Advances in Brief

Immune Response to a Carcinoembryonic Antigen Polynucleotide Vaccine

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Abstract

We have constructed a DNA plasmid encoding the full length complementary DNA for human carcinoembryonic antigen (CEA) driven by the cytomegalovirus early promoter/enhancer (plasmid DNA encoding human CEA) and demonstrated that this plasmid can function as a polynucleotide vaccine. This polynucleotide vaccine induced humoral and/or cellular immune responses specific for human CEA in all 5 immunized mice. Lymphoblastic transformation data with the use of enriched T-cell populations detected the presence of CEA-specific memory T-cells in 3 of 5 mice. Lymphocytes from 2 of 5 mice had interleukin 2/interleukin 4 release in response to CEA. CEA specificity was confirmed by the absence of reactivity to a control antigen and lack of CEA reactivity among mice vaccinated with a control plasmid encoding chloramphenicol acetyltransferase. Four of 5 mice vaccinated with plasmid DNA encoding human CEA demonstrated anti-CEA antibody responses. This immune response compared favorably with a positive control group of mice immunized with vaccinia-CEA by a dose and schedule previously shown to induce immunoprotection and therapy against a human CEA expressing syngeneic murine colon carcinoma model. Studies are ongoing to establish the construct, dose, and schedule to elicit optimal CEA-specific immune response as well as immunoprotection and therapy against human CEA expressing syngeneic murine adenocarcinoma models.

Introduction

CEA2 is probably the most extensively characterized human tumor associated antigen (1, 2). This M, 180,000 glycoprotein was originally thought to be present only in adenocarcinomas and fetal intestine but subsequently was found in small amounts in normal colonic mucosa. A large number of monoclonal antibodies to CEA have allowed extensive epitope mapping which define CEA-specific and cross-reactive epitopes which reside on CEA-like molecules in normal tissues (3). The expression of CEA by adenocarcinoma cells is characteristic of human colon, breast, and non-small cell lung cancer (1, 2).

The cloning of the CEA gene from a human colon tumor cell library was recently described by one of us (4). The 2.4-kilobase cDNA clone contained the complete coding sequence for CEA and has been successfully inserted into a vaccinia virus genome (New York City attenuated strain) (5). This recombinant viral vaccine has been administered to mice (6) and rhesus monkeys (7) with induction of CEA-specific humoral and cellular immune responses as well as immunoprotection and therapeutic effects in a syngeneic CEA-expressing colon carcinoma line (4) in mice. These preclinical observations clearly demonstrate the ability of CEA to function as an effective target for tumor immunity. Currently, clinical trials of the recombinant vaccinia-CEA vaccine are under way.

Interest in alternative mechanisms of achieving immune response to CEA has led us to consider direct cDNA immunization. This novel approach to gene transfer was discovered in 1990 when myofiber cells in the context of infectious diseases as a novel approach to vaccination, hereafter referred to as polynucleotide vaccination. A polynucleotide vaccine encoding influenza A nucleoprotein administered to mice produced influenza nucleoprotein-specific antibodies and cytolytic T-cells as well as protection from subsequent challenge with influenza A virus (9). This vaccine strategy was able to induce neutralizing antibodies to influenza A hemagglutinin in rhesus monkeys (10), and neutralizing antibodies to human immunodeficiency virus type 1 infection in mice (11). The goal of this report is to describe the murine immune response to a polynucleotide vaccine composed of the cDNA for a human tumor-associated antigen (CEA).

Materials and Methods

Plasmid DNAs for Vaccination

An expression plasmid was constructed encoding the human CEA cDNA. The vector utilized was pcDNA3 (Invitrogen), which contains the cytomegalovirus early promoter/enhancer driving expression of the heterologous gene (Fig. 1). As control, we utilized the plasmid DNA pcDNA-CAT (Invitrogen), which contains the chloramphenicol acetyltransferase reporter gene in the pcDNA3 vector. For simplicity, the resulting plasmids were designated pCEA and pCAT. Large scale preparation of the plasmid DNAs was carried out by the procedure of alkaline lysis followed by cesium chloride density gradient centrifugation (12). After extraction with 2-propanol to remove all residual ethidium bromide, the DNA was precipitated in large lots (5 mg) and stored at −70°C as pellets. For experimental use, the DNA was reconstituted in sterile saline at a concentration of 1 mg/ml and stored in aliquots at −20°C for direct use in injection/vaccination protocols. These specific methods were used as published works had noted: (a) DNA of a high level of purity is most efficacious for eliciting an immunological response by the direct injection method, and (b) individual batch-to-batch variations of DNA require the development of sufficient amounts of homogeneous reagent for each distinct experiment (13). The ability of pCEA to direct synthesis and cell surface expression of CEA was demonstrated through transduction of NIH 3T3 fibroblasts by the adenovirus/polylysine conjugate method (14) and immunohistochemical staining with the CEA-specific Col-1 monoclonal antibody (15).

Experimental Design

Groups of 5 mice were vaccinated with pCEA or pCAT as a negative control. Polynucleotide vaccination was performed according to the dose and schedule reported by Ulmer et al. (9) for influenza A nucleoprotein, i.e., 200 μg of DNA i.m. every 3 weeks for 3 injections. Five additional mice were vaccinated with a recombinant vaccinia virus encoding human CEA by scarification every 3 weeks for 3 immunizations to provide a positive control for
Antigen-presenting cells consisted of irradiated (2000 rads) bulk splenic mononuclear cells from naive syngeneic mice added at 5 × 10^6/well. Stimulated wells received purified human CEA over a range of concentrations (1–100 μg/ml); ovalbumin (100 μg/ml) as a negative control antigen; or a positive control mitogen, PHA (5 μg/ml). The range of CEA concentrations described above provided optimal stimulation in our prior studies of recombinant vaccinia-CEA-immunized mice (6). Control wells received cells only. Cells in all wells were cultured in a total volume of 200 μl of complete RPMI with 10% fetal calf serum. After 5 days in culture, cells were pulsed with tritiated thymidine (1 μCi/well) overnight, harvested with a Skatron automatic cell harvester, and incorporated radioactivity was determined by using a β scintillation counter. The mean cpm for quadruplicate wells correlates with the cellular proliferation rate (17). The stimulation ratio was calculated as mean cpm of the stimulated wells divided by mean cpm of the control wells. A positive assay was defined as a stimulation ratio ≥ 2.0.

Lymphokine Release

T-cell responses were examined through biological assessment of IL-2/IL-4 release (20). Briefly, mononuclear cells were cultured exactly as above with the same panel of antigens or mitogen over the same range of concentrations with the exception that after 3 days in culture, cell-free supernatants were harvested and assayed immediately or stored at ~70°C. Supernatant IL-2 and IL-4 activity was quantitated based on their ability to support proliferation of CTLL-2 cells sensitive to both cytokines (20). CTLL-2 cells were maintained in media containing 40% rat growth factor as a cytokine source prepared per American Type Culture Collection recommendations, and cells in log phase growth at least 48 h after addition of fresh rat growth factor were selected for assay. CTLL-2 cells were washed 5 times in phosphate-buffered saline to remove exogenous cytokines. These cells were added at 5000 cells/well in 100 μl of complete RPMI-10% fetal calf serum. Each well also received 100 μl of cell culture supernatant to be assayed for IL-2/IL-4 activity. Following 24 h in culture, cells were pulsed with tritiated thymidine (1 μCi/well) for 8 h, harvested with a Skatron automatic cell harvester, and incorporated radioactivity was determined by using a β scintillation counter. Results were expressed as mean cpm of quadruplicate wells. The stimulation ratio was calculated as mean cpm of wells receiving supernatants from stimulated wells divided by mean cpm of wells receiving supernatants from control wells. A positive assay was defined as a stimulation ratio ≥ 2.0. Standard curves were also generated for each assay by using recombinant mouse IL-2 or IL-4.

Antibody Assay

Anti-human CEA antibody was quantitated by using a double antigen immunoradiometric assay as previously described (21). Briefly, polystyrene beads were coated with purified human CEA (2 μg bead) in PBS, washed 3 times with PBS containing 1% bovine serum albumin, and stored in wash buffer at 4°C until use. Twenty μl of mouse sera (normal control or post-vaccination) were diluted to 100 μl with PBS and incubated with a single coated bead (in duplicate) for 2 h on a laboratory oscillator at room temperature, washed with 4 ml of PBS and incubated with 100 μl of 125I-labeled human CEA (2 μg/ml) for 1 h (approximately 200,000 cpm bead), rewash with PBS, and counted on a Micromedic automatic gamma counter. Background nonspecific binding of 1% of the 125I-CEA was subtracted from cpm bound and the ng of CEA bound to the bead per ml of sera was calculated from the known specific activity of the 125I-CEA. A positive assay (>20 ng/ml) has been defined as exceeding 2 SD above the mean value of 10 normal mouse sera.

Reagents

Purified human CEA was obtained from hepatic metastases of human colonic adenocarcinoma (Vitro Diagnostics, Denver, CO) (6). Ovalbumin and PHA were obtained from Sigma Chemical Co., St. Louis, MO. CTLL-2 and NIH 3T3 cells were obtained from the American Type Culture Collection. Polystyrene beads (6.4 mm) were obtained from Precision Plastic Ball, Chicago, IL.
CARCINOEMBRYONIC ANTIGEN POLYNUCLEOTIDE VACCINE

Table 1 Lymphoblastic transformation response to polynucleotide vaccination with pCEA

<table>
<thead>
<tr>
<th>Antigen (µg/ml)</th>
<th>Mouse 1</th>
<th>Mouse 2</th>
<th>Mouse 3</th>
<th>Mouse 4</th>
<th>Mouse 5</th>
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<tbody>
<tr>
<td>Control</td>
<td>110 ± 10^4</td>
<td>290 ± 30</td>
<td>330 ± 70</td>
<td>320 ± 60</td>
<td>310 ± 50</td>
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<tr>
<td>CEA (100)</td>
<td>970 ± 110</td>
<td>360 ± 70</td>
<td>26,000 ± 3,000</td>
<td>1,200 ± 290</td>
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<td>CEA (30)</td>
<td>920 ± 460</td>
<td>280 ± 70</td>
<td>26,000 ± 2,800</td>
<td>1,600 ± 600</td>
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<td>CEA (10)</td>
<td>200 ± 30</td>
<td>320 ± 60</td>
<td>12,000 ± 1,900</td>
<td>370 ± 80</td>
<td>330 ± 90</td>
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<tr>
<td>CEA (3)</td>
<td>230 ± 30</td>
<td>140 ± 40</td>
<td>6,100 ± 1,600</td>
<td>470 ± 200</td>
<td>260 ± 60</td>
</tr>
<tr>
<td>OVA* (100)</td>
<td>210 ± 50</td>
<td>370 ± 100</td>
<td>280 ± 50</td>
<td>360 ± 120</td>
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<td>PHA</td>
<td>36,000 ± 1,100</td>
<td>34,000 ± 4,600</td>
<td>72,000 ± 8,000</td>
<td>29,000 ± 1,200</td>
<td>39,000 ± 3,800</td>
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</tbody>
</table>

* Mice (5) received 200 µg of pCEA i.m. every 3 weeks for 3 doses.

Results

Lymphoblastic transformation response of the 5 mice receiving the CEA polynucleotide vaccine is listed in Table 1. Mice 1, 3, and 4 demonstrated dose-dependent lymphocyte proliferative responses to human CEA with peak stimulation ratios ranging from 5 to 78. Mice 2 and 5 were negative. All 5 mice failed to respond to ovalbumin included as a control antigen with stimulation ratios ranging from 0.8 to 1.9. Responses to PHA ranged from 91 to 327, indicating that the T-cells from each mouse were not generally impaired.

To control for the nonspecific effects of plasmid DNA injection on CEA-specific immune response, 5 mice were vaccinated with acetyltransferase pCAT according to the same dose and schedule used for pCEA vaccination. The lymphoblastic transformation response of these mice is provided in Table 2. All 5 mice failed to respond to human CEA with stimulation ratios ranging from 0.5 to 1.5. Responses to PHA ranged from 25 to 62, again indicating that the T-cells from each mouse were not generally impaired.

Five mice were vaccinated with recombinant vaccinia-CEA according to the optimized dose and schedule used to induce immunoprotection and therapy against a syngeneic CEA-expressing murine colon carcinoma model (6). These mice were included to provide a positive control for CEA-specific immune response as well as a standard of reference against which polynucleotide vaccination could be compared. Table 3 provides lymphoblastic transformation data from these recombinant vaccinia-CEA vaccinated mice. All 5 mice demonstrated dose-dependent lymphocyte proliferative responses to human CEA with peak stimulation ratios ranging from 2.5 to 12. All 5 mice failed to respond to ovalbumin and responded well to PHA.

Lymphocytes from the five pCEA vaccinated mice were evaluated for lymphokine release to CEA. Mice 3 and 4 demonstrated dose-dependent IL-2/IL-4 release in response to human CEA with stimulation ratios of 25 and 8, respectively. Mouse 1 had a borderline response with a stimulation ratio of 2.7. Mice 2 and 5 were negative for IL-2/IL-4 release in response to CEA but demonstrated appropriate lymphokine release to mitogens.

Anti-CEA antibody response was evaluated in pCEA, pCAT, and recombinant vaccinia-CEA-immunized mice by radiometric assay with results provided in Table 4. Four of 5 mice vaccinated with pCEA demonstrated anti-CEA antibody responses. Mice 2 and 5, which were negative for lymphoblastic transformation (Table 1), had substantial anti-CEA antibody responses, while mouse 4 had no anti-CEA antibody response despite positive lymphoblastic transformation and lymphokine release assays to CEA. All pCAT-vaccinated mice were negative, and all recombinant vaccinia-CEA vaccinated mice were positive for CEA-specific antibody response.

Discussion

We have constructed a DNA plasmid encoding the full length cDNA for human CEA driven by the cytomegalovirus early promoter/enhancer (pCEA) and demonstrated that this plasmid can function as a polynucleotide vaccine when delivered intramuscularly. This polynucleotide vaccine induced humoral and/or cellular immune responses specific for human CEA in all 5 immunized mice. The lymphoblastic transformation data using enriched T-cell populations was able to detect the presence of CEA-specific memory T-cells in 3 of 5 mice. Lymphocytes from 2 of 5 mice had IL-2/IL-4 release in response to CEA. Lymphokine release in response to CEA correlated well with proliferative response as 2 of 3 mice positive for lymphoblastic transformation demonstrated lymphokine release and both mice negative for lymphoblastic transformation lacked evidence of lymphokine release. CEA-specificity was verified by the absence of reactivity to a control antigen (ovalbumin), and lack of CEA reactivity among mice vaccinated with pCAT control plasmid. Among those vaccinated with pCEA, mice 2 and 5 had anti-CEA antibody responses without detectable lymphoblastic transformation to CEA, while mouse 4 demonstrated lymphoblastic transformation to CEA without an antibody response. These observations could be explained by activation of different functional T-helper cell subsets among the mice (22, 23). Mice 2 and 5 with antibody responses in the absence of lymphoblastic transformation may well represent T-helper 2 responses which support

Table 2 Lymphoblastic transformation response to polynucleotide vaccination with pCAT

<table>
<thead>
<tr>
<th>Antigen (µg/ml)</th>
<th>Mouse 1</th>
<th>Mouse 2</th>
<th>Mouse 3</th>
<th>Mouse 4</th>
<th>Mouse 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>390 ± 50^c</td>
<td>260 ± 30</td>
<td>300 ± 50</td>
<td>480 ± 80</td>
<td>320 ± 40</td>
</tr>
<tr>
<td>CEA (100)</td>
<td>310 ± 60</td>
<td>130 ± 20</td>
<td>310 ± 60</td>
<td>530 ± 150</td>
<td>240 ± 40</td>
</tr>
<tr>
<td>CEA (30)</td>
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<td>140 ± 20</td>
<td>280 ± 40</td>
<td>710 ± 240</td>
<td>360 ± 50</td>
</tr>
<tr>
<td>CEA (10)</td>
<td>320 ± 120</td>
<td>180 ± 40</td>
<td>240 ± 40</td>
<td>420 ± 130</td>
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</tr>
<tr>
<td>CEA (3)</td>
<td>470 ± 160</td>
<td>120 ± 20</td>
<td>150 ± 20</td>
<td>520 ± 160</td>
<td>290 ± 50</td>
</tr>
<tr>
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<td>440 ± 90</td>
<td>180 ± 50</td>
<td>110 ± 20</td>
<td>270 ± 40</td>
<td>170 ± 30</td>
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<tr>
<td>PHA</td>
<td>9,600 ± 1,200</td>
<td>16,000 ± 1,900</td>
<td>9,000 ± 1,900</td>
<td>18,000 ± 670</td>
<td>18,000 ± 770</td>
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</table>

* Mice (5) received 200 µg of pCAT i.m. every 3 weeks for 3 doses.

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antibody production rather than cell-mediated immunity (delayed hypersensitivity). Conversely, mouse 4 with a positive lymphoblastic transformation assay in the absence of antibody production may represent a T-helper 1 response favoring cell-mediated immunity over antibody response. Mice 1 and 3 could represent balanced participation by both T-helper 1, and T-helper 2 cells generating CEA-specific lymphoblastic transformation and antibody responses. The variability of immune response among animals may also be due to the technique of injection. For comparison, we examined a known dose and schedule for a CEA vaccine, recombinant vaccinia-CEA (6). This reagent and regimen provide immunoprotection and therapy against a human CEA expressing syngeneic murine colon cancer model. It is encouraging that our pilot study of the polynucleotide vaccine encoding human CEA without optimization generated similar immune response data.

Polynucleotide vaccination offers a number of advantages in the context of tumor vaccination. First, it elicits both antibody and cell-mediated immune responses by using a non-replicating vaccine without adjuvants. Second, intracellular synthesis of the tumor-associated antigen favors major histocompatibility complex class I peptide display and may represent a pivotal role of the generation of cytolytic T-cells (24). Third, gene expression in skeletal muscle following plasmid injection has been detected for up to 19 months post-injection with the foreign antigen favoring major histocompatibility complex class I peptide display that our pilot study of the polynucleotide vaccine encoding human CEA without optimization generated similar immune response data.

Studies are ongoing to establish the construct, dose, and schedule to elicit optimal CEA-specific immune response, including induction of cytolytic T-cells. The ability of CEA polynucleotide vaccination to induce immunoprotection and therapy of human CEA-expressing syngeneic mouse colon and breast carcinoma models is also being examined.

References


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