine every 7 days for a total of four injections and also

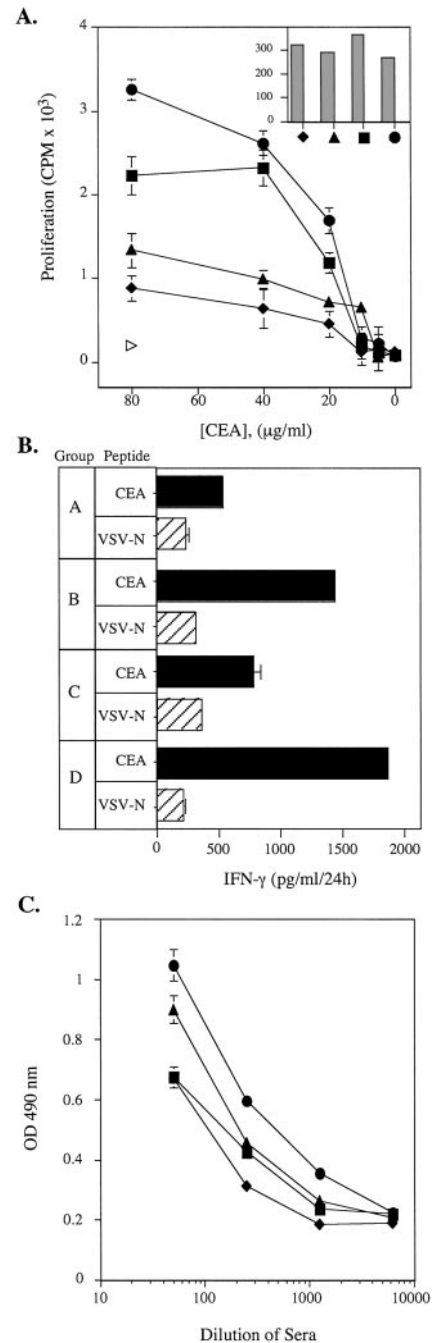
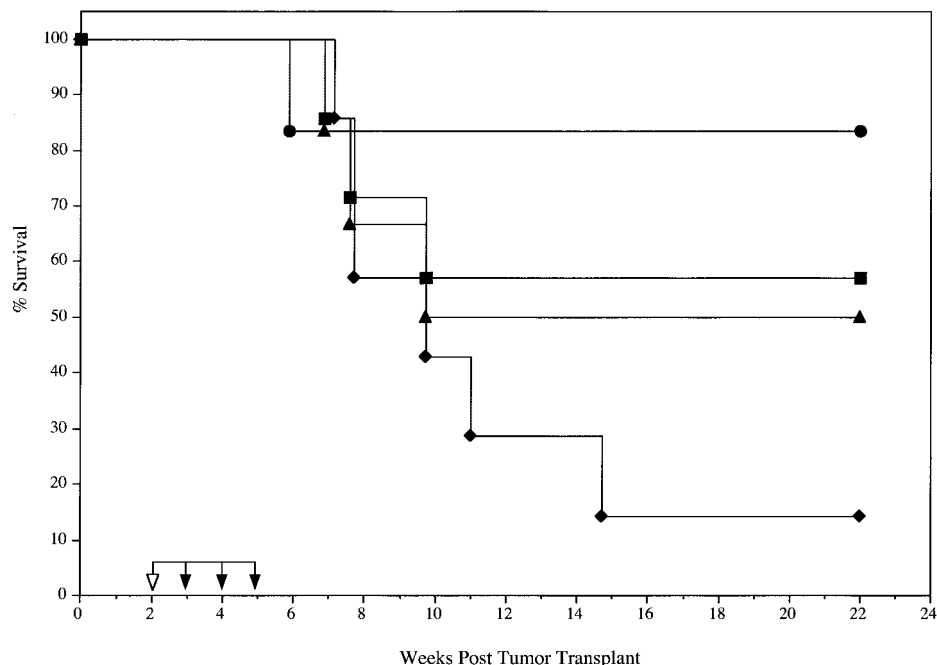


Fig. 1. Effect of cytokines on CEA-specific immune responses in mice vaccinated with CEA/TRICOM vectors. CEA Tg mice, three/group, were vaccinated s.c. with  $10^8$  pfu of rV-CEA/TRICOM, followed by three weekly boosts with  $10^8$  pfu of rF-CEA/TRICOM (Table 1). Group A ( $\blacklozenge$ ) received no additional cytokines. Group B ( $\blacksquare$ ) received GM-CSF in addition to CEA/TRICOM vectors, as described in "Materials and Methods." Group C ( $\blacktriangle$ ) received low-dose IL-2 in addition to CEA/TRICOM vectors, as described in "Materials and Methods." Group D ( $\bullet$ ) received both GM-CSF and low-dose IL-2 in addition to the CEA/TRICOM vectors. A, lymphoproliferation of splenic T cells in response to CEA protein. T-cell proliferation was measured by [<sup>3</sup>H]thymidine incorporation and graphed as cpm versus protein concentration. Proliferation of all of the groups in response to the control protein mouse albumin ( $80 \mu\text{g/ml}$ ) is also shown ( $\Delta$ ). SDs are based on the mean of triplicate wells. Inset, proliferation of splenic T cells from vaccinated mice in response to the T-cell mitogen ConA. Equal numbers of T cells from mice vaccinated as described above were cultured with  $2.5 \mu\text{g/ml}$  ConA (gray bars) or without  $2.5 \mu\text{g/ml}$  ConA (below the limit of detection) and naive splenocytes as APC. B, IFN- $\gamma$  production by T cells in response to the 8-mer CEA peptide (solid bars) or an irrelevant control peptide, VSV (hatched bars). Splenocytes were bulk-cultured for 7 days with  $10 \mu\text{g/ml}$  CEA peptide. Purified T cells were restimulated for 24 h with either CEA peptide or VSV peptide and irradiated naive splenocytes as APCs. Supernatant was harvested and assayed by ELISA for the presence of IFN- $\gamma$ . SDs are based on the mean of triplicate wells in the ELISA assay. C, CEA-specific IgG antibodies in serum of vaccinated CEA Tg mice, as assayed by ELISA. SDs are based on the mean value of serum samples from five mice.

Fig. 2. Effect of cytokines on the survival of CEA Tg mice bearing 14-day established experimental metastases treated by immunization with CEA-TRICOM vectors. Tumors were transplanted by intrasplenic injection of MC-38 colon carcinoma cells that were transduced with CEA. Two weeks after tumor injection, mice in all groups (A–D) were vaccinated s.c. with  $10^8$  pfu of rV-CEA/TRICOM as prime vaccinations (*open arrow*), followed by three weekly boosts with  $10^8$  pfu of rF-CEA/TRICOM (*closed arrows*). Group A (◆) received no additional cytokines. Group B (■) received GM-CSF. Group C (▲) received low-dose IL-2. Group D (●) received both GM-CSF and low-dose IL-2. See “Materials and Methods” for details. Each group consisted of seven mice. Mice were monitored weekly for survival.



received GM-CSF and low-dose IL-2 (group C, □). As seen in Fig. 3A, priming with rV-CEA/TRICOM and boosting with rF-CEA/TRICOM significantly enhanced CEA-specific lymphoproliferation as compared with homogeneous priming and boosting with rF-CEA/TRICOM (Fig. 3A,  $P < 0.001$  at  $50 \mu\text{g/ml}$  CEA). T cells from all of the groups failed to react with the negative control protein, ovalbumin ( $\Delta$ ). Next, responses of  $\text{CD8}^+$  T cells were examined.  $\text{IFN-}\gamma$  production by T cells on stimulation with CEA peptide was significantly enhanced in rV-CEA/TRICOM-primed animals compared with rF-CEA/TRICOM-primed animals ( $P < 0.001$ ; Fig. 3B). T cells from rF-CEA/TRICOM-primed and -boosted mice, however, produced greater levels of  $\text{IFN-}\gamma$  than did T cells from mice that were given HBSS vaccine in the presence of cytokines ( $P = 0.042$ ). No significant differences in antibody titers were found between mice that received the diversified prime and boost and mice that received rF-CEA/TRICOM for all of the vaccinations (Fig. 3C). In the serum of mice of both groups, however, a significant amount of CEA-specific antibodies was found, compared with serum from mice that received HBSS and cytokines ( $P < 0.001$ ; titer  $< 50$ ).

A tumor therapy study was then carried out in CEA Tg mice to determine whether diversified prime and boost was more effective than the continued use of rF-CEA/TRICOM. CEA Tg mice received MC-38-CEA cells 14 days before vaccinations, as described in the “Materials and Methods” section. Mice were vaccinated in a manner identical to that described above (Fig. 3) and were then monitored weekly for survival. The data depicted in Fig. 4 clearly show that the diversified prime and boost strategy with rV-CEA/TRICOM followed by rF-CEA/TRICOM boosting significantly enhanced the survival of mice over a homologous prime and boost vaccination regimen with rF-CEA/TRICOM ( $P = 0.032$ ). Seventy % of mice that were vaccinated with rV-CEA/TRICOM and boosted with rF-CEA/TRICOM survived during the 23-week observation period (Fig. 4, ●), whereas only 20% of mice that received the homologous prime and boost with rF-CEA/TRICOM survived after 21 weeks (Fig. 4, ▲). Ten % of the mice that received only cytokines and no vaccine (HBSS) survived after 15 weeks (Fig. 4, □). Although the homogeneous prime and boost regimen showed a trend toward increased survival (7-week difference in survival time at the 50% survival point), this difference

was not quite significant ( $P = 0.0546$ ) because of the small group size. However, if mice from study 4, vaccinated with WT virus and cytokines (group B, Fig. 5), are combined with the cytokine-only group (group C, Fig. 4), the trend of homologous prime and boost with cytokines *versus* the use of cytokines alone becomes significant ( $P = 0.027$ ).

**Vector Control Studies.** A tumor therapy study was initiated to determine the effect of WT vectors, vectors containing only costimulatory molecules (and not CEA) as vaccines, and the use of cytokines on the survival of tumor-bearing mice (Table 1, study 4). Mice received CEA-expressing tumor cells 14 days before vaccination, as described in “Materials and Methods.” Untreated mice died by 10 weeks post-tumor transplant (Fig. 5, group A, ◆). Only 10% of mice treated with WT viruses (priming with V-WT and boosting with FP-WT) and GM-CSF and IL-2 (group B, ○), and 11% of mice treated with rV-TRICOM prime and rF-TRICOM boosts, and GM-CSF and IL-2 (group C,  $\Delta$ ) survived during the 20-week observation period. Mice that were vaccinated with rV-CEA/TRICOM and boosted with rF-CEA/TRICOM, but did not receive cytokines, died by 10 weeks post-tumor transplant (group D, ■), again demonstrating the synergy in the use of vaccine plus cytokines. However, 60% of mice that were vaccinated with rV-CEA/TRICOM, boosted with rF-CEA/TRICOM, and received GM-CSF and low-dose IL-2 (group E, ●) survived for the duration of the study (20 weeks), which was significantly different from mice from other groups, including those that received control vectors with or without TRICOM ( $P = 0.02$ ).

## DISCUSSION

Combinations of different vaccine strategies were examined to determine the maximal therapeutic response in a stringent tumor model, using Tg mice that expressed human CEA as self-antigen (CEA Tg mice). The vast majority of tumor therapy models to date have initiated vaccine therapy 3–6 days post-tumor transplant (23–27). Vaccine therapy was initiated 14 days post-tumor transplant. To our knowledge, this is the longest time interval between tumor transplant and vaccine therapy for a tumor therapy model in which the vaccine is directed against a self-antigen.

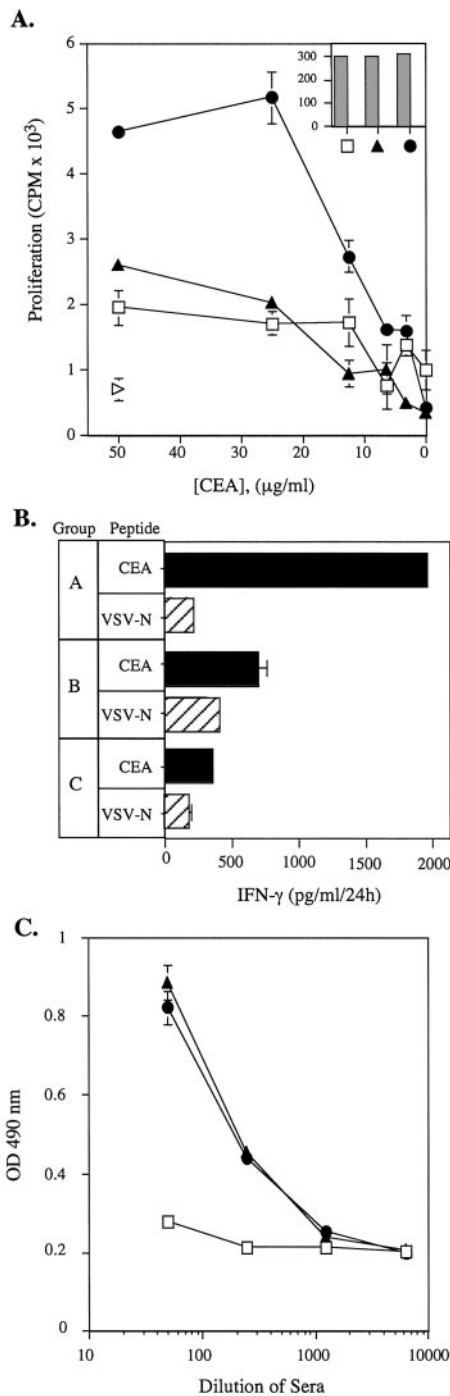


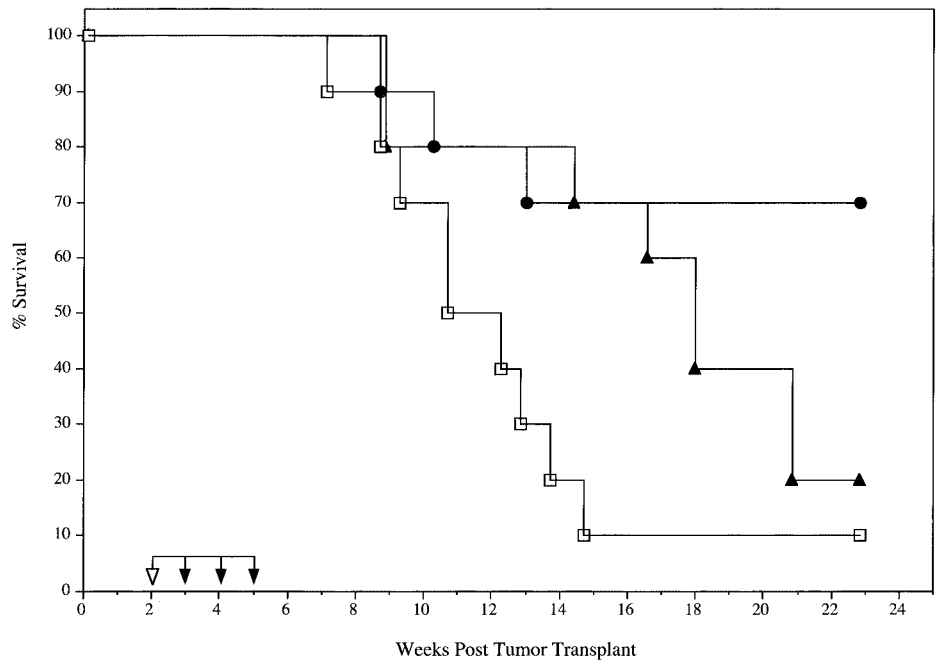
Fig. 3. Effect of the diversified prime and boost on CEA-specific immune responses in mice. CEA Tg mice, three/group, were vaccinated s.c. with either  $10^8$  pfu of rV-CEA/TRICOM (group A, ●) or  $10^8$  pfu of rF-CEA/TRICOM (group B, ▲). Mice then received three weekly boosts with  $10^8$  pfu of rF-CEA/TRICOM. Control mice received only HBSS (group C, □). GM-CSF and low-dose IL-2 were given with all of the vaccinations as indicated in "Materials and Methods." A, T-cell proliferation was measured by [ $^3$ H]thymidine incorporation and graphed as cpm versus protein concentration. Proliferation of all of the groups in response to ovalbumin (80  $\mu$ g/ml) is also shown ( $\Delta$ ). SDs are based on the mean of triplicate wells. *Inset*, proliferation of splenic T cells from vaccinated mice in response to the T-cell mitogen ConA. Equal numbers of T cells from mice vaccinated as described were cultured with 2.5  $\mu$ g/ml ConA (gray bars) or without 2.5  $\mu$ g/ml ConA (below the limit of detection) and naive splenocytes as APCs. B, IFN- $\gamma$  production by T cells in response to CEA peptide (solid bars) or an irrelevant control peptide, VSV (hatched bars). Splenocytes were bulk-cultured for 7 days with 10  $\mu$ g/ml CEA peptide. Purified T cells were restimulated for 24 h with either CEA peptide or VSV peptide and irradiated naive splenocytes as APCs. Supernatant was harvested and assayed by ELISA for the presence of IFN- $\gamma$ . SDs are based on the mean of triplicate wells in the ELISA assay. C, CEA-specific IgG antibodies in serum of vaccinated CEA Tg mice, as assayed by ELISA. SDs are based on the mean value of serum samples from five mice.

The TRICOM vectors used in these studies have been shown previously to activate T cells to greater levels than the use of any one or two of these costimulatory molecules in recombinant vectors (3). However, it has been hypothesized that high levels of costimulation coupled with high levels of antigen expression may down-regulate immune responses, because of extensive signaling either via the TCR and CD28 or via up-regulation of CTLA-4 on the activated T cells (28). Sabzevari *et al.* (29) has reported that high peptide concentrations of PCC could induce apoptosis in effector/memory T cells, possibly related to CD80 acquisition by these cells. Therefore, we sought to investigate whether repeated boosting with a vector containing three costimulatory molecules would be more effective than continued boosting with vaccines containing the TAA but devoid of costimulatory molecules. To that end, CEA Tg mice were primed and boosted with rV-CEA/TRICOM and rF-CEA/TRICOM; then four additional boosts were given with rF-CEA/TRICOM, rF-CEA, or HBSS (Table 1). Interestingly, even though all of the mice were primed and boosted with rV-CEA/TRICOM and rF-CEA/TRICOM, CEA-specific immune responses remained relatively low in the mice that were administered HBSS for boosts 3–6 (Table 2). This indicates that repeated boosting with antigen was required to maintain CEA-specific immune responses. In addition, these data demonstrate that repeated boosting with CEA/TRICOM is not detrimental for CEA-specific immune responses. In fact, CEA/TRICOM boosting appeared to be superior to repeated boosting with rF-CEA. This may seem to be in contrast with data described above showing that stimulation of effector/memory T cells with high doses of peptide resulted in apoptosis (29). However, an important difference with that study is that, in CEA Tg mice, in which human CEA is a self-antigen, only low-affinity T cells are likely to exist. Sabzevari *et al.* (29) used PCC-specific T cells from PCC T-cell receptor Tg mice, which have a high affinity for PCC. It is, therefore, hypothesized from the data reported here that in the CEA Tg mice, greater levels of signals 1 and 2 are required to activate CEA-specific T cells, and, thus, overstimulation is not likely to occur.

The level of CEA-specific antibodies was not influenced by repeated boosting with rF-CEA/TRICOM as opposed to rF-CEA. This could be because the TRICOM vector was originally designed to enhance type 1 T-cell responses. Few type 2 cytokines, if any, were produced by T cells stimulated with TRICOM vectors (3). Alternatively, the high number of vaccinations may have caused optimal antibody levels that could not be altered by repeated boosting with rF-CEA/TRICOM.

To potentially further enhance CEA-specific immune responses, the effect of GM-CSF and IL-2 in addition to CEA/TRICOM viral vectors was investigated in CEA Tg mice. IL-2 has been shown to promote the proliferation and differentiation of T and B cells and to enhance cytolytic activity of natural killer cells and lymphokine-activated killer cells (30). However, serious toxicity has been associated with high doses of IL-2 (31, 32). Therefore, we chose to administer low doses of IL-2. The dose used (16,000 IU/injection, twice a day for 4 days) has been defined as low-dose IL-2 treatment, because it has no direct antitumor effect (Ref. 14; also see Fig. 5). IL-2 has also been previously shown to enhance the vaccine efficacy of a rV-CEA vaccine in conventional mice (14). In addition, the effect of GM-CSF was investigated. The infiltration of APCs to regional lymph nodes has been shown to be enhanced by locally administered GM-CSF (33–35), which also potentiates T-cell responses (10). In the studies presented here, when used with vaccine, both IL-2 and GM-CSF significantly enhanced CEA-specific T-cell proliferation and IFN- $\gamma$  production in response to peptide (Fig. 1) and increased the survival of CEA Tg mice that received CEA-expressing tumors (Fig. 2). The addition of low-dose IL-2 only to the poxvirus-based CEA/TRICOM vaccine strategy described here (rV-CEA/TRICOM, rF-CEA/TRICOM) failed to significantly increase CEA-specific T-cell proliferation, or specific IFN- $\gamma$  secretion from CD8 $^+$  T-cells from immunized mice (Fig. 1). However, survival of tumor-bearing mice was enhanced

Fig. 4. Effect of the diversified prime and boost on the survival of CEA Tg mice bearing 14-day established experimental metastases. Tumors were transplanted by intrasplenic injection of MC-38 colon carcinoma cells that were transduced with CEA. Two weeks after tumor injection, mice were primed (*open arrow*) s.c. either with  $10^8$  pfu of rV-CEA/TRICOM (●) or with  $10^8$  pfu of rF-CEA/TRICOM (▲). Mice then received three weekly boosts (*closed arrows*) with  $10^8$  pfu of rF-CEA/TRICOM. Control mice received only HBSS (□). GM-CSF and low-dose IL-2 were given with all of the vaccinations as indicated in "Materials and Methods." Each group consisted of 10 mice. Mice were monitored weekly for survival.



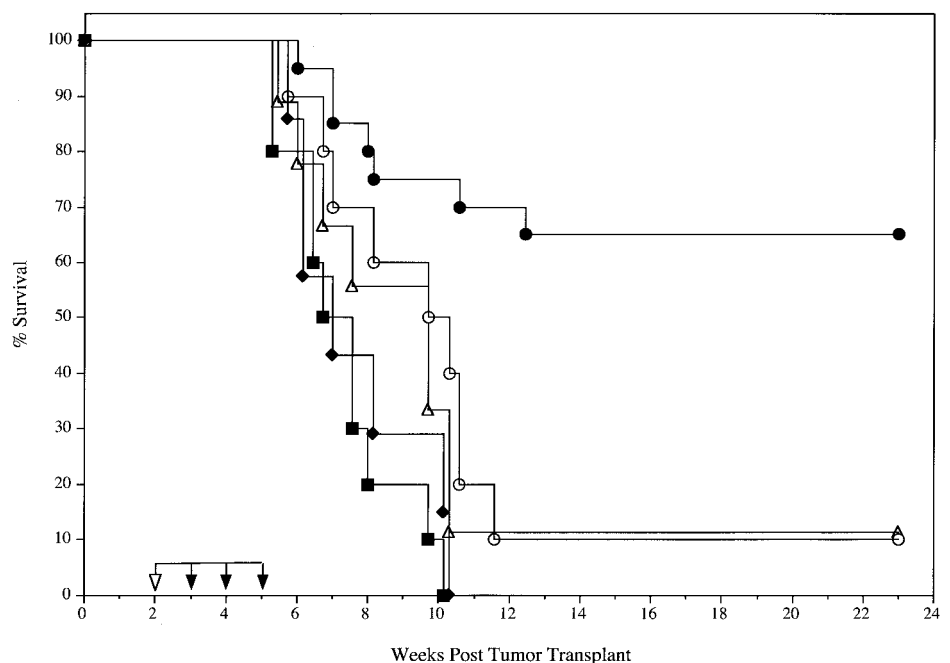
by a combination of IL-2 and GM-CSF (Fig. 2). Mice that were cured of tumors showed no evidence of overt toxicity. However, studies are ongoing to examine such mice for any evidence of autoimmune phenomena as well as to determine the relevant effector cells that mediate antitumor therapy.

In contrast to the use of IL-2, the use of GM-CSF in addition to the vaccine regimen resulted in significantly higher levels of CEA-specific lymphoproliferation (Fig. 1A) and greater levels of specific IFN- $\gamma$  secretion from immune CD8<sup>+</sup> T-cells (Fig. 1B), as well as a marked and significant increase in survival (Fig. 2). It has been previously shown that a single administration of rF-GM-CSF is as potent as four daily administrations of rGM-CSF (10). rGM-CSF was used in the primary vaccination with rV-CEA/TRICOM in the antitumor studies reported because of the possibility that the replication competent rV-CEA/TRICOM would

lyse cells infected with rF-GM-CSF and, thus, limit the paracrine effect of the expressed GM-CSF over the 2 or 3 weeks that fowlpox vectors have been shown to express their transgene. Because recombinant fowlpox is nonreplicating, rF-GM-CSF was used in conjunction with the boost vaccinations. The combination of GM-CSF and IL-2, when used with vaccine, resulted in further increased levels of IFN- $\gamma$  secretion and further increased survival.

A diversified prime and boost vaccination regimen, with other viral or DNA vectors, has also been shown in other studies to be more effective than a homologous prime and boost (4, 25, 36, 37). In our studies, we used the rV-vector for priming and recombinant fowlpox vectors for boosting, which has previously been shown to be very effective (4, 5, 8). We sought to investigate whether increased T-cell responses via the use of CEA/TRICOM vectors and the addition of cytokines,

Fig. 5. Effect of control viruses and cytokines on the survival of CEA Tg mice bearing 14-day established experimental metastases. Tumors were transplanted by intrasplenic injection of MC-38 colon carcinoma cells that were transduced with CEA. Fourteen days after tumor transplant, mice were vaccinated s.c. weekly for 4 weeks. *Open arrow*, the prime vaccination; *closed arrows*, the boosts. Group A (◆; see Table 1) received injections with only HBSS. Group B (○) received  $10^8$  pfu of V-WT for the prime and  $10^8$  pfu of FP-WT for the three weekly boosts. In addition, GM-CSF and low-dose IL-2 were given, as described in "Materials and Methods." Group C (△) received  $10^8$  pfu of rV-TRICOM for the prime and  $10^8$  pfu of rF-TRICOM for the three weekly boost immunizations. In addition, GM-CSF and low-dose IL-2 were given. Group D (■) received  $10^8$  pfu of rV-CEA/TRICOM as a prime and  $10^8$  pfu of rF-CEA/TRICOM for the boosts with no addition of cytokines. Group E (●) received  $10^8$  pfu of rV-CEA/TRICOM as a prime and  $10^8$  pfu of rF-CEA/TRICOM for the boosts. Both GM-CSF and low-dose IL-2 were given in addition to the CEA/TRICOM vectors. Each group consisted of 10 mice. Mice in each group were monitored weekly for survival.





in CEA Tg mice, would eliminate the need for the diversified prime and boost regimen. Vaccination with rF-CEA/TRICOM vectors and cytokines, without a primary vaccination with rV-CEA/TRICOM, increased the survival time of mice bearing CEA-expressing tumors (Fig. 4). Our studies clearly showed, however, that the diversified prime and boost, with rV-CEA/TRICOM for the prime and rF-CEA/TRICOM for all boosts, was far more effective than the homologous prime and boost, using only rF-CEA/TRICOM vectors, in both *in vitro* and *in vivo* readouts (Figs. 3 and 4).

The studies here demonstrate for the first time that a diversified prime and boost regimen (with CEA/TRICOM poxviral vectors) and the addition of cytokines (GM-CSF and low-dose IL-2) are most effective in treating tumors in a rigorous setting (a 14-day tumor therapy model in CEA Tg mice). These data underscore the use of (a) potent vaccines containing a tumor antigen gene and three T-cell costimulatory molecule transgenes, and (b) the use of these vaccines in combination with two different cytokines to enhance therapeutic responses. These studies thus provide a proof of concept for the design of vaccine clinical protocols for use in patients with cancer.

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## Vector-based Vaccine/Cytokine Combination Therapy to Enhance Induction of Immune Responses to a Self-Antigen and Antitumor Activity

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