Vaccine Therapy of Established Tumors in the Absence of Autoimmunity

James W. Hodge, Douglas W. Grosenbach, Wilhelmina M. Aarts, Diane J. Poole, and Jeffrey Schlom

Laboratory of Tumor Immunology and Biology, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland 20892-1750

ABSTRACT

Purpose: Many current clinical trials involve vaccination of patients with vaccines directed against tumor-associated antigens, which are, in actuality, “self-antigens” over-expressed in tumors as compared with normal tissues. As tumor vaccines become more potent through the addition of costimulatory molecules and cytokines and the use of diversified prime and boost regimes, the level of concern rises regarding the balance between antitumor immunity and pathological autoimmunity. Studies were conducted using mice bearing a transgenic self-antigen [human carcinoembryonic antigen (CEA)], which is expressed in some normal adult tissues, and tumor expressing the same self-antigen. These mice were vaccinated with recombinant poxviral vectors [recombinant vaccinia, recombinant fowlpox (rF)] encoding the CEA transgene as well as a triad of costimulatory molecules [B7-1, ICAM-1, and LFA-3 (TRICOM)]. Here we investigate the mechanism of tumor therapy and evaluate the safety of such a regimen in a self-antigen system. To our knowledge, the study reported here is the first description of the safety of such a regimen in a self-antigen system. To investigate the mechanism of tumor therapy and evaluate the safety of such a regimen in a self-antigen system. To our knowledge, the study reported here is the first description of the safety of such a regimen in a self-antigen system.

Experimental Design: CEA transgenic mice were transplanted with CEA-expressing tumors. Fourteen days later, mice were vaccinated with recombinant vaccinia-CEA/TRICOM admixed with recombinant murine granulocyte macrophage colony-stimulating factor and then given low-dose interleukin 2. Mice were boosted on days 21, 28, and 35 with rF-CEA/TRICOM admixed with rF-granulocyte macrophage colony-stimulating factor and then given low-dose interleukin 2. Mice were monitored for survival and compared with groups of mice vaccinated in a similar manner with poxviral vectors containing CEA/B7-1 or CEA transgenes. To determine the mechanism of antitumor therapy, mice were depleted of T-cell subpopulations before vaccination with the CEA/TRICOM regimen. Mice successfully cured of tumor and age-matched control mice were monitored for 1 year. At 1 year, several clinical assays were carried out involving analysis of 9 serological parameters, 11 urinalysis parameters, and 14 immunological parameters. In addition, histopathology was performed on 42 tissues/mouse.

Results: The CEA/TRICOM vaccination regimen induced a therapeutic antitumor response as measured by increased survival, which was due largely to induced T-cell responses (both CD4+ and CD8+) as determined by selective T-cell subset depletion. The CEA/TRICOM vaccination regimen induced a significant increase in proliferation of CD4+ T cells to CEA protein and a significant increase in secretion of IFN-γ from CD8+ T cells in response to a defined CEA epitope. Despite CEA expression in normal adult gastrointestinal tissues, no toxicity was observed in the CEA/TRICOM-vaccinated group when an array of clinical serum and urine chemistry assays was conducted 1 year after vaccination. Moreover, a comprehensive histopathological evaluation of all tissues from these groups also showed no evidence of toxicity.

Conclusions: Activation of T cells directed against a tumor-associated self-antigen, sufficient to mediate therapeutic antitumor immunity, was observed in vivo without the development of autoimmunity as analyzed by a comprehensive evaluation of biochemical, immunological, and histopathological criteria. These studies demonstrate that the use of vectors containing as many as three costimulatory molecules does not induce autoimmunity or other pathology. These studies thus demonstrate that a balance can indeed be achieved between the induction of an immune response to a self-antigen, which is capable of antitumor therapy, and the absence of autoimmunity.

INTRODUCTION

The vast majority of human TAAs that are now being or plan to be targeted in vaccine clinical trials have been defined as “self-antigens” due to overexpression on tumor as compared with normal tissue or expression on tumor tissue and a nonvital organ. Many of these TAAs have also been shown to be ex-
pressed during fetal development. Thus, one would expect that a host immune response to such “self-TAA”s would be extremely weak in the tumor-bearing host (1–5). Because of this, many experimental and clinical tumor vaccine studies have focused on various strategies to enhance T-cell responses to specific TAAs. However, the induction of more potent immune responses to a self-antigen raises the question of induction of autoimmunity. Indeed, the induction of autoimmunity has been demonstrated the following: (a) the advantages of vaccinia or avipox [fowlpox and/or canarypox (ALVAC)] for delivery of the TAA to the immune system (5, 12–14); (b) rV vectors can be used only once due to host-limiting immune responses directed against the vector (4, 15–18); (c) recombinant avipox vectors can be used multiple times without inhibition of transgene expression, as demonstrated in preclinical studies (15, 16, 19) and clinical studies (17, 20); (d) the advantages of diversified vaccine prime and boost regimens (15, 17, 21); (e) the use of T-cell costimulation either by antibody-mediated blockade mechanisms (22, 23) or via insertion of costimulatory molecules into vectors (24–26) enhances T-cell responses to the TAA; moreover, the use of rV or rF vectors containing TRICOM has been shown to activate T cells to greater levels than the use of any one or two of these costimulatory molecules in recombinant vectors (25); and (f) the use of cytokines to enhance APC function and/or T-cell function for antitumor immunity (27–32).

As tumor vaccines become more potent (for example, by expression of the TAA via poxviral vectors, the addition of costimulatory molecules and cytokines, and the use of diversified prime and boost regimens), the level of concern rises regarding the balance between antitumor immunity and pathological autoimmunity. A combination of all of the strategies noted above has been shown to greatly amplify antigen-specific immune responses to CEA 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 (5, 33). 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 (5). 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 (5). These mice thus provide a model for peripheral tolerance to a self-TAA. To evaluate the effectiveness, mechanism, and potential coincident autoimmunity of different vaccine regimens directed against a self-TAA, we have used this model. 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 (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 (TRICOM; Ref. 25). Here, the CEA/TRICOM vaccination regimen is compared with regimens using only one costimulatory molecule (CEA/B7-1) or no costimulatory molecules (CEA). The results demonstrate the following: (a) vaccination with CEA/TRICOM vaccines is more efficacious than regimens using CEA/B7-1 or CEA vectors in increasing survival and in the induction of CEA-specific T-cell responses; (b) both CD4+ and CD8+ T-cell responses and NK cells contribute to the antitumor effect; and (c) CEA+ tumor-bearing CEA-Tg mice that were treated with the CEA/TRICOM regimen and cured of tumor showed no evidence of autoimmune pathology or other pathology at 1 year.

These studies demonstrate for the first time that activation of T cells to a level sufficient to mediate therapeutic antitumor immunity to a self-antigen in vivo can occur without the development of adverse autoimmunity or other pathology at 1 year postvaccination. They also demonstrate that, in this model, the multiple use of vectors containing three costimulatory molecules does not induce any evidence of autoimmunity.

MATERIALS AND METHODS

Recombinant Poxviruses. rF viruses were constructed by the insertion of foreign sequences into the BamHIJ region of the genome of the POXVAC-TC (Schering-Plough Corp., Kenilworth, NJ) strain of fowlpox virus as described previously (34). The rF virus designated rF-CEA contains the human CEA gene under the control of the 40k promoter (35). The rF virus designated rF-CEA/B7-1 contains the human CEA gene under the control of the 40k promoter and the murine B7-1 gene under the control of the synthetic early/late promoter (36). The rF virus containing the human CEA gene and the murine B7-1, ICAM-1, and LFA-3 genes (designated rF-CEA/TRICOM) has been described previously (25). The rV virus designated rV-CEA, which contains the human CEA gene under the control of the 40k promoter, has been described previously (37). The rV virus designated rV-CEA/B7-1, which contains the human CEA gene under the control of the 40k promoter and the murine B7-1 gene under the control of the synthetic early/late promoter, has been described previously (38). The rV virus containing the human CEA gene and the murine B7-1, IFN-γ, GM-CSF, and LFA-3 genes (designated rV-CEA/TRICOM) has been described previously (25). The rF virus containing the gene for murine GM-CSF under control of the 40k promoter has been described previously (39). Nonrecombinant wild-type fowlpox virus was designated FP-WT, whereas non-rV virus (Wyeth strain) was designated V-WT. Drs. Dennis Pandozzi, Gail Mazzara, and Linda Gritz of Therion Biologics Corp. (Cambridge, MA) kindly provided all Orthopox viruses as part of an ongoing Collaborative Research and Development Agreement between the National Cancer Institute/NIH and Therion.

Animals/Cells. C57BL/6 mice Tg for human CEA (designated CEA-Tg) were originally 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 have been described previously (33). PCR of DNA from whole blood to detect the CEA gene was used to screen for CEA+ mice as described previously (5). Mice were housed and maintained under pathogen-free conditions in microisolator cages. For experiments, 8–12-week-old 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 (39). Before transplantation to...
mice, the cells were trypsinized, dispersed through a 70-μm cell strainer (Falcon; Becton Dickinson, Franklin Lakes, NJ), and washed twice in HBSS before final suspension in HBSS.

**Tumor Therapy Studies.** CEA-Tg mice were transplanted with 50,000 MC-38-CEA cells to form experimental peripancreatic metastases, as described previously (40). Briefly, the spleens of anesthetized mice were exteriorized by means of a small subcostal incision. Cells were directly injected in 100 μl of HBSS using 26-gauge 5/8-inch needles. Splenectomy was performed approximately 2 min after tumor cell injection by cautery 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 environment (40).

Fourteen days after tumor transplant, mice were vaccinated s.c. once with 1 × 10⁸ pfu of V-WT, rV-TRICOM, rV-CEA, rV-CEA/B7-1, or rV-CEA/TRICOM admixed with recombinant murine GM-CSF (20 μg; PeproTech, Rocky Hill, NJ) and human IL-2 (16,000 IU; Hoffmann-La Roche, Nutley, NJ). Concurrently, GM-CSF (20 μg) was administered at the injection site for the following 3 days. Concurrently, IL-2 (16,000 IU) was administered twice a day for 3 days. This dose of IL-2 is designated low dose (32). Seven days after the primary vaccination, mice were boosted with 1 × 10⁷ pfu of the corresponding fowlpox vector (FP-WT, rF-TRICOM, rF-CEA, rF-CEA/B7-1, or rF-CEA/TRICOM) admixed with 1 × 10⁷ pfu of rF-GM-CSF. IL-2 (16,000 IU) was administered twice a day for 4 days. This booster vaccination regimen was repeated two additional times at 7-day intervals. Mice were monitored weekly for survival. In a duplicate set of mice, certain mice were sacrificed, and tumors were collected when present. These tumors were frozen, sectioned at 5 μm, and stained with the CEA-specific antibody COL-1 (41).

**Lymphoproliferative and Cytokine Release Assays.** In an additional study, CEA-Tg mice were vaccinated in a manner identical to that described above, but they did not receive a tumor transplant. CEA-specific T-cell responses of vaccinated mice were analyzed 3 weeks after the final vaccinations. For consistency in terms of comparison with previous vaccine and vaccine strategy studies (14–16), proliferation was used to measure CD4⁺ T-cell responses, and cytokine production was used to measure CD8⁺ T-cell responses. CEA-specific lymphoproliferation was evaluated as described previously (42). To evaluate CD8⁺ T-cell responses, spleens from 3 animals/group were removed 3 weeks after the last immunization, dispersed into single-cell suspensions, pooled, and cocultivated with 10 μg/ml of the H-2Db-restricted 8-mer peptide CEA526-533 [EAQNNTYLL; referred to henceforth as CEA peptide (5, 38)] for 6 days. Bulk lymphocytes were recovered by centrifugation through a Ficoll-Hypaque gradient. T cells were restimulated with fresh irradiated naive splenocytes and 10 μg/ml of either CEA peptide or VSV-N52-59 peptide [vesicular stomatitis virus; RGYVYQGL; referred to henceforth as VSV-N peptide (43)] for 24 h. Supernatant was collected and analyzed for murine IFN-γ by capture ELISA as described previously (44).

**T-cell Depletion Experiments.** Twelve-week-old CEA-Tg mice were transplanted with experimental metastases on day 0. On day 7, animals were depleted of CD4⁺ T cells, CD8⁺ T cells, NK cells, or both CD4⁺ and CD8⁺ T cells by i.p. injection of anti-CD4 (GK1.5 hybridoma), anti-CD8 (Lyt 2.2 hybridoma), or anti-NK (P136 hybridoma) ascitic fluid. Antibodies (100 μl diluted ascitic fluid/dose) were injected on days 7, 8, 9, 10, and 13 relative to the tumor transplant and then injected every week for the duration of the experiment. These depletion conditions were validated by flow cytometry analysis of peripheral blood using phycoerythrin-conjugated MAb s anti-CD4, anti-CD8, and anti-NK (PharMingen, San Diego, CA); 99% of the relevant cell subset was depleted, whereas all other subsets remained within normal levels. On day 14, mice were vaccinated once with 1 × 10⁸ pfu of rV-CEA/TRICOM admixed with recombinant murine GM-CSF (20 μg) and human IL-2 (16,000 IU). GM-CSF (20 μg) was administered at the injection site for the following 3 days. Concurrently, IL-2 (16,000 IU) was administered twice a day for 3 days. Seven days after the primary vaccination, mice were boosted with 1 × 10⁸ pfu of rF-CEA/TRICOM admixed with 1 × 10⁷ pfu of rF-GM-CSF. IL-2 (16,000 IU) was administered twice a day for 4 days. This booster vaccination regimen was repeated two additional times at 7-day intervals.

**Toxicology.** Twelve-week-old CEA-Tg mice were transplanted with experimental metastases on day 0. On day 14, mice were vaccinated once with 1 × 10⁸ pfu of rV-CEA/TRICOM admixed with recombinant murine GM-CSF (20 μg) and human IL-2 (16,000 IU). GM-CSF (20 μg) was administered at the injection site for the following 3 days. Concurrently, human IL-2 (16,000 IU) was administered twice a day for 3 days. Seven days after the primary vaccination, mice were boosted with 1 × 10⁸ pfu of rF-CEA/TRICOM admixed with 1 × 10⁷ pfu of rF-GM-CSF. IL-2 (16,000 IU) was administered twice a day for 4 days. This booster vaccination regimen was repeated two additional times at 7-day intervals. Mice that were successfully treated for experimental metastases were monitored for 1 year. Control mice consisted of CEA-Tg mice that did not receive tumor and were not vaccinated. The mice in the control group were age-matched to within 4 weeks of each other. Mice were monitored daily for clinical signs such as respiratory distress and mobility and weighed weekly. Urine was collected each day for the final 3 days before sacrifice. At the 1-year time point, mice were sacrificed by Pathology Associates (Frederick, MD). The portions of the study performed by Pathology Associates were conducted by United States Food and Drug Administration Good Laboratory Practice Standards, 21 CFR Part 58. Upon sacrifice, serum was collected for analysis of CEA protein, antibodies to CEA, vaccinia, fowlpox, B7-1, ICAM-1, LFA-3, GM-CSF, as well as for autoantibodies nRNP, histone, scl-70, dsDNA, ssDNA, and circulating immune complexes. Blood samples were sent to Laboratory Corporation of America (Chevy Chase, MD) for clinical chemistry parameters (blood urea nitrogen, creatinine, total protein, albumin, globulin, bilirubin, serum aspartate aminotransferase, serum alanine aminotransferase, and alkaline phosphatase). Urine samples (72-h collection) were sent to Laboratory Corporation of America for urinalysis parameters (specific gravity, pH, protein, glucose, ketones, blood, bilirubin, nitrates, WBC, RBC, and sediment). Pathology was performed by Pathology Associates. Sections of the left lateral lobe of the liver, left lobe of the lung, stomach (including tunica and margo plicatus), small intestine (jejenum), cecum, colon, adrenal glands, bone (femur), bone marrow (fe-
mur), brain, duodenum, esophagus, eyes, gallbladder, gross lesions, heart, ilium, jejunum, kidneys, lymph nodes (mandibular and mesenteric), mammary glands, ovaries, pancreas, parathyroid gland, pituitary gland, salivary gland (mandibular, sublingual, and parotid), sciatic nerve, skeletal muscle, skin (ventral abdomen), spinal cord (thoracolumbar), stomach (foregastrointestinal and glandular), thymus, thyroid gland, trachea, urinary bladder, and uterus were collected into 10% neutral buffered formalin. These tissues were trimmed, embedded in paraffin, sectioned at 5 µm, and stained with H&E. Kidney sections were stained for deposited antigen/antibody complexes. Two board-certified veterinary pathologists (American College of Veterinary Pathologists) independently examined all tissues from all animals.

Circulating serum CEA protein levels were quantified by ELISA (AMDL, Tustin, CA) according to the manufacturer’s instructions. The detection limit for CEA protein was 1 ng/ml. Anti-CEA antibody (IgG) was quantified in the serum of each animal by ELISA as described previously (16). Anti-vaccinia and anti-fowlpox antibodies were quantified by ELISA as described previously (16). Antibody vaccinia and anti-fowlpox antibody titers were reported as the reciprocal of the highest serum dilution yielding a positive signal. Anti-B7-1, anti-ICAM-1, and anti-LFA-3 antibodies were quantified by fluorescence-activated cell-sorting capture assay as described previously (45). Detection limits were 0.49, 7.8, and 15.6 ng/ml, respectively. Anti-GM-CSF antibody was quantitated by ELISA as described previously (19). Antibody levels to nRNP, histone, scl-70 (DNA topoisomerase I), dsDNA, ssDNA, and circulating immune complexes were determined in a qualitative or semiquantitative manner (Alpha Diagnostic International, San Antonio, TX) according to the manufacturer’s instructions.

Statistical Analysis of the Data. Where indicated, the results of tests of significance are reported as Ps and are derived from Student’s t test using a two-tailed distribution. Ps were calculated at 95%. Evaluation of survival patterns in mice bearing peripancreatic tumors was performed by the Kaplan-Meier method, and results were 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 representing ±1 SE are depicted. In some cases, the variation was such that the error bars were obscured by the plot symbol.

RESULTS

Treatment of Tumor-bearing CEA-Tg Mice with CEA/TRICOM Vectors Results in Increased Survival. A tumor therapy study was initiated to determine the therapeutic potency of different vaccine regimens; the CEA/TRICOM vaccine regimen was compared with the CEA/B7-1 or CEA regimens. In these studies, MC-38 murine colon carcinoma cells, which had been transduced with the CEA gene using a retroviral vector, were inoculated intrasplenically into CEA-Tg mice, and mice were splectomized. As shown in previous studies, large peripancreatic metastases develop at 4–5 weeks after tumor transplant (46, 47). This tumor is lethal in 80–100% of mice by 12 weeks after transplant if left untreated (Fig. 1). Mice in all groups received tumor at day 0 (Fig. 1, open arrow). Fourteen days after tumor transplant, vaccination was initiated as indicated (Fig. 1, closed arrows). Six groups of mice were used in these studies. Group 1 (Fig. 1, ) received a primary vaccination of rV-CEA/TRICOM followed by three weekly booster vaccinations with rF-CEA/TRICOM. Group 2 (Fig. 1, ) received a primary vaccination of rV-CEA/TRICOM followed by three weekly booster vaccinations with rF-CEA/B7-1. Group 3 (Fig. 1, ) received a primary vaccination of rV-CEA followed by three weekly booster vaccinations with rF-CEA/B7-1. The antigen control group (group 4; Fig. 1, ) received a primary vaccination of rV-TRICOM (no CEA in vector) followed by three weekly booster vaccinations with rF-TRICOM (no CEA in vector). The vector control group (group 5; Fig. 1, ) received V-WT as the primary vaccination and FP-WT for the subsequent three booster vaccinations. All of the above-mentioned groups received recombinant GM-CSF protein with the prime vaccination and rF-GM-CSF with subsequent boost; all groups also received low-dose systemic IL-2. A control group (group 6; Fig. 1, ) received a primary injection of HBSS (buffer) followed by three weekly administrations of HBSS.
As can be seen in Fig. 1, 60% of the mice in group 1 receiving the recombinant CEA/TRICOM vaccines remained alive and apparently healthy through the 25-week observation period. However, only 25% of the mice that received CEA/B7-1 vaccines (group 2) survived past 16 weeks. Of the mice that received CEA vaccines (group 3), 20% survived. Treatment of mice with TRICOM vectors (group 4) or wild-type vectors (group 5) resulted in 11% and 10% survival, respectively. No mice that were treated with buffer or cytokine only survived past 10 weeks.

The \( P \) for survival between mice receiving no treatment and mice receiving treatment with wild-type control vectors or antigen control vectors was not significant (\( P = 0.498 \) and 0.442, respectively). The \( P \) for the survival of mice receiving CEA/B7-1 vaccines versus buffer was statistically significant (\( P = 0.022 \)). Furthermore, survival of mice receiving the CEA/TRICOM vaccines (group 1) was significant as compared with survival of mice receiving buffer (group 6; \( P = 0.026 \)). The overall trend was that vaccination with CEA/TRICOM vaccines resulted in greater survival than vaccination with CEA/B7-1 or CEA vaccines; however, due to small group size in this one experiment, significance was not achieved (\( P = 0.2331 \), and 0.1004, respectively). This experiment was conducted several times. When data from this experiment were combined with data from other survival experiments for statistical meta-analysis, combined survival data increased the population for the CEA/TRICOM group (group 1; \( n = 82 \)). Using the meta-analysis, survival of mice receiving the CEA/TRICOM vectors (group 1) was significant as compared with survival of mice receiving CEA/B7-1 (group 2; \( P = 0.0494 \)). In addition, the \( P \) for the survival of mice receiving CEA/TRICOM vaccines versus CEA (group 3) was statistically significant (\( P = 0.0077 \)).

As noted, 60% of the mice in group 1 receiving the recombinant CEA/TRICOM vaccines remained alive through the 25-week observation period and demonstrated no evidence of tumor at 10 weeks (Fig. 2B). However, mice receiving CEA/TRICOM vaccines and failing therapy (40%; Fig. 1, F) exhibited extensive tumor formation (Fig. 2A). The livers of both treated and control animals were identical. The tumors from CEA/TRICOM-treated mice that failed therapy (Fig. 2D) expressed levels of CEA similar to those in tumors from untreated mice (Fig. 2C), thereby ruling out the immune selection of antigen escape variants as a cause for vaccine failure.

**Induction of CD4\(^+\) and CD8\(^+\) T-cell Responses Correlates with Survival.** A duplicate set of CEA-Tg mice were vaccinated as described above, except that they were not transplanted with peripancreatic metastases. Twenty-one days after the last vaccination, splenocytes were analyzed for CEA-specific immune responses (Fig. 3). CD4\(^+\) CEA-specific responses (Fig. 3A) observed in the group receiving the CEA/TRICOM vaccines (Fig. 3A, ◆) were significantly greater than those observed in the groups receiving CEA/B7-1 vaccines (\( P = 0.002 \)), CEA vaccines (\( P < 0.001 \)), TRICOM (no CEA) vaccines (\( P < 0.0001 \)), and wild-type control vaccines (\( P = <0.0001 \)).
A.

Immune responses to CEA vaccines in CEA-Tg mice. CEA-Tg mice were divided into six vaccination groups. Group 1 (■) received a rV-CEA/TRICOM prime vaccination followed by three weekly boosts with rF-CEA/TRICOM. Group 2 (○) received a rV-CEA/B7-1 prime vaccination followed by three weekly boosts with rF-CEA/B7-1. Group 3 (△) received a rV-CEA prime vaccination followed by three weekly boosts with rF-CEA. Group 4 (○) received a rV-TRICOM prime vaccination followed by three weekly boosts with rF-TRICOM. Group 5 (△) received a V-WT prime vaccination followed by three weekly boosts with FP-WT. For groups 1–5, all prime vaccinations were administered with recombinant GM-CSF and low-dose IL-2, and all boost vaccinations were admixed with rF-GM-CSF and low-dose IL-2. Group 6 (□) received only HBSS buffer injections. In vitro assays were performed 3 weeks after the last vaccination. Each group contains splenic T cells pooled from three mice. A, lymphoproliferation of splenic T cells in response to CEA protein. Purified splenic T cells were tested for reactivity to CEA protein in an in vitro lymphoproliferative assay using naive splenocytes as APCs and whole CEA protein as antigen. Proliferation in response to the negative control protein ovalbumin (80 µg/ml) is indicated by the open triangle. Proliferation in response to the T-cell mitogen concanavalin A (2.5 µg/ml) is shown in the inset. B, IFN-γ production in response to the 8-mer CEA peptide. Splenic T cells from vaccinated mice were bulk-cultured for 1 week with 10 µg/ml CEA peptide. T cells were purified and restimulated for an additional 24 h with CEA (■) or VSV-N (□) peptide. Culture supernatants were assayed for IFN-γ production by ELISA.

Fig. 3 Immune responses to CEA vaccines in CEA-Tg mice. CEA-Tg mice were divided into six vaccination groups. Group 1 (●) received a rV-CEA/TRICOM prime vaccination followed by three weekly boosts with rF-CEA/TRICOM. Group 2 (○) received a rV-CEA/B7-1 prime vaccination followed by three weekly boosts with rF-CEA/B7-1. Group 3 (△) received a rV-CEA prime vaccination followed by three weekly boosts with rF-CEA. Group 4 (○) received a rV-TRICOM prime vaccination followed by three weekly boosts with rF-TRICOM. Group 5 (△) received a V-WT prime vaccination followed by three weekly boosts with FP-WT. For groups 1–5, all prime vaccinations were administered with recombinant GM-CSF and low-dose IL-2, and all boost vaccinations were admixed with rF-GM-CSF and low-dose IL-2. Group 6 (□) received only HBSS buffer injections. In vitro assays were performed 3 weeks after the last vaccination. Each group contains splenic T cells pooled from three mice. A, lymphoproliferation of splenic T cells in response to CEA protein. Purified splenic T cells were tested for reactivity to CEA protein in an in vitro lymphoproliferative assay using naive splenocytes as APCs and whole CEA protein as antigen. Proliferation in response to the negative control protein ovalbumin (80 µg/ml) is indicated by the open triangle. Proliferation in response to the T-cell mitogen concanavalin A (2.5 µg/ml) is shown in the inset. B, IFN-γ production in response to the 8-mer CEA peptide. Splenic T cells from vaccinated mice were bulk-cultured for 1 week with 10 µg/ml CEA peptide. T cells were purified and restimulated for an additional 24 h with CEA (■) or VSV-N (□) peptide. Culture supernatants were assayed for IFN-γ production by ELISA.

or buffer (P < 0.0001). In addition, vaccination with CEA/B7-1 vaccines was superior to vaccination with CEA vaccines (P = 0.0033). Thus, the generation of antigen-specific CD4+ T-cell responses increased in a statistically significant fashion with the insertion of one and three costimulatory molecules into the vaccine and also correlated with survival (P = 0.022 and 0.026, respectively).

CD8+ T-cell responses were measured using a CEA 8-mer peptide (CEA peptide), and IFN-γ production was measured. A control peptide (VSV-N) was also used. As seen in Fig. 3B, CD8+ CEA-specific T-cell responses could be observed in the CEA-Tg mice receiving the recombinant CEA/TRICOM vaccines (group 1), and these were significantly greater than the responses observed for the groups receiving CEA/B7-1 vaccines (P < 0.0001), CEA vaccines (P < 0.0001), TRICOM vaccines (P < 0.0001), wild-type control vaccines (P = <0.0001), or buffer (P < 0.0001). In addition, vaccination with CEA/B7-1 vaccines produced a superior amount of IFN-γ as compared with CEA vaccines (P = 0.0008). No responses were seen to the control peptide. As seen in Fig. 3B, superior CD8+ responses induced by the CEA/TRICOM vaccine regimen correlated with increased survival (Fig. 1).

CEA/TRICOM-mediated Antitumor Therapy Is Dependent on CD4+ T Cells, CD8+ T Cells, and NK Cells. We next characterized the effector cells involved in the in vivo antitumor therapy after vaccination with CEA/TRICOM vectors. After tumor administration, but before vaccine administration, mice were depleted of CD4+ and/or CD8+ T cells or NK cells by MAbFs. Antibodies were given during and after the vaccination regimen to ensure continued depletion of the relevant T-cell subsets. As seen in Fig. 4, therapy with CEA/TRICOM vectors plus cytokine (Fig. 4, ●) resulted in a marked and significant increase in survival as compared with mice receiving no therapy (Fig. 4, □; P = 0.0017). Both CD4+ and CD8+ T cells were found to contribute to the antitumor therapy induced by this vaccination regimen because tumor-free survival was reduced in those treated animals that were depleted of both CD4+ and CD8+ T cells (P = 0.0073). Depletion of CD4+ T cells alone (Fig. 4, ○), CD8+ T cells alone (Fig. 4, ■), or NK cells alone (Fig. 4, ♦) each had a negative effect on survival.

Analysis of Pathological Effects Coincident with the Positive Tumor Therapeutic Effect Induced by Vaccination with CEA/TRICOM Vectors. Because CEA is a self-antigen expressed in gastrointestinal tissue and, to a lesser extent, in other tissues of CEA-Tg mice, comprehensive studies were conducted to examine the potential pathology coincident to CEA-Tg mice successfully treated for CEA-expressing tumors by the CEA/TRICOM vaccination regimen. Mice underwent tumor therapy as described above and were then monitored for 1 year. Control mice consisted of age-matched CEA-Tg mice that did not receive tumor and were not vaccinated. During the course of the study, no deaths occurred. There were no remarkable clinical observations noted throughout the study in either group. Mice in both the CEA/TRICOM-treated group and the age-matched control group gained weight during the study, and the treatments had no effect on body weights (P = 0.86).

At 1 year, studies were conducted to assess potential toxicities. These included: (a) 9 serological parameters; (b) 11
urinalysis parameters; and (c) 14 immunological parameters. In addition, histopathology was performed on 42 tissues/mouse.

Table 1 summarizes the nine serological parameters measured in the treatment group and the age-matched control group. The two groups were virtually identical in the laboratory parameters measured. The serum values for each mouse within each group fell within the normal range defined for healthy mice. In addition, the mean values for each serum parameter for the CEA/TRICOM treatment group fell within the SDs of the values obtained for the age-matched control mouse group ($P > 0.5$).

Urine was collected from each mouse for the final 3 days before termination of the experiment. Table 1 depicts the values of the 11 urologic parameters measured. Again, the two groups were virtually identical in the laboratory parameters measured. The slightly elevated specific gravity values in both groups were attributed to sample evaporation over the 3-day collection period, thus increasing the apparent solute concentration and saturation. The mean value for specific gravity for the CEA/TRICOM treatment group fell within the SDs of the values obtained for the age-matched control mouse group ($P > 0.5$). Both the CEA/TRICOM treatment group and the age-matched control group had qualitative increases in urine protein (1+), ketones (trace), blood (trace), and bilirubin (1+). However, it should be noted that the laboratory-defined “normal range” was derived from 8–12-week-old mice (Laboratory Corporation of America). Trace increases in blood/bilirubin, protein, and ketones in both groups can be attributed to the age of these groups (15 months).

Table 2 summarizes the 14 serological parameters measured in the CEA/TRICOM treatment group and age-matched control group. Both groups expressed serum CEA levels comparable with normal CEA-Tg mice (5). The mice vaccinated with the CEA/TRICOM regimen developed high-titer IgG antibody levels specific for CEA (1:1640); these results demonstrate that the immune response developed after CEA/TRICOM vaccination was maintained at 1 year of age and 11 months after the final vaccination. Antibodies specific for the poxviral vectors used in the vaccination regimen, vaccinia and fowlpox, were also detected (1:200 and 1:150, respectively). Sera were examined for signs of neo-immune reactivities, namely, reactions against the vaccine costimulatory components murine B7-1, murine ICAM-1, and murine LFA-3 and murine GM-CSF. There were no detectable levels of IgG, IgA, or IgM antibodies against these four proteins present in sera from mice.

Table 1  Mean serum chemistry and urinalysis values in CEA-Tg mice that were cured of tumor with CEA/TRICOM vectors

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal ranges</th>
<th>Treatment group $^a$ (n = 5)</th>
<th>Age-matched control group $^a$ (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum assays</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUN $^b$ (mg/dl)</td>
<td>11–29</td>
<td>24.7 ± 3.2</td>
<td>24.0 ± 6.2</td>
</tr>
<tr>
<td>Creatine (mg/dl)</td>
<td>0.0–0.3</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Total Protein (g/dl)</td>
<td>5.2–6.9</td>
<td>5.7 ± 0.1</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.6–5.1</td>
<td>3.5 ± 0.2</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>Globulin (g/dl)</td>
<td>1.1–2.5</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.1–0.4</td>
<td>0.1 ± 0.0</td>
<td>0.15 ± 0.1</td>
</tr>
<tr>
<td>ALT (IU/liter)</td>
<td>35–121</td>
<td>67 ± 8.6</td>
<td>110 ± 20</td>
</tr>
<tr>
<td>ALP (IU/liter)</td>
<td>91–304</td>
<td>224 ± 41.6</td>
<td>162 ± 16</td>
</tr>
<tr>
<td>AST (IU/liter)</td>
<td>30–300</td>
<td>162 ± 23</td>
<td>274 ± 9</td>
</tr>
<tr>
<td>Urinalysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific gravity</td>
<td>1.001–1.035</td>
<td>1.059 ± 0.13</td>
<td>1.054 ± 0.01</td>
</tr>
<tr>
<td>pH</td>
<td>5–7</td>
<td>6.0 ± 0.0</td>
<td>6.1 ± 0.25</td>
</tr>
<tr>
<td>Protein</td>
<td>Negative</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>Glucose</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Ketones</td>
<td>Negative</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Blood</td>
<td>Negative</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Negative</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>Nitrites</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>WBC</td>
<td>0–2/field</td>
<td>1–2</td>
<td>1–2</td>
</tr>
<tr>
<td>RBC</td>
<td>0–2/field</td>
<td>1–3</td>
<td>1–3</td>
</tr>
<tr>
<td>Sediment</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

$^a$ Twelve-week old CEA-Tg mice bearing 14-day experimental peripancreatic metastases were vaccinated once with $1 \times 10^7$ pfu of rV-CEA/TRICOM admixed with recombinant murine GM-CSF (20 μg) and human IL-2 (16,000 IU). GM-CSF (20 μg) was administered at the injection site for the following 3 days. Concurrently, IL-2 (16,000 IU) was administered twice a day for 3 days. Seven days after the primary vaccination, mice were boosted with $1 \times 10^8$ pfu of rF-CEA/TRICOM admixed with $1 \times 10^7$ pfu of rF-GM-CSF. IL-2 (16,000 IU) was administered twice a day for 4 days. This booster vaccination regimen was repeated two additional times at 7-day intervals. Serum samples were taken after 1 year. Urine was collected for 3 days before sacrifice.

$^b$ Serum samples were taken from age-matched CEA-Tg mice (15 months).

$^c$ BUN, blood urea nitrogen; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase.
Table 2  Serum immunological parameters of CEA-Tg mice that were cured of tumor with CEA/TRICOM vectors

<table>
<thead>
<tr>
<th>Test</th>
<th>Normal</th>
<th>Treatment group&lt;sup&gt;a&lt;/sup&gt; (n = 5)</th>
<th>Age-matched control group&lt;sup&gt;b&lt;/sup&gt; (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA Protein</td>
<td>13–100 ng/ml</td>
<td>37.1 ± 12.1</td>
<td>24.3 ± 11.2</td>
</tr>
<tr>
<td>CEA Ab&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;50</td>
<td>1640 ± 250</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Vaccinia Ab&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Negative</td>
<td>200</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Fowlpox Ab&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Negative</td>
<td>150</td>
<td>&lt;50</td>
</tr>
<tr>
<td>BT-1 Ab&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Negative</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>ICAM-1 Ab&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Negative</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>LFA-3 Ab&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Negative</td>
<td>&lt;100</td>
<td>&lt;100</td>
</tr>
<tr>
<td>GM-CSF Ab&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>nRNP Ab&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Histone Ab&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>ScI-70 Ab&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>dsDNA Ab&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>ssDNA Ab&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Positive&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Positive (2+)</td>
<td>Positive (1+)</td>
</tr>
<tr>
<td>CIC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

<sup>a</sup> Twelve-week-old CEA-Tg mice bearing 14-day experimental peripancreatic metastases were vaccinated once with 1 × 10<sup>8</sup> pfu of rV-CEA/TRICOM admixed with recombinant murine GM-CSF (20 μg) and human IL-2 (16,000 IU). GM-CSF (20 μg) was administered at the injection site for the following 3 days. Concurrently, IL-2 (16,000 IU) was administered twice a day for 3 days. Seven days after the primary vaccination, mice were boosted with 1 × 10<sup>6</sup> pfu of rF-CEA/TRICOM admixed with 1 × 10<sup>7</sup> pfu of rF-GM-CSF. IL-2 (16,000 IU) was administered twice a day for 4 days. This booster vaccination regimen was repeated two additional times at 7-day intervals. Serum samples were taken after 1 year.

<sup>b</sup> Serum samples were taken from age-matched CEA-Tg mice (15 months).

<sup>c</sup> Ab titer reported as the highest reciprocal serum dilution yielding positive signal.

<sup>d</sup> Anti-costimulatory molecule Ab titer reported as the highest reciprocal serum dilution yielding positive signal. Detection limits: anti-B7-1 0.49 ng/ml; anti-ICAM-1 7.8 ng/ml; anti-LFA-3 15.6 ng/ml.

<sup>e</sup> Anti-GM-CSF Ab titers determined by capture ELISA. Detection limit, 3 ng/ml.

<sup>f</sup> ANAs: nSCL-70 (DNA topoisomerase 1); CIC, circulating immune complexes. Results are semiquantitative and are expressed as 0 to 4+.

<sup>g</sup> Normal value for mice of this age.

that received CEA/TRICOM therapy or age-matched control mice. We then examined these samples for levels of ANAs specific for nRNP, histone, topoisomerase-1 (sel-70), dsDNA, ssDNA, or circulating immune complexes. Again, there were no detectable antibody levels present in sera samples from mice that received CEA/TRICOM therapy or age-matched control mice, with the exception of ssDNA, which was present in both the age-matched control group (1+) and the CEA/TRICOM treatment group (2+). This will be discussed below.

At 15 months of age, necropsies were conducted on control CEA-Tg mice and on CEA-Tg mice that were vaccinated and cured of tumor. Tissues were processed through paraffin, rendered to H&E-stained sections, and evaluated for histopathological changes. Table 3 summarizes the histopathological findings noted in the 42 tissues examined for each mouse. Any changes in these two groups of mice were remarkably similar. Both groups exhibited a common lesion of older mice, that of multiple lymphoid aggregates/nodules in multiple organs (diagnosed as lymphocytic infiltrate of, primarily, liver, lung, kidney, and urinary bladder in these animals); this presence of lymphocytic infiltration is consistent with the generalized lymphocytic infiltration reported to occur in aging C57BL/6 mice (48). In both groups, there was minimal to mild lymphocytic depletion of the mandibular and mesenteric lymph nodes. This was noted to probably reflect both the age of the animals and the relatively immunologically sterile environment in which they lived. There were changes in both groups noted in the femoral head of the control animals (compaction and occasional minimal erosion of the articular cartilage). A corresponding change in articular cartilage was seen in only one animal in the treated group. The change was noted to be minimal in intensity and coincident with the advanced age of these mice.

Photomicrographs of tissues expressing the self-antigen CEA (cecum and colon) are depicted in Fig. 5. Cecum tissue from an age-matched control mouse (Fig. 5A) and a CEA/TRICOM-treated mouse (Fig. 5B) was determined to be normal. Colon tissue from an age-matched control mouse depicts normal lymphocytic infiltrate (Fig. 5C) and is indistinguishable from colon tissue obtained from a CEA/TRICOM-treated mouse (Fig. 5D).

Because kidney involvement is frequently seen as a sequela of autoimmune deposition of immune complexes, we examined the kidneys of CEA-Tg mice treated with CEA/TRICOM (Fig. 5F) and age-matched control mice (Fig. 5E). Again, the samples were virtually indistinguishable from one another, with focal minimal lymphocytic infiltrate noted for both groups. Thus, despite the fact that a vigorous immune response (sufficient in intensity to cure mice of CEA-expressing tumor) directed against CEA was mounted in CEA-Tg mice receiving CEA/TRICOM vaccines, no evidence of autoimmunity was observed in such CEA-Tg mice.

**DISCUSSION**

The studies reported here examined the potential long-term pathological and autoimmune consequences of a vaccination regimen of sufficient potency to eradicate tumors expressing a self-antigen. CEA-Tg mice were successfully treated for CEA-expressing tumors by the CEA/TRICOM vaccination regimen and monitored for 1 year; numerous parameters were analyzed: (a) 9 serological parameters (Table 1); (b) 11 urinalysis parameters (Table 1); and (c) 14 immunological parameters (Table 2). In addition, histopathology was performed on 42 tissues (Table 3; Fig. 5). The vaccination regimen produced no significant adverse biological effects in CEA-Tg mice. There were essentially no differences between the treated group and the age-matched control group. Although serology, urinalysis, and histology were not performed at points earlier than 1 year, there were no observed treatment-related effects on mortality, body weight, or clinical signs in vaccinated mice as compared with the control mice throughout the 12-month observation period. It should be pointed out, however, that the sera obtained at sacrifice (1 year) demonstrated that an immune response to CEA was maintained 11 months after the final vaccination.

It should be noted that the model (40) used in the studies reported here involves transplant of tumor cells to the peripancreatic environment by way of intrasplenic injection followed by removal of the spleen after 2 min. Previous studies have shown that if the spleen is left intact after tumor cell administra-
tration, mice will rapidly die of splenic tumor. Thus, the possibility exists that particular immune cell populations, such as suppressor T cells, might have been disproportionately removed during the splenectomy process. Nonetheless, mice vaccinated with control vaccine or unvaccinated mice died rapidly of their tumor (Fig. 1), and the effect of the vaccine was demonstrated to be mediated by both CD4+ and CD8+ T cells (Figs. 3 and 4).

In humans, there is a clear relationship between ANAs and autoimmune disease (49). The biological significance of the presence of ANAs and their correlation with pathology, however, depends on the specific antigen recognized by the ANAs, with the strongest predictor of autoimmune pathology being antibodies specific for dsDNA. No detectable antibody levels were present in mice that received CEA/TRICOM therapy or age-matched control mice specific for dsDNA, nRNP, histone, topoisomerase-1 (scl-70), or circulating immune complexes. There were slightly higher levels of serum antibodies specific for ssDNA of mice that had received the CEA/TRICOM vaccine regimen as compared with the age-matched control mice. However, analysis of naive aged mice clearly showed an age-related ANA response in the C57BL/6 strain of mice, confirming previous reports of increased autoimmune reactivity in C57BL/6 mice as they age (48). In humans and in mice, anti-ssDNA can be present for many years and is not known to be associated with pathological lesions (50). Because normal C57BL/6 mice express increased ANA titers with age (45, 48), this finding was not considered significant.

Several groups have previously examined active-specific immunotherapy-induced antitumor responses in the context of autoimmunity. These studies can be broadly divided into two areas: (a) tumor prevention or protection studies; and (b) tumor therapy studies. In tumor prevention studies, the level of immune response required to reject a transplanted tumor is much lower than that required to eliminate an established tumor (42). Nonetheless, some tumor protection studies using vaccines directed against melanoma antigens tyrosinase-related protein-1 and tyrosinase-related protein-2 resulted in autoimmune vitiligo (6–8, 51, 52). Vitiligo has also been observed in melanoma patients receiving high-dose IL-2 or IL-2 plus peptide vaccine (53).

In a tumor therapy model using Tg mice in which an immunogenic lymphocytic choriomeningitis virus epitope was coexpressed on tumor cells and pancreatic β-islet cells, treatment with peptide-pulsed dendritic cells resulted in the induction of high avidity lymphocyte choriomeningitis virus-specific T cells, and tumor growth was controlled initially. However, the antitumor effect was ultimately accompanied by fatal autoimmune diabetes (9). In contrast, some tumor therapy studies (54, 55) have shown that MUC-1 self-antigen-specific T cells can selectively eradicate tumors in mice without damaging normal tissues expressing the same antigen. However, in all those studies, mice were examined by histopathology of selected tissues at 30 days postvaccination. Heukamp et al. (10) demonstrated that C57BL6 mice vaccinated with a MUC-1 DNA vaccine prime followed by a rV MUC-1 boost developed MUC-1-specific CTLs. These CTLs were adoptively transferred to MUC-1-Tg mice to examine autoimmunity. No mice displayed any detectable signs of immune-mediated tissue destruction (10). Again, however, in these studies, tissues were examined at only 30 days postvaccination, and no biochemical or immunological analyses were performed.

The main distinction between the studies reported here and the above-noted studies is that in those models, vaccination of MUC-1-Tg mice with dendritic cell vaccines or MUC-1 DNA/vaccinia regimen did not develop detectable CTL responses, necessitating the adoptive transfer of CTLs from non-Tg mice to the MUC-1-Tg mice to examine autoimmunity pathway. Here, we examined long-term autoimmune pathology of CEA-Tg mice bearing CEA+ tumors successfully treated 14 days posttumor transplant with CEA/TRICOM vectors. Here we also
demonstrate: (a) the increase in potency of vectors containing three costimulatory molecules (B7-1, ICAM-1, and LFA-3) versus one costimulatory molecule (B7-1) in both antitumor activity (Fig. 1) and the induction of both CD4+ and CD8+ T-cell responses (Fig. 3); and (b) that the use of such vectors is safe.

Active specific immunotherapy has been associated with autoimmunity in certain experimental systems. In a rat model,
severe destructive autoimmune prostatitis was observed as a consequence of vaccination with prostatic steroid-binding protein (56). In a similar model, vaccination with rV prostatic acid phosphatase also mediated tissue-specific prostatitis (57). Administration of antibodies specific for CTLA-4 has been reported to modulate costimulatory molecule function through a negative signal blockade that results in antitumor therapy. However, a large percentage of mice cured of melanoma lesions by anti-CTLA-4 therapy developed vitiligo, starting at the site of vaccination and in most cases progressing to distant locations (58, 59). In addition, in a prostate cancer model, treatment with CTLA-4 resulted in marked prostatitis accompanied by destruction of epithelium (23). These results are in contrast to those reported here, where antitumor therapy is associated with no autoimmune phenomenon. The disparity between the results reported here and those above could be attributable to either the targeted tissue (prostate or melanoma versus colon) or the modality of T-cell stimulation through CTLA-4 blockade versus the use of multiple T-cell costimulatory molecules. It should be stressed that the multiple costimulatory molecules used here (B7-1, ICAM-1, and LFA-3) are normally expressed on professional APCs such as dendritic cells with no induction of autoimmunity. Therefore, the use of CEA/TRICOM vectors to create a temporary costimulatory hot spot for T-cell induction would not be expected to be associated with autoimmune sequela.

How is it possible to selectively target tumor tissue while sparing normal tissues expressing the same self-antigen? Several hypotheses have been suggested, including immune cell access, antigen density (overexpression of the TAA expressed on tumor as compared with normal tissue (60, 61)), and, as discussed above, low avidity of tumor-reactive T cells (11, 60, 62, 63). These hypotheses could apply to the model system described here: (a) polar CEA expression on normal tissues in CEA-Tg mice is restricted to the luminal surface of the gut, whereas the peripancreatic tumors expressing CEA express the antigen in a nonpolar manner (64, 65); (b) with regard to antigen density, CEA is overexpressed on tumor cells as compared with normal mouse tissue (5); (c) CEA-reactive T cells that have escaped the thymus during ontogeny may have a low avidity for this self-epitope (5); and (d) the self-antigen-reactive T cells might be held in check by CD4+/CD25+ suppressor T cells (66). Finally, it has been suggested by Gilboa et al. (11) that the immune response to tumors will remain self-contained because tumors are unable to channel antigens to the professional APC system that is needed to activate and reactivate memory T cells. Thus, tumor immunity and autoimmunity would not become a “runaway” response, and it should be possible to control the development or severity of any autoimmune reactions simply by stopping the vaccination (and therefore terminating the immune response; Ref. 11). Alternatively, autoimmunity may be able to be controlled by immunosuppressive drugs. Nonetheless, the studies reported here demonstrate that one can use potent vaccines containing transgenes for multiple costimulatory molecules and a self-TAA and obtain therapeutic responses of established tumors in the absence of autoimmunity.

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James W. Hodge, Douglas W. Grosenbach, Wilhelmina M. Aarts, et al.


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