A Pilot Study of CTLA-4 Blockade after Cancer Vaccine Failure in Patients with Advanced Malignancy

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Abstract Purpose: Eleven patients with progressive advanced malignancy after administration of a cancer vaccine received a fully human anti-CTLA-4 monoclonal antibody (ipilimumab). The primary end point was to determine drug toxicity. Tumor response, tumor-specific CD8+ T-cell immune responses, and modulation of CD4+ CD25+ FoxP3+ regulatory T-cell (Treg) numbers were secondary end points.

Experimental Design: Three patients with colon cancer, four with non–Hodgkin’s lymphoma, and four with prostate cancer were treated. The first dose was given at 3 mg/kg and subsequent doses were administered monthly at 1.5 mg/kg for a total of four cycles.

Results: Tumor regression was observed in two patients with lymphoma; one of which obtained a partial response of 14-month duration. Ipilimumab was well tolerated with predominantly grade 1/2 toxicities. One drug-related grade 3 toxicity was observed. One patient died within 30 days of treatment due to progressive colon cancer. No increase in vaccine-specific T-cell responses was observed after therapy. Tregs as detected by expression of CD4+ CD25+ CD62L+ declined at early time points but rebounded to levels at or above baseline values at the time of the next infusion.

Conclusions: Ipilimumab treatment depressed Treg numbers at early time points in the treatment cycle but was not accompanied by an increase in vaccine-specific CD8+ T-cell responses in these patients previously treated with a variety of investigational anticancer vaccines. A partial response was observed in one patient with follicular lymphoma. A phase I/II trial evaluating ipilimumab in patients with follicular lymphoma is currently ongoing.

The T-cell receptor CD28 interacts with members of the B7 costimulatory molecule family to activate T cells and stimulate their growth and expansion (1). Its actions are opposed by its inhibitory counterpart, CTLA-4 (CTLA-4). CD28 is constitutively expressed on T cells, whereas CTLA-4 expression is induced following antigen activation of T cells. Due to its higher affinity for B7 family members, CTLA-4 decreases T-cell responses to maintain T-cell homeostasis as antigenic challenges are controlled. In the absence of CTLA-4, T cells undergo unrestricted expansion that results in the death of animals lacking its expression due to autoimmunity (2–4).

Inhibition of CTLA-4 interaction with its ligands B7.1 (CD80) and B7.2 (CD86) with monoclonal antibodies may potentiate T-cell responses by facilitating T-cell activation or prolonging T-cell activity. Inhibition of signaling through CTLA-4 exacerbates the development of autoimmune diseases and enhances the activity of cancer vaccines (5, 6). Preclinical studies showed that CTLA-4 blockade alone protects animals against challenge with immunogenic tumors, whereas weakly or nonimmunogenic tumors require additional signals for protection against tumor growth (7–10). The combination of antitumor vaccination and CTLA-4 blockade protected the animals from progressive tumor growth but induced autoimmunity to normal tissues that expressed the antigen to which the animals were vaccinated. These observations of tumor regression prompted the initiation of clinical trials using fully human antibodies directed against CTLA-4 in cancer patients. Two antibodies with specificity for CTLA-4 have entered clinical trials and showed early efficacy against tumors sensitive to immune modulation, including malignant melanoma and renal cell cancer. Clinically, the administration of both antibodies is associated with the development of autoimmune phenomena. However, the mechanism responsible for autoimmunity and tumor response is unclear (11–13).
We report the results of a clinical trial using ipilimumab in patients with progressive tumor following administration of a cancer vaccine. The primary end point of the trial was to determine the clinical toxicity of multiple doses. Secondary end points included assessment of disease response, effects on regulatory T-cell (Treg) numbers, and vaccine-specific CD8+ T-cell responses.

Materials and Methods

Eligibility criteria. Patients with progressive disease previously treated on National Cancer Institute–approved antitumor vaccine studies were eligible. Patients with non–Hodgkin’s lymphoma immunized with patient-specific idiotype vaccines, prostate cancer patients immunized with a recombinant vaccinia virus vector expressing prostate-specific antigen, and colon cancer patients immunized with a mutant ras peptide-pulsed dendritic cell vaccine were eligible. Eligibility criteria also included age ≥18 years, life expectancy of ≥2 months, Karnofsky performance status ≥70%, treatment with one of the cancerspecific vaccines within 14 months of study entry, white cell count ≥2,500/µL, granulocytes ≥1,500/µL, platelet ≥100,000/µL, hemoglobin ≥10 g/dL, hematocrit ≥30%, creatinine ≤2.0 mg/dL, serum glutamic oxaloacetic transaminase/serum glutamate pyruvate transaminase value <3.0-fold greater than the upper limit of normal, and glutamic oxaloacetic transaminase/serum glutamate pyruvate transaminase value <3.0-fold greater than the upper limit of normal, and bilirubin <3.0 g/dL. Exclusion criteria included corticosteroid use, history of autoimmune disease, or active infection. Patients with HIV, hepatitis B, or hepatitis C were excluded. This protocol was approved by the Institutional Review Board of the National Cancer Institute, and all patients provided written informed consent before enrollment on protocol.

Study design. This was a single-center, open-label phase II trial to assess the safety and activity of ipilimumab in selected solid tumor patients that had progressed following antitumor vaccination. Ipilimumab is a fully human IgG1 antibody obtained from transgenic HuMab mice, strain HC2/Kco7 (Medarex, Bloombury, NJ), immunized with the extracellular domain of CTLA-4. Ipilimumab was administered to patients for four cycles. The initial dose of 3 mg/kg was administered i.v. over 90 min, and the subsequent three doses were administered at 1.5 mg/kg every 4 weeks.

Patient assessments. After initial eligibility screening, evaluations were done at 4-week intervals to assess clinical response. Measurable disease was recorded using the Response Evaluation Criteria in Solid Tumors criteria for patients with prostate or colon cancer (14). Prostate-specific antigen criteria for response and progression were based on the Working Group Consensus (15). Patients with lymphoma were evaluated using the International Workshop Standardized Response Criteria (16).

To screen for the development of autoimmunity, all patients were required to have a negative serum rheumatoid factor and an antinuclear antibody of ≤1:80 before study. If the antinuclear antibody was positive, anti-dsDNA, anti-SSA, anti-SSB, antiphospholipid, antineutrophil, and anti-islet cell antibodies were done. While on study, serum autoantibodies and immune complexes were monitored regularly. In addition, all patients underwent a regular screening examination by an ophthalmologist.

Immunologic assessment. Flow cytometry was used to assess the surface expression of selected T-cell markers on peripheral blood mononuclear cells (PBMC) before and 72 h after each cycle of therapy using a flow cytometer (FACScan, Becton Dickinson, San Jose, CA). Leucocytes (CD45/PE/CD14 PE, Becton Dickinson), IgG1 subclass controls (FITC, PE, and PerCP, Becton Dickinson), CD4 (FITC, Beckman Coulter, Fullerton, CA), CD8 (PE, Beckman Coulter), CD3 (FITC, Becton Dickinson), CD20 (PerCP, Becton Dickinson), CD25 (PE, Becton Dickinson), CD69 (FITC, Becton Dickinson), CD71 (PE, Becton Dickinson), CD62L (FITC, Becton Dickinson), CD45RA (FITC, Beckman Coulter), CD45RO (PE, DAKO, Carpenteria, CA), and natural killer cells by CD3+CD16 and CD56+ (PE, Becton Dickinson) were used as reagents. CD4+CD25+CD62L− T cells were used as a marker for Tregs, as the CD62L+ population is more potent, proliferates, and maintains suppressive function far better than CD62L− populations (17–19).

Prostate-specific antigen immunologic response. Patients were evaluated for a change in their vaccine-specific CD8+ T-cell responses compared with their baseline using a peptide-specific enzyme-linked immunospot assay measuring IFN-γ as described previously (20, 21). The assay was done in the Laboratory of Tumor Immunology and Biology (National Cancer Institute, Bethesda, MD).

Idiotype vaccine immunologic response. Idiotype-specific T-cell cytokine responses, IFN-γ enzyme-linked immunospot, and anti-idiotype antibody responses were measured as described previously (22, 23).

Statistical analysis. The flow cytometry data were transformed to the log scale because observations were approximately Gaussian on this scale, and relative change is a natural way to evaluate change for these measurements. We analyzed the data in several ways. First, we concentrated on changes in these measurements over the first cycle of treatment. We fit a linear mixed model to test for global changes in flow measurements over the first cycle of treatment (24). For flow measurements with a significant global change, we tested for pairwise differences between measurements using paired t tests. Second, we analyzed data corresponding to all available treatment cycles by fitting linear mixed models to all available longitudinal flow cytometry data. All statistical tests were two sided and a P value of ≤0.05 was used as the cutoff for declaring statistical significance.

Results

Patient characteristics. Eleven patients (median age, 56 years; range, 34–72), 4 patients with non–Hodgkin’s lymphoma (2 follicular and 2 mantle cell), 4 patients with prostate cancer, and 3 patients with colon cancer, were treated with ipilimumab between October 2002 and July 2003 (Table 1). All patients had advanced-stage disease, and 8 (73%) had visceral metastases. Patients had a median of 5 prior therapies (range, 2–7). All colon cancer and lymphoma patients had received chemotherapy before vaccine therapy.

Antitumor activity. The only objective responses were seen in the non–Hodgkin’s lymphoma patients. Two patients experienced tumor regression: a partial response in a follicular lymphoma patient and a mixed response in a mantle cell lymphoma patient. The 54-year-old male with symptomatic stage IVE (mantle cell lymphoma) received one cycle of therapy, in which clinical and radiological improvement occurred in a large abdominal mass; however, progression of disease was noted at other sites (Fig. 1). A partial response was seen in one patient with stage IV follicular lymphoma after two cycles of therapy, which was maintained for 14 months after completion of therapy. A lymph node biopsy obtained after ipilimumab administration showed persistent follicular lymphoma with a meaningful CD3+ T-cell infiltrate (Fig. 2). A mixture of CD4+ and CD8+ T cells was present with a predominance of CD4+ cells. No objective tumor responses were seen in the prostate or colon cancer patients.

Toxicities following ipilimumab therapy. Common adverse events possibly related to the study drug included fatigue, desquamating skin rash, urticaria, allergic rhinitis, diarrhea, constipation, and ocular symptoms, all of which were grade 1 or 2 (Table 2). Although there were 13 grade 3 and 4 adverse events, 11 of these occurred in patients 7 and 11, both of whom had metastatic colorectal cancer with evidence of rapid disease.
progression while on therapy, including 1 patient who died within 30 days of their second treatment. These events were not believed to be related to ipilimumab but rather due to tumor progression. An episode of grade 3 diarrhea in a patient with mantle cell lymphoma of the gut and chronic constipation, who self-administered large quantities of laxatives and stool softeners, was also believed to be unrelated to ipilimumab. In light of the association of constipation with ipilimumab administration, it is possible that this patient and the patients with colorectal cancer experienced gastrointestinal toxicity.

Patient 2 developed a grade 2 rash on his trunk and extremities after the second cycle of therapy. A biopsy revealed nonspecific epidermal spongiosis with a mild perivascular lymphocytic infiltrate and occasional eosinophils. The patient

<table>
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<th>Patient</th>
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<th>Prior therapy</th>
<th>Time from last vaccine (mo)</th>
<th>Cycle no.</th>
<th>Response (mo)</th>
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Abbreviations: CRC, colorectal cancer; C, chemotherapy; E, embolization; H, hormonal therapy; M, monoclonal therapy; MCL, mantle cell lymphoma; SD, stable disease; PD, progressive disease; PR, partial response; S, surgery; V, vaccine therapy; XRT, radiation therapy.

*Off study to pursue other therapy with no evidence of disease progression.

**Fig. 1.** Computerized tomographic images of the patients showing tumor regression. Before (A) and after (B) cycle 1 in the mantle cell lymphoma patient showing a reduction in size of a pelvic mass after treatment with one cycle. Pretreatment (C) and posttreatment (D) images in the follicular lymphoma patient who achieved a partial response of 14-mos duration.
received antihistamines, and after 1 week of delay, further doses of ipilimumab were administered without complication.

After completion of four cycles of ipilimumab, patient 4 with known bilateral adrenal lesions developed adrenal insufficiency requiring the initiation of replacement cortisone therapy. Biopsy of the adrenal mass showed only metastatic adenocarcinoma, suggesting that progressive tumor growth rather than autoimmune adrenalitis was responsible for the adrenal insufficiency.

Patient 5 developed a transient low positive antinuclear antibody after the first cycle; however, this reverted to normal after cycle 2. All other autoantibody screens remained negative.

Flow cytometry. Flow cytometry was done on the patients’ PBMCs to define surface marker expression changes in response to therapy. Tregs express CTLA-4, and although this antigen is primarily intracellular, cell surface expression of CTLA-4 is also observed. The major group of Tregs expresses CD4 and CD25; to more specifically identify these cells, CD62L expression was identified. Average CD4+CD25+CD62L+ T cells seemed to change over the first cycle of treatment ($P < 0.001$ using a linear mixed model). Specifically, there was a bimodal response following therapy; CD4+CD25+CD62L+ T cells decreased in 0 to 3 days (geometric mean ratio, 0.82) after the infusion ($P = 0.003$) with a rebound by day 28 ($P < 0.001$, for comparing day 3 with day 28; Fig. 3). Further, values at day 28 (measurement immediately before second treatment cycle) were significantly larger than pretreatment values ($P = 0.03$). We also analyzed all CD4+CD25+CD62L+ T cells longitudinal data throughout all four cycles and found an overall reduction from immediately before treatment to day 3, with a subsequent rebound at the end of the treatment cycle ($P = 0.01$).

We examined the changes in mean CD4+CD25+, CD4+CD3+, and CD3+CD8+ cells over each cycle of therapy (data not shown). The bimodal pattern of a decrease in cells at day 3 in the cycle with a rebound at the end of each cycle was observed for CD4+CD25+ ($P = 0.005$) and CD4+CD3+ ($P = 0.02$) but to a less degree for CD3+CD8+ ($P = 0.09$).

As an additional measure of Tregs, expression of FoxP3 in PBMCs was quantitated by Taqman analysis for a single patient (Fig. 4). The effects of ipilimumab administration on peripheral blood T cells were similar with a decrease in FoxP3 expression at early time points after ipilimumab administration and with a rebound increase by the time of the next treatment.

Immunologic response. Consistent increases in prostate-specific antigen–specific T-cell responses in association with therapy were not identified. An IFN-γ enzyme-linked immunospot assay done on the responding lymphoma patient (patient 8) failed to show any significant differences in the frequency of tumor-reactive T cells before and after treatment with ipilimumab over the course of this study (Fig. 5).
A variety of immunotherapies have shown tumor responses in a small segment of the patients with malignant melanoma and renal cell cancer. A review of all cancer vaccine trials conducted at the National Cancer Institute showed a low overall objective response rate of 2.6%, which was comparable with the results obtained by others (25). The lack of response may be related to the suppressive regulatory mechanisms of the immune system, which normally help prevent the development of autoimmune disease. The inhibition of T-cell expansions through the effect of the CTLA-4 receptor on T cells plays a major role in the maintenance of immunologic tolerance and therefore represents a target amenable to clinical intervention in the induction of antitumor immunity.

The importance of the inhibitory function of CTLA-4 is highlighted in the studies on knockout mice (26, 27). In the absence of CTLA-4, almost all of the peripheral T cells display an activated phenotype (CD25+, CD69+, and CD62L+) and a 4-fold increase in the proportion of cycling T cells. This T-cell activation and proliferation uncontrolled in CTLA-4 knockout mice leads to a fatal lymphoproliferative disorder within weeks of birth.

When this trial was initiated, there were two hypotheses about the possible mechanisms of action of ipilimumab on the immune response. The first was that blockade of CTLA-4 on CD8+ T cells would result in an expansion of vaccine-specific T-cell responses. It might be anticipated that an expansion of both CD4+ and CD8+ T-cell populations could occur as is seen in the CTLA-4 knockout mouse. However, no increase in peripheral blood CD3+, CD8+ T cells was detected following ipilimumab administration in man. Similarly, the number of vaccine-specific CD8+ T cells in the peripheral blood did not increase. These results are consistent with those of Phan et al. (13) and Attia et al. (12) who showed no increase in vaccine-specific T-cell responses in vaccinated patients with melanoma compared with historical controls. In another study in which a melanoma peptide vaccine was combined with CTLA-4 blockade, a higher level of CD8+ T-cell responses to the gp100 peptide vaccine, but not to the MART-1 peptide, was observed. The level of the immune response to the gp100 peptide was low in this study, with only one patient showing a level of gp100-specific tetramer-positive cells of >0.2% (28). In contrast, Smith et al. (29) showed that 28% of patients vaccinated with gp100...
The other hypothesis was the potential for ipilimumab to deplete Tregs through antibody-dependent cell-mediated cytotoxicity. Tregs express surface as well as intracellular CTLA-4; whereas immune response 2 signature includes genes encoding T-cell markers and genes that are highly expressed in macrophages, whereas immune response 2 signature includes genes planned. One possible explanation for the tumor regression observed is that the transient decrease in Tregs allows for immune activation that in turn produced the tumor regression. The prominent T-cell infiltrate in the posttreatment sample from the patient who achieved a partial remission is consistent with this hypothesis. Maintaining the reduction in Tregs may improve the antitumor activity. Denileukin diftitox, a fusion molecule between interleukin-2 and diphtheria toxin, or other immunotoxins, such as LMB-2 or RFT5.dgA, can potentially reduce the number of CD25+ cells and provide a rationale for agent combination. In contrast to the PC61 antibody that depletes murine CD25+ T cells, basiliximab and daclizumab as nondepleting antibodies would not be expected to augment the antitumor activity by removing Tregs. There was no difference in the degree of reduction of Tregs in the responding patients compared with nonresponders, suggesting that other factors in addition to the change in Treg numbers is important for inducing autoimmune reactions and antitumor effects.

This study did not show significant autoimmune toxicity, such as the autoimmune phenomenon reported elsewhere. A recent publication reported by Yang et al. documented grade 3/4 gastrointestinal toxicity in 21% of patients with metastatic melanoma and renal cell carcinoma (31). The reason for the relative lack of enterocolitis in our patients, albeit a small population, is unclear. It may be related to prior chemotherapy regimens or perhaps to differential frequency of autoimmune toxicity in different tumor types.

It is of interest that, in the early interleukin-2 studies, responses were observed in follicular lymphoma patients as well as those with melanoma and renal cell cancer. Gene expression profiles from follicular lymphoma samples identify two signatures that determine prognosis (32). In contrast to patients with diffuse large cell lymphoma and mantle cell lymphoma where prognosis is based on the genetic signature of the malignant cells, the prognosis in follicular lymphoma depends on the signature of the infiltrating cells in the node. The immune response 1 signature includes genes encoding T-cell markers and genes that are highly expressed in macrophages, whereas immune response 2 signature includes genes encoding FOXp3.

The observation that the number of Tregs declines but then increases is confusing. Although the number of Tregs increased 1 month after treatment, the functional characteristics of these cells have not been examined. There may be a defect in the function of these cells, and assays to determine this are needed. It is of interest that, in the early interleukin-2 studies, responses were observed in follicular lymphoma patients as well as those with melanoma and renal cell cancer. Gene expression profiles from follicular lymphoma samples identify two signatures that determine prognosis (32). In contrast to patients with diffuse large cell lymphoma and mantle cell lymphoma where prognosis is based on the genetic signature of the malignant cells, the prognosis in follicular lymphoma depends on the signature of the infiltrating cells in the node. The immune response 1 signature includes genes encoding T-cell markers and genes that are highly expressed in macrophages, whereas immune response 2 signature includes genes encoding FOXp3.
expressed in macrophages and dendritic cells. We are currently evaluating the pretreatment and posttreatment effects of ipilimumab to correlate with tumor response. Microarray analysis will allow us to determine whether it is possible to alter the immune response signature of the patient or if tumor response is more likely with one signature than another.

Conclusions

This study showed the safety of ipilimumab in this patient population. There was tumor regression observed in 2 of 11 patients studied. We observed a decrease in CD4+CD25+CD62L− T-cell numbers at 3 days after ipilimumab infusion, which was followed by a subsequent increase in their number. Treg number expansion following therapy may abrogate the potential anti-tumor effects of ipilimumab; methods to reduce T regulatory populations may further augment its efficacy.