Induction of Mucosal and Systemic Immune Responses against Human Carcinoembryonic Antigen by an Oral Vaccine

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Abstract

Carcinoembryonic antigen (CEA) is a tumor-associated antigen targeted for the development of colorectal tumor vaccines. In this study, we developed papillomavirus pseudoviruses encoding the truncated CEA without NH₂-terminal signal peptide (PV-CEA) as an oral vaccine to induce CEA-specific CTL responses. In CEA transgenic (CEA-Tg) mice orally immunized with PV-CEA, the immunologic tolerance to CEA as a "self-antigen" was overcome and both mucosal and systemic CEA-specific cytolytic activities were detected by *in vitro* ⁵¹Cr release assays. In a tumor prevention model, the growth rate of CEA⁻ tumors was significantly delayed in CEA-Tg mice orally immunized with PV-CEA when compared with the control vaccine. Further, the IFN-γ enzyme-linked ImmunoSPOT and *in vitro* ⁵¹Cr release assay results showed that HLA-A2-restricted, CEA-specific CTL responses were induced in both mucosal and systemic lymphoid tissues in A2 transgenic mice after oral immunization with PV-CEA. Finally, we showed that coadministration of papillomavirus pseudoviruses encoding interleukin-2 with PV-CEA enhanced the generation of A2-restricted, CEA-specific CTLs in aged CEA/A2 double transgenic mice, which were more clinically relevant. Our data suggest that PV-CEA pseudovirus vaccine is a promising oral CEA vaccine for humans to induce CEA-specific CTLs at the site of colorectal tumors (i.e., intestinal mucosa), which might efficiently eliminate CEA⁺ colorectal tumor cells in the mucosa. (Cancer Res 2005; 65(15): 6990–9)

Introduction

Carcinoembryonic antigen (CEA) is a 180-kDa cell surface glycoprotein extensively expressed in the majority of colon, rectal, stomach, and pancreatic cancers, 70% of non–small cell lung cancers and 50% of breast cancers (1–3). It is usually expressed at lower concentrations in normal adult colonic epithelia. Due to its unique expression pattern, it has been targeted as a tumor-associated antigen to induce CEA-specific antibodies, CD4⁺ T helper cells, and CD8⁺ CTLs to treat CEA⁺ tumors (4). However, like many other tumor-associated antigens, CEA is a "self-antigen" usually expressed during fetal development and later in normal colonic epithelia at low levels. To evaluate vaccines directed against CEA as a self-antigen, a useful animal model was provided by the establishment of mice that carry the transgene for human CEA and express CEA in a tissue-specific manner similar to humans (5–9).

Materials and Methods

Mice. C57BL/6 mice and A2-Tg breeder mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice carrying the gene for human CEA (CEA-Tg mice) were originally obtained from Dr. John Thompson (University of Freiburg, Freiburg, Germany) and kindly provided by Drs. John W. Greiner and Jeffrey Schlom (National Cancer Institute, NIH, Bethesda, MD). The CEA-Tg mouse colony was maintained with continuous backcrossing with C57BL/6 mice. A2-Tg mice were generated by in-house breeding. CEA/A2 double transgenic mice were generated by crossing male CEA-Tg mice with female A2-Tg mice. Mice were maintained under specific conditions.

In CEA transgenic (CEA-Tg) mice, peripheral T-cell tolerance to CEA was shown by the absence of any immune response to either endogenous CEA or after vaccination with the CEA protein in adjuvant (9). However, the immunologic tolerance to CEA is not absolute. When CEA-Tg mice were immunized with recombinant poxvirus-based vaccines (9–13), CEA-transfected syngeneic fibroblasts in combination with *Corynebacterium parvum* (14), dendritic cells pulsed with anti-idiotypic antibody mimicking CEA (15, 16), DNA vaccines encoding CEA absorbed onto cationic microparticles (17), or an oral vaccine using attenuated bacteria encoding CEA (18–20), tolerance to this self-tumor–associated antigen was overcome as evidenced by the development of CEA-specific antibody, CD4⁺ T helper cells, and CD8⁺ CTL responses.

In humans, CEA-specific immune responses were also elicited under certain circumstances. It has been shown that CEA contains multiple HLA-A2-restricted CTL epitopes (21–26). CD8⁺ MHC-restricted CTLs capable of lysing autologous tumors expressing CEA have been isolated from patients vaccinated with recombinant poxviruses expressing CEA (27–29). CEA-specific CTLs can also be generated *in vitro* by antigen-presenting cells (APC) pulsed with synthetic CEA peptides or CEA RNA (24–26, 30). Papillomaviruses are a group of small DNA viruses that infect mucosa and skin. Their major structural protein L1 can be assembled spontaneously into virus-like particles (VLP) when expressed in insect cells, yeasts, and bacteria (31–36). Papillomavirus VLPs can be used to package unrelated plasmids to form papillomavirus pseudoviruses (37–39). We have shown that VLPs can be used as a mucosal vaccine carrier to deliver plasmids encoding the genes of interest into mucosal and systemic lymphoid tissues, and oral immunization with papillomavirus pseudoviruses induces mucosal and systemic CTL responses specific for the target antigens (39–41). Therefore, in this study, we developed papillomavirus pseudoviruses encoding the truncated CEA without NH₂-terminal signal peptide (PV-CEA) as an oral vaccine to induce intestinal mucosal and systemic CEA-specific CTL responses, which are critical for treating CEA⁺ colorectal tumors. We determined the immunogenicity of PV-CEA pseudoviruses in CEA-Tg mice and HLA-A2 transgenic (A2-Tg) mice. Furthermore, we explored the approach to induce CEA-specific CTLs in aged CEA/A2 double transgenic mice, which provides a more clinically relevant animal model.
pathogen-free conditions in the Loyola University Comparative Medicine Facility (Maywood, IL). All experimental procedures were carried out according to the protocols approved by the Institutional Animal Care and Use Committee.

Screening of carcinoembryonic antigen, A2, and carcinoembryonic antigen/A2 double transgenic mice. PCR was used to identify CEA(-) and A2(-) transgenic mice. Mouse genomic DNA was extracted from mouse tails. DNA (100–200 ng) was used for PCR in a 50 µL reaction volume. The CEA PCR was done as described previously (9). At the end of experiments, the CEA expression in CEA-Tg mice was confirmed by CEA ELISA (Calbiotech, Inc., Spring Valley, CA) using the mouse sera. For the A2 PCR, the sequences for A2-specific primers were GAGGTATTTCTCATACCTCGTG and CGCTCTGGTTTATGAGTCCGGCG. The amplification program consisted of 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and a final 72°C for 7 minutes. The A2 expression on the cell surface was confirmed by flow cytometry after immunostaining using BB7.2 hybridoma culture supernatant (American Type Culture Collection, Manassas, VA).

Cell lines. The human colorectal adenocarcinoma cell line SW1116 was obtained from American Type Culture Collection and cultured in complete Leibovitz’s L-15 medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). RMA (H-2b) is a murine lymphoma cell line and was maintained in complete RPMI 1640 containing 10% FBS, 2 mmol/L glutamine, 5 × 10−5 mol/L β-mercaptoethanol, and 100 units/mL penicillin-100 µg/mL streptomycin (all from Invitrogen). RMA-CEA cells are RMA cells transfected with the plasmid DNA pCI-CEA, which encodes the truncated CEA without NH2-terminal signal peptide. RMA-CEAF cells are RMA cells transfected with the plasmid DNA pCI-CEAF, which encodes the full-length CEA polypeptide. RMA-neo cells are RMA cells transfected with the empty vector pCI-neo. EL4-A2Kb is a transfectant of the EL4 thymoma that expresses the HLA-A2 and is derived from EL4 and was obtained from Dr. W.M. Kast (Loyola University Chicago; ref. 42). All these cell lines were maintained in complete RPMI 1640 containing 5% FBS.

Construction of expression plasmid pCI-CEA encoding human carcinoembryonic antigen without the NH2-terminal signal peptide and plasmid pCI-CEAF encoding the full-length carcinoembryonic antigen polypeptide with NH2-terminal signal peptide. RNA was isolated from the human colorectal cancer cell line SW1116 using a RNeasy Mini Kit (Qiagen). The cDNA was cloned into the TA vector pCR II-TOPO (Invitrogen). Then, the CEA sequence was amplified by PCR using KlenTaq LA Polymerase Mix (Clontech, Palo Alto, CA). The primers for the CEA without the NH2-terminal signal peptide were CCAGAATTCACACCACTGAGACCTTAGCT- GAATTCAACCATGAGCTCATATGATCCACGCACGGCCAA, and AATGGGCCGCCCTATATCAAGGACACCCCAA; the primers for the full-length CEA were ACCCATGAGCTCCTCCTGCGGC and AATGGGCCGCCCTATATCAAGGACACCCCAA. The CEA or CEAFL PCR product was cloned into the TA vector pCR II-TOPO (Invitrogen). Then, the CEA or CEAF fragment was cut out from the pCR II-CEA or pCR II-CEAFL vector and cloned into mammalian expression vector pCI-neo (Promega) using EcoRI and NotI sites to generate CEA expression plasmid pCI-CEA or pCI-CEAF. To determine the CEA expression of pCI-CEA in mammalian cells, we transfected 5 × 104 murine RMA cells with 5 µg pCI-neo or pCI-CEA using the Superfect transfection reagent (Qiagen). The transfectants were selected with 1 mg/mL G418. To determine the CEA gene expression in the transfectant RMA-CEA, reverse transcription-PCR (RT-PCR) was done using CEA-specific primers (CCA-GAATTCAACCATGAGCTCATATGATCCACGCACGGCCAA and CGGATTTACACCCCTATATGATCCACGCACGGCCAA) and Omnisci RT-PCR (Qiagen). β-actin RT-PCR was done as the control using the primers TCGACACAGGCTCCGGCAGT and TCGGGTACAGCAGGATG.

Generation of papillomavirus pseudoviruses PV-CEA, PV-CEAF, and PV-IL-2. Bovine papillomavirus-1 (BPV-1) VLPs were generated using the recombinant baculovirus expression system and purified as described before (39). After dialysis against 10 mmol/L HEPES solution, VLPs (40 µg) were incubated at the condition of 25 mmol/L Tris-HCl (pH 8.0), 15 mmol/L NaCl, 10 mmol/L EGTA, and 20 mmol/L DTI in a final volume of 200 µL at room temperature for 60 minutes. Under this condition, the VLPs were completely disrupted. Then, 1 µg/µL plasmid pCI-CEA or pCI-CEAF (20 µL) was added and the mixture was diluted by 220 µL reassembly buffer containing 25 mmol/L CaCl2 and 20% DMSO to form VLPs at room temperature for 1 hour. To determine the efficiency of plasmid DNA encapsidation in the VLPs, PV-CEA or PV-CEAFV pseudovirus preparation (100 µL) was treated with 80 units Benzonase (Sigma) for 1 hour at 20°C to digest the DNA outside of VLPs. The sample was then heated at 100°C for 10 minutes to inactivate Benzonase and digested with proteinase K (1 mg/mL) at 55°C for 3 hours. The remaining plasmid DNA was extracted by phenol/chloroform. The amount of plasmid DNA was determined by spectrophotometry and ethidium bromide fluorescent quantitation. As a control, PV-CEA or PV-CEAFV pseudovirus preparation (100 µL) without Benzonase digestion was treated in the same way to extract the DNA to estimate the loss of DNA during the extraction. According to the DNA amounts before and after the extraction in the control without Benzonase treatment and the DNA amount after the extraction for the sample treated with Benzonase, we calculated the amount of the DNA packaged inside of VLPs. The amount of the plasmid DNA package inside of VLPs was used to determine the copy numbers of the plasmid DNA. Because one VLP can package only one plasmid (40), we concluded that the copy number of plasmid DNA was equal to the number of PV-CEA or PV-CEAFV pseudovirions. Papillomavirus interleukin (IL)-2 pseudoviruses (PV-IL-2) were generated in the same way using the plasmid DNA pCDNA/puriLL-2 encoding human IL-2 (41).

Immunizations and tumor challenge. Six- to 8-week-old mice were used for the experiments. For oral immunization, mice were immunized twice at a 2-week interval by oral gavage with either 20 µg BPV VLPs or 0.8 × 1011 PV-CEA pseudovirions generated from 20 µg BPV VLPs. For tumor challenge experiments, 1 week after the second immunization, 1 × 106 RMA-CEAFV cells were s.c. injected in the right flank. Tumor size was measured every 2 days as a tumor area (mm2) with the largest perpendicular diameters. For s.c. vaccinations, the same dose and immunization interval were used as oral immunizations but by s.c. injection into the lower right flank. For co-administration of PV-IL-2 with PV-CEA orally, 12-month-old mice were given both 0.8 × 1011 PV-CEA generated from 20 µg BPV VLPs and 0.8 × 1011 PV-IL-2 generated from 20 µg BPV VLPs; as the controls, we used 40 µg BPV VLPs or 0.8 × 1011 PV-CEA generated from 20 µg BPV VLPs plus 20 µg BPV VLPs. Ten days after the second immunization, mice were sacrificed for the subsequent experiments.

Peptide synthesis. CEAS25-33 (EAQNTYTL, peptide 1), CEAS5-363 (LWVVNNQSL, peptide 2), CEAS90-108 (IYIPNASL, peptide 3), CEAS167-355 (PEIQNTYTL, peptide 4), CEAS171-279 (CGIQNVSZA, peptide 5), CAP-1 (YLSGANL), LCMV Gp33 peptide (KAVYNFLATC), and HIV-1 gp16020-128 peptide (KLTPLCVTL) were synthesized (>95% pure) in BioSynthesis (Lewisville, TX) or Sigma-Genosys (The Woodlands, TX). All peptides were dissolved in DMSO to a stock concentration of 5 mg/mL and stored at −70°C.

Isolation of lymphocytes from spleen and Peyer’s patches. To isolate splenocytes, mouse spleen was removed and homogenized. RBGs were lysed by ACK lysis buffer. The single-cell suspension was collected by passing splenocytes through 70 µm cell strainers (BD Discovery Labware, Bedford, MA). Then, the cell suspension was incubated in nylon-wool columns (Polysciences, Warrington, PA) for 1 hour at 37°C. The enriched T cells were collected by washing cells through the columns with complete RPMI 1640. To isolate lymphocytes from Peyer’s patches, small intestines were flushed with ice-cold PBS to remove fecal contents and visible Peyer’s patches were

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Papillomavirus-CEA Pseudoviruses as an Oral Vaccine

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harvested. After homogenization, Peyer's patch cells were passed through 70 μm cell strainers and lymphocytes were isolated by Ficoll gradient separation (Amersham, Piscataway, NJ). There were ~30% of CD8+ T cells and 50% of CD4+ T cells in T-cell-enriched splenocytes used for the subsequent experiments; there were ~5% of CD8+ T cells and 15% of CD4+ T cells in Peyer's patch lymphocytes. There was no significant difference in cell composition for splenocytes and Peyer's patch lymphocytes from different treatment groups (flow cytometry data not shown).

**In vitro cytotoxicity assay.** The cytotoxicity was measured by a standard 6-hour 51Cr release assay. To determine the mucosal CTL response, Peyer's patch lymphocytes were pooled from four mice per group and were directly used as effector cells for the assay. To determine the systemic CTL response, splenocytes enriched for T cells from each individual mouse were in vitro stimulated for 5 days and then used as effector cells for the assay. Splenocytes enriched for T cells were seeded into 96-well U-bottomed plates (BD Discovery Labware) at 5 × 10^4 cells per well in 200 μL complete RPMI 1640 with 5 μg/mL CEA peptides and 5% T-stim without concanavalin A (BD Discovery Labware). Irradiated (5,000 rad) splenocytes (1 × 10^5 cells per well) from naive mice were used as feeder cells for in vitro stimulation. RMA cells (1 × 10^4) were used as target cells by incubating at 37°C with 200 μM sodium chromate (Perkin-Elmer, Boston, MA) for 1 hour. After washing thrice, target cells were resuspended in complete RPMI 1640 with 5 μg/mL CEA peptides and incubated for 30 minutes. Target cells were seeded into 96-well V-bottomed plates (Nunc, Rochester, NY) at 2 × 10^4 cells per well in 100 μL complete RPMI 1640. Effector cells from Peyer's patches or spleen were seeded into triplicate wells containing the target cells at various effector/target (E:T) cell ratios, making a final volume of 200 μL. Plates were incubated at 37°C in a humidified incubator with 5% (v/v) CO2 for 6 hours. Plates were centrifuged, and the supernatant (100 μL) was removed from each well to assess the 51Cr release using a gamma radiation counter (Perkin-Elmer). The percentage of specific lysis (%) was calculated as described previously (40).

**IFN-γ enzyme-linked ImmunoSPOT assay.** An enzyme-linked ImmunoSPOT (ELISPOT) assay was used to detect CEA-specific, INF-γ-secreting cells after stimulation with the synthetic CEA peptides. Multiscreen 96-well-plates (Millipore, Bedford, MA) were coated with 100 μL/well of 5 μg/mL anti-IFN-γ capture antibody (PharMingen, San Diego, CA) in PBS at 4°C overnight. Plates were washed once with 200 μL/well complete RPMI 1640 and blocked with 200 μL/well complete RPMI 1640 at room temperature for 2 hours. Splenocytes enriched for T cells and Peyer's patch lymphocytes were added starting at 1 × 10^6 or 1 × 10^5 cells, respectively, per well in triplicate wells with 1/3 serial dilutions. Irradiated splenocytes (1 × 10^6 cells per well) from naive mice were used as the feeder cells. Cells were cultured in complete RPMI 1640 containing 50 units IL-2 (R&D Systems, Minneapolis, MN) and 5 μg/mL CEA peptides. LCMV-specific, D0-restricted peptide Gp33-41 was used as the control for D0 expression, the RMA cell line was transfected with pCI-CEA and pCI-CEAFL. Then, the truncated or full-length CEA fragment was inserted into a mammalian cell expression vector pCI-neo to construct plasmid pCI-CEA or pCI-CEAFL. To test the CEA protein expression, the RMA cell line was transfected with pCI-CEA or pCI-CEAFL to generate RMA-CEA or RMA-CEAFL cells. As shown in Fig. 1A, an 180-kDa CEA protein was detected in RMA-CEAFL cells but not in RMA-CEA cells. By immunohistochemistry staining (data not shown), the cell surface CEA expression was detected in RMA-CEAFL cells but not in RMA-CEA cells. Because the CEA polypeptide without NH2-terminal signal peptide should not be glycosylated without entering ER, we expected that the CEA protein in RMA-CEA cells would be ~80 kDa in molecular weight as reported previously (47). To confirm the truncated CEA polypeptide expression, pCR II-CEA was used for the in vitro transcription and translation assay with the rabbit reticulocyte lysate system. An ~80-kDa protein was detected after the in vitro transcription and translation using pCR II-CEA (Fig. 1B). Furthermore, the truncated CEA expression was detected in RMA-CEA cells by RT-PCR (Fig. 1C) and Western blotting analysis (Fig. 1D). The molecular weight of the CEA protein expressed in RMA-CEA cells was ~80 kDa.

**Generation of papillomavirus pseudoviruses PV-CEA and PV-CEAFL and comparison of immunogenicities of PV-CEA and PV-CEAFL.** BPV-1 VLPs were produced in Spodoptera frugiperda (Sf9) insect cells using a recombinant baculovirus encoding BPV-1 L1. BPV-1 VLPs were isolated and purified as we described previously (39). After dialysis, VLPs were disrupted in the buffer containing EGTA and DTT. The complete disruption of VLPs
was confirmed by electronic microscopy as reported previously (39, 40). Plasmid pCI-CEA or pCI-CEAFL was added and the mixture was diluted by the reassembly buffer containing 25 mmol/L CaCl₂ and 20% DMSO to form VLPs. Thus, L1 proteins were reassembled into VLPs and the plasmid DNA pCI-CEA or pCI-CEAFL was packaged inside of VLPs. The reassembled VLPs were confirmed by electronic microscopy as reported previously (39, 40).

To confirm that the plasmid DNA was packaged inside of VLPs, the PV-CEA or PV-CEAFL pseudovirus preparation was treated with Benzonase to digest the DNA outside of VLPs. The plasmid DNA packaged inside of VLPs would not be digested by Benzonase. The sample then was heated to inactivate Benzonase and treated with proteinase K and SDS to digest the VLPs. The remaining plasmid DNA was extracted by phenol/chloroform and detected by running agarose gel and staining with ethidium bromide (39, 40). Because the PV-CEA and PV-CEAFL pseudoviruses are replication defective without the papillomavirus genome, we cannot determine the pseudovirus titers by traditional assays. Considering one VLP can package only one plasmid (40), we conclude that the copy number of the plasmid DNA inside of VLPs is equal to the number of pseudovirions. Therefore, we estimated the titers of PV-CEA and PV-CEAFL pseudoviruses as described in Materials and Methods.

When CEA was delivered in the context of viral forms, we expected peripheral immunologic tolerance to CEA in CEA-Tg mice would be broken as seen by other viral and bacterial vector-based CEA vaccines. To compare the immunogenicities of PV-CEA and PV-CEAFL pseudoviruses as described in Materials and Methods.

Figure 1. Construction of expression plasmid pCI-CEA encoding human CEA without NH₂-terminal signal peptide and plasmid pCI-CEAFL encoding the full-length CEA polypeptide with NH₂-terminal signal peptide. A, soluble cell lysates of RMA, RMA-CEA, and RMA-CEAFL cells in the lysis buffer [1% Triton X-100, 20 mmol/L Tris-HCl (pH 7.6), 5 mmol/L EDTA] were separated by 8% SDS-PAGE and 1:1,000 diluted mouse anti-CEA antibody was used for the Western blotting assay. An ~180-kDa CEA protein was detected in RMA-CEAFL cells but not in RMA-CEA cells. B, the CEA fragment without a signal sequence was cloned into TA vector pCR II-TOPO to generate pCR II-CEA in which CEA is downstream of SP6 promoter. We use the plasmid pCR II-CEA to determine the CEA protein expression in vitro. The plasmid with the CEA fragment in the reverse orientation was used as the negative control. In TNT lysate reaction in the presence of rabbit reticulocyte lysate, SP6 RNA polymerase, [³⁵S]methionine was used to label the newly synthesized protein from the DNA templates. ³⁵S-labeled protein was detected by exposure to the X-ray film after separation by 8% SDS-PAGE. An ~80-kDa CEA protein was detected. C, RNA was extracted from RMA-CEA cells and RT-PCR was done to determine the mRNA expression of the truncated CEA. RNA cells transfected with empty vector (RMA-neo) were used as the negative control. The plasmid pCI-CEA was used as the positive control for PCR, β-actin RT-PCR was done as the control. D, soluble cell lysates of RMA-neo and RMA-CEA were separated by 8% SDS-PAGE and 1:500 diluted mouse anti-CEA antibody was used for the Western blotting assay. An ~80 kDa CEA protein was detected in RMA-CEA cells. E, both PV-CEA and PV-CEAFL induced systemic CEA-specific CTL response in CEA-Tg mice by s.c. immunizations. Splenocytes isolated from CEA-Tg mice immunized with control VLPs (squares), PV-CEA (diamonds), or PV-CEAFL (triangles) were in vitro stimulated for 5 days with peptide 1 and used as effector cells for the ⁵¹Cr release assay at the different E:T ratios. After peptide 1 stimulation, effort cells were used to lyse target cells pulsed with peptide 1 (filled symbols) or no peptide (open symbols). Splenocytes isolated from individual mice were used for the experiments. Representative of six mice per group in two sets of experiments. P1-specific killing in both PV-CEA and PV-CEAFL groups was significantly higher than that in the control VLPs group (P < 0.05). The difference between PV-CEA and PV-CEAFL was not statistically significant.
PV-CEAFL, CEA-Tg mice were s.c. immunized twice at a 2-week interval with 20 μg BPV VLPs or ~0.8 × 10³ PV-CEA or PV-CEAFL pseudovirions generated from 20 μg BPV VLPs. Ten days after the second immunization, splenocytes were isolated to test systemic CEA-specific CTL responses. CEA₅₂₆₋₅₃₃ is a known CEA-specific murine CTL epitope capable of inducing a CD₈⁺ CTL response restricted by murine Dᵇ MHC class I molecules (9, 10, 48). We used this known epitope (peptide 1) to test CEA-specific, Dᵇ-restricted CTL response. As shown in Fig. 1E, both PV-CEA and PV-CEAFL induced systemic CEA-specific CTL response in CEA-Tg mice by s.c. immunizations. It suggests that PV-CEA and PV-CEAFL pseudoviruses can break the self-antigen tolerance to CEA. However, there was no significant difference in eliciting CEA-specific CTL response between PV-CEA and PV-CEAFL. We chose PV-CEA for the subsequent oral immunization experiments.

Carcinoembryonic antigen–specific CTL and antibody responses in carcinoembryonic antigen transgenic mice after oral immunization with PV-CEA pseudoviruses and antitumor immunity after tumor challenge. Previously, we showed that papillomavirus pseudoviruses encoding a target antigen pseudoinfected mucosal and systemic lymphoid tissues and induced mucosal and systemic CTL responses specific for the target antigen after oral immunization (39). Therefore, we hypothesized that PV-CEA pseudoviruses encoding human CEA could be used as an oral vaccine to induce mucosal and systemic CTL responses against CEA. To determine the immunogenicity of PV-CEA by oral immunization in CEA-Tg mice, mice were immunized twice at a 2-week interval by oral gavage with 20 μg BPV VLPs or ~0.8 × 10¹¹ PV-CEA pseudovirions generated from 20 μg BPV VLPs. Ten days after the second immunization, mice were sacrificed and used to test CEA-specific CTL responses. If PV-CEA pseudoviruses induce mucosal and systemic CEA-specific CTL responses, we expect that CEA-specific CTLs induced in the Peyer’s patches and spleen can lyse the H-2ᵇ target cells pulsed with H-2ᵇ-restricted, CEA-specific epitope peptides.

In addition to CEA₅₂₆₋₅₃₃ (peptide 1), we searched for more Dᵇ-restricted CEA-specific CTL epitopes in this study. The amino acid sequence of human CEA (Genbank accession no. M17303) was scanned to predict Dᵇ-restricted CTL epitopes (nonamers) by using Peptide Binding Predictions (http://bimas.dcr.tni.nih.gov) and Peptide Predictions (www.syfpeithi.de). We combined the search results by two different methods and tested the following peptides as the CEA-specific, Dᵇ-restricted CTL epitope candidates: CEA₅₅₅₋₅₆₃ (peptide 2), CEA₁₀₀₋₁₀₈ (peptide 3), CEA₃₄₇₋₃₅₅ (peptide 4), and CEA₅₇₁₋₅₇₉ (peptide 5).

To test mucosal and systemic CEA-specific CTL responses in CEA-Tg mice, the lymphocytes isolated from Peyer’s patches and spleen, respectively, were used for the in vitro ⁵¹Cr release assay.

**Figure 2.** Mucosal CEA-specific cytolytic activity in CEA-Tg mice after oral immunization with PV-CEA pseudoviruses. Peyer’s patch lymphocytes freshly isolated from CEA-Tg mice immunized with PV-CEA (❒) or VLPs (▲) were pooled and directly used as the effector cells at different E:T ratios for the 6-hour in vitro ⁵¹Cr release assay. RMA cells pulsed with peptides 1 to 5, respectively, were used as target cells. All experiments were done thrice. Representative results.
Peyer’s patch lymphocytes were pooled from the mice (four mice per group) and were directly used as effector cells for the $^{51}$Cr release assay. As shown in Fig. 2, Peyer’s patch lymphocytes isolated from mice orally immunized with PV-CEA pseudoviruses lysed RMA target cells pulsed with peptide 1 (CEA526-533) and peptide 5 (CEA571-579). In contrast, Peyer’s patch lymphocytes from the control mice orally immunized with VLPs did not. RMA target cells pulsed with peptides 2 to 4 were not lysed by Peyer’s patch lymphocytes from mice orally immunized with PV-CEA pseudoviruses. Our data showed that oral immunization of PV-CEA induced mucosal CEA526-533-specific and CEA571-579-specific CTL responses in CEA-Tg mice. It indicates that CEA571-579 is a H-2b-restricted, CEA-specific CTL epitope.

We found that splenocytes from mice orally immunized with PV-CEA lysed RMA target cells pulsed with peptide 1 (CEA526-533) or peptide 5 (CEA571-579) after in vitro stimulation with peptide 1 or 5, respectively (Fig. 3A). However, splenocytes stimulated with other peptides did not (data not shown). It suggests that oral immunization with PV-CEA pseudoviruses also induces systemic CEA-specific CTL response in CEA-Tg mice. Therefore, PV-CEA pseudoviruses can induce both mucosal and systemic CEA-specific CTL responses in CEA-Tg mice by oral immunization. Our data also suggest that peptide 5 (CEA571-579) is a novel H-2$^d$ restricted, CEA-specific CTL epitope.

We also used the IFN-γ ELISPOT assay to determine CEA-specific IFN-γ-secreting cells in Peyer’s patches and spleen in CEA-Tg mice after oral immunization with VLPs or PV-CEA pseudoviruses. Using CEA-specific CTL epitope peptides to stimulate lymphocytes, we expected responder cells secreting INF-γ should be CEA epitope-specific CTLs. With or without exogenous D$^b$-restricted, CEA-specific epitope peptide stimulation, there were significantly more IFN-γ-secreting cells in the Peyer’s patch lymphocytes from CEA-Tg mice orally immunized with PV-CEA compared with the control mice immunized with VLPs (data not shown). For the splenocytes, we also found that there were more IFN-γ-secreting cells in mice immunized with PV-CEA with or without exogenous CEA-specific CTL epitope peptides (data not shown). We reasoned that endogenous CEA from CEA-Tg mice were processed and presented by CEA$^+$ cells or APCs, which stimulated CEA-specific CD4$^+$ and CD8$^+$ T cells to secret IFN-γ during in vitro culture, even without adding exogenous D$^b$-restricted, CEA-specific epitope peptides. Actually, CEA-Tg mice even have a high level of CEA in the serum besides the CEA expression on the normal intestinal epithelial cells (9).

We did not expect that oral immunization of PV-CEA could induce a significant level of anti-CEA antibody response because the truncated CEA polypeptide without NH2-terminal signal peptide was not expressed on the cell surface and the protein was not stable. As shown in Fig. 3B, no anti-CEA IgG/IgM levels in sera (left) or IgA levels in intestinal washings (right) were detected in CEA-Tg mice orally immunized with BPV VLPs or PV-CEA. However, as we expected, there were anti-CEA antibodies in both sera and intestinal washings in CEA-Tg mice orally immunized with PV-CEAFL, because the full-length CEA was expressed on the cell surface.

Oral immunization of PV-CEA induced CEA-specific CTL response but no antibody responses. It was of interest to determine whether oral immunization with PV-CEA provides antitumor effect against a challenge with CEA$^+$ tumors. We compared the growth of CEA$^+$ tumor cells in CEA-Tg mice orally immunized with PV-CEA...
or control BPV VLPs. One week after the second immunization, 1 x 10^6 RMA-CEAFL cells were s.c. injected into mice. As shown in Fig. 4, the tumor growth rates were significantly delayed in CEA-Tg mice orally immunized with PV-CEA when compared with mice treated with the control BPV VLPs (P < 0.05).

The truncated carcinoembryonic antigen delivered by PV-CEA pseudoviruses was immunogenic in the context of human MHC HLA-A2. To develop a human vaccine, our ultimate goal is to induce human CEA-specific T-cell responses. Several human CEA-specific, A2-restricted epitopes have been identified. Therefore, we wanted to determine whether CEA-specific antigenic epitopes can be presented on human MHC class I molecules and induce CEA-specific human epitope-specific CTL responses in an animal model. A2-Tg mice express both murine H-2^b molecules and transgenic human HLA-A2 molecules, which provides a critical preclinical screening model for a human vaccine. Additionally, PV-CEA pseudoviruses encode a truncated CEA without the signal peptide, which alters the CEA protein synthesis, modification, degradation, and antigen processing pathways. Therefore, it is important to determine whether the truncated CEA can be processed to present human A2-restricted epitopes on A2 molecules. CAP-1 is a HLA-A2-restricted CEA-specific CTL epitope (49). Thus, we used CAP-1 to determine mucosal and systemic A2-restricted CTL responses against CEA in A2-Tg mice after oral immunization with PV-CEA pseudoviruses.

To determine whether CAP-1-specific CTLs were induced in A2-Tg mice orally immunized with PV-CEA pseudoviruses, we did the in vitro 51Cr release assay for the cytolytic activity of CTLs using lymphocytes isolated from Peyer's patches and spleen as effector cells (Fig. 5A). We found that CAP-1-specific cytolytic activity was only marginally above the background for the Peyer's patch lymphocytes from A2-Tg mice orally immunized with PV-CEA pseudoviruses compared with the controls. However, CAP-1-specific cytolytic activity was strong for the splenocytes from A2-Tg mice orally immunized with PV-CEA pseudoviruses after in vitro stimulation for 5 days with CAP-1. In contrast, there were no CAP-1-specific cytolytic activities for the lymphocytes of Peyer's patches and spleen from the mice orally immunized with VLPs.

Furthermore, Peyer's patch lymphocytes and splenocytes isolated from A2-Tg mice immunized with VLPs or PV-CEA were used as responder cells for the IFN-γ ELISPOT assays. CAP-1 was used for in vitro stimulation for 40 hours and A2-restricted HIV-1 gp160120-128 peptide was used as the control. The results (Fig. 5B) showed that there were more A2-restricted, CAP-1-specific, INF-γ-secreting cells for lymphocytes obtained from both Peyer's patches and spleen in mice orally immunized with PV-CEA than those from mice orally immunized with VLPs. After in vitro stimulation with the control HIV peptide, there were only the background levels of INF-γ-secreting cells for both Peyer's patches and spleen.

The combination of the ELISPOT and cytotoxicity assay data showed that oral immunization with PV-CEA pseudoviruses induced mucosal and systemic A2-restricted CAP-1-specific CTL responses in A2-Tg mice. However, the cytolytic activity of mucosal Peyer's patch lymphocytes was only at a marginal level in the 51Cr release assay without in vitro stimulation to amplify CAP-1-specific CTLs.

The immunogenicity of PV-CEA pseudoviruses in aged carcinoembryonic antigen/A2 double transgenic mice. Many patients with CEA+ colorectal tumors are elderly individuals. Therefore, it is important to test whether PV-CEA pseudoviruses are immunogenic in aged CEA/A2 double transgenic mice, which provides a more clinically relevant model. CEA/A2 double transgenic mice express human CEA and carry both murine H-2^b and human HLA-A2 molecules (data not shown). There were neither H-2^b-restricted nor A2-restricted, CEA-specific CTL responses to endogenous CEA in 6- to 8-week-old CEA/A2 double transgenic mice (data not shown), which suggests a peripheral CD8+ T-cell tolerance to CEA (data not shown). Previously, we showed that aged mice did not respond well to immunization with papillomavirus pseudoviruses, which was most likely due to age-related reduction of IL-2 production by T cells (41). Delivery of IL-2 by papillomavirus pseudoviruses encoding IL-2 restored the immune responses to papillomavirus pseudovirus vaccinations (41).

We defined mice >1 year old as aged mice. Aged mice were orally immunized twice with PV-CEA pseudoviruses, PV-CEA pseudoviruses plus PV-IL-2 pseudoviruses, or VLPs alone. Ten days after the second immunization, mice were sacrificed and splenocytes were isolated to determine whether PV-CEA pseudoviruses were immunogenic in aged mice and whether coadministration of PV-IL-2 would enhance CEA-specific CTL response induced by PV-CEA pseudoviruses (Fig. 6). We found that there was only a marginal level of CAP-1-specific CTL response for the splenocytes from aged CEA/A2 double transgenic mice orally immunized with PV-CEA pseudoviruses, and coadministration of PV-IL-2 pseudoviruses with PV-CEA pseudoviruses enhanced CAP-1-specific CTL response for the splenocytes from aged CEA/A2 double transgenic mice.

**Discussion**

The rationale for a therapeutic tumor vaccine is to induce and maintain tumor-specific CTLs capable of eliminating tumor cells. CEA is a tumor-associated antigen expressed at a high level in the majority of colorectal tumor cells but at a very low level in the normal intestinal epithelia. Therefore, CEA is used as a target antigen for the development of a therapeutic vaccine against CEA+ colorectal tumors. CEA+ colorectal tumors are mainly located on the gastrointestinal mucosal surface. Ideally, CEA-specific CTLs induced by a CEA vaccine should be able to localize in the local mucosal area and efficiently lyse neighboring CEA+ tumor cells.
In addition, the vaccine has to break the tolerance to CEA as a self-antigen. Considering systemic immunization in general does not induce mucosal CTLs (50), we delivered plasmid DNA encoding CEA in the context of mucosatropic papillomavirus VLPs (PV-CEA pseudovirus) to mucosal lymphoid tissues by oral immunization. We have shown that oral immunization with PV-CEA induced both mucosal and systemic CTLs in CEA-Tg mice, indicating that PV-CEA pseudoviruses can break the peripheral self-tolerance to CEA. We believe that APC in Peyer’s patches and other lymphoid tissues can take up PV-CEA pseudoviruses and the encoded CEA is expressed by these APCs, which induce both mucosal and systemic CEA-specific CTLs. We also believe that the VLPs render APCs more immunogenic so that the tolerance to CEA is overcome in CEA-Tg mice. To our knowledge, this is the first report directly showing the CEA-specific CTL response in the mucosa after oral immunization with an oral CEA vaccine.

In this study, we identified a novel H-2b-restricted, CEA-specific CTL epitope CEA526-533 after screening four predicted D1-restricted CTL epitope candidates for CEA. This new epitope is similar to the known epitope CEA526-533 in that the extent of the CTL responses specific for them is similar. Although PV-CEA induced CTLs that lysed RMA target cells pulsed with these two epitope peptides, neither RMA-CEA nor RMA-CEAFL cells expressing CEA were lysed when used as target cells (data not shown). The data were similar to the observation that a H-2 D1-restricted CD8+ CTL line specific for CEA526-533 peptide efficiently lysed peptide-pulsed targets but not CEA-expressing tumor cells (48). These two epitope-specific CTLs might be low-avidity cytotoxic T cells, which are able to lyse target cells directly pulsed with peptides but not tumor cells expressing CEA (48). In an in vivo tumor challenge model, we showed that oral immunization with PV-CEA could delay the tumor growth rate after CEA+ tumor challenge. It suggests that CEA-specific CD8+ CTLs induced by PV-CEA can kill CEA+ tumors in vivo and provide antitumor effect against CEA+ tumors. However, no CEA-Tg mice orally immunized with PV-CEA completely reject tumors. It might be due to a very high dose of tumor cells used for the challenge. The multiple immunizations and/or the coadministration of effective vaccine adjuvants might be required to enhance immune responses for successful tumor prevention.

The IFN-γ ELISPOT assay was used for the functional detection of T-cell activation. CEA-specific epitope peptides were added to stimulate T cells. However, we found the endogenous CEA expressed in CEA-Tg mice interfered with the results of addition of exogenous CEA-specific CTL peptides in the ELISPOT assay. In that case, IFN-γ-secreting cells should include both CEA-specific CD4+ and CD8+ T cells. In Zhou et al.’s ELISPOT assays (51), there

Figure 5. Mucosal and systemic CEA-specific CTL response in HLA-A2-Tg mice after oral immunization with PV-CEA pseudoviruses. A, mucosal and systemic CEA-specific cytolytic activity: Peyer’s patch lymphocytes and splenocytes were isolated from CEA-Tg mice immunized with PV-CEA (squares) or VLPs (diamonds). Left, freshly isolated Peyer’s patch lymphocytes were pooled in each group and directly used as effector cells to lyse EL4-A2K target cells pulsed with CAP-1 (filled symbols) or the control HIV peptide (open symbols). Representative of two different experiments. Right, splenocytes were in vitro stimulated with CAP-1 for 5 days and then used as the effector cells. EL4-A2K cells pulsed with CAP-1 (filled symbols) or the HIV peptide (open symbols) were used as the target cells. Splenocytes isolated from individual mice were used for the experiments. Representative of >12 mice per group in three sets of experiments. B, mucosal and systemic HLA-A2-restricted, CAP-1-specific, IFN-γ-secreting cells were detected by the IFN-γ ELISPOT assay. Peyer’s patch lymphocytes (left) and splenocytes (right) were isolated from CEA-Tg mice orally immunized with PV-CEA (filled column) or VLPs (open column) and used as the responder cells. The splenocytes isolated from naïve mice were used as the feeder cells in the presence of CAP-1 or the control HIV peptide. Left, numbers of IFN-γ-secreting cells in 1 x 10^5 Peyer’s patch lymphocytes pooled from four mice per group. The difference between VLPs and PV-CEA is significant in response to CAP-1 stimulation (P < 0.05). Right, numbers of IFN-γ-secreting cells in 1 x 10^5 splenocytes from individual mice. The difference between VLPs and PV-CEA is significant in response to CAP-1 stimulation (P < 0.05). All experiments were done thrice. Representative results.
immunization with PV-CEA in A2-Tg mice induced CAP-1-monitor CEA-specific CTLs. Double transgenic mice, we did not use the IFN-CEA in CEA-Tg mice. In the subsequent experiments using CEA/A2 for one particular epitope very well in the presence of endogenous experiments.

Figure 6. Coadministration of PV-IL-2 enhances the CEA-specific CTL response in aged CEA/A2 double transgenic mice after oral immunization with PV-CEA pseudoviruses. Twelve-month-old CEA/A2 double transgenic mice were orally immunized with VLPs (diamonds), PV-CEA (squares), or PV-CEA plus PV-IL-2 (triangles). The splenocytes isolated from immunized mice were in vitro stimulated with CAP-1 peptide for 5 days and used as the effector cells for the in vitro 51Cr release assay. Top, EL4-A2Kb cells pulsed with the control HIV peptide were used as the target cells; bottom, EL4-A2Kb cells pulsed with CAP-1 were used as the target cells. Representative of two different experiments.

were a very high number of IFN-γ-secreting cells in CEA-Tg mice vaccinated with CEA vaccines even without CEA peptide stimulation when compared with the control vaccination groups. It was similar to our findings in CEA-Tg mice. Therefore, the IFN-γ ELISPOT assay cannot be used to indicate the CD8+ CTLs specific for one particular epitope very well in the presence of endogenous CEA in CEA-Tg mice. In the subsequent experiments using CEA/A2 double transgenic mice, we did not use the IFN-γ ELISPOT assay to monitor CEA-specific CTLs.

CAP-1 is an A2-restricted CEA-specific CTL epitope. Oral immunization with PV-CEA in A2-Tg mice induced CAP-1-specific cytolytic activity among splenocytes after in vitro stimulation with CAP-1 peptide for 5 days. However, in mice orally immunized with PV-CEA pseudoviruses, no significant CAP-1-specific cytolytic activity was detected among lymphocytes freshly isolated from Peyer's patches without in vitro CAP-1 stimulation. Mice and humans harbor different T-cell receptor repertoires. CAP-1 might not be an immunodominant CTL epitope of CEA in A2-Tg mice. Therefore, not enough CAP-1-specific CTLs were induced by immunization. Without in vitro amplification of CAP-1-specific CTLs by CAP-1 stimulation, no significant cytolytic activity can be detected. We further used the IFN-γ ELISPOT assay to determine the number of CAP-1-specific, IFN-γ-secreting cells in Peyer's patch lymphocytes and splenocytes after in vitro stimulation with CAP-1 for 40 hours. There were more CAP-1-specific, IFN-γ-secreting cells in Peyer's patches and spleen from mice orally immunized with PV-CEA pseudoviruses compared with the control mice immunized with VLPs. Our 51Cr release assay data combined with the ELISPOT data suggest that oral immunization with PV-CEA pseudoviruses can induce mucosal and systemic CAP-1-specific CTL response in A2-Tg mice.

Finally, we used a more clinically relevant animal model, aged CEA/A2 double transgenic mice, to test our human CEA vaccine. PV-CEA pseudoviruses induced only a marginal level of CAP-1-specific cytolytic activity in aged CEA/A2 double transgenic mice by oral immunization. Coadministration of PV-IL-2 pseudoviruses enhanced CAP-1-specific CTL response to PV-CEA pseudovirus vaccine. IL-2 production is deficient in CD4+ T cells in aged mice (52, 53). It was reported that combination with IL-2 improved the immunotherapy of a recombinant CEA vaccinia vaccine even in young mice (11, 54). Our data suggest that oral immunization with PV-CEA pseudovirus vaccine can induce HLA-A2-restricted, CEA-specific CTL response in CEA/A2 double transgenic mice. It implies that PV-CEA pseudovirus vaccine is a promising oral CEA tumor vaccine for humans. Coadministration of PV-IL-2 pseudoviruses might enhance the effect of PV-CEA pseudovirus vaccine in the elderly who do not respond well to vaccinations.

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