Vaccine-based Therapy Directed against Carcinoembryonic Antigen Demonstrates Antitumor Activity on Spontaneous Intestinal Tumors in the Absence of Autoimmunity

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ABSTRACT

By virtue of its tissue-specific expression, carcinoembryonic antigen (CEA) is an important self, tumor-associated antigen, which is expressed by different human adenocarcinomas and also serves as a target for active-specific immunotherapy. Similar to humans, CEA expression in mice transgenic for the human CEA gene (CEA.Tg) occurs predominantly along the gastrointestinal tract. CEA.Tg mice were crossed with mice bearing a mutation in the Apc gene (MIN mice), and the CEA.Tg/MIN progeny developed multiple intestinal neoplasms, which overexpress CEA to levels that are reminiscent of those reported for tubulovillous intestinal adenomas from patients. CEA.Tg/MIN mice were vaccinated with an aggressive diversified prime/boost vaccine regimen: (a) a primary vaccine consisting of recombinant vaccinia virus-expressing CEA and a triad of costimulatory molecules (TRICOM); B7.1, ICAM-1, and LFA-3 (rV-CEA-TRICOM); and (b) a booster vaccine using CEA-TRICOM in a recombinant avipox virus (fowlpox) virus (r-F-CEA-TRICOM). Granulocyte/macrophage colony-stimulating factor was administered as a biological adjuvant with all vaccinations, either as a recombinant protein (with rV-CEA-TRICOM) or as a recombinant avipox virus (with r-F-CEA-TRICOM). That vaccine regimen generated strong CEA-specific host immune responses in CEA.Tg/MIN mice, which resulted in (a) a delayed onset of adult anemia and weight loss, (b) a significant reduction in the number of intestinal tumors, and (c) improved overall survival. No evidence of autoimmunity directed against normal tissues expressing CEA was observed in mice in which the CEA-based vaccine significantly reduced intestinal tumor load. The CEA.Tg/MIN mice present a clinically relevant model in which different CEA-based vaccine strategies can be tested on the spontaneous onset of intestinal tumorigenesis.

INTRODUCTION

CEA, a Mr 180,000–200,000 oncofetal antigen, is a member of the Ig superfamily and expressed on a high percentage of adenocarcinomas, particularly those of the colon, pancreas, breast, lung, rectum, and stomach (1, 2). Because of its limited normal tissue expression and its overexpression on carcinomas, CEA is also considered a self, tumor-associated antigen and a target for passive (3) and active (4–10) immunotherapy (4–10). Recent clinical data have established that CEA is a target for eliciting immune responses in CEA.Tg/MIN mice, which resulted in (a) a delayed onset of adult anemia and weight loss, (b) a significant reduction in the number of intestinal tumors, and (c) improved overall survival. No evidence of autoimmunity directed against normal tissues expressing CEA was observed in mice in which the CEA-based vaccine significantly reduced intestinal tumor load. The CEA.Tg/MIN mice present a clinically relevant model in which different CEA-based vaccine strategies can be tested on the spontaneous onset of intestinal tumorigenesis.

MATERIALS AND METHODS

Mice. Mice expressing the gene for human CEA (CEA.Tg, Line 2682, C57BL/6 (H-2b), heterozygous) were obtained from John Thompson (University of Freiburg, Freiburg, Germany). MIN (C57BL/6-ApcMIN–/–) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Both colonies were maintained with continuous backcrossing with C57BL/6 mice. Mice with spontaneous arising CEA-expressing intestinal tumors were derived by breeding female CEA.Tg with male MIN mice. All mice entered onto this study were the offspring of the CEA.Tg x MIN cross and maintained in microisolator cages and fed Purina Rodent Certified Chow 5002 ad libitum. Animal care was in compliance with recommendations of the Guide for Care and use of Laboratory Animals, National Research Council.

Genotyping. Fecal and blood samples were taken from the CEA.Tg x MIN F1 offspring at weaning. Mice carrying the CEA transgene were identified by the presence of fecal CEA protein detected using a solid-phase, double-determinant, anti-CEA ELISA kit (AMDL, Inc., Tuscan, CA; Ref. 15), and addition to CEA expression in normal tissues, CEA.Tg mice used in this study have high serum levels of CEA that presents additional peripheral tolerance to the host immune system (14). Yet, tolerance to CEA has been overcome by vaccinating CEA.Tg mice with recombinant orthopox or avipox-CEA viruses, murine fibroblasts expressing CEA, and an oral CEA-based DNA vaccine, as shown by the generation of anti-CEA Ig antibodies, Ig class switching, Th1 type CEA-specific CD4+ responses, and CD8-dependent cytotoxicity (14–18). Besides generating CEA-specific host immunity, CEA-based vaccines have elicited antitumor immunity against CEA-expressing tumors (14–18). The use of transplanted CEA-expressing tumor cells in those studies presents some important limitations, including the retroviral insertion of CEA without associated regulatory elements into cells that normally do not express the gene and the growth of those tumors cells at ectopic sites (s.c., lung, liver) not authentic to colorectal cancer. From an immunological perspective, the rapid growth rate of the transplanted tumors not only fails to mimic the growth characteristics of carcinomas but also requires a shortened interval between cancer vaccine administrations, which probably does not permit optimal host immunity.

In this study, CEA.Tg mice were bred with ApcMIN (MIN) mice that are heterozygous for a mutant allele of the mouse homologue of the human APC gene (19). MIN is a fully penetrant, autosomal dominant, nonsense mutation (codon 850) believed to initiate tumorigenesis in utero and results in multiple spontaneous intestinal neoplasms in adult mice (19, 20). Mice (CEA.Tg/MIN) carrying both the MIN and human CEA genes develop numerous intestinal neoplasms with strong CEA expression in all tumor cells, as well as CEA expression in normal GI tissues (21, 22). An immunotherapeutic protocol, consisting of recombinant poxviruses expressing CEA and TRICOM (B7.1, ICAM-1, and LFA-3) (23), combined with GM-CSF, induced strong anti-CEA host immune responses that significantly suppressed intestinal tumor load and improved long-term survival of CEA.Tg/MIN mice. CEA.Tg/MIN mice can serve as an important preclinical model for the evaluation of cancer vaccines alone or in combination with other anticancer therapeutic modalities.
Materials and Methods. For peptide extraction, the MIN mutation, and those mice that were designated CEA.Tg/MIN. Mice expressing MIN, but not CEA, were designated MIN and those expressing CEA, but not MIN mutation, were designated CEA.Tg. Mice that were negative for both CEA and MIN were designated C57BL/6. Genotypes of all mice were rechecked at the completion of the study.

Vaccines, Adjuvants, and Injection Schema. Details for the construction and production of the recombinant vaccinia (23) and avipox (fowlpox) viruses (23, 25) have been published. Vaccines are referred to as either CEA-based or non-CEA-based vaccines. The CEA-based vaccines were comprised of (a) a recombinant vaccinia virus or a (b) recombinant avipox (fowlpox) virus containing genes encoding human CEA and three murine costimulatory molecules B7.1, ICAM-1 and LFA-3 and designated either rV-CEA-TRICOM or rF-CEA-TRICOM. The non-CEA-based vaccines are the same recombinant vaccinia and avipox viruses expressing the TRICOM but not the human CEA gene (designated either rV-TRICOM or rF-TRICOM). A description of the construction of the recombinant avipox (fowlpox) virus expressing murine GM-CSF (rF-GM-CSF) has been reported (15). The parental virus for the generation of recombinant fowlpox-GM-CSF was plaque purified from a tissue culture-adapted vaccine strain of fowlpox virus. rF-GM-CSF was constructed via homologous recombination in vivo between the parental fowlpox DNA and a plasmid vector that contains the murine GM-CSF gene.

All vaccines were given s.c. in 100 μl of HBSS at the base of the tail. Recombinant vaccinia-based vaccines, rV-CEA-TRICOM or rV-TRICOM, were the primary vaccines and administered at a dose of 10^7 pfu in combination with 20 μg of rGM-CSF (PeproTech, Inc., Rock Hill, NJ). Recombinant GM-CSF protein was then injected for an additional 3 consecutive days at the vaccination site. Booster vaccinations were also administered s.c. in 100 μl containing 10^7 pfu of either rF-CEA-TRICOM or rF-TRICOM combined with 10^7 pfu of rF-GM-CSF. A group of mice received injections of the vehicle (HBSS) alone and were designated vehicle control.

Intestinal Extract Preparation and Solid-Phase RIAs. Three to five mice from each group listed in Table 1 were sacrificed at 150–160 days of age. The entire intestine was removed and its contents flushed with cold DPBS. For the CEA.Tg and C57BL/6 mice, 3–5 cm sections were isolated from the proximal/distal jejunum and ileum. For the CEA.Tg/MIN and MIN mice, neoplastic lesions (n = 20–30) were microisolated using a dissecting microscope from those same intestinal areas. Extracts were prepared and the protein concentrations determined as described previously (26). Forty μg of extract protein were dried to each well of a 96-well plate, and the binding of 125I-COL-1 and an irrelevant, isotype-matched antibody, BL3, was detected with horseradish peroxidase-conjugated goat antimouse IgG (Kirkegaard & Perry Labs., Inc., Gaithersburg, MD) using an ELISA microplate autoreader at A405nm. Triplicates of positive and negative controls and serum samples were run for all assays.

Proliferation Assay. Complete details of the T-cell proliferation assay have been described previously (15). Splenocytes were enriched for T cells that were incubated in flat-bottomed, 96-well plates at a cell density of 1.0–1.5 x 10^6 cells/well with 5 x 10^5 irradiated (2000 rad) syngeneic CEA.Tg mouse splenocytes containing 50–312.5 μg/ml CEA or ovalbumin. Proliferation was measured after 5 days of incubation at 37°C by adding [3H]thymidine (1 μCi/well; Amersham Corp., Chicago, IL) to the wells 18 h before harvesting. Cells were harvested and counted by liquid scintillation spectrometry as described previously (15).

Cytokine Production Assays. Splenic T cells from CEA.Tg/MIN mice were isolated and grown in flat-bottomed, 96-well plates at a cell density of 2 x 10^5 cells/well, 5 x 10^5 irradiated (2000 rad) syngeneic CEA.Tg mouse splenocytes/well and 50 μg CEA/ml. Supernatants from designated wells for each treatment group were harvested after 48 h, and IFN-γ levels were measured using the appropriate ELISA assay (Endogen, Inc., Cambridge, MA).

Table 1  CEA expression levels in normal/neoplastic intestinal tissues and sera

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GI tissue</th>
<th>RIAa (cpm 125I-COL-1 bound/40 μg protein)</th>
<th>EIAb (μg CEA/mg protein)</th>
<th>IHCc</th>
<th>Serum CEAd (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA.Tg/MIN</td>
<td>Neoplastic</td>
<td>44,400 ± 2,150</td>
<td>3.27-4.45</td>
<td>+ +</td>
<td>42.2 ± 7.6</td>
</tr>
<tr>
<td>CEA.Tg</td>
<td>Normal</td>
<td>12,160 ± 990</td>
<td>1.35-1.80</td>
<td>+</td>
<td>60.8 ± 14.9</td>
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<tr>
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<td>Neoplastic</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>Normal</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
<td></td>
</tr>
</tbody>
</table>

*Mice from each group (3–5/group) were sacrificed at 150 days of age, and normal and neoplastic intestinal tissues were removed and protein extracts prepared as outlined in “Materials and Methods.” Forty μg of extract protein were dried to each well of a 96-well plate, and the binding of 125I-COL-1 and an irrelevant, isotype-matched antibody, BL3, was performed as a solid-phase RIA. Data represent the mean ± SE of the total 125I-COL-1 (â 125I)BL3 for each group of mice. NEG, <500 cpm 125I-COL-1 bound.

b CEA levels were measured in the same protein extracts (diluted to 1.0 mg protein/ml) using the anti-CEA ELISA kit (AMDL, Inc.). Data represent the range of CEA levels for 3–5 individual mice/group. NEG, <5 ng CEA/mg protein.

c IHC staining intensity of COL-1 in intestinal tumors of CEA.Tg/MIN and normal intestines of CEA.Tg mice. For each slide, 3–5 different fields were scored independently by a board-certified pathologist. Scoring was based on either the absence of staining, NEG, or relative staining intensities: +, weak, pale brown; + +, strong, dark brown immunoprecipitate.

d Sera CEA levels were measured as described in the “Materials and Methods.” Data represent the mean ± SE of 5–8 mice/group. NEG, <5 ng CEA/ml serum.

In each assay, cpm from wells to which no antibody or 125I-BL3 (400–600 cpm) was subtracted from the cpm from the wells that received 125I-COL-1. CEA levels were also measured in the same protein extracts using the anti-CEA ELISA kit (AMDL, Inc.) according to the manufacturer’s instructions. Internal low and high standards for CEA were included in all assays.

Serum CEA and Anti-CEA Antibody Responses. CEA antibodies were measured by ELISA as described previously (14). Microtiter plates were sensitized overnight at 4°C with 100 ng/well CEA (International Enzymes, Fallbrook, CA) or ovalbumin (Sigma Chemicals, St. Louis, MO). Wells were blocked with DPBS containing 5% BSA, followed by a 1-h incubation of diluted mouse serum (1:10–1:3,125). Antibodies bound to the wells were detected with horseradish peroxidase-conjugated goat antimouse IgG (Kirkegaard & Perry Labs., Inc., Gaithersburg, MD) using an ELISA microplate autoreader at A405nm. Triplicates of positive and negative controls and serum samples were run for all assays.
significant. Meier test. All test. Significance differences in overall survival were evaluated using the Kaplan-
tumor burden.

Any mouse whose weight fell for 4 consecutive weeks and whose hematocrit
of age, and booster vaccines were given monthly for the duration of the study.
that were given the CEA-based vaccine. Mice were genotyped immediately
P
s two-tailed
P
0.05 was considered

Fig. 1. Number of tumors along the GI tract of MIN mice that were either CEA.Tg (■) or CEA negative (●). Data are the mean ± SE. 5 mice/group.

RESULTS
CEA.Tg/MIN Experimental Model. Initial studies were designed to determine the approximate levels of CEA expression in normal versus neoplastic intestinal tissue. Normal intestinal tissue was isolated from CEA.Tg and C57BL/6 mice, whereas neoplastic intestinal tissue (predominately adenomas) was taken from CEA.Tg/MIN and MIN mice as outlined in the “Materials and Methods.” CEA levels in protein extracts, as measured using an anti-CEA monoclonal antibody (COL-1) in a direct-binding, solid-phase RIA and CEA EIA, were ~3-fold higher in the neoplastic tissue when compared with levels in extracts from normal intestines (Table 1). Higher CEA expression levels in the intestinal neoplastic tissues were also observed with IHC staining. Both CEA.Tg/MIN and CEA.Tg mice had measurable serum CEA levels, and the presence of intestinal tumors in the CEA.Tg/MIN mice was not associated with increased serum CEA levels. Comparison of both the number and location of intestinal and colonic tumors found in MIN mice were approximately the same whether those mice were either CEA.Tg or CEA negative (Fig. 1).

Vaccine Effects on Body Weight and Hematocrit Levels of CEA.Tg/MIN and MIN Mice. CEA.Tg/MIN or MIN (CEA-negative) mice received the CEA-based, the non-CEA-based vaccine, or the vehicle control. As explained in the “Materials and Methods,” the CEA-based vaccine was comprised of rV-CEA-TRICOM combined with rGM-CSF followed by monthly boosts with rF-CEA-TRICOM combined with rF-GM-CSF, whereas the non-CEA-based vaccine was rV-TRICOM combined with rGM-CSF followed by monthly boosts with rF-TRICOM combined with rF-GM-CSF. Vehicle control mice received injections of HBSS. CEA.Tg/MIN mice, like MIN mice (19, 20), developed adult-onset anemia accompanied by severe, progressive weight loss, overt changes that are linked with the formation of intestinal tumors. Individual mouse body weights from five groups of mice were measured weekly, and hematocrit levels were determined every 4–6 weeks. As shown in Table 2, the average total weight gain for CEA.Tg/MIN mice that received either the non-CEA-based vaccine or the vehicle control was similar to that of MIN mice given the CEA-based vaccine or vehicle control. Mice in all four control groups achieved their maximum weight before 120 days of age, usually gaining 5–6 g. In addition, by 120 days of age, the mice in those four groups were either anemic or borderline anemic as indicated by their hematocrit values (≤36; Table 2). In contrast, CEA.Tg/MIN mice that received the CEA-based vaccine gained weight and by 150 days of age, their overall average weight gain was 10.1 ± 1.6 g; hematocrit levels remained in the normal range, suggesting that vaccine administration helped maintain normal health status of those mice (Table 2).

Comparative Anti-CEA Immunity Generated in CEA.Tg/MIN and Other Mice. It was of interest to examine whether the weight gain and normal hematocrits of the CEA.Tg/MIN mice might be associated with the presence of CEA-specific host immunity. After receiving the primary vaccination followed by 3 monthly booster vaccinations of either the CEA-based or non-CEA-based vaccine, groups of CEA.Tg/MIN mice were evaluated for the presence of anti-CEA humoral and cellular immune responses. CEA.Tg/MIN mice vaccinated with the CEA-based vaccine had developed strong anti-CEA serum Ig levels (Fig. 2A) and CEA-specific CD4 T-cell

![Image](cancerres.aacrjournals.org)

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proliferative responses (Fig. 2B). Furthermore, CD8 T-cell responses after vaccination using the CEA-based vaccines were also present as indicated by CEA-peptide-specific IFN-γ production (Fig. 2C) and lysis of peptide-pulsed targets (Fig. 2D). No measurable CEA-specific immune responses were detected in CEA.Tg/MIN that were vaccinated with the non-CEA-based vaccine or those that received vehicle control. CEA-specific humoral, CD4 and CD8 cellular responses were also found in MIN that were vaccinated with the CEA-based vaccine (data not shown).

Vaccine Effects on Tumor Formation and Overall Survival. Total number of GI tumors in CEA.Tg/MIN mice that were vaccinated with the CEA-based vaccine, non-CEA-based vaccine, or the vehicle control were determined at ~150 days of age (Fig. 3). The average number of tumors found in either CEA.Tg/MIN mice treated with the vehicle control or the non-CEA-based vaccine was 36.7 ± 6.2 and 46.8 ± 6.2, respectively. There was a significant reduction in the average number of tumors (22.4 ± 6.8) found in CEA.Tg/MIN mice that received the CEA-based vaccine when compared with either control group (Fig. 3).

In a separate long-term survival study (Fig. 4), CEA.Tg/MIN mice were vaccinated with the CEA-based vaccine, non-CEA-based vaccine, or the vehicle control, whereas a group of MIN mice (CEA-negative littermates) received the CEA-based vaccine. All mice in the three control groups, CEA.Tg/MIN mice given either the non-CEA-

### Table 2. Effect of CEA-based vaccine on weight gain and hematocrit levels

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Vaccine</th>
<th>No. of mice</th>
<th>Δ Body weight (g)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>90 days</td>
<td>120 days</td>
</tr>
<tr>
<td>CEA.Tg/MIN</td>
<td>CEA-based</td>
<td>12</td>
<td>10.1 ± 1.6 b</td>
<td>46.9 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>Non-CEA-based</td>
<td>10</td>
<td>6.0 ± 1.1</td>
<td>39.7 ± 5.9</td>
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<tr>
<td></td>
<td>None</td>
<td>12</td>
<td>5.8 ± 0.9</td>
<td>42.9 ± 2.8</td>
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<tr>
<td>MIN</td>
<td>CEA-based</td>
<td>13</td>
<td>5.5 ± 0.7</td>
<td>43.3 ± 2.1</td>
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<tr>
<td></td>
<td>None</td>
<td>12</td>
<td>5.1 ± 0.4</td>
<td>44.1 ± 2.7</td>
</tr>
</tbody>
</table>

* Maximum amount of gram body weight gained. Mean ± SE.

b P ≤ 0.05 (versus CEA.Tg/MIN mice given either the non-CEA-based vaccine or the vehicle control, and MIN mice that received either the CEA-based vaccine or the vehicle control).
based vaccine or vehicle control, and the MIN mice vaccinated with the CEA-based vaccine, were sacrificed by 25–27 weeks because of progressive weight loss and anemia. For comparison, at 27 weeks of age, 80% of the CEA.Tg/MIN mice that received the CEA-based vaccine were alive with stable body weights and normal hematocrits. At 40 weeks, 50% of those mice remained alive; however, by week 49, all mice were anemic with progressive weight loss that required sacrifice. Macroscopic examination revealed numerous neoplastic lesions.

**Histopathology, Hematology, and IHC.** Spleen, pancreas, lung, liver, and kidneys from individual CEA.Tg/MIN mice that received either the CEA-based vaccine, non-CEA-based vaccine, or the vehicle control were examined for gross and microscopic lesions (Table 3). In all three groups, spleens were enlarged and most other organs were pale when taken from mice that had multiple intestinal proliferative lesions. Histopathological analyses revealed that the enlarged spleens were attributable to increased extramedullary hematopoiesis, and the paleness of other organs seemed associated with severe anemia because no other pathology was found (Table 3). Sporadic hydronephrosis was found in all three groups of mice. Serum samples from individual mice were analyzed for the presence of anti-DNA and ANA titers, and no changes were observed in any of the three groups of CEA.Tg/MIN mice (Table 3).

**Intestinal tissues from CEA.Tg/MIN mice treated with the CEA-**

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**Table 3** Histopathology and serum anti-DNA and ANA titers of CEA.Tg/MIN mice

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Mouse no.</th>
<th>Vaccine (n)</th>
<th>S</th>
<th>P</th>
<th>LU</th>
<th>L</th>
<th>K</th>
<th>Anti-DNA</th>
<th>ANA</th>
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<tbody>
<tr>
<td>CEA-based</td>
<td>1</td>
<td>5</td>
<td>EH</td>
<td>N</td>
<td>N</td>
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<td>N</td>
<td>1.6</td>
<td>2</td>
</tr>
</tbody>
</table>

*a Mice were sacrificed between 150–175 days of age.
*b Tissues examined for histopathology: S, spleen; P, pancreas; LU, lungs; L, liver; K, kidneys.
*c Anti-DNA titers were measured using sodium sulfate precipitates of undiluted serum samples in a RIA. Titers > 2.5 IU/ml were considered positive.
*d An ELISA test was used to measure antinuclear antibody using two pools of nuclear antigens: pool A consisted of single-stranded DNA, SSA, SSB, and Jo-1; pool B consisted of double-stranded DNA, ribonucleoprotein, histones, samarium, and scl-70. Results were scored as to the overall reactivity: 0, negative; 1–4, weakly, moderately, strong, very strongly reactive.
*e Histopathological findings: N, normal; EH, extramedullary hematopoiesis; HN, hydronephrosis.

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Fig. 5. H&E (A, C, E, and G) and COL-1 immunohistochemical staining (B, D, F, and H) of intestinal tissues from CEA.Tg/MIN mice that received the CEA-based vaccine. A and C are examples of adenomas in the small intestine (jejunum) from CEA.Tg/MIN mice in which the CEA-based vaccine did not reduce adenoma formation. A and C, adenoma (arrows) with compression of adjacent villi (v; ×10X). B, CEA expression in the adenoma illustrated in A (×10). D, strong CEA expression in adenoma illustrated in C (×10). E, cells in the adenoma illustrated in C are basophilic with hyperchromatic, piled-up nuclei with scattered mitotic figures (arrows; ×20). F, strong CEA expression in tumor cells (×20). G, normal gut architecture in a CEA.Tg/MIN mice in which the CEA-based vaccine prevented tumor formation (×25). H, crypts in the normal intestine shown in G express CEA (arrow; ×40).
based vaccine were analyzed histopathologically (H&E staining) and by IHC for CEA expression using anti-CEA monoclonal antibody COL-1 (Fig. 5). H&E staining revealed typical intestinal adenomas (Fig. 5, A and C) from CEA.Tg/MIN mice in which the CEA-based vaccines did not reduce tumor load. Fig. 5E illustrates basophilic cells with hyperchromatic, piled-up nuclei and the presence of mitotic figures within an adenoma. Overexpression of CEA as evidenced by relative COL-1 staining intensity was found in adenomas (Figs. 5, B and D) when compared with adjacent normal epithelial. Fig. 5F is a higher magnification of CEA expression in an intestinal adenoma from a CEA.Tg/MIN mouse in which the CEA-based vaccine did not suppress adenoma formation. Histopathological analyses of the intestines from CEA.Tg/MIN mice in which the CEA-based vaccine suppressed adenoma formation revealed normal intestinal architecture (Fig. 5G) and CEA expression in intestinal crypts (Fig. 5H).

DISCUSSION

MIN mice carry a germ-line mutation of the murine Apc gene, which results in the formation of multiple intestinal adenomas (19, 20). In humans, a homologous germ-line mutation in the tumor suppressor gene, adenomatous polyposis coli (APC), predisposes individuals to an inherited form of colon cancer, familial adenomatous polyposis, characterized by the early development of multiple colorectal adenomas, some of which can subsequently form carcinomas (31). Somatic mutations of the APC gene are found in the early stages of ~85–90% of sporadic colorectal cancers (31, 32). In this study, MIN mice bred with mice carrying the human CEA gene result in offspring that spontaneously develop multiple intestinal tumors that overexpress CEA. Previous reports from this laboratory have shown the ability to generate (a) host immune responses to CEA, a self, tumor antigen, and (b) antitumor immunity against transplantable CEA-expressing tumors in the CEA.Tg mice vaccinated with different vaccine regimens (14, 15). Introduction of the MIN genotype presents an experimental murine model that (a) develops spontaneous intestinal neoplasms expressing CEA in an authentic tissue site and (b) provides a 4–5 month life span during which cancer vaccines can be administered at intervals that better mimic those being used in clinical studies.

Besides its restricted expression in normal tissues, overexpression of CEA in neoplastic tissues is regarded as an important property that allows CEA to be a target for immunotherapy (1, 2). CEA expression levels have been reported to be ~2–6-fold higher in tubulovillous adenomas of patients when compared in protein extracts from histologically normal mucosa from healthy donors (33). In agreement with previous studies (21, 22), CEA expression was ~3-fold higher in the intestinal tumors of CEA.Tg/MIN mice when compared with normal intestinal samples from CEA.Tg mice. Not only does this overexpression of CEA offer an opportunity to be exploited by the host immune system, the similarities between humans and the CEA.Tg/MIN mice provides additional evidence for the use of this experimental model in preclinical cancer vaccine studies.

Recombinant orthopox vectors, both vaccinia and avipox, have been preferred candidates for cancer vaccines because numerous genes can be inserted and the vector-driven immunogen elicits strong immune responses against weak immunogens such as CEA (34, 35). Diversified prime/boost vaccine regimens in this study used two vectors: (a) a replication competent, recombinant vaccinia-CEA-TRICOM virus that elicits a strong immune response to CEA but whose repeated use is limited because of antivaccinia host immunity; and (b) a nonreplicating, recombinant avipox-CEA-TRICOM virus that can be administered multiple times as a boost. The diversified prime/boost vaccine protocol has been shown to be superior to administering multiple injections of the same recombinant vaccine (36). Insertion of TRICOM into both the recombinant vaccinia and avipox vaccines results in a more vigorous anti-CEA immune response as well as antitumor immunity (23, 25). GM-CSF was included as a biological adjuvant because of its ability to increase antigen-presenting cell infiltration at the vaccination site (37) and regional lymph nodes (15), resulting in a more vigorous T-cell response to CEA (15). Moreover, recent evidence demonstrates that GM-CSF elicits optimal adjuvant effects when delivered as a recombinant avipox virus.4

CEA.Tg/MIN mice present a formidable challenge for the immune system to generate not only anti-CEA host immunity but also to affect intestinal tumor formation. From the MIN standpoint, tumorigenesis is believed to initiate in utero (38), followed by aberrant crypt foci formation (ACFMin, 38) at 2 weeks of age, and the large tumor burden as a result of numerous intestinal tumors. CEA expression in normal tissues and its presence in circulating serum also presents a considerable degree of peripheral tolerance for the immune system to overcome. Therefore, an aggressive vaccination schema, primary vaccination by ages 30–35 days with monthly booster vaccinations, was adopted. The first indications that the CEA-based vaccine was eliciting favorable results was the health status of the CEA.Tg/MIN mice. CEA.Tg/MIN mice, like MIN mice, develop adult-onset anemia (hematocrit values < 35) commensurate with progressive weight loss. Anemia and progressive weight loss typically appeared before 120 days of age in the CEA.Tg/MIN that were treated with the vehicle control or vaccinated with the non-CEA-based vaccine. Overall weight gain in those mice as well as MIN mice rarely exceeded 5–6 g, whereas CEA.Tg/MIN mice vaccinated with the CEA-based vaccine maintained normal hematocrit levels and often gained >10 gms of body weight. When analyzed at necropsy, those CEA.Tg/MIN mice that received the CEA-based vaccine had a significant reduction in the number of intestinal tumors when compared with CEA.Tg/MIN mice that received the non-CEA-based vaccine (P < 0.01) or vehicle control (P < 0.05). Of the 13 CEA.Tg/MIN mice that received the CEA-based vaccine, 5 had dramatic responses (0–3 tumors), 4 had partial responses (4–25 tumors), and the remaining 4 had no response. The administration of the vaccine devoid of the CEA transgene did not suppress tumor formation, thus providing a compelling argument that the generation of anti-CEA host immunity was, indeed, involved in tumor formation. Additional study is needed to elucidate the exact mechanism as well as those events that result in incomplete suppression of tumor formation and/or escape from immune regulation. The absence of any antitumor effects in some mice could be attributable to the inability of the vaccine to induce sufficient antitumor immunity and/or the development of tumor escape mechanisms. Tumor escape can occur because of tumor-related events (i.e., loss/reduction of expression of major histocompatibility antigens, transforming growth factor β production, Fas-FasL interactions) and/or changes within the immune system (i.e., down-regulation of T cell ζ-chain, generation of T suppressor cells). The present findings argue that CEA loss variants do not explain why some CEA.Tg/MIN mice were unresponsive to the CEA-based vaccine (i.e., no reduction in tumor load) because IHC staining revealed strong CEA expression in those tumors (Fig. 5, B, D, and F). Nonetheless, the CEA.Tg/MIN mice present an excellent model to investigate those other phenomena of immune evasion.

Vaccines that target tissue-specific, self-antigens in mouse models are capable of activating autoreactive T cells that elicit autoimmune pathology (39–42). Other animal studies have provided ample evi-

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dence that vaccination protocols can generate substantial antitumor immunity with little, or no, autoimmunity (43, 44). One of the more intriguing issues of this and previous studies (14, 15, 25) has been the ability of the orthopox-based vaccines to induce significant antitumor host immunity with little, if any, autoimmunity directed against normal CEA-expressing tissues in CEA.Tg mice. Histopathology and IHC staining of normal intestinal tissues revealed no significant changes in intestinal architecture or CEA expression levels in CEA.Tg/MIN mice in which the CEA-based vaccine dramatically reduced the number of intestinal tumors (Fig. 5, G and H). The most common pathological finding was enlarged spleens associated with extramedullary hematopoiesis, which, probably, was a compensation for anemia. Neither anti-DNA nor ANA serum antibodies were elevated in any mice receiving the CEA-based vaccine. Those results were underscored by the long-term survival of CEA.Tg/MIN mice that received the CEA-based vaccine. At 40 weeks of age, ~50% of those mice were still alive. By week 49, however, all were dead, not because of any autoimmune-associated pathology, but rather, because those mice developed anemia, progressive weight loss, and intestinal tumors, the same disease progression that occurred by weeks 25–27 in the different control groups of mice. The absence of autoimmunity in those mice in which the CEA-based vaccine mediated significant antitumor immunity remains to be determined. Some possible explanations include (a) differential susceptibility of tumor and normal tissues to the immune effector arms and (b) braking of the autoreactive T-cell activity by tolerizing antigen-presenting cells, the presence of regulatory T cells or terminating vaccination (45). Investigating those possibilities will be crucial for the ongoing development of overexpressed self, tumor antigens as immunotherapy targets.

ACKNOWLEDGMENTS

We thank Donald Hill, Garland Davis, and Keith Rogers for their excellent technical assistance. We also thank Debra Weingarten for editorial assistance in the preparation of this manuscript.

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Vaccine-based Therapy Directed against Carcinoembryonic Antigen Demonstrates Antitumor Activity on Spontaneous Intestinal Tumors in the Absence of Autoimmunity

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