Induction of Protective Host Immunity to Carcinoembryonic Antigen (CEA), a Self-Antigen in CEA Transgenic Mice, by Immunizing with a Recombinant Vaccinia-CEA Virus

Erik Kass, Jeffrey Schlom, John Thompson, Fiorella Guadagni, Paolo Graziano, and John W. Greiner

ABSTRACT

Human carcinoembryonic antigen (CEA) is a well-characterized oncofetal glycoprotein whose overexpression by human carcinomas has been a target for cancer immunotherapy. Transgenic mice that express CEA as a self-antigen with a tissue distribution similar to that of humans have been developed. This study investigates: (a) the responsiveness of the CEA transgenic (CEA.Tg) mice to endogenous CEA or CEA administered as a whole protein in adjuvant; and (b) whether the presentation of CEA as a recombinant vaccinia virus could generate CEA-specific host immunity. By and large, the CEA.Tg mice were unresponsive to CEA, as shown by the lack of detectable CEA-specific serum antibodies and the inability to prime an in vitro splenic T-cell response to CEA. Furthermore, the administration of whole CEA protein in adjuvant to CEA.Tg mice failed to elicit either anti-CEA IgG titters or CEA-specific T-cell responses. Only weak anti-CEA IgM antibody titters were found in those mice. In contrast, CEA.Tg mice immunized with recombinant vaccinia virus expressing CEA generated relatively strong anti-CEA IgG antibody titers and demonstrated evidence of immunoglobulin class switching. These mice also developed Td4-type CEA-specific CD4+ responses and CEA peptide-specific cytotoxicity. The ability to generate CEA-specific host immunity correlated with protection of the CEA.Tg mice against a challenge with CEA-expressing tumor cells. Protection against tumor growth was accomplished with no apparent immune response directed at CEA-positive normal tissues. The results demonstrate the ability to generate an effective antitumor immune response to a tumor self-antigen by immunization with a recombinant vaccinia virus. CEA.Tg mice should be an excellent experimental model to study the effects of more aggressive immunization schemes directed at established tumors with the possible development of accompanying autoimmune responses involving normal tissues.

INTRODUCTION

CEA, which was first described in 1965 (1), is a M, 180,000–200,000 oncofetal antigen expressed in normal epithelial tissues as well as in a high percentage of adenocarcinomas, particularly those of the colon, pancreas, breast, and lung (2, 3). Its presence in patient serum is used for disease staging and as an indicator of residual disease and/or tumor recurrences (4). Although the functional nature of CEA has yet to be clearly established, molecular and cellular studies have shown that: (a) CEA belongs to a family of 29 genes that is part of the immunoglobulin gene superfamily (5); (b) its expression has been linked with cell adhesion (6), cell surface recognition by bacteria (7), and possibly with metastatic spread (8); and (c) CEA expression levels can be augmented by IFNs (9). As a membrane antigen, CEA has been targeted by CEA-specific radioimmunoconjugates in experimental and clinical immuno detection and therapy protocols (10).

The question of whether CEA is immunogenic in humans and whether it could be a target for active immunization has recently been reexamined. Healthy individuals and cancer patients were considered unresponsive to CEA because most of the experimental data on host immunity to CEA were, by and large, equivocal (11–14). More recent reports have provided intriguing new insights into the immunogenicity of CEA. In vitro studies have reported the generation of human anti-CEA antibodies (15) and the proliferation of tumor-infiltrating lymphocytes from patients diagnosed with colorectal cancer by an anti-CEA anti-idiotypic antibody (16). Several clinical studies provide additional support that CEA can be immunogenic in humans. The administration of an anti-CEA anti-idiotypic antibody to patients diagnosed with colorectal cancer generated anti-CEA antibodies and idiotype-specific T-cell proliferation (17). The immunization of patients with rV-CEA, combined with subsequent peptide-based in vitro stimulations, generated CD8+ MHC-restricted CTLs capable of lysing autologous tumors (18). Recently, immunization of colorectal carcinoma patients after surgery with recombinant CEA induced weak antibody and cellular responses to recombinant CEA (19). Therefore, under defined circumstances, CEA is capable of evoking an immunological response in humans.

We have developed rV-CEA, a recombinant vaccinia virus that expresses CEA at high quantity and fidelity (20, 21). Using CEA as a target antigen in a C57Bl/6 murine model, rV-CEA immunization generated CEA-specific cellular immune responses that correlated with the host protection from tumor challenge and regression of CEA-positive tumors (21). Furthermore, the addition of selected cytokines (IL-2 and granulocyte macrophage colony-stimulating factor) or costimulatory molecules (i.e., B7; Refs. 22 and 23) has been shown to improve the antitumor effects of rV-CEA immunization. The present study investigates the efficacy of generating CEA-specific host immunity after the rV-CEA immunization of CEA.Tg mice (24). Findings suggest that CEA.Tg mice are unresponsive to whole CEA protein but mount significant anti-CEA antibody and cellular responses after rV-CEA immunization. The findings are reminiscent of those from the clinical trials and indicate that the CEA.Tg murine model may be critical to the investigation of effective antitumor vaccine strategies against a self-antigen.

MATERIALS AND METHODS

CEA.Tg Mice. A breeding pair of CEA.Tg mice (H-2b; line 2682) were provided by Dr. John Thompson (Institute of Immunobiology, University of Freiburg, Freiburg, Germany). Mice were housed and maintained in microisolator cages under pathogen-free conditions. A complete explanation of the generation of CEA.Tg mice has been published previously (24). Lines were established from founder animals by continuous backcrossing with C57Bl/6 mice.

Screening of CEA.Tg Mice. PCR analysis was used to identify CEA-positive transgenic mice. Mice were weaned at 4 weeks and bled approximately 2 weeks later. DNA was extracted from 100 μl of whole blood using a Genomic DNA Purification kit (Promega, Madison, WI) according to the

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2 The abbreviations used are: CEA, carcinoembryonic antigen; rV-CEA, recombinant vaccinia virus expressing CEA; CEA.Tg, CEA transgenic; OVA, ovalbumin; IL, interleukin; mAb, monoclonal antibody; HRP, horseradish peroxidase; puf, plaque-forming units; SI, stimulation index
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manufacturer’s instructions. Approximately 100–200 ng of DNA were amplified in a 50-μl reaction volume in Perkin-Elmer Gene Amp tubes using a Perkin-Elmer 9600 Gene Amp system (PE Applied Biosystems, Perkin-Elmer Corp., Branchburg, NJ). The following CEA-specific primers were used: primer A, (5’ primer) 5′-GGACTTTTTACACAGAATTGGG-3′; and primer B, 5′-CTCTGTGCCTGAACTGAGAC-3′. DNA was added to 1× PCR buffer, 3 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates, and 100 mM primers A and B; heated to 95°C for 10 min; and placed on ice for 5 min. AmpliTaq polymerase (2 units) was added, and the amplification program consisted of 35 cycles at 94°C for 1 min, 55°C for 1 min for annealing, 72°C for 1 min, and a final 72°C elongation step for 10 min. The PCR product of each reaction was analyzed by size fractionation using a 1% agarose gel. Amplification of CEA-positive DNA resulted in a 485-bp fragment. DNA from human colorectal tumor cells was used as a positive control.

CEA levels were measured in the serum of CEA.Tg mice, CEA-negative littermates, and wild-type B6 mice. Blood samples (200 μl) were collected, and the sera were isolated by centrifugation at 1500 × g. CEA levels were determined using the europium-based Delfia CEA assay. The assay is a solid-phase, double determinant fluorometric assay (Wallac, Inc., Gaithersburg, MD) and was used according to the manufacturer’s instructions. The cutoff value for positive serum CEA was 5.0 ng/ml. In all assays, internal low and high CEA standards were included.

**Cell Culture.** The CEA-expressing MC-38 cells, which were designated MC-38-CEA-2, were produced by transducing the murine colon adenocarcinoma cell line MC-38 (H-2b) with human CEA cDNA using retroviral expression vector pBNC (25). The cell line was subsequently cloned and routinely examined for stable CEA expression as measured by the cell surface reactivity using anti-CEA mAb 5C4 antibody (Lonza, Walkersville, MD) and was used according to the manufacturer’s instructions. The complete description of rV-CEA has been described previously (20, 21). CEA was produced by Western blot analysis using murine mAb COL-1 (26). V-Wyeth was obtained from Therion Biologics and used as the wild-type control. Lyophilized CEA (Vitro Diagnostics, Littleton, CO) or OVA (Sigma, St. Louis, MO) was initially dissolved in HBSS and admixed with OVA were administered s.c. near the base of the tail.

V-Wyeth was by tail scarification or s.c. near the base of the tail. CEA and rV-CEA-immunized wild-type B6 mice.3 Immunization with rV-CEA and COL-1 (26). The MC-38-CEA-2 cell line was grown in DMEM containing high glucose and 10% heat-inactivated fetal bovine serum. rV-CEA, Proteins, CEA326–533, and Immunizations. Briefly, rV-CEA was produced by homologous recombination of a plasmid (provided by The rV-CEA was produced by homologous recombination of a plasmid (provided by The rV-CEA was produced by homologous recombination of a plasmid (provided by Therion Biologics Corp., Cambridge, MA) containing a human CEA cDNA inserted into the HindIII M site of the Wyeth strain of vaccinia virus (V-Wyeth). The complete description of rV-CEA has been described previously (20, 21). CEA was detected by Western blot analysis using murine mAb COL-1 (26). V-Wyeth was obtained from Therion Biologics and used as the wild-type control. Lyophilized CEA (Vitro Diagnostics, Littleton, CO) or OVA (Sigma, St. Louis, MO) was initially dissolved in HBSS and assayed with an equal volume of a modified stable formulation of Detox-PC adjuvant (provided by RIBI ImmunoChem Research, Inc., Hamilton, MT, Ref. 27) just before administration. Detox-PC has been reported to function as an effective adjuvant in a study demonstrating the immunogenicity of ras peptides in a murine model (28). CEA326–533 was synthesized in our laboratory on a 432A solid-phase, double determinant fluoroimmunometric assay (Wallac, Inc., Winooski, VT) at 490 nm. A

**Cytokine Production Assays.** Splenic T cells from CEA.Tg mice were isolated and grown in flat-bottomed 96-well plates at a density of 2 × 10⁶ cells/well. 5 × 10⁵ irradiated (2000 rad) syngeneic CEA.Tg mouse splenocytes containing CEA (100–6.25 μg/ml), UV-inactivated V-Wyeth (2.0 × 10⁵ pfu/ml), or ovalbumin (100 μg/ml). Proliferation was measured after 5 days of incubation at 37°C by adding [1H]thymidine (1 μCi/well; Amersham) to the wells 18 h before harvesting. Cells were harvested and counted by liquid scintillation spectroscopy (Wallac, Inc.).

**CTL Line and Target Cells.** Approximately 2 weeks after the second immunization with V-Wyeth or rV-CEA, spleens from two to three mice/group were pooled, and single cell suspensions were generated. Splenocytes were suspended in complete medium containing RPMI 1640 supplemented with 15 mM HEPES (pH 7.4), 2 mM l-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mg/ml gentamicin, 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), and 50 μM 2-mercaptoethanol. A total of 25 × 10⁶ splenocytes were added in 10 ml to T-25 flasks along with a CEA 8-mer peptide (referred to as CEA peptide; 10 μg/ml) corresponding to amino acid positions 526–533 (EAQNTTYL). CEA peptide was added to five times with buffer, followed by the addition of 50 μl/well streptavidin-HRP conjugate (PharMingen; 1:2,000 dilution) and further incubated at 37°C for 1 h. Plates were washed three to five times with buffer, and the absorbance was developed and measured as outlined above. Positive wells indicating the presence of a particular immunoglobulin subtype were scored when the mean absorbance of the CEA-expressing wells was at least double the mean of the OVA- or BSA-sensitized wells by three SDs. All experiments included wells sensitized with immunoglobulin subtypes (100 ng/well) to assess whether any cross-reactivity was present with the secondary biotinylated antibodies.

**T-Cell Proliferation Assay.** Complete details of the T-cell proliferation assay have been described previously (23). Splenocytes were enriched for T cells by magnetic murine pan B (B220) Dynabeads (Dynal, A.S., Oslo, Norway), and fluorescence-activated cell-sorting analysis showed that the resulting cell population was >95% CD3⁺. Isolated T cells were incubated in flat-bottomed 96-well plates at a cell density of 1.5 × 10⁵ cells/well with 5 × 10⁵ irradiated (2000 rad) syngeneic CEA.Tg mouse splenocytes containing CEA (100–6.25 μg/ml), UV-inactivated V-Wyeth (2.0 × 10⁵ pfu/ml), or ovalbumin (100 μg/ml). Proliferation was measured after 5 days of incubation at 37°C by adding [1H]thymidine (1 μCi/well; Amersham) to the wells 18 h before harvesting. Cells were harvested and counted by liquid scintillation spectroscopy (Wallac, Inc.).

**Cytolytic Assays.** Cytolytic activity was assessed 6 days later using EL-4, a murine lymphoma cell line, ± CEA326–533. Cytotoxicity Assays. CTL activity was assessed in a standard 4-h chromium release assay. Target cells (2–3 × 10⁶) were radiolabeled with 250 μCi of Na₂⁵¹CrO₄ (Amersham) in Opti-MEM (Life Technologies, Inc., Gaithersburg, MD) at 37°C for 90 min and then washed. Viable T cells were recovered from culture by centrifugation over a Ficoll-Hypaque gradient. Effector and target cells were coincubated in 96-well round-bottomed plates at graded E:T ratios in the presence or absence of peptide. Plates were centrifuged at 100 × g for 2 min to initiate contact between cells. Incubation was carried out at 37°C for 4 h, after which the plates were centrifuged at 400 × g for 5 min, and supernatant was harvested using a Supernant Collection System (Skatron Systems, Norway), and fluorescence-activated cell-sorting analysis showed that the resulting cell population was >95% CD3⁺. Isolated T cells were incubated in flat-bottomed 96-well plates with 5 × 10⁵ irradiated (2000 rad) syngeneic CEA.Tg mouse splenocytes, and 50 μg/ml CEA. Supernatant from designated wells for each treatment group was harvested after 48 h, and IFN-γ and IL-4 levels were measured using the appropriate ELISA assay (Endogen, Inc., Cambridge, MA).

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Tumor Prevention Studies. The 6–8-week-old male and female CEA.Tg mice and CEA-negative littermates were immunized as outlined in Table 3. Fourteen days after the second immunization, mice were injected in the right flank (s.c.; 100 μl) with 3 × 10^5 MC-38-CEA-2 tumor cells. Routine fluorescence-activated cell-sorting analysis of the MC-38-CEA-2 tumor cells revealed CEA expression (COL-1 binding) by ≥85% of the cells, strong MHC class I expression, and no MHC class II (I-Aβ) expression. Tumors were measured weekly, and the volumes were calculated as follows: volume (mm^3) = [(short axis)^2 × (long axis)]/2. Mice bearing tumors of ≥2 cm^3 were sacrificed by cervical dislocation for humane reasons, and the day of death was recorded.

Immunohistochemistry. Tissues were removed from CEA.Tg mice, fixed in 10% buffered formalin, and embedded in low-melting point paraffin. Immunohistochemical staining of CEA was performed on 4-μm tissue sections air-dried on poly-L-lysine-coated slides using a modification of the avidin-biotin complex method (29). Briefly, tissue sections were deparaffinized in xylene, rehydrated in graded ethanols, and treated for 20 min at room temperature with methanol containing 0.3% H2 O2 to inhibit endogenous peroxidase activity. After rinsing in PBS (pH 7.4), the sections were incubated in 10% normal horse serum for 15 min, and a biotinylated anti-CEA mAb (COL-1) was added at a concentration of 20 μg/ml (200 μg/slide) and incubated overnight at 4°C. An isotype-matched mAb with irrelevant antigen specificity was used as a negative control. After a PBS rinse, slides were incubated with avidin DH-biotinylated HRP H complex for 30 min at room temperature. The slides were then washed in PBS, and the peroxidase reaction was initiated using 0.06% diaminobenzidine (Sigma) and 0.01% H2 O2 for 2 min. After the final PBS rinse, the sections were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted under a coverslip using Permount.

A routine histological examination was also conducted for each specimen on sections stained with H&E.

Statistical Analysis. Statistical significance of T-cell proliferation/lysis data was based on Student’s two-tailed t test. Differences in the growth rate of the MC-38-CEA-2 tumors as measured by changes in tumor volume for each treatment group were compared using the Mann-Whitney U test. All P values reported are two-sided and have not been adjusted for the multiplicity of evaluation performed on the data. P < 0.05 was considered significant.

RESULTS

Identification of CEA.Tg Mice. C57Bl/6 mice bearing CEA as a transgene were initially identified with PCR using CEA-specific oligonucleotide probes. CEA.Tg mice and their CEA-negative littermates were also analyzed for the presence of serum CEA. As summarized in Table 1, there was 100% concordance with the presence of the CEA transgene and detectable serum CEA levels in 25 mice. The range of serum CEA levels was 12.8–133.0 ng/ml, with a mean level of 60.8 ng CEA/ml serum. No CEA was present in the serum of either CEA-negative littermates or wild-type B6 mice.

Humoral Response to CEA in CEA.Tg Mice. Initial studies determined whether naive CEA.Tg mice had circulating serum levels of anti-CEA antibodies. Analysis of serum samples from 10 individual untreated CEA.Tg mice failed to detect the presence of either anti-CEA IgM (data not shown) or IgG (Fig. 1A) antibody titers. In these experiments, groups of CEA.Tg mice were treated twice with either whole CEA or OVA protein in adjuvant, and serum samples were analyzed for the presence of anti-CEA or anti-OVA antibody titers. As summarized in Fig. 1A, CEA.Tg mice immunized with OVA in adjuvant developed significant anti-OVA IgG antibody titers that ranged between 5000 and 6000. However, as also shown in Fig. 1A, there was an inability to detect anti-CEA IgG antibody responses in CEA.Tg mice that were treated twice with whole CEA in adjuvant. These findings underscore the ability of the CEA.Tg mice to mount a humoral response to a foreign antigen, but not to CEA, a self-antigen. Next, CEA.Tg mice were given 10^7 pfu of either rV-CEA or V-Wyeth in adjuvant twice at 2-week intervals. Two weeks after the final treatment, mice were bled, and serum was tested for the presence of anti-CEA or anti-OVA IgG antibodies as outlined in “Materials and Methods.” Data are the mean ± SE of the A_490 values for each group. In some cases, the error bars are covered by the symbol. In the same experiment, CEA.Tg mice (four mice/group) were given 10^7 pfu (100 μl s.c.) of V-Wyeth or rV-CEA. Unimmunized mice (A) received an equal volume of HBSS. Data are the mean (SE < 10%) ± A_490 values for four mice in the HBSS- and V-Wyeth-treated groups. A_490 values are shown for the four individual rV-CEA-immunized CEA.Tg mice (average of triplicate determinations). Results in A and B are from a representative experiment, two to three experiments were performed for each immunogen with similar outcomes.

<table>
<thead>
<tr>
<th>Table 1. Screening of CEA.Tg mice*</th>
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<tr>
<td>Genotype</td>
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<tr>
<td>----------</td>
</tr>
<tr>
<td>CEA.Tg mice</td>
</tr>
<tr>
<td>CEA-negative littermates</td>
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<tr>
<td>Wild-type B6 mice</td>
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* Individual mice were bled between 28–35 days. Approximately 100 μl of whole blood were used for PCR-based detection of the CEA transgene. Serum was collected from the remainder and tested for CEA levels as described in “Materials and Methods.”

** Neg. < 5 ng CEA/ml serum.
serum anti-CEA IgM titers of 200–300. No anti-CEA antibody levels were detected in CEA.Tg mice treated with the control vaccinia virus, V-Wyeth. Since CEA.Tg mice immunized with either rV-CEA or OVA in adjuvant developed measurable antigen-specific IgG serum titers, the sera of mice from each group with the highest titers were further analyzed to identify the immunoglobulin subtypes. As summarized in Table 2, CEA.Tg mice immunized with rV-CEA developed IgG1a, IgG2a, and IgG2b responses, whereas both CEA.Tg mice immunized with OVA generated a strong IgG1 antibody response. Four CEA-negative littersmates immunized with rV-CEA were also analyzed and found to develop strong IgG1a, IgG2a, and IgG2b responses. No IgG3 responses were detected in any of the mice.

Of interest were the comparative strengths of the anti-CEA IgG responses generated in CEA.Tg mice versus those of CEA-negative littersmates after multiple rV-CEA immunizations. Groups of CEA.Tg mice and CEA-negative littersmates were immunized three times with rV-CEA at 2-week intervals, and serum samples were analyzed for anti-CEA IgG responses (Fig. 2). Anti-CEA IgG titers generated in the CEA.Tg mice (approximately 250) were approximately 40-fold lower than those measured for the corresponding CEA-negative littersmates (approximately 10,000).

**Cellular Immune Response to CEA in CEA.Tg Mice.** Subsequent studies focused on whether the immunization of CEA.Tg mice could also result in the generation of CEA-specific cellular immune responses. Initial studies investigated whether splenic T cells isolated from naive CEA.Tg mice could be primed in vitro to respond to soluble CEA. As shown in Fig. 3A, no measurable incorporation of \( ^{3} \)H]thymidine was found when splenic T cells isolated from naive CEA.Tg mice were grown in vitro in the presence of soluble CEA. These cells incorporated significant levels of \( ^{3} \)H]thymidine if grown in concanavalin A, affirming their competence to respond to proper stimuli. CEA.Tg mice and their CEA-negative littersmates were given

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**Table 2** Analyses of immunoglobulin isotypes in immunized CEA.Tg mice and CEA-negative littersmates

<table>
<thead>
<tr>
<th>Mice</th>
<th>Immunogen</th>
<th>No. of Serum dilution</th>
<th>Serum dilution</th>
<th>Immunoglobulin analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA.Tg mice</td>
<td>rV-CEA</td>
<td>1-10</td>
<td>10</td>
<td>++ ++ ++ ++ Neg</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>OVA + SDT</td>
<td>1-50</td>
<td>50</td>
<td>++ Neg Neg Neg Neg</td>
</tr>
<tr>
<td>CEA-negative littersmates</td>
<td>rV-CEA</td>
<td>×4</td>
<td>1000</td>
<td>++ ++ ++ ++ Neg</td>
</tr>
</tbody>
</table>

* Wells in 96-well plates were sensitized with the appropriate immunoglobulin subtype as outlined in “Materials and Methods.” Serum samples from immunized CEA.Tg mice were diluted 1:10 to 1:1250, and the assay was carried out as described. Identification of different immunoglobulin subtypes was based on relative \( A_{490} \)nm using the mean \( \bar{A}_{490} \)nm + 3 SD for the appropriate immunoglobulin subtype as cutoff between negative and positive. For example, wells sensitized with IgG1 and incubated in the presence of serum from nonimmunized CEA.Tg mice had a mean \( A_{490} \)nm + 3 SD of 0.115 + 0.026 = 0.146. Therefore, all IgG-sensitized wells in which the \( A_{490} \)nm was >0.15 were scored +. Moreover, wells in which \( A_{490} \)nm exceeded 0.5 were scored as ++. Data represent the results from duplicate determinations.

* SDT, super DETOX adjuvant.
Groups of CEA.Tg mice (two to three mice/group) were immunized twice with 20 μg of CEA in adjuvant ( ), 10^7 pfu of V-Wyeth ( ), or 10^7 pfu of rV-CEA ( ) as described in “Materials and Methods.” Unimmunized CEA.Tg mice received HBSS ( ). At sacrifice, spleens from each group were removed and pooled. T cells were isolated, and the lymphoproliferative assay was carried out. The SI was calculated as follows: [cpm (antigen-stimulated cells)]/ [cpm (unstimulated cells)]. Each data point represents the mean ± SE of triplicate determinations from a representative experiment; two to three separate experiments were carried out with similar results.

10^7 pfu of either rV-CEA or V-Wyeth by tail scarification. When the splenic T cells were isolated 2 weeks later, CEA-specific splenic T-cell proliferation was detected in the rV-CEA-immunized CEA.Tg mice. The amount of [3H]thymidine incorporated by splenic T cells from rV-CEA-immunized CEA.Tg mice was significantly higher (P < 0.05) than that measured for splenic T cells isolated from either untreated or V-Wyeth-immunized CEA.Tg mice (Fig. 3A). As reported in previous studies, rV-CEA immunization of wild-type C57Bl/6 mice (in this case, the CEA-negative littersmates) also resulted in significant [3H]thymidine uptake by splenocytes when cultured in the presence of CEA (Fig. 3B). As expected, a comparison of the splenic T-cell SI's revealed a higher SI for rV-CEA-immunized CEA-negative littersmates than for the rV-CEA-immunized CEA.Tg mice (15.7 versus 6.8, respectively; P < 0.05). CEA specificity of T-cell proliferation was determined by the lack of any measurable [3H]thymidine uptake when those same cells were incubated in the presence of OVA (data not shown). CEA.Tg mice and the CEA-negative littersmates treated with either rV-CEA or V-Wyeth were immunized against the virus as demonstrated by comparable splenic T-cell-proliferative responses in the presence of UV-inactivated vaccinia virus.

In a prime and boost immunization protocol, CEA.Tg mice were given rV-CEA, V-Wyeth, or whole CEA protein in adjuvant, and splenic T cells were analyzed for CEA-specific proliferation and cytokine production. It was previously observed that no humoral (Fig. 1A) or cellular (Fig. 3A) immune response to CEA was detectable in naive CEA.Tg mice, and that the administration of CEA in adjuvant generated only an anti-CEA IgM response in these mice. In this study, CEA.Tg mice were given whole CEA in adjuvant twice, and the splenic T cells were assayed for CEA-specific proliferation. The SI (i.e., [3H]thymidine incorporation) calculated for the CEA-specific proliferation of splenic T cells from CEA.Tg mice given whole CEA in adjuvant was not significantly different from that for splenic T cells from unimmunized CEA.Tg mice (Fig. 4). However, CEA.Tg mice immunized twice with rV-CEA developed a strong CEA-specific T-cell-proliferative response with a SI of approximately 19.4 that was significantly higher (P < 0.05) than the SIs for CEA.Tg mice treated with V-Wyeth or whole CEA.

Functionally specific subsets of CD4^+ T cells produce and secrete selective sets of cytokines in response to antigen. IFN-γ, IL-2, and tumor necrosis factor α are produced in a type 1 response and promote cell-mediated immunity, whereas IL-4, IL-5, and IL-10 production promote a type 2, or humoral, immunity (30). Supernatant was collected from splenic T cells isolated from either untreated mice or CEA.Tg mice treated with rV-CEA, V-Wyeth, or whole CEA protein in adjuvant and subsequently stimulated in vitro in the presence or absence of soluble CEA. Measurement of IFN-γ and IL-4 production was used to indicate the presence of either a type 1 or type 2 response, respectively. Splenocytes from CEA.Tg mice immunized twice with rV-CEA produced substantial quantities of IFN-γ when cultured in the presence of CEA (Fig. 5). These cells also produce low but detectable levels of IL-4. In contrast, splenic T cells from CEA.Tg mice treated with whole CEA in adjuvant produced barely detectable levels of IFN-γ. Interestingly, these T cells produced levels of IL-4 that were approximately 5–6-fold higher than those found in the supernatants from splenic T cells of rV-CEA-immunized CEA.Tg mice. No appreciable amounts of either cytokine were detected after the incubation of splenic T cells from unimmunized or V-Wyeth-immunized CEA.Tg mice in the presence of CEA. No measurable levels of IL-10 were found in any of the cultures (data not shown).

Subsequent studies were carried out to determine whether rV-CEA immunization of CEA.Tg mice could also generate CEA-specific cytotoxicity. Splenic T cells were isolated from CEA.Tg mice that had been previously treated twice with either rV-CEA, V-Wyeth, CEA, or HBSS. Splenocytes were isolated and grown in vitro with two rounds of stimulation in the presence of CEA (Fig. 6). At an E:T ratio of 50:1, peptide-specific lysis was observed for T cells isolated from CEA.Tg mice that had been immunized previously with rV-CEA (Fig. 6). An E:T ratio of 50:1, peptide-specific lysis was approximately 18%. No appreciable lysis of EL-4 cells was observed in any other T-cell population.

**In Vivo Prevention of CEA-expressing Tumors.** Previous findings argue that CEA.Tg mice could be immunized against CEA with rV-CEA, as evidenced by the development of measurable CEA-
CEA-SPECIFIC HOST IMMUNITY IN CEA.Tg MICE

Fig. 6. Cytolytic activity of CEA126-533-derived CTLs. The cytolytic activity was tested against EL-4 target cells incubated in the presence of 0.2 μg of CEA126-533. CEA.Tg mice (two mice/group) were previously immunized with HBSS (○), CEA in adjuvant (■), V-Wyeth (●), or rV-CEA (△) as outlined previously. Data presented are the mean ± SE from a representative experiment that was repeated once with similar results.

specific humoral and cellular host immune responses. It was of interest to determine whether the presence of the CEA-specific host immunity could, in turn, protect CEA.Tg mice against a challenge with CEA-expressing tumors. CEA.Tg mice and CEA-negative littermates were immunized three times with rV-CEA or V-Wyeth every 2 weeks and challenged with CEA-expressing tumor cells 2 weeks after the final immunization. Tumor appearance and subsequent growth were monitored weekly, and the results are summarized in Fig. 7. CEA.Tg mice treated with HBSS or immunized with V-Wyeth developed progressively growing tumors (>2000 mm³); hence, 80–90% of these mice were sacrificed by week 7. In contrast, 50% (5 of 10) of the CEA.Tg mice immunized with rV-CEA were protected against tumor growth and remained tumor free at week 7. Of the five rV-CEA-immunized CEA.Tg mice that developed tumors, the time of tumor appearance and the subsequent growth rates were delayed when compared with the CEA.Tg mice given HBSS or immunized with V-Wyeth (P < 0.05). Protection of CEA-negative C57Bl/6 mice with rV-CEA is well documented (21, 22). Therefore, it was expected that 80% (8 of 10) of the CEA-negative littermates immunized with rV-CEA were tumor free at week 7. No protection was afforded to CEA-negative littermates treated with HBSS or V-Wyeth.

In the experiment summarized in Fig. 7, all of the CEA.Tg mice immunized with rV-CEA or V-Wyeth were monitored weekly for overt signs of toxicity (i.e., weight loss, diarrhea, feeding abnormalities, basic neurological parameters, and changes in fur coloration). All appeared healthy and maintained normal weight when compared with CEA-negative littermates that were similarly immunized. At 1 month after tumor challenge, blood chemistries were performed on CEA.Tg mice and CEA-negative littermates immunized with rV-CEA or V-Wyeth (Table 3). Elevations in creatinine phosphokinase levels were noted in 2 of 10 rV-CEA-immunized CEA.Tg mice. In addition, 16 of 20 of the CEA.Tg mice and CEA-negative littermates treated with rV-CEA and V-Wyeth had elevated blood amylase levels (Table 3). The significance of elevated amylase levels in mice immunized with vaccinia virus is unknown at this time. In another experiment, CEA.Tg mice were immunized with either rV-CEA or V-Wyeth, and normal tissues were analyzed for changes in histology and relative levels of CEA expression (Table 4). Gross and microscopic examinations of H&E-stained tissue sections of the intestinal tract, esophagus/stomach, kidneys, and liver revealed no major alterations in tissue/cellular architecture in either rV-CEA- or V-Wyeth-immunized CEA.Tg mice when compared with untreated CEA.Tg mice. Immunohistochemical analysis confirmed the presence of CEA in the intestinal tract and the esophagus/stomach of the CEA.Tg mice. Moreover, no substantial alterations in the cellular staining patterns for CEA expression were observed in the intestinal tracts and esophagus/stomach of either V-Wyeth- or rV-CEA-immunized CEA.Tg mice when compared with those from untreated CEA.Tg mice (Table 4).

DISCUSSION

CEA is one of several self-antigens expressed by carcinomas that have been identified as potential targets for active immunotherapy (31). Several approaches, such as immunizing with rV-CEA, have successfully generated CEA-specific host immunity in murine and primate models (20, 32) and, as outlined earlier, provided additional support to the observation that CEA can be immunogenic in humans (18). Mice expressing CEA as a transgene have been developed in three separate laboratories (24, 33, 34). Whereas those CEA.Tg mice have differences in CEA expression levels, they serve as experimental models with which to address critical questions regarding the effect(s) of the endogenous expression of CEA on the generation of CEA-specific host immunity. It is recognized that although normal tissue expression of CEA was primarily confined to the gastrointestinal tract, other sites of ectopic CEA expression, such as the trachea, esophagus, small intestine, and lung, do exist (35). These CEA.Tg mice also have circulating serum CEA levels that were confirmed in the present study (Ref. 25; Table 1). In fact, with additional study, circulating serum CEA could perhaps replace PCR-based screening for identifying CEA.Tg mice. In any case, whereas it has been argued (34) that the presence of CEA in CEA.Tg mouse serum may not be optimal for immunotherapy studies, it also presents a worst-case scenario for generating CEA-specific host immune responses. In humans, normal CEA serum levels are less than 2–5 ng/ml, whereas the mean value in the CEA.Tg mice is 60 ng/ml (Table 1). Therefore, if the concentration of the self-antigen impacts the development of an immune re-
response to that antigen (i.e., breaking tolerance), then CEA serum levels in the CEA.Tg mice might make the generation of an anti-CEA immune response that much more arduous. However, from another viewpoint, the serum CEA levels in the CEA.Tg mice are somewhat analogous to those found in patients diagnosed with late-stage colorectal cancer (36), which may allow for some extrapolation of experimental data in future clinical study.

The present study was designed to determine whether: (a) naive CEA.Tg mice were responsive to endogenous CEA; (b) CEA-specific host immunity could be induced by administering CEA in an adjuvant or as a recombinant vaccinia virus; and (c) immunization of CEA.Tg mice could be protective against tumor challenge. Elevated serum CEA levels could be a danger signal for the immune system (37) and result in a resident humoral and/or cellular response to CEA in naive CEA.Tg mice. However, no measurable anti-CEA antibody titers were found in the serum of naive CEA.Tg mice, suggesting that CEA overexpression does not induce a CEA-specific humoral immunity. This finding agrees with those from another CEA.Tg murine model (34) in which the expression of CEA by tumors in vivo did not result in the appearance of CEA antibodies. Another explanation would be that low titers of anti-CEA antibodies are present in the naive CEA.Tg mice in this study but are undetectable because of complexing with serum CEA. From a cellular immunology standpoint, attempts to prime splenic T cells in vitro from the same CEA.Tg mice with the addition of exogenous CEA failed. Combining these observations suggests that the naive CEA.Tg mice were unresponsive to endogenous CEA. In subsequent experiments, CEA.Tg mice developed anti-CEA IgM antibodies but not detectable CEA-specific IgG or cell-mediated responses after immunization with whole CEA in adjuvant. CEA.Tg mice in this study should be considered weakly responsive to whole CEA. In another study, administration of a CEA peptide to CEA.Tg mice failed to induce any CEA-specific CTL response (34). It remains to be determined whether the inability of CEA.Tg mice to mount an immune response after the administration of either CEA in adjuvant or CEA peptides (38) involves in vivo mechanisms of immune tolerance.

Recombinant vaccinia vaccines that express a foreign gene are excellent candidates for active immunization. Vaccinia has a wide host range and is capable of accepting large inserts of a foreign gene, whereas the co-presentation of a weakly immunogenic gene product with highly immunogenic vaccinia proteins can boost the immune response to the inserted gene product (39–41). Indeed, the data presented here indicate that the vehicle of immunization against self-antigen is critical. In the present study, presentation of CEA by a recombinant vaccinia virus proved to be a highly efficient mode of immunization. Immunization of CEA.Tg mice with rV-CEA induced anti-CEA IgG antibody titers (Fig. 1B), mediated immunoglobulin class switching (Table 2), and generated $T_{H1}$-type CEA-specific CD4$^+$ response (Figs. 4 and 5) and CEA peptide-specific cytotoxicity (Fig. 6). Moreover, multiple rV-CEA immunizations protected CEA.Tg mice from challenge with CEA-expressing tumor cells (Fig. 7). Whereas it would be attractive to focus on the advantages gained by immunizing CEA.Tg mice with rV-CEA, it is equally important to point out that the overall immune response to CEA in the CEA.Tg mice is relatively weak when compared with that generated in CEA-negative littermates immunized with rV-CEA. These differences seem to be inherently predictable, given the apparent differences between immunizing against a self-antigen versus a non-self-antigen. However, subsequent studies should address whether the observed differences between generating a CEA-specific immune response in CEA.Tg mice and CEA-negative littermates are solely quantitative or are also qualitative in nature. Investigators have suggested that the unresponsiveness of lymphocytes may not be determined solely by the self and non-self nature of the antigen but by the specific conditions in which the antigen is presented to the immune system. For example, immunizing using different doses of antigen, altering the adjuvant or type of antigen-presenting cell, and the use of heterokaryons of dendritic and tumor cells (42–45) have stimulated host immunity directed at self-antigens. Within the context of the present findings, rV-CEA immunization of CEA.Tg mice combines some of those same conditions (i.e., local inflammation and high production levels of a weak immunogen) that might contribute to the generation of a CEA-specific host immune response. This and other CEA.Tg murine models will provide an excellent experimental setting in which to identify

### Table 3. Blood chemistries of CEA.Tg mice and CEA-negative littermates immunized with rV-CEA

<table>
<thead>
<tr>
<th>Marker</th>
<th>Reference (normal) range</th>
<th>Untreated (n = 3)</th>
<th>V-Wyeth (n = 3)</th>
<th>rV-CEA (n = 10)</th>
<th>CEA-negative littermates (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST$^a$</td>
<td>72–288 U/liter</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Not tested</td>
</tr>
<tr>
<td>ALT$^a$</td>
<td>24–140 U/liter</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Creatine phosphokinin</td>
<td>0–800 U/liter</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Amylase</td>
<td>602–2311 U/liter</td>
<td>Normal</td>
<td>↑ (3/3)</td>
<td>↑ (7/10)</td>
<td>↑ (2/3)</td>
</tr>
<tr>
<td>BUN</td>
<td>9–28 mg/dl</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.2–0.7 mg/dl</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Bilirubin (total)</td>
<td>0.0–0.9 mg/dl</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Uric acid</td>
<td>2.2–4.6 mg/dl</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Albumin</td>
<td>2.6–4.6 mg/dl</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Protein (total)</td>
<td>4.0–6.2 mg/dl</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

$^a$ CEA.Tg mice and CEA-negative littermates were immunized and subsequently challenged with CEA-expressing tumor cells as outlined in Fig. 7. Blood chemistries were performed on individual mice at approximately 1 month after challenge.

### Table 4. Analyses of normal tissues from untreated and rV-CEA- and V-Wyeth-treated CEA.Tg mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal tissue</th>
<th>Histochemical analysis</th>
<th>CEA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Intestinal tract</td>
<td>Normal</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Esophagus/stomach</td>
<td>Normal</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Kidneys</td>
<td>Normal</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>Normal</td>
<td>–</td>
</tr>
<tr>
<td>V-Wyeth</td>
<td>Intestinal tract</td>
<td>Normal</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Esophagus/stomach</td>
<td>Normal</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Kidneys</td>
<td>Normal (ND)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>Normal</td>
<td>–</td>
</tr>
<tr>
<td>rV-CEA</td>
<td>Intestinal tract</td>
<td>Normal</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Esophagus/stomach</td>
<td>Normal</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Kidneys</td>
<td>Normal</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>Normal (ND)</td>
<td>–</td>
</tr>
</tbody>
</table>

$^a$ Groups of CEA.Tg mice (two mice/group) were treated twice at 2-week intervals with 10$^7$ pfu of rV-CEA or V-Wyeth by tail scarification. Two weeks after the second treatment, mice were sacrificed, and spleens were used to generate the data summarized in Fig. 3. Normal tissues were also removed and prepared for immunohistology. Data presented represent the results from both of the CEA.Tg mice in each group.

$^b$ Routine histological examinations were conducted for each specimen on H&E-stained sections.

CEA expression as indicated by the presence of dark brown DAB cellular precipitate. ND, not done.
cytokines, costimulatory molecules, and other factors that augment the immune response to a self-antigen. Also important in subsequent studies will be a more detailed account of the toxicology that might accompany the generation of a stronger immune response to CEA associated with successful tumor therapy. One issue requiring further investigation is the apparent ability of rV-CEA immunization to generate an anti-CEA response that elicits antitumor immunity with little or no effect on CEA-positive normal tissues. With the hypothesis that the antitumor immunity elicited by rV-CEA immunization is cell mediated, subsequent studies should determine the exact mechanism, and whether relative expression levels of CEA and MHC class I antigens on tumor versus normal tissues or the location of those tissues might provide a basis for the apparent selectivity. Other studies should focus on the elucidation of principles, not only for the generation of tumor-reactive T cells recognizing a self-antigen, but also for the evaluation of their therapeutic efficacy.

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Induction of Protective Host Immunity to Carcinoembryonic Antigen (CEA), a Self-Antigen in CEA Transgenic Mice, by Immunizing with a Recombinant Vaccinia-CEA Virus

Erik Kass, Jeffrey Schlom, John Thompson, et al.


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