Clinical and Immune Responses in Advanced Colorectal Cancer Patients Treated with Anti-Idiotype Monoclonal Antibody Vaccine That Mimics the Carcinoembryonic Antigen

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ABSTRACT
Carcinoembryonic antigen (CEA) is expressed in a wide variety of adenocarcinomas, and it is well recognized that cancer patients are immunologically "tolerant" to CEA. The purpose of this study was to determine whether we could break immune tolerance to CEA by vaccinating patients with a monoclonal anti-idiotype antibody that is the internal image of CEA and to determine what impact this might have on patient survival.

Twenty-four patients with advanced CEA-positive colorectal cancer who failed standard therapies except for two were entered into this Phase Ib trial. One patient was considered not assessable, because on the day of entering into the study, she was diagnosed with acute myelogenous leukemia. Patients were treated with 1, 2, or 4 mg of aluminum hydroxide-precipitated 3H1 anti-idiotype antibody every other week for four injections and then monthly until tumor progression was observed. Immunological monitoring included humoral and cellular idiotypic and CEA responses, and all patients were evaluated for toxicity, response, and survival.

Hyperimmune sera from 17 of 23 patients demonstrated an anti-anti-idiotype Ab3 response, and 13 of these responses were demonstrated to be true anti-CEA responses (Ab1'). The antibody response was polyclonal, and 11 mediated antibody-dependent cellular cytotoxicity. Ten patients had idiotypic T-cell responses, and five had specific T-cell responses to CEA. None of the patients had objective clinical responses, but overall median survival for the 23 evaluable patients was 11.3 months, with 44% 1-year survival (95% confidence interval, 23–64%). Toxicity was limited to local swelling and minimal pain.

Anti-idiotype monoclonal antibody 3H1 that mimics CEA was able to break immune tolerance in the majority of treated patients. Overall survival of 11.3 months was comparable to other phase II data with advanced colorectal cancer patients treated with a variety of chemotherapy agents, including irinotecan, with considerably less toxicity. Although it is not clear that the vaccine itself had an impact on survival, this should be determined in a Phase III randomized trial.

INTRODUCTION
CEA is a Mr 180,000 glycoprotein tumor-associated antigen present on endodermally derived neoplasms of the gastrointestinal tract as well as other adenocarcinomas (1). CEA is also found in the digestive organs of the human fetus and, thus, the name CEA was derived. Circulating CEA can be detected in the majority of patients with CEA-positive tumors. Specific monoclonal antibodies have been raised against CEA (2–4), and some have been radiolabeled for diagnostic and clinical studies (5). As with most tumor-associated antigens that are seen as self-antigens by the immune system, cancer patients are immunologically "tolerant" to CEA, which is likely related to its oncofetal origin. However, a limited number of reports from the 1970s suggested that some patients with CEA-positive tumors may have minimal humoral and cellular immunity to CEA (6–10); these results are controversial.

The network hypothesis of Lindenmann (11) and Jerne (12) offers an elegant approach to transforming epitope structures into idiotypic determinants expressed on the surface of antibodies. According to the network concept, immunization with a given tumor-associated antigen will generate production of antibodies against this tumor-associated antigen, which are termed Ab1; Ab1 is then used to generate a series of anti-idiotype antibodies against the Ab1, termed Ab2. Some of these Ab2 molecules can effectively mimic the three-dimensional structure of the tumor-associated antigen identified by the Ab1. These particular anti-idiotypes, called Ab2β, fit into the paratopes of Ab1 and express the internal image of the tumor-associated antigen. The Ab2β can induce specific immune responses similar to those induced by the original tumor-associated antigen and can, therefore, be used as surrogate tumor-associated antigens. Immunization with Ab2β can lead to the generation of anti-anti-idiotype antibodies (Ab3) that recognize the corresponding original tumor-associated antigen identified by Ab1.

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The abbreviations used are: CEA, carcinoembryonic antigen; ADCC, antibody-dependent cellular cytotoxicity; CI, confidence interval.
Because of this Ab1-like reactivity, the Ab3 is also called Abl’ to indicate that it might differ in its other idiotopes from Ab1.

For several reasons, we consider CEA an excellent tumor-associated antigen for active immunotherapy with an anti-idiotypic antibody. First of all, CEA is typically present at high levels on the tumor cell surface. CEA is one of the most well-characterized antigens, its gene sequence is known, and its three-dimensional structures have been identified (13). CEA is a member of the immunoglobulin supergene family (14) located on chromosome 19, which is thought to be involved in cell-cell interactions. Because CEA is considered an adhesion molecule (15, 16), it might play an important role in the metastatic process by mediating attachment of tumor cells to normal cells. Thus, active immunotherapy targeted to CEA might be particularly beneficial in preventing metastasis.

Highly purified CEA is available from several sources, and it can be used in serological assays. Inasmuch as some of the epitopes on CEA are shared by normal tissues, immunization with intact CEA molecules might trigger potentially harmful autoimmune reactions, whereas an Ab2β generated against an anti-CEA monoclonal antibody that recognizes a CEA-specific epitope would be theoretically safer and more effective. Furthermore, Ab2β expressed in a different molecular environment has been shown to overcome the immunosuppression in the host by stimulating “silent clones” and/or allowing T-cell help to become active, making the overall immune response stronger, which the nominal antigen (e.g., CEA) is unable to do (17, 18). Therefore, an appropriate anti-idiotypic antibody could be an excellent candidate to induce antitumor immunity in CEA-positive cancer patients.

A number of investigators have generated anti-idiotypic antibodies in rats, mice, baboons, and humans that mimic CEA (19–27). We have generated and characterized an anti-idiotypic murine monoclonal antibody to a murine monoclonal antibody designated 8019 that identifies a specific epitope on CEA (28). This is a highly restricted CEA epitope that is not found on normal adult tissues and hematopoietic cells, including granulocytes. The IgG1 anti-idiotypic antibody generated to 8019, designated 3H1, was shown to be an internal image by generating anti-anti-idiotypic (Ab3) responses that recognized CEA in mice, rabbits (29), and monkeys (30). The 3H1 anti-idiotypic antibody was used to treat the patients reported in this Phase lb clinical trial. In a preliminary report, we described polyclonal anti-CEA (Abl’) responses in 9 of the first 12 patients treated in this study as well as idiotypic and CEA-specific T cell-proliferative responses in the majority of these patients (31). In this report, we present the clinical and immune responses in the 24 patients entered into this Phase lb trial.

PATIENTS AND METHODS
Selection of Patients. All of the patients had CEA-positive advanced colorectal carcinoma and failed standard therapies except two patients, who refused standard chemotherapy (Table I). One patient was considered not assessable because she was diagnosed with acute myelogenous leukemia on the day of entering into the trial. Baseline studies included complete physical examination; computer axial tomography examination of the chest, abdomen and pelvis; serum CEA level; and routine blood counts and chemistries. All of the patients had been off...
prior therapy for at least 4 weeks, and staging was repeated 1 month after the fourth immunizations and then every 3 months. All of the patients signed informed consent forms approved by the University of Kentucky Institutional Review Board.

**Treatment Schedule.** Patients were treated intracutaneously with either 1, 2, or 4 mg of aluminum hydroxide-precipitated 3H1 anti-idiotype antibody every other week for four injections. Patients were randomized in groups of three to 1-, 2-, or 4-mg doses rather than a dose-escalation schema. If there was no tumor progression at the end of the four injections, they were then continued on a monthly basis and evaluated every 3 months. Patients were removed from the study for tumor progression.

**Generation of the Anti-Idiotype Antibody for the Clinical Trial.** The murine monoclonal antibody 8019 was used to immunize syngeneic BALB/c mice for the production of the anti-idiotype antibody. Immunization of BALB/c mice, hybridoma fusion and cloning, selection of anti-idiotype (Ab2), and production of ascites in bulk quantities in mice were done as described previously (32, 33). The Ab2 anti-idiotype 3H1 (IgG1) was purified from ascites by affinity chromatography on a protein A-CL Sepharose 4B column. The purity of the isolated immunoglobulin (>95%) was determined by SDS-PAGE and high-pressure liquid chromatography techniques. Sterility, pyrogenicity, polynucleotides, mycoplasma, and adventitious virus were tested for inhibition in the presence of different patients' hyperimmune Ab3 sera and Ab1. This demonstrated whether Ab3 in patients' sera shared idiotopes with 8019 (Ab1). Also, this inhibition assay between Ab1-Ab2 binding by Ab3 sera indicated whether Ab3 is a true anti-anti-idiotype. Inhibition greater than 20% by Ab3 sera at a 1:10 dilution was considered positive.

**Inhibition RIA of the Binding between Ab1 and Ab2 by Patients’ Ab3 Antibodies.** Preimmune and hyperimmune patient sera samples were treated with unrelated murine immunoglobulins to remove anti-idiotypic and allotypic activities. Serial dilutions of sera were then tested for inhibition in the Ab1-Ab2 binding assay. All assays were performed in triplicate. For direct binding inhibition assay between Ab1 and Ab2, purified Ab2 3H1 was used to coat plates (500 ng/well), and the binding of radiolabeled 8019 (Ab1) to Ab2 was tested for inhibition in the presence of different patients' hyperimmune Ab3 sera and Ab1. This demonstrated whether Ab3 in patients' sera shared idiotopes with 8019 (Ab1). Also, inhibition assay between Ab1-Ab2 binding by Ab3 sera indicated whether Ab3 is a true anti-anti-idiotype. Inhibition greater than 20% by Ab3 sera at a 1:10 dilution was considered positive.

**Detection of Anti-CEA Antibodies in Patients Immunized with Ab2 3H1.** This assay was conducted to determine whether some of the Ab3 induced in patients by monoclonal murine Ab2 were of the Ab' type and would bind to CEA. Purified CEA (Rougier Bio-tech, Montreal, Quebec, Canada) was radioiodinated with 125I by the chloramine T method. Radiolabeled CEA (1 x 10^6 cpm) was reacted with 0.5 ml of 2% heat-inactivated FCS and penicillin and streptomycin. The nonspecific mitogen phytohemagglutinin P was
1270 Anti-Idiotype Antibody That Mimics CEA

**Table 2** Immune responses and disease progression

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dose (mg)*</th>
<th>No. of injections</th>
<th>Ab3b (anti-anti-Id)</th>
<th>Ab1' (anti-CEA)</th>
<th>ADCC</th>
<th>Idiotypic T cell</th>
<th>CEA T cell</th>
<th>Time to progression (days)</th>
<th>Survival (days)</th>
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<td>–</td>
<td>43</td>
<td>118</td>
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<tr>
<td>2</td>
<td>1</td>
<td>8</td>
<td>+, 53%</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>161</td>
<td>694</td>
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<td>–</td>
<td>–</td>
<td>70</td>
<td>156</td>
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* Patients were entered into the study randomized in groups of three to 1-, 2-, or 4-mg doses of 3H 1 . They are presented in this table at the dose they received rather than the chronological order in which they were entered into the study.

b Inhibition of binding of Ab1 to Ab2 greater than 20% at a 1:10 dilution of Ab3 patient serum is considered positive (+). Percentage shown is maximum inhibition at a 1:10 dilution.

used as a positive control at 2 and 1 µg per well. After the cells were incubated for 5 days at 37°C in an atmosphere containing 5% carbon dioxide, they were pulsed with [14C]thymidine (1 µCi per well) for 20 h. Data were expressed as mean counts (triplicate wells) per minute of [14C]thymidine incorporation. The SD of the data was <10% for each determination.

Peripheral blood mononuclear cells isolated from some selected patients were also incubated with different concentrations of purified CEA (10–250 ng) according to the protocol above.

**ADCC Assay.** LS/174-T cells were labeled in 1 ml of complete DMEM containing 200 µCi of 51Cr by incubation for 1 h at 37°C. The cells were then washed with DMEM without FCS and suspended in the same medium. Cells (1 × 10^4 in 25 µl) were added to individual wells of a 96-well microtiter plate together with different dilutions of crude immune serum (neat and 1:5). Peripheral blood mononuclear cells, isolated by Ficoll/Hypaque gradient centrifugation from normal human donors, were then added to the plate at the E:T ratio of 100:1. The plates were centrifuged at 400 × g for 2 min and incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO₂. After incubation, the plates were centrifuged at 400 × g for 5 min and cpm released in the supernatant were measured. The amount of spontaneously released 51Cr was also measured in wells that contained only labeled target cells. Total released cpm was assessed by lysing labeled cells with 1% NP40. The percentage of lysis was calculated by the formula given below. To calculate lysis specifically attributable to ADCC, the percentage of lysis due to effector cells (i.e., natural killer cells) in the absence of the antibody was subtracted from each value obtained above.

\[
\text{Lysis} = \frac{\text{Experimental lysis} - \text{spontaneous lysis}}{\text{Maximum lysis} - \text{spontaneous lysis}} \times 100
\]

\[
\% \text{ specific lysis} = \frac{\text{Experimental lysis} - \text{spontaneous lysis}}{\text{Maximum lysis} - \text{spontaneous lysis}} \times 100
\]

**Assay for Circulating CEA in Serum.** CEA was quantified in heat-extracted serum. For this, 1 ml of 0.2 m sodium acetate buffer (pH 5.0) was added to 0.5 ml of serum, vortex-mixed, incubated for 15 min at 90°C, and centrifuged (1200 × g) for 10 min. The supernatants were assayed the same day or stored frozen at −20°C until assay. One hundred µl of supernatant were then assayed by the enzyme immunoassay for CEA.

**Statistical Analysis.** Survival was estimated by the method of Kaplan and Meier (35).

**RESULTS**

**Immune Responses to Anti-Idiotype Antibody Vaccine.**

The development of humoral immunity induced by immunization with aluminum hydroxide-precipitated Ab2 3H1 assay was achieved by testing serum obtained from patients before therapy and after their last 3H1 vaccination. Hyperimmune sera from 17 of 23 patients demonstrated an anti-anti-idiotypic Ab3 response (Table 2) as determined by the inhibition of Ab1 (8019) binding to Ab2 (3H1) by serial dilutions of patient sera (Fig. 1). Inhibition of greater than 20% at a 1:10 dilution was considered positive. Five of the six patients who did not have an Ab3 immune response were patients who had rapid growth of their tumor within the first 3 months of therapy and were removed from the study. Thirteen of the 17 patients who had an anti-anti-
Fig. 2 Reactivity of patient Ab3 with purified radiolabeled CEA. Patients' sera (0.5 ml) obtained after the last immunization was absorbed onto protein G-Sepharose 4B beads and reacted with $1 \times 10^6$ cpm radiolabeled CEA. After the reaction, the mixture was centrifuged, and the precipitate was washed and counted in a gamma counter. Each sample was performed in duplicate, and the mean of the cpm bound is shown. Preimmune sera and PBS-BSA were used as controls. Ab1 8019 was used at a 10-μg concentration. No reactivity (cpm bound) greater than PBS-BSA was observed with preimmune sera from these patients. Patients 5 and 14 had a high reactivity with CEA, whereas patient 22 demonstrated minimal positivity.

Fig. 1 Inhibition RIA of Ab1 (8019) binding to Ab2 (3H1) by patients' Ab3 sera. Purified 3H1 was used to coat the plate (500 ng/well), and the binding of radiolabeled 8019 (~90,000 cpm) to 3H1 was tested for inhibition in the presence of various dilutions of Ab3 sera obtained from patients after the last immunization. Twenty % inhibition at a 1:10 dilution was considered positive.

Idiotype Ab3 response also had true anti-CEA responses (Ab1'). Representative data are shown in Figs. 2 and 3. All of the antibody responses were polyclonal, primarily IgG, and 1 mediated ADCC (Fig. 4). Interestingly, patients whose sera mediated in vitro ADCC always contained anti-CEA antibodies. Ten patients had idiotype T-cell responses, and five had specific T-cell responses to CEA. Representative data from two patients are shown in Fig. 5.

Time to Progression and Survival. None of the patients had objective clinical responses to the 3H1 vaccine. However, 13 patients continued on therapy from 3 to 21 months (median, 5 months or 7 immunizations) and were discontinued at the time of tumor progression. Median time to progression for all 23 patients was 2.4 months (95% CI, 1.9–4.3 months), and survival was 11.3 months (95% CI, 7.8–13.7 months) with a 44% 1-year survival (95% CI, 28–64%; Fig. 6A and B). We compared the time to progression in patients who had inhibition of binding of Ab1 to Ab2 less than 20% at a 1:10 dilution of Ab3 patient serum, 20–50%, and greater than 50%; results were 1.3, 2.4, and 5.3 months, respectively. Survival times for the same three groups of patients were 5.5, 11.4, and 16.1 months, respectively (Fig. 7A and B).

Toxicity. Toxicity was typically minimal, with only local reactions at the injection site with mild erythema and induration. A few patients developed large, local reactions with swelling that resolved within a few days. Mild fever and chills relieved by acetaminophen occurred in only a few patients. The antiidiotype treatment did not have any deleterious effects on hematopoietic cells, or renal or hepatic function. There was no clinical or laboratory evidence of serum sickness.

Serial Monitoring of Circulating CEA. Indirect measurement of extent of disease (CEA level) was recorded prior to immunization and determined after each immunization and then once monthly following completion of the immunization schedule. For this, patients' sera were heat inactivated to precipitate the immunoglobulins that would interfere with the CEA-monitoring assays, which involve murine monoclonal antibodies that bind to CEA. CEA is heat stable and was measured in the clear,
centrifuged supernatant by routine assay. The serial monitoring of CEA correlated with disease progression, and all patients who progressed clinically had a rise in their serum CEA levels except patients 5 and 10, who did not secrete CEA. None of the patients demonstrated a significant drop in their CEA levels during the course of this therapy, although those patients with stable disease had no demonstrable rise in their CEA until disease progression.

**DISCUSSION**

We have demonstrated in 17 of 23 patients vaccinated with the 3H1 anti-idiotype antibody that mimics CEA the ability to generate an anti-anti-idiotypic Ab3 response. None of the patients in the study had a pre-existing antibody to CEA, and 13 patients were able to generate an anti-CEA Ab1’ response. Eleven of the patients’ antibodies mediated ADCC, and 10 had idiotypic T cell-proliferative responses, five of which were CEA specific. We do not believe that the immune responses in this Phase Ib study related necessarily to the dose of the antibody but rather to the number of injections patients received and likely, to a lesser extent, prior cytotoxic chemotherapy and radiation therapy that may have been immunosuppressive. Interestingly, many of these patients had increasing levels of circulating immune complexes determined by routine Raji cell assay (data not shown), but none of the patients had symptoms of immune complex disease. To our knowledge, this is the only clinical trial in the world’s literature demonstrating the ability to generate specific and reproducible immunity to CEA in patients with CEA-positive malignancies. In another study, patients were vaccinated with vaccinia virus transduced with the CEA gene (37). Postimmunization peripheral blood was stimulated with interleukin-2 and 9- to 11-mer CEA peptides selected to conform to human HLA class 1-A2 motifs. Three lines were stab-
It was also of interest that the 3H1 anti-idiotype antibody was effective in eliciting immune responses using aluminum hydroxide precipitation rather than a potent adjuvant. Aluminum hydroxide precipitation is considered a very weak immunogen, yet it was quite adequate in generating potent immune responses. This has been particularly true in our more recent

Fig. 6  A, Kaplan-Meier time to progression curve for all 23 eligible patients. Median time to progression was 2.4 months (95% CI, 1.9–4.3 months). B, Kaplan-Meier survival curve for all 23 eligible patients. Median survival time was 11.3 months (95% CI, 7.8–13.7 months); 1-year survival rate was 44% (95% CI, 28–64%).

lished from patients, and one of these lines was studied for detailed analysis and demonstrated HLA-A2-restricted cytotoxicity. Other investigators have demonstrated active immunity to non-CEA antigens generated to anti-idiotype vaccines in patients with melanoma (38, 39), B-cell lymphoma (40), and T-cell lymphoma (41).
Fig. 7  A. Kaplan-Meier time to progression curve for patients with less than 20% inhibition of binding of Ab1 to Ab2 at a 1:10 dilution of Ab3 patient serum (solid line), 20–50% inhibition (dotted line), and greater than 50% inhibition (dashed line). Median times to progression were 1.3 (n = 6), 2.4 (n = 7), and 5.3 (n = 10) months, respectively. B. Kaplan-Meier survival curve for patients with less than 20% inhibition (solid line), 20–50% inhibition (dotted line), and greater than 50% inhibition (dashed line). Median survival times were 5.5 (n = 6), 11.4 (n = 7), and 16.1 (n = 10) months, respectively.
studies treating patients in the clinical adjuvant setting, who do not have measurable metastatic disease and also have normal serum CEA levels. We suspect that aggregation of soluble idiotypic determinants by aluminum hydroxide precipitation may have helped increase antigenicity. Also, because the antibody is a foreign protein and was injected as an intact immunoglobulin, the Fc portion of the murine immunoglobulin may have served as a “carrier” to help induce immune responses. It was also of interest that our anti-idiotype antibody and purified CEA were able to stimulate an in vitro CD4+ T cell-proliferative response post vaccination. We believe that the response observed against the purified CEA was based on the recognition of processed idiotypic peptides that have homology to the CEA sequence. We have identified a peptide sequence region of CEA which has homology to the CDR of the light chain of our 3H1 anti-idiotype antibody and have demonstrated reproducible stimulation of T cells in 3H1-immunized patients.4 We are currently generating T-cell lines and CEA-transduced EBV B-cell lines from vaccinated patients. Cytotoxicity studies will be carried out when these lines are established.

The level of immune response clearly correlated directly with time to progression and survival (Fig. 7, A and B). However, these data can be interpreted in two ways. One interpretation is that increased immunity led to delayed progression and prolonged survival. However, the other possibility is that those patients with delayed progression and prolonged survival were healthier and more immunoresponsive. In addition, these patients received more injections of vaccines, because patients with progression were removed from the study at the time of progression. In future trials, we will consider continuing immunizations even in the setting of progressive disease. Although the vaccine may not have a major role in inhibiting the growth of disease, it may still have a role in preventing metastases.

The median time to progression was 2.4 months. The duration of survival of 11.3 months and 1-year survival of 44% (95% CI, 23–64%) was equal to or greater than those in similar trials, in which patients were treated with Phase II cytotoxic drugs. Indeed, the recent Phase II trial of irinotecan in patients with recurrent colorectal cancer demonstrated a median survival of 10.4 months and 1-year survival of 46% (95% CI, 39–53%; Ref. 36). However, a distinct difference in our patient population was that the patients on the irinotecan trial had either evidence of tumor progression while on 5-fluorouracil-based chemotherapy or progressed within 6 months of receiving such a regimen. Our patient population was more diverse, and a number of our patients had multiple prior chemotherapies with or without radiation therapy. Clearly, the quality of life in our patients was superior to those who received irinotecan, given that the only toxicity in our patients was a local skin reaction. Most chemotherapy agents have significant toxicity, and the major toxicity of irinotecan has been diarrhea, which in some cases has been severe. Trials are being planned with 3H1 to study patients with advanced disease, but of greater interest will be trials for patients in the adjuvant clinical setting that will include resected Dukes D patients and patients with Dukes B and C colorectal cancer. We have already determined in patients with Dukes B and C colorectal cancer that 5-fluorouracil with either levamisole or leucovorin has no measurable impairment of the immune response to 3H1.

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4 Manuscript in preparation.


Clinical and immune responses in advanced colorectal cancer patients treated with anti-idiotype monoclonal antibody vaccine that mimics the carcinoembryonic antigen.

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