

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

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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 titers or CEA-specific T-cell responses. Only weak anti-CEA IgM antibody titers 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 T_H1-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,² which was first described in 1965 (1), is a M_r 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 radioimmunoconju-

gates in experimental and clinical immunodetection 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 idiotypic-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-2^b; 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 μ l 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; pfu, plaque-forming units; SI, stimulation index

manufacturer's instructions. Approximately 100–200 ng of DNA were amplified in a 50- μ l 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'-GGACTTTTAAACACAGAATTGGG-3'; and primer B, 5'-CCTTGTGCCCATGGAACACAGAC-3'. DNA was added to 1 \times 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 μ l) were collected, and the sera were isolated by centrifugation at 1500 \times g. CEA levels were determined using the europium-based DELFIA CEA assay. The assay is a solid-phase, double determinant fluoroimmunoassay (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-2^b) 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 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, CEA₅₂₆₋₅₃₃, and Immunizations. Briefly, 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 *Hind*III 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 admixed 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). CEA₅₂₆₋₅₃₃ was synthesized in our laboratory on a 432A peptide synthesizer (Applied Biosystems, Foster City, CA) using F-moc chemistry. CEA₅₂₆₋₅₃₃ was purified, analyzed by reverse-phase high-pressure liquid chromatography using a C₁₈ column (>90% pure), dissolved in distilled water, filter sterilized, and stored in aliquots at -80°C. CEA₅₂₆₋₅₃₃ was chosen from >50 CEA peptides because of its recognition and subsequent lysis of peptide-pulsed EL-4 cells in a primary CTL using splenic T cells isolated from rV-CEA-immunized wild-type B6 mice.³ Immunization with rV-CEA and V-Wyeth was by tail scarification or s.c. near the base of the tail. CEA and OVA were administered s.c. near the base of the tail.

Serum Antibody Responses. Serum samples were collected from unimmunized and immunized CEA.Tg mice as well as from CEA-negative littermates and analyzed for the presence of antibodies to the appropriate target antigen by ELISA. Microtiter plates were sensitized overnight at 4°C with 100 ng/well CEA (International Enzymes, Fallbrook, CA), OVA (Sigma), or BSA. Wells were blocked with PBS containing 5% BSA, followed by a 1-h incubation in diluted mouse serum (dilution, 1:10 to 1:31,250). After incubation, excess liquid was aspirated, and plates were washed three to five times with buffer (PBS containing 1% BSA). Antibodies bound to the wells were detected with HRP-conjugated goat antimouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) or IgM (Jackson ImmunoResearch, West Grove, PA). After a 1-h incubation, the level of reactivity was detected with the addition of chromogen, o-phenylenediamine, for 10 min and read using an ELISA microplate autoreader EL310 (Bio-Tek Instruments, Inc., Winooski, VT) at A_{490 nm}.

³ J. Hodge, personal communication.

Triplicates of positive and negative controls and serum samples were run for all assays. Antibody titers were determined as the reciprocal of the serum dilution that results in an A_{490 nm} value of 0.5.

To determine the different immunoglobulin subtypes, plates were sensitized with CEA, OVA, or BSA (100 ng/well) as outlined above. Biotin-labeled secondary antibodies specific for the different subtypes (*i.e.*, IgG1, IgG2a, IgG2b, and IgG3; PharMingen, San Diego, CA) were added at a 1:250 dilution with 50 μ l/well and incubated for 1 h at 37°C. The plates were washed three to five times with buffer, followed by the addition of 50 μ l/well streptavidin-HRP conjugate (PharMingen; 1:2,000 dilution) and further incubated at 37°C for 1 h. Plates were washed four 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 for CEA exceeded those of 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 (Dyna, 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 \times 10⁵ cells/well with 5 \times 10⁵ irradiated (2000 rad) syngeneic CEA.Tg mouse splenocytes containing CEA (100–6.25 μ g/ml), UV-inactivated V-Wyeth (2.0 \times 10⁷ pfu/ml), or ovalbumin (100 μ g/ml). Proliferation was measured after 5 days of incubation at 37°C by adding [³H]thymidine (1 μ Ci/well; Amersham) to the wells 18 h before harvesting. Cells were harvested and counted by liquid scintillation spectroscopy (Wallac, Inc.).

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 \times 10⁴ cells/well, 5 \times 10⁵ irradiated (2000 rad) syngeneic CEA.Tg mouse splenocytes/well, and 50 μ g/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).

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 μ M 2-mercaptoethanol. A total of 25 \times 10⁶ splenocytes were added in 10 ml to T-25 flasks along with a CEA 8-mer peptide (referred to as CEA peptide; 10 μ g/ml) corresponding to amino acid positions 526–533 (EAQNNTTYL). T-cell cultures were restimulated 6 days later in 24-well plates. T cells (200,000) were added along with 5 \times 10⁶ irradiated syngeneic splenocytes, 10 μ g/ml CEA₅₂₆₋₅₃₃, and 10 units/ml recombinant human IL-2 (Proleukin; Chiron Corp., Emeryville, CA). Cytolytic activity was assessed 6 days later using EL-4, a murine lymphoma cell line, \pm CEA₅₂₆₋₅₃₃.

Cytotoxicity Assays. CTL activity was assessed in a standard 4-h chromium release assay. Target cells (2–3 \times 10⁶) were radiolabeled with 250 μ Ci of Na₂[⁵¹Cr]O₄ (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 \times 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 \times g for 5 min, and supernatant was harvested using a Supernatant Collection System (Skatron Co., Sterling, VA). Radioactivity was read in a gamma counter (Cobra Auto-gamma; Packard Instruments, Downers Grove, IL). The percentage of specific lysis was calculated as the mean \pm SE of triplicate wells according to the following formula: percentage of specific lysis = [(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)] \times 100. Maximum ⁵¹Cr release was obtained by adding Triton X-100 to target cells (0.2%). Spontaneous ⁵¹Cr release was obtained from target cells incubated in the absence of T cells and in the presence or absence of CEA₅₂₆₋₅₃₃.

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 μ l) 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-A^b) expression. Tumors were measured weekly, and the volumes were calculated as follows: volume (mm) = [(short axis)² \times (long axis)]/2. Mice bearing tumors of >2 cm³ 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- μ m 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% H₂O₂ 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 μ g/ml (200 μ g/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% H₂O₂ 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

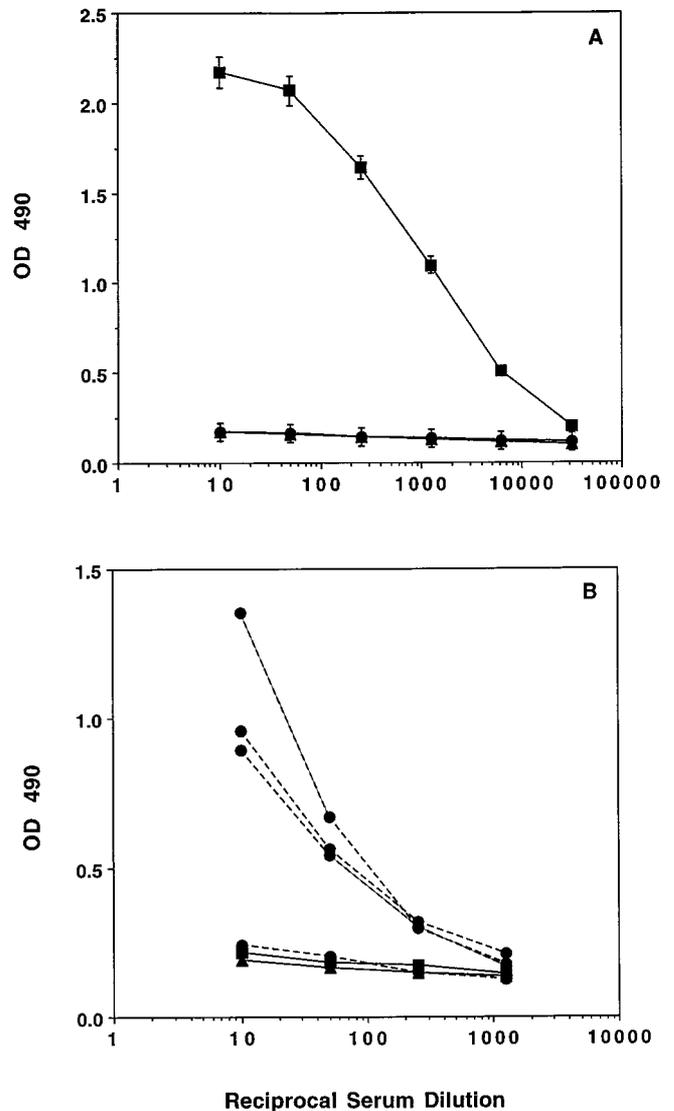


Fig. 1. Induction of anti-CEA/OVA IgG serum titers in CEA.Tg mice. A, CEA.Tg mice (two mice/group) were given 20 μ g of either CEA (▲), OVA (■), or HBSS (●) in adjuvant twice at 2-week intervals. Two weeks after the final treatment, mice were bled, and serum was tested for the presence of anti-CEA or anti-OVA IgG antibodies as outlined in "Materials and Methods." Data are the mean \pm SE of the $A_{490\text{ nm}}$ value for each group. In some cases, the error bars are covered by the symbol. B, in the same experiment, CEA.Tg mice (four mice/group) were given 10^7 pfu (100 μ l; s.c.) of V-Wyeth (■) or rV-CEA (●). Unimmunized mice (▲) received an equal volume of HBSS. Data are the mean (SE < 10%) $A_{490\text{ nm}}$ values for four mice in the HBSS- and V-Wyeth-treated groups. $A_{490\text{ nm}}$ 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.

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 to investigate whether presentation of CEA in a recombinant vaccinia virus could induce a humoral response to the self-antigen. Indeed, as shown in Fig. 1B (dashed lines), three of four CEA.Tg mice given rV-CEA developed measurable anti-CEA IgG antibody titers (range, 50–100). Two of four rV-CEA-immunized CEA.Tg mice also had

Table 1. Screening of CEA.Tg mice^a

Genotype	No. of mice	RT-PCR ^d	Serum CEA (ng/ml)		
			No. of mice	Range	Mean \pm SE
CEA.Tg mice	25	+	25	12.8–133.0	60.8 \pm 14.9
CEA-negative littermates	25	Neg ^b	10	Neg	Neg
Wild-type B6 mice	10	Neg	10	Neg	Neg

^a Individual mice were bled between 28–35 days. Approximately 100 μ l 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."

^b 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 IgG1, IgG2a, and IgG2b responses, whereas both CEA.Tg mice immunized with OVA generated a strong IgG1 antibody response. Four CEA-negative littermates immunized with rV-CEA were also analyzed and found to develop strong IgG1, 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 littermates after multiple rV-CEA immunizations. Groups of CEA.Tg mice and CEA-negative littermates 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

Table 2 Analyses of immunoglobulin isotypes in immunized CEA.Tg mice and CEA-negative littermates^a

Mice	Immunogen	No. of mice	Serum dilution	Immunoglobulin analysis ^a			
				IgG1	IgG2a	IgG2b	IgG3
CEA.Tg mice	rV-CEA	1	10	++	++	++	Neg
		2	10	Neg	+	+	Neg
	OVA + SDT ^b	1	50	++	Neg	Neg	Neg
		2	50	++	Neg	Neg	Neg
CEA-negative littermates	rV-CEA	×4	1000	++	++	++	Neg

^a 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 $A_{490\text{ nm}}$ using the mean ($A_{490\text{ nm}}$) + 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 $A_{490\text{ nm}}$ + 3 SD of $0.115 + 0.026 = 0.146$. Therefore, all IgG-sensitized wells in which the $A_{490\text{ nm}}$ was >0.15 were scored +. Moreover, wells in which $A_{490\text{ nm}}$ exceeded 0.5 were scored as ++. Data represent the results from duplicate determinations.

^b SDT, super DETOX adjuvant.

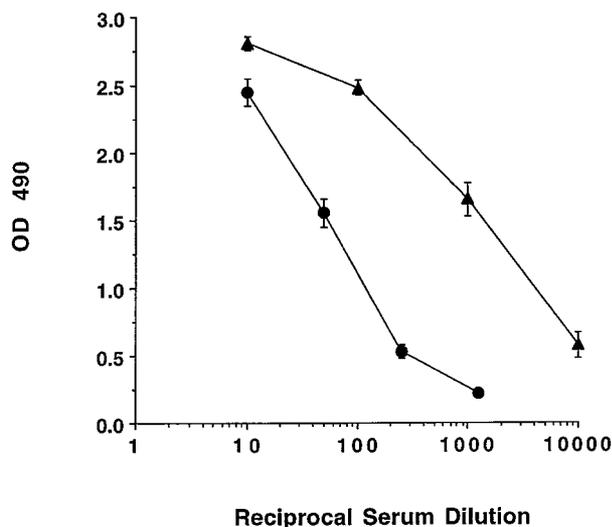


Fig. 2. Comparison of anti-CEA IgG responses in CEA.Tg mice and CEA-negative littermates. Groups (four mice/group) of CEA.Tg mice (●) and CEA-negative littermates (▲) were immunized three times (s.c.) with rV-CEA (10^7 pfu) at 2-week intervals. Serum samples were collected from individual mice 2 weeks after the final immunization and assayed for the presence of anti-CEA IgG antibodies. Results presented are the mean \pm SE of the four mice in each group. In some instances, the error bars are covered by the symbol. Data are from a representative experiment that was repeated once with similar results.

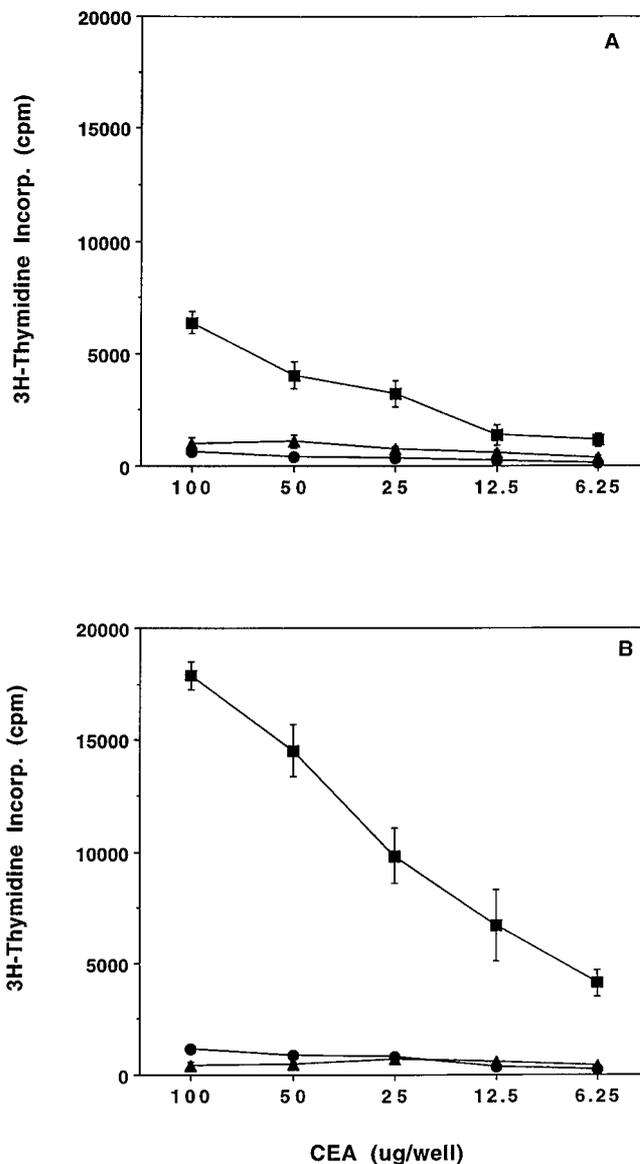


Fig. 3. Comparison of CEA-specific CD4⁺ proliferative responses from CEA.Tg mice (A) and CEA-negative (B) littermates. Mice (two to three mice/group) were given 10^7 pfu of rV-CEA (■) or V-Wyeth (▲) by tail scarification ($10\ \mu\text{l}$), whereas control mice (four mice/group) were given an equal volume of HBSS (●). Fourteen days later, mice were sacrificed, spleens were pooled, T cells were isolated, and the proliferative responses to soluble CEA were measured by [³H]thymidine incorporation as described in "Materials and Methods." Data are presented as the mean \pm SE from a representative experiment. The experiment was repeated three to four times with similar results. Note that some error bars are covered by the symbol.

the CEA.Tg mice (approximately 250) were approximately 40-fold lower than those measured for the corresponding CEA-negative littermates (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 [³H]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 [³H]thymidine if grown in concanavalin A, affirming their competence to respond to proper stimuli. CEA.Tg mice and their CEA-negative littermates were given

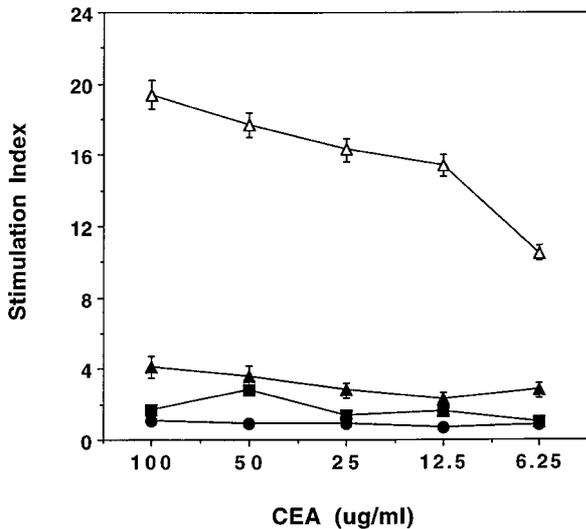


Fig. 4. Splenic CD4⁺ proliferation following a prime and boost immunization strategy. Groups of CEA.Tg mice (two to three mice/group) were immunized twice with 20 μ g of CEA in adjuvant (▲), 10⁷ pfu of V-Wyeth (■), or 10⁷ pfu of rV-CEA (△) as described in "Materials and Methods." Unimmunized CEA.Tg mice 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 \pm SE of triplicate determinations from a representative experiment; two to three separate experiments were carried out with similar results.

10⁷ 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 littermates) 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 SIs revealed a higher SI for rV-CEA-immunized CEA-negative littermates 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 littermates 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_{526–533}. At that time, T cells were evaluated for their ability to lyse EL-4 cells \pm CEA_{526–533}. Peptide-specific cell-mediated lysis was observed for T cells isolated from CEA.Tg mice that had been immunized previously with rV-CEA (Fig. 6). At an E:T ratio of 50:1, peptide-specific lysis was approximately 18%. No appreciable lysis of EL-4 cells \pm CEA_{526–533} 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-

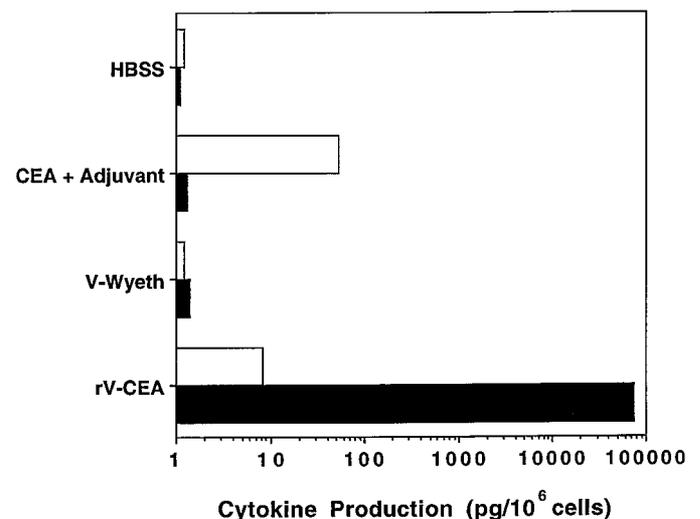


Fig. 5. IFN- γ (■) and IL-4 (□) production during CEA-mediated CD4⁺ T-cell proliferation. CEA.Tg mice (two to three mice/group) were immunized with HBSS, CEA, V-Wyeth, or rV-CEA as outlined in the legend of Fig. 4. Spleens were pooled, and T cells were isolated and incubated with freshly isolated, irradiated antigen-presenting cells and 50 μ g of CEA for 48 h. Results shown are the mean from triplicate wells (SE \pm 15%) from a representative experiment. The experiment was repeated with similar results. No measurable amounts of either IFN- γ and IL-4 were found when splenic T cells from each group were cultured in the absence of CEA. Concanavalin A-stimulated splenic T cells from each group produced approximately 1–2 μ g of IFN- γ and 20–30 ng of IL-4/10⁶ cells during the same time period.

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

Marker	Reference (normal) range	CEA.Tg mice			CEA-negative littermates		
		Untreated (n = 3)	V-Wyeth (n = 3)	rV-CEA (n = 10)	Untreated	V-Wyeth (n = 3)	rV-CEA (n = 4)
AST ^b	72–288 U/liter	Normal	Normal	Normal	Not tested	Normal	Normal
ALT	24–140 U/liter	Normal	Normal	Normal		Normal	Normal
Creatine phosphokinase	0–800 U/liter	Normal	Normal	↑ (2/10)		Normal	Normal
Amylase	602–2311 U/liter	Normal	↑ (3/3)	↑ (7/10)		↑ (2/3)	↑ (4/4)
BUN	9–28 mg/dl	Normal	Normal	Normal		Normal	Normal
Creatinine	0.2–0.7 mg/dl	Normal	Normal	Normal		Normal	Normal
Bilirubin (total)	0.0–0.9 mg/dl	Normal	Normal	Normal		Normal	Normal
Uric acid	2.2–4.6 mg/dl	Normal	Normal	Normal		Normal	Normal
Albumin	2.6–4.6 g/dl	Normal	Normal	Normal		Normal	Normal
Protein (total)	4.0–6.2 g/dl	Normal	Normal	Normal		Normal	Normal

^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 tumor challenge.

^b AST, aspartate aminotransferase; ALT, alanine aminotransferase; ↑, elevation in blood markers; (n, n), number of mice with elevated marker, number of mice tested.

sponse 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 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 4 Analyses of normal tissues from untreated and rV-CEA- and V-Wyeth-treated CEA.Tg mice^a

Treatment	Normal tissue	Histochemical analysis ^b	CEA expression ^c
Untreated	Intestinal tract	Normal	+
	Esophagus/stomach	Normal	+
	Kidneys	Normal	–
	Liver	Normal	–
V-Wyeth	Intestinal tract	Normal	+
	Esophagus/stomach	Normal	+
	Kidneys	Normal	ND
	Liver	Normal	ND
rV-CEA	Intestinal tract	Normal	+
	Esophagus/stomach	Normal	+
	Kidneys	Normal	–
	Liver	Normal	ND

^a Groups of CEA.Tg mice (two mice/group) were treated twice at 2-week intervals with 10⁷ 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.

^c 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

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