Durable Carcinoembryonic Antigen (CEA)-Specific Humoral and Cellular Immune Responses in Colorectal Carcinoma Patients Vaccinated with Recombinant CEA and Granulocyte/Macrophage Colony-Stimulating Factor

Gustav J. Ullenhag,1 Jan-Erik Frödin,2 Mahmood Jeddi-Tehrani,2,3 Karin Strigård,4 Emma Eriksson,2 Ali Samanci,2 Aniruddha Choudhury,2 Bo Nilsson,5 Eva D. Rossmann,2 Szilvia Mosolits,2 and Håkan Mellstedt2

1Department of Oncology, Radiology and Clinical Immunology, Section of Oncology, Uppsala University Hospital, Uppsala, Sweden; Department of Oncology and Cancer Centre Karolinska, Karolinska Hospital, Stockholm, Sweden; 2Department of Immunology, Monoclonal Antibody Research Center, Avesina Research Center, Tehran, Iran; 3Department of Surgery, Huddinge University Hospital, SE-141 86 Stockholm, Sweden; 4Unit of Cancer Epidemiology, Institute of Oncology-Pathology, Radiumhemmet, Stockholm, Sweden

ABSTRACT

Purpose: Previous studies have indicated that carcinoembryonic antigen (CEA) might be a suitable immunotherapeutic target in colorectal carcinoma (CRC). The aim of the present study was to analyze the immunological and clinical effects of vaccination with CEA together with the adjuvant granulocyte/macrophage colony-stimulating factor (GM-CSF).

Experimental Design: Twenty-four resected CRC patients without macroscopic disease were immunized seven times with recombinant CEA at four different dose levels over a 12-month period. Half of the patients received GM-CSF (80 μg/day for 4 consecutive days) at each immunization. Patients were monitored immunologically for 36 months and clinically for 76 months. T-cell response was evaluated by a [3H]thymidine incorporation assay, and IgG response was determined by ELISA.

Received 12/10/03; revised 2/16/04; accepted 2/18/04.
Grant support: The Swedish Cancer Society, the Cancer Society in Stockholm, the King Gustaf V Jubilee Fund, the Cancer and Allergy Foundation, the Gunnar Nilsson Foundation, Ragnar Söderberg Foundation, Tornspiran, Hesselman, the Research Fund of the Department of Oncology, Uppsala University Hospital, Swedish Medical Society, and the Karolinska Institute Foundations. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: Håkan Mellstedt, Department of Oncology (Radiumhemmet), CancerCentre Karolinska, Karolinska Hospital, SE-171 76 Stockholm, Sweden. Phone: 46-8-5177 4641 (direct) or 46-8-5177 4308 (secretary); Fax: 46-8-318327; E-mail: hakan.mellstedt@ks.se.

Results: Minor local side effects were common. All 12 patients (100%) in the GM-CSF group developed a CEA-specific T-cell as well as an IgG response. The corresponding figures in the CEA alone group were 9 of 12 (75%) and 8 of 12 (66%), respectively. GM-CSF significantly augmented the amplitude of the T-cell response and the IgG titers. No dose–response relationship was noted. The immune responses at 12 months persisted 24 months after the last vaccination. Anti-CEA IgG titers were associated with increased survival (P < 0.05), whereas standard prognostic factors had no relationship, with the exception of serum CEA value.

Conclusions: Vaccination with recombinant CEA and GM-CSF appears to be a nontoxic regimen inducing potent and durable antigen-specific IgG and T-cell response. The results of this study justify more extensive trials with recombinant CEA protein for immunotherapy of CRC.

INTRODUCTION

Despite major efforts at therapy, the prognosis remains poor for stage II, III, and IV colorectal carcinoma (CRC), with 5-year survival rates of 60–80, 30–55, and <3%, respectively (1). New treatment modalities are urgently required. Specific immunotherapies including cancer vaccines and monoclonal antibodies may be complementary alternatives. Monoclonal antibodies have been shown to induce tumor regression (2, 3) and improve clinical outcome was also noted in some of the studies (11, 14).

These preliminary data suggested that CEA might be a useful candidate vaccine to further explore as an approach to immunotherapy of CRC. Use of the whole protein as the recombinant antigen has distinct advantages, such as the potential availability of a large repertoire of epitopes, naturally processed and presented by the antigen-presenting cells of the immunized individual. Exogenous antigens may use both the class II and I presentation pathways through cross-presentation (15). This process is facilitated by granulocyte/macrophage colony-stimu-
Vaccination in Colorectal Carcinoma

Clinical characteristics of colorectal carcinoma patients immunized with the recombinant carcinoembryonic antigen protein with or without the adjuvant cytokine granulocyte/macrophage colony-stimulating factor

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Table 1

- rCEA, recombinant carcinoembryonic antigen; GM-CSF, granulocyte/macrophage colony-stimulating factor.
- Dukes’ staging system.
- Time from start of immunization.

The aim of the present study was to vaccinate CRC patients free of macroscopic disease with rCEA and assess the anti-CEA-specific humoral and cellular immune responses over a 3-year period. The prognostic effect of an induced immune response to CEA was also correlated with clinical outcome over a follow-up period of >6 years.

MATERIALS AND METHODS

Patients. Twenty-four patients (14 males and 10 females) were entered in the present study. The median age was 61 years (range, 33–80 years), and all of the patients had undergone surgery for CRC Dukes’ stage A (n = 1), B (n = 12), or C (n = 11). None of the patients had macroscopic tumors at the start of immunizations (Table 1). Seventeen patients had colon cancer, whereas in 7 patients the primary tumor was located in the rectum. The Karnofsky index was ≥90 in all patients. Only one of the patients had received adjuvant chemotherapy in addition to surgery. The median time from surgery to start of immunization was 5.7 months (range, 1–39 months). The study was approved by the Ethics Committee of the Karolinska Institute, and informed consent was obtained from each patient.

The first patient was included in December 1994 and the last in April 1996. At that time adjuvant chemotherapy was not standard treatment for colon cancer stage C in Sweden. All patients were monitored for immunological responses for 36 months. The last clinical follow-up was done in the spring of 2003.

Vaccination Protocol. The trial was as an open-labeled, randomized, dose-ranging study. Six consecutive patients were enrolled at each of the four rCEA dose levels: 100, 316, 1000, or 3000 µg/immunization, respectively. Patients receiving 100 or 316 µg of rCEA were considered as the low-dose group, and those receiving 1000 or 3000 µg formed the high-dose group. rCEA was administered s.c. at days 1 and 14 and at months 2, 4, 6, 9, and 12, i.e., the total vaccination period was 1 year. At each dose level of rCEA, half of the patients (n = 3) were randomized to concomitant administration of recombinant human GM-CSF (Leucomax; Schering-Plough, Kenilworth, NJ; GM+ group), whereas the other half received CEA alone (GM− group). With each immunization, 80 µg/day GM-CSF was administered s.c. at the same site as the CEA. GM-CSF was administered once daily for 4 consecutive days starting with the day of CEA immunization.
**Vaccine Preparation and Antigens for in Vitro Test.**
The rCEA used in this trial was produced by Protein Sciences Corp. (Meriden, MA) according to a previously described process (7). The gene for CEA was cloned from human colon adenocarcinoma cells (LS174T) and introduced into the baculovirus Autographa californica nuclear polyhedrosis virus (Ac-NPV) under the transcriptional control of the AcNPV polyhedrin promoter. The rCEA used in these studies had an amino acid sequence identical to the naturally occurring protein but was engineered to prevent the addition of glycosylphosphatidylinositol at the COOH terminus. This modification facilitated the secretion of the rCEA out of the insect cells. The rCEA was N-linked glycosylated, with a molecular mass of 120 kDa, which is smaller than the 180 kDa molecular mass of natural CEA. This was due to the lack of complex glycosylation within insect cells. The rCEA was purified by a series of hydrophobic interactions and anion-exchange chromatography under non-denaturing conditions to at least 99% purity. The rCEA was additionally tested for purity by quantitative scanning densitometry, for safety in mice and guinea pigs, and for sterility and pyrogenicity. The purified rCEA for immunization was formulated with aluminum phosphate (alum; 0.5 mg/ml aluminum ion as AlPO4). rCEA without alum was used for in vitro tests.

A baculovirus control protein (BCP) was also produced for in vitro tests (7). Control preparations contained a mixture of insect cells and baculovirus proteins from cells infected with a control baculovirus expression vector. The procedure involved extracting essentially all of the insect cells and baculovirus proteins in infected cells, binding them to a single anion-exchange chromatography column, and recovering the control protein preparation in a phosphate-buffered solution. This procedure gave a BCP preparation that contained all of the major insect cells and baculovirus proteins to serve as a control in the in vitro assays against any of the low-level contaminants found in purified rCEA.

**Clinical Examination and Follow-Up.** Before immunization, all participants underwent a complete case history, physical examination, and laboratory tests [hemoglobin concentration; WBC with a differential; platelet count; blood chemistries, including blood urea nitrogen, creatinine, electrolytes, albumin, and total protein; liver function tests; serum tumor markers (CEA, CA19-9, and CA50); and standard urine analysis]. Side effects were graded according to the WHO adverse effects criteria (22). The patients were followed clinically every second month during the first 9 months as described above, then every third month for 24 months, and after that every 6 months. Blood was drawn for immunological analyses before vaccine administration and at months 1, 2, 4, 6, 7, 9, 12, 15, 18, 21, 24, 30, and 36 postvaccination.

**Isolation of Blood Cells.** Peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation of heparinized venous blood on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden; density, 1.077 g/ml). Adherent cells were obtained by incubation of PBMCs for 30 min at 37°C in tissue culture flasks (Nunc, Roskilde, Denmark), and nonadherent cells, peripheral blood lymphocytes, were collected. Adherent cells were removed from the flasks with gentle scraping and used as antigen-presenting cells. The cells were resuspended in HEPES-buffered RPMI 1640 (Life Sciences, Inc., Paisley, Scotland) supplemented with antibiotics (100 IU/ml penicillin; 100 μg/ml streptomycin), 1-glutamine (2 mm), and 10% heat-inactivated normal human AB serum. The detailed method has been described previously (10).

**Proliferation Assay (DNA Synthesis).** Nonadherent peripheral blood lymphocytes were cocultured with autologous adherent monocytes at a ratio of 10:1. A total of 105 cells/well were cultured in 96-well round-bottomed microtiter plates (Göteborgs Termometerfabrik, Stockholm, Sweden) at 37°C in humidified air with 5% CO2 for 5 days. rCEA and the control protein (BCP) were added at concentrations of 1–100 ng/ml, respectively. Concanavalin A (80 μg/ml; Pharmacia Biotech, Uppsala, Sweden), phytohemagglutinin (10 μg/ml; Sigma-Aldrich Sweden AB, Stockholm, Sweden), purified protein derivative of tuberculin (2.5 μg/ml; Statens Seruminstitut, Copenhagen, Denmark), and tetanus toxoid (50 ng/ml; SBL Vaccine, Stockholm, Sweden) were used as positive controls. We added 1 μCi/well [3H]thymidine (Amersham, Biosciences United Kingdom Ltd., Buckinghamshire, United Kingdom; specific activity, 5 Ci/mmol) for the final 18 h of culture, and the cells were harvested by use of an automatic cell harvester (Skatron, Lier, Norway). Radioactivity was measured with a liquid scintillation counter (LKB 1212 Rackbeta; Pharmacia, Stockholm, Sweden). The results are expressed as the mean of triplicates. A stimulation index (SI) was calculated for each triplicate by dividing the mean radioactivity (cpm) of stimulated cells by that of unstimulated cells. The highest SI induced by the different concentrations in each test was used.

The SIs of 23 retested, peripheral blood lymphocyte, nonimmunized CRC patients were 1.64 + 1.22 (mean ± 2 SD) against rCEA and 1.63 + 1.18 against BCP. The corresponding values of healthy control donors (n = 21) were 1.41 + 0.85 against CEA and 1.42 + 0.76 against BCP. On the basis of these data, we considered a SI value >3.0 a positive value. A patient was considered to have developed a CEA-specific proliferative response if a positive response could be detected at a minimum of two different time points with a value at least twice of the baseline.

Before the start of vaccination there was no statistically significant difference with regard to proliferative responses against rCEA, mitogens, or recall antigens between patients designated for immunization with or without GM-CSF (data not shown).

**Antibody-Dependent Cellular Cytotoxicity (ADCC).** Cells from the SW48 cell line (American Type Culture Collection, Manassas, VA), a human CRC cell line expressing surface-bound CEA, were used as target cells in the ADCC assays. We incubated 3 × 104 viable SW48 cells with 100 μl of 10 mCi/ml 31Cr solution (Amersham) at 37°C for 1 h, washed the cells twice with PBS, and resuspended them at a concentration of 5 × 105 cells/ml. We added 104 labeled target cells to round-bottomed wells of a 96-well microtiter plate (Nunc). Effector cells (PBMCs) from healthy control donors were added at E:T cell ratios of 50:1, 25:1, 12:1, and 6:1. Five μl of undiluted heat-inactivated (56°C for 30 min) sera from patients or healthy control donors were then added. The cells were cultured for 18 h at 37°C in humidified air with 5% CO2. A 100-μl portion of supernatant was removed from each well, and the radioactivity was measured in a gamma counter (1480 Wizard 3; Wallac,
Turku, Finland). Spontaneous release of target cells alone and maximum release after lysis with 0.1% Triton X-100 were determined. The percentage of cytotoxicity was calculated by the following formula: Lysis (\%) = (release in sample – spontaneous release)/(maximum release – spontaneous release) × 100.

**ELISA.** Flat-bottomed microtiter ELISA plates (Costar, Cambridge, MA) were coated at 4°C overnight with 100 μl of CEA (2.5 μg/ml) or BCP (2.5 μg/ml) in 0.05 M carbonate buffer (pH 9.7). After three washes in PBS containing 0.05% Tween 20, the wells were blocked with 1% BSA (Sigma-Aldrich) for 30 min at 37°C and washed three times. Serum samples diluted 1:50 in PBS containing 1% BSA and 0.05% Tween were added to the coated wells and incubated overnight at 4°C. After washing, the wells were incubated for 2 h at 37°C with alkaline-phosphatase-conjugated goat anti-human IgG (1:1000 dilution; Sigma). We then added 100 μl of p-nitrophenyl phosphate (1 mg/ml; Sigma) in 1 M diethanolamine buffer (pH 9.8) containing 0.5 mmol MgCl₂ as the color substrate and incubated the plates at room temperature for 20 min. The absorbance was measured at 405 nm by an automatic ELISA reader. Results are expressed as the mean absorbance of triplicate wells after subtraction of background values.

The absorbance (mean ± SD) values for rCEA and BCP in healthy control donors (n = 19) were 0.133 ± 0.145 and 0.059 ± 0.042, respectively. The change in absorbance between CEA and BCP was calculated for each individual sample. A postimmune serum was considered positive if the Δabsorbance value was at least 2-fold higher than that of the preimmune serum as well as more than the mean + 2 SD of healthy controls.

**Flow Cytometry.** Immunofluorescence analyses were performed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The cell line SW48 (10⁶ cells/sample) was suspended in PBS and incubated with undiluted heat-inactivated sera (50 μl) of patients or healthy control donors on ice for 45 min. Cells were then washed in PBS twice and incubated with FITC-conjugated rabbit antihuman immunoglobulin (5 μl; diluted 1:10; DAKO, Glostrup, Denmark) on ice for 30 min in the dark. After two washes, flow cytometric analysis was performed. For each sample, 15,000 events were analyzed. The relative frequencies of stained cells were determined using the CellQuest software program (Becton Dickinson).

**Delayed-Type Hypersensitivity (DTH).** The DTH reaction was evaluated before the start of immunization and at month 12 after start of immunization. rCEA without alum in sterile PBS (0.1 ml of a 100 μg/ml solution) was injected intradermally into the forearm. A positive reaction was defined as induration and/or redness at least 10 mm in diameter measured 24–72 h after the injection (23).

**Statistical Methods.** Medians of continuous parameters were compared by Mann–Whitney U test. Univariate Cox regression analysis was applied to evaluate prognostic factors for survival. Multivariate Cox regression analyses were performed for factors significant in the univariate analyses.

The relationships between survival and anti-CEA IgG titers as well as survival and T-cell responses (SI values) were analyzed as time-dependent covariates in Cox’s regression analysis as described previously (24). The relative hazard and 95% confidence interval was determined. Values for antibody titers and SI, respectively, defined at times 0, 6, 12, 18, 24, 30, and 36 months from start of immunization were used.

**RESULTS**

**Patients and Safety.** Patient characteristics at study entry are shown in Table 1. Twenty patients were given all seven immunizations, whereas four patients were withdrawn because of relapse of the disease before the entire vaccination schedule had been completed. Patient 17 (GM⁻) received three vaccinations, patient 9 (GM⁺) and patient 19 (GM⁺) received five vaccinations each, and patient 10 (GM⁻) received six vaccinations.

Nine patients in the GM⁺ group experienced side effects compared with 5 in the GM⁻ group (Table 2). Six patients experienced side effects only at one immunization, four experienced side effects on two occasions, and one patient each experienced side effects at the third, fourth, fifth, and sixth immunizations, respectively.

All systemic as well as local side effects were grade I, except for one patient in the GM⁺ group who had a large grade II erythema immediately after the first immunization. Local side effects consisted of irritation at the injection site (12 patients); the frequency decreased with repeated immunizations. Systemic adverse events included fever (four patients), headache (three patients), myalgia (four patients), allergic reactions (dyspnea, urticaria; two patients), nausea (two patients), dyspepsia (one patient), fatigue (one patient), and vertigo (one patient). All systemic side effects except for one were in the GM-CSF group. The difference was statistically significant (P = 0.015). The frequency of systemic adverse events declined with repeated immunizations. One patient transiently developed Parkinson-like symptoms at the fourth and fifth immunizations as well as diarrhea at the last immunization, but this was considered unrelated to treatment.

Hematological parameters as well as renal and hepatic functions remained within the normal ranges during the observation period. No abnormalities were found in urine analysis. No clinical manifestations of autoimmune reactions were observed.

**In Vitro T-Cell Response against CEA.** The induction of an anti-CEA proliferative T-cell response was noted as early as after the first immunization (Fig. 1). The magnitude of the response gradually increased with repeated vaccinations. The magnitude was significantly augmented by the adjuvant cytokine GM-CSF. All patients in the GM-CSF group developed a CEA-specific T-cell response, compared with nine in the CEA-alone group. Interestingly, in the GM-CSF group, a high level proliferative cellular response was sustained for 2 years after the last immunization. There was no statistically significant difference in the magnitude of the immune response between the patients who received a low dose of rCEA (100 or 316 μg immunization) or a high dose (1000 or 3000 μg immunization; data not shown).

**DTH.** Seventeen patients were evaluated for an in vivo T-cell response against rCEA at month 12. Four of eight tested patients who had received GM-CSF (50%) developed a positive DTH reaction compared with two of nine patients (22%) who...
had received CEA alone. The difference was statistically not significant.

**Anti-CEA IgG Antibodies.** All patients (12 of 12) in the GM-CSF group developed a CEA-specific IgG response compared with 8 of 12 patients in the CEA-alone group. Repeated immunizations induced a gradual increase in the anti-CEA IgG titers (Fig. 2). Significantly higher anti-CEA titers were induced in patients receiving GM-CSF compared with the CEA-alone group. Maximum titers occurred at the last immunization, followed by a gradual decline. High levels of anti-CEA antibodies continued to be detected 2 years after the last immunization in both groups of patients. There was no relationship between the CEA dose used for immunization and the magnitude of the anti-CEA IgG titers (data not shown).

**ADCC.** Sera from four patients (patients 2, 8, 3, and 7) obtained at months 12–15 were tested for binding (flow cytometry) to a CEA-expressing human tumor cell line (SW48). The individual sera stained 18, 87, 1.9, and 5.7%, respectively, of the SW48 cells, whereas sera from healthy control donors stained a mean (± SE) 0.57 ± 0.04% of cells (n = 9).

Sera were also analyzed for opsonization activity in ADCC by use of the SW48 tumor cell line. As effector cells, PBMCs from a healthy control donor were used. The lytic activity in the presence of sera from patients 2 and 8, which showed significant binding to the tumor cells, was 64% and 50%, respectively, whereas for sera from patients 3 and 7, which stained few tumor cells, the corresponding values were 36 and 26%, respectively, similar to the opsonization capacity of control sera that stained few SW48 cells (Fig. 3).

**Immune Response and Relation to Clinical Outcome.** Five patients in the CEA-alone group and six patients in the GM-CSF group relapsed and died during follow-up (Table 1). Two additional patients in the CEA-alone group died from causes unrelated to their malignancy.

To analyze the influence of the anti-CEA IgG response on survival, we compared the antibody titers of patients who were alive (n = 11) at last follow-up with those of patients who had succumbed to disease in the same period (n = 13). Antibody titers at months 0, 6, 12, 18, 24, 30, and 36 postvaccination were compared. Higher anti-CEA IgG titer values over time, which were statistically significant, were noted in those who were alive compared with those who had died during follow-up (P < 0.05; Fig. 4). Univariate analyses of standard prognostic factors for survival (age, sex, Dukes’ stage, tumor localization, and grade of differentiation; Ref. 25) and immune status before vaccination (proliferative T-cell response against tetanus toxoid and purified protein derivative of tuberculin) did not reveal any statistically significant difference between those patients who

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**Table 2** Frequency (number of patients) of side effects considered related to treatment in relation to immunization time in colorectal carcinoma patients vaccinated with recombinant carcinoembryonic antigen with (GM+) or without (GM−) the adjuvant cytokine granulocyte/macrophage colony-stimulating factor

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<td>Vertigo</td>
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<td>Dry skin</td>
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* There were 12 patients in the GM+ group and 12 patients in the GM− group.

b One grade II reaction; all others were grade I (WHO).
were alive or had died with the exception of serum CEA value (Table 3). In multivariate Cox regression analyses, however, none of the factors was statistically significant.

The magnitude of the proliferative T-cell response (SI value) was also tested for survival. No statistically significant influence of the anti-CEA proliferative T-cell response could be established (data not shown).

DISCUSSION

One of the salient features of this study is the relatively long period over which the patients were evaluated clinically and immunologically. Several clinical trials using CEA as a target have been conducted previously (26). However, the long time interval over which the patients were followed in the present study provides incontrovertible evidence that CEA can be used as a target tumor antigen without associated immunopathological disease arising from autoimmune responses. It is noteworthy that autoimmune manifestations were absent despite the use of a strong adjuvant, such as GM-CSF, and potent T-cell responses and antibody titers exhibited by some of the patients.

In the present study, a sustained and high IgG antibody titer against CEA was significantly related to survival, whereas no statistical relationship was noted with proliferative T-cell response. The clinical importance of the induced anti-CEA antibodies was further supported by Cox regression analyses showing a statistically significant relationship between antibody titers and survival, which was not the case for standard prognostic factors. An issue that needs to be taken into consideration when interpreting the results is the comparatively few patients in this study. This in part may account for the lack of association by Cox’s analysis between survival and disease stage, an established factor associated with prognosis. The statistically significant association between antibody titer and survival within these patients suggests that this relationship was particularly strong. Nevertheless, although this is an interesting observation, the few patients in the present study precludes it from being presented as a definitive conclusion. The manner in which such an anti-CEA antibody response may influence tumor growth has not been established experimentally. Our ADCC experiments imply that antibodies may enhance lysis of tumor targets by immune cells. Some of the antibodies used in cancer therapy are known to mediate their effects by this mechanism (27). Alternatively, antibodies against CEA may impair the function of this molecule. It is known that CEA is an intercellular adhesion molecule and promotes the aggregation of colon adenocarcinoma cells. It is likely that the increased adhesion and aggregation of colon carcinoma cells facilitate their survival at a site...
distal to the primary tumor, thereby promoting metastasis (28). Antibodies against CEA that neutralize the intercellular adhesion function may thus restrict tumor survival and impede metastasis.

The significantly longer survival of patients who developed sustained high anti-CEA antibody titers is in keeping with previous published reports. Vaccination of metastatic CRC patients with a β-human chorionic gonadotropin peptide (37-amino acids long) induced anti-human chorionic gonadotropin antibodies in most patients. High antibody titers were associated with a longer overall survival (29). Patients with stage III melanoma, free of disease after surgery, were randomized to receive treatment with a ganglioside (GM2) in combination with BCG, whereas the control group received BCG only. There was a significant increase in overall survival of patients who developed anti-GM2 IgM antibodies (30). In another study, surgically resected stage III melanoma patients were immunized with a polyvalent melanoma vaccine consisting of antigens shed by four melanoma cell lines. Improved survival was noted for patients developing a melanoma-vaccine-specific humoral response (31). Patients with recurrent ovarian carcinoma treated with a murine monoclonal anti-idiotypic antibody (ACA125) mimicking the tumor-associated antigen CA125 developed a humoral anti-CA125 immune response and survived significantly longer than patients without detectable CA125-specific antibodies (32).

No statistical relation between the proliferative T-cell response and survival was noted in this study. Other vaccination studies in cancer patients, however, have reported the correlation of T-cell responses to improved prognosis. A positive correlation between survival and DTH reaction was observed in other studies in melanoma patients vaccinated with a polyvalent melanoma vaccine (33, 34). Renal cell carcinoma patients vaccinated with tumor cells who developed a positive skin test reaction survived significantly longer than patients who remained skin test negative (35). Patients with pancreatic carcinoma vaccinated with mutant ras peptides who developed a peptide-specific DTH reactivity and/or an in vitro proliferative T-cell response also had a longer survival than patients not developing a cellular response (36).

Table 3: Univariate Cox regression analyses of prognostic factors for overall survival

<table>
<thead>
<tr>
<th>Factors</th>
<th>Evaluable patients (n)</th>
<th>Relative hazard</th>
<th>Degrees of freedom</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CEA&lt;sup&gt;a&lt;/sup&gt; IgG titer (absorbance)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24</td>
<td>0.10</td>
<td>1</td>
<td>0.01–0.96</td>
<td>0.045</td>
</tr>
<tr>
<td>Anti-CEA T-cell response&lt;sup&gt;c&lt;/sup&gt; (SI)</td>
<td>24</td>
<td>0.968</td>
<td>1</td>
<td>0.900–1.040</td>
<td>0.37</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>24</td>
<td>1.03</td>
<td>1</td>
<td>0.99–1.08</td>
<td>0.177</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>24</td>
<td>2.18</td>
<td>1</td>
<td>0.73–6.54</td>
<td>0.166</td>
</tr>
<tr>
<td>Dukes’ stage&lt;sup&gt;d&lt;/sup&gt;</td>
<td>24</td>
<td>1.35</td>
<td>1</td>
<td>0.45–4.03</td>
<td>0.592</td>
</tr>
<tr>
<td>Tumor site (rectum vs. colon)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>24</td>
<td>1.73</td>
<td>1</td>
<td>0.48–6.31</td>
<td>0.405</td>
</tr>
<tr>
<td>Differentiation&lt;sup&gt;f&lt;/sup&gt;</td>
<td>24</td>
<td>0.19</td>
<td>1</td>
<td>0.04–1.05</td>
<td>0.056</td>
</tr>
<tr>
<td>1 vs. 2</td>
<td>24</td>
<td>0.30</td>
<td>1</td>
<td>0.04–1.97</td>
<td>0.207</td>
</tr>
<tr>
<td>CEA (μg/l)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>24</td>
<td>1.24</td>
<td>1</td>
<td>1.001–1.525</td>
<td>0.048</td>
</tr>
<tr>
<td>Anti-T&lt;sub&gt;T&lt;/sub&gt; T-cell response (SI)</td>
<td>22</td>
<td>0.973</td>
<td>1</td>
<td>0.931–1.018</td>
<td>0.234</td>
</tr>
<tr>
<td>Anti-PPD&lt;sup&gt;h&lt;/sup&gt; T-cell response (SI)</td>
<td>21</td>
<td>1.005</td>
<td>1</td>
<td>0.988–1.022</td>
<td>0.579</td>
</tr>
</tbody>
</table>

<sup>a</sup> CEA, carcinoembryonic antigen; SI, stimulation index; TT, tetanus toxoid; PPD, purified protein derivative.

<sup>b</sup> Specific anti-carinoembryonic antigen response over a 3-year period.

<sup>c</sup> At surgery.

<sup>d</sup> Degree of differentiation of the tumor: 1 = high, 2 = medium, 3 = low.

<sup>e</sup> At start of vaccination.

<sup>f</sup> No statistical relation between the proliferative T-cell response and survival was noted in this study. Other vaccination studies in cancer patients, however, have reported the correlation of T-cell responses to improved prognosis. A positive correlation between survival and DTH reaction was observed in other studies in melanoma patients vaccinated with a polyvalent melanoma vaccine (33, 34). Renal cell carcinoma patients vaccinated with tumor cells who developed a positive skin test reaction survived significantly longer than patients who remained skin test negative (35). Patients with pancreatic carcinoma vaccinated with mutant ras peptides who developed a peptide-specific DTH reactivity and/or an in vitro proliferative T-cell response also had a longer survival than patients not developing a cellular response (36). A positive correlation between a specific IFN-γ T-cell response and clinical outcome has been demonstrated in some studies (10, 12, 37–45), whereas such a relationship has not been noted in others (42–45). The lack of correlation between T-cell proliferative response and clinical outcome in our study does not have an obvious reason. The induction of a proliferative response is in itself evidence that the T cells in the patients are not anergized to CEA. The explanation may then relate to the nature of the induced T-cell subsets and to the susceptibility of CRC tumor cells to T-cell-mediated lysis in vivo.

In summary, vaccination with rCEA in combination with low-dose local administration of GM-CSF induced a strong, long-lasting anti-CEA-specific T-cell as well as IgG antibody response. There was a statistically significant association of antibody titers with survival of the patients in this study. However, a more extensive study with larger patient numbers would be required to definitely establish the relationship between antibody titers and patient survival. The results of the study support the further exploration of CEA protein as a cancer vaccine. Improvements in the immune and clinical responses may be achieved by alternative approaches such as the prime-boost concept, i.e., prime with DNA and boost with the protein (46), which may represent an effective method to induce tumor-antigen-specific CD4 and CD8 T cells as well as antibodies.

ACKNOWLEDGMENTS

We thank Lena Virving, Birgitta Hagström, and Ingrid Eriksson for expert technical assistance. For skillful secretarial help we thank Gerd Ståhlberg and Gunilla Burén. The rCEA was a kind gift from Protein Sciences Corp. (Meriden, CT).

REFERENCES

2. Sacchi S, Federico M, Dastoli G, et al. Treatment of B-cell non-
Hodgkin’s lymphoma with anti CD 20 monoclonal antibody Rituximab. 
plus a monoclonal antibody against HER2 for metastatic breast cancer 
therapy for resected Dukes’ C colorectal cancer: seven-year outcome of 
5. Fields AL, Keller AM, et al. Edrecolomab (17–1A antibody) in 
combination with 5-fluorouracil (5-FU) based chemotherapy in the 
adjuvant treatment of stage III colon cancer: results of a randomized 
128a.
6. Okawa S, Nakazato H, Kosaki G. Primary structure of human 
carcinoembryonic antigen (CEA) deduced from cDNA sequence. 
gene for carcinoembryonic antigen: analysis of its promoter indicates a 
region conveying cell type-specific expression. Mol Cell Biol 1990;10: 
2738–48.
8. Thompson JA, Grunert F, Zimmermann W. Carcinoembryonic anti-
Antitumor activity and immune responses induced by a recombinant 
carcinoembryonic antigen-vaccinia virus vaccine. J Natl Cancer Inst 
of granulocyte/macrophage-colony-stimulating factor is of significant 
importance for the induction of a strong humoral and cellular 
response in patients immunized with recombinant carcinoembryonic 
responses in resected colon cancer patients treated with an idiotype 
monoclonal antibody vaccine that mimics the carcinoembryonic anti-
advanced cancer patients of a diversified prime-and-boost vaccination 
protocol using recombinant vaccinia virus and recombinant nonreplicat-
ing avipox virus to elicit anti-carcinoembryonic antigen immune 
13. Conry RM, Allen KO, Lee S, Moore SE, Shaw DR, LoBuglio AF. 
Human autoantibodies to carcinoembryonic antigen (CEA) induced by 
cyte macrophage colony-stimulating factor and prior chemotherapy on 
the immunological response to a vaccine (ALVAC-CEA B7.1) in 
Antigen presentation and T cell stimulation by dendritic cells. Annu Rev 
cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes 
17. Salgaller ML, Lodge PA. Use of cellular and cytokine adjuvants in 
18. Ullenhag GJ, Frodin JE, Strigard K, Mellstedt H, Magnusson CG. 
Induction of IgG subclass responses in colorectal carcinoma patients 
vaccinated with recombinant carcinoembryonic antigen. Cancer Res 
19. Markowicz S, Engleman EG. Granulocyte-macrophage colony-
stimulating factor promotes differentiation and survival of human 
peripheral blood dendritic cells in vitro. J Clin Investig 1990;85: 
955–61.
20. Sallusto F, Lanzavecchia A. Efficient presentation of soluble 
antigen by cultured human dendritic cells is maintained by granulo-
cyte/macrophage colony-stimulating factor plus interleukin 4 and 
1109–18.
cyte/macrophage-colony-stimulating factor produced by recombiant 
avian poxviruses enriches the regional lymph nodes with antigen-present-
14.
22. Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of 
23. Clay TM, Hoheika AC, Mosca PJ, Lyerly HK, Morse MA. Assays 
for monitoring cellular immune responses to active immunotherapy of 
24. Kalbfleisch JD, Prentice RL. The statistical analysis of failure time 


Clinical Cancer Research

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Gustav J. Ullenhag, Jan-Erik Frödin, Mahmood Jeddi-Tehrani, et al.


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