Lack of Effect of Cyclophosphamide on the Immunogenicity of a Melanoma Antigen Vaccine

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ABSTRACT

Melanoma antigen vaccines are a conceptually attractive approach to prevent or delay disease recurrence in patients with surgically resected malignant melanoma. However, the immunogenicity of current vaccines is relatively low. Cyclophosphamide, when given in low doses prior to antigen exposure, is an immunomodulator which has been shown to enhance both humoral and cellular antitumor responses in animals and humans. We conducted a prospective, randomized, clinical trial to study whether pretreatment with cyclophosphamide augments the immunogenicity of a polyvalent, allogeneic, melanoma antigen vaccine in patients with melanoma and low tumor burden.

Forty-five patients with resected stage II melanoma (regional metastases) were randomly allocated to treatment with melanoma vaccine or melanoma vaccine plus cyclophosphamide. All patients received the same dose and schedule of vaccine immunizations; those randomized to cyclophosphamide received 300 mg/m² i.v. 3 days prior to each vaccine immunization. Cellular immune responses were evaluated by delayed-type hypersensitivity (DTH) skin reactivity to a test dose of vaccine at baseline (prior to treatment) and following the fourth immunization. Humoral immune responses were measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiographic analysis of indirect immunoprecipitates of patients' sera at the same time points.

Twenty-four patients were randomized to cyclophosphamide pretreatment and 21 to vaccine alone; 22 and 18 patients were evaluable in each group, respectively.

Differences were statistically nonsignificant with respect to either cellular (DTH) or humoral (antibody) responses between the two groups. DTH responses were induced in 16 of 22 (73%) and 15 of 18 (83%) patients treated with cyclophosphamide plus vaccine and vaccine alone, respectively. The mean posttreatment augmentation in DTH response in the cyclophosphamide group was 9.5 mm, compared with 9.9 mm in the vaccine-only group. Eight of 12 (66%) cyclophosphamide-pretreated patients and 9 of 12 (75%) vaccine-only patients produced increased titers of antimelanoma antibodies following treatment. No differences were observed between the groups in disease-free or overall survival.

In summary, low-dose cyclophosphamide pretreatment failed to augment the immunogenicity of a polyvalent, allogeneic, melanoma vaccine in patients with completely resected early-stage melanoma.

INTRODUCTION

Recent advances in adoptive immunotherapy of metastatic malignant melanoma have demonstrated the clinical utility of biological antitumor agents. A theoretically attractive alternative to the passive administration of high doses of exogenous biological response modifiers is the use of tumor vaccines to specifically and actively induce host antitumor immune responses. There is ample preclinical evidence that melanoma vaccines can prevent or delay disease progression in mice (1–3), and preliminary clinical studies suggest that first-generation melanoma vaccines can induce immune responses and may slow the progression of melanoma in some patients (4–12). Advantages to vaccine immunotherapy are (a) the ability to induce ongoing and long lasting immunity, (b) the preferential stimulation of cellular rather than humoral antitumor responses, (c) the ease and safety of administration and repeated treatment, and (d) the unique potential to prevent cancer.

One strategy to potentiate the immunogenicity of vaccines is the use of cyclophosphamide prior to immunization (16). Low-dose cyclophosphamide administered 2–4 days prior to antigen administration may augment immune responsiveness, presumably by inactivating suppressor lymphocytes which down-regulate immune responses to new antigens (17, 18). Cyclophosphamide pretreatment enhances both humoral and cellular responses to vaccine immunization in tumor-bearing animals (19, 20), including those to tumor vaccines (21). In patients with advanced disease, cyclophosphamide has been reported to augment cellular immunity induced by a melanoma vaccine (16, 17).

Based on these data, we conducted a prospective, randomized, clinical trial to study whether pretreatment with a low dose of cyclophosphamide augments the immunogenicity of a polyvalent, allogeneic, melanoma antigen vaccine in patients with melanoma and low tumor burden.

MATERIALS AND METHODS

Patients. The study population consisted of 45 patients with resected stage II (regional) melanoma. Patients were randomly allocated to treatment with melanoma vaccine or melanoma vaccine plus cyclophosphamide and were entered into the study between June 1, 1987, and October 30, 1989. Eligibility criteria included: histologically confirmed regional metastases; adequate bone marrow function (WBC ≥4000, hematocrit ≥30, platelets ≥100,000), renal function (creatinine ≤2), and liver function (bilirubin ≤2, normal serum glutamate oxalate transaminase); and absence of distinct metastases, as documented by physical examination and normal computer-tomography scans of brain, chest, abdomen, and pelvis. The patients were older than 18 years and had no second malignancy or other immunosuppressive diseases. No concomitant steroids or other potentially immunosuppressive medications were permitted. Patients were required to have intact cellular immunity, as defined by skin test reactivity (≥5-mm induration at 48 h) to at least one of a standard panel of recall antigens (PPD intermediate strength, mumps, Candida, SK-SD). No prior treatment for
melanoma, other than surgery, was permitted. All patients signed written informed consent prior to randomization, as approved by the New York University Medical Center Institutional Review Board.

Study Design. Patients began immunotherapy no earlier than 4 weeks and no later than 10 weeks following complete surgical resection of all evident disease. Baseline physical examination, laboratory tests, chest X-ray, computer-tomography scans, and skin tests were obtained prior to entry into the study. Randomization was conducted according to sequential assignment from sets of five coded cards. Following randomization, patients were treated with 40 μg of vaccine, which was bound to alum as an adjuvant, divided into four equal aliquots, and administered intradermally, in 0.05 ml of saline, into each extremity. An extremity which had undergone lymph node dissection was not treated; rather, two doses of vaccine were administered in the contralateral extremity. Immunizations were given every 3 weeks for four cycles during the induction phase. Booster immunizations were then administered every 4 weeks for three cycles, every 12 weeks for three cycles, and then every 24 weeks. In addition to therapeutic immunizations, a skin test dose of 10 μg was administered intradermally on the volar aspect of the forearm at the first and fourth immunization. Patients assigned to the cyclophosphamide arm received i.v. cyclophosphamide (300 mg/m² in 250 ml 5% dextrose/water, over 20 min) 3 days prior to each vaccine treatment. Prochlorperazine was permitted as an antiemetic. Dexamethasone was not used.

Preparation of Vaccine. A soluble, partially purified, allogeneic, polyclonal melanoma antigen vaccine was prepared from material shed into serum-free culture medium by four lines of melanoma cells, according to a method which has previously been described (22). Three of the cell lines (SFSK-Mel 28, SFM14, and SFM20) were human and one (SFMHS54) was of hamster origin. The cells were selected because they expressed different patterns of cell surface melanoma-associated antigens. The cells were adapted to grow and were maintained in serum-free medium, to prevent contamination of the vaccine with fetal calf serum proteins. For vaccine production, shed material was collected, concentrated by vacuum dialysis, pooled, treated with 0.5% Nonidet P-40 to break up aggregates, and ultra centrifuged at 100,000 × g for 90 min, to remove transplantation alloantigens. The supernatant was concentrated by vacuum dialysis, filter sterilized, adjusted to a protein concentration of 200 μg/ml, bound to alum, dispersed into sterile glass vials, and stored at −4°C until used. The biochemical and antigenic properties of the vaccine have been published (22). It contains the >240-kDa melanoma-associated antigens defined by MoAb 118.1 (25), the 26-, 29-, 95-, and 116-kDa antigens defined by MoAb Nu4b (26), and the 75-, 95-, 120-, 140-, 150-, and 240-kDa antigens defined by a polyclonal antiserum raised in our laboratory (27) and is free of fetal calf serum proteins. For vaccine production, shed material was collected, concentrated by vacuum dialysis, pooled, treated with 0.5% Nonidet P-40 to break up aggregates, and ultra centrifuged at 100,000 × g for 90 min, to remove transplantation alloantigens. The supernatant was concentrated by vacuum dialysis, filter sterilized, adjusted to a protein concentration of 200 μg/ml, bound to alum, dispersed into sterile glass vials, and stored at −4°C until used. The biochemical and antigenic properties of the vaccine have been published (22). It contains the >240-kDa melanoma-associated antigens defined by MoAb 118.1 (25), the 26-, 29-, 95-, and 116-kDa antigens defined by MoAb Nu4b (26), and the 75-, 95-, 120-, 140-, 150-, and 240-kDa antigens defined by a polyclonal antisera raised in our laboratory (27) and is free of fetal calf serum proteins. Skin test material was prepared in a manner identical to treatment vaccine but was not bound to alum.

Evaluation of Cellular Immune Responses. Cellular immune response to the vaccine was evaluated by DTH reactions, in intradermal skin tests, to 10 μg of vaccine without alum. The diameter of erythema and induration at 24 h after immunization was measured with vernier calipers at the first vaccine immunization (baseline) and at the fourth vaccine immunization. Vaccine-induced augmentation of DTH induration was calculated by subtracting the baseline value from the fourth-immunization value in each patient.

Evaluation of Humoral Immune Responses. Antibodies to melanoma cell surface antigens were assayed by indirect immunoprecipitation of detergent extracts of lactoperoxidase-radioiodinated melanoma cells with Protein A-sepharose, as previously described (23). The immunoprecipitates were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and bound antigens were visualized by autoradiography. The presence of a vaccine-induced antibody response was indicated by the presence of a labeled antigen band bound by postbut not preimmunization serum obtained from the same patient.

Clinical Follow-up and Criteria for Tumor Progression. Patients were evaluated for disease recurrence every 3 months during the first year and at 3-6-month intervals thereafter. This evaluation consisted of a complete history and physical examination, complete laboratory blood tests including serum chemistries, liver function tests, and complete blood count, and urinalysis. Chest X-rays were obtained every 6 months, and other diagnostic tests were performed as clinically indicated. Whenever possible, suspicious lesions were biopsied for histological confirmation of recurrence.

Disease-free and overall survival were calculated as the number of months from the date of first vaccine treatment until the first documented evidence of tumor recurrence or death, respectively.

Statistical Methods. The principal immunological response variables in this study were cellular responses (as measured by skin test reactivity, in mm of induration) and antibody responses (as measured by antigen band bound to a greater extent by post- versus pretreatment sera). Patients' before-treatment values were compared to their own posttreatment values. For each treatment group and for each time point evaluation, the one-sample paired t test and one-sample paired Mann-Whitney test were used to test the significance of the augmentation of the patient's response. Analysis of variance was used to examine differences in augmentation of immune response between the treatment groups.

Clinical Follow-up. Overall and disease-free survival curves were calculated according to the Kaplan-Meier method.

RESULTS

Patient Characteristics. Forty-five patients were entered into the study. Twenty-four patients were randomized to receive cyclophosphamide in addition to vaccine treatment and 21 were randomized to vaccine alone. The clinical characteristics at time of entry into the study are described in Table 1. The treatment groups were balanced in terms of major prognostic factors, including site and thickness of primary lesion, number of patients presenting with clinically involved lymph nodes, and number of histologically positive nodes counted on histopathological specimens. Five patients were not evaluable for DTH responses, two in the cyclophosphamide arm and three in the vaccine-only arm. Three of these patients developed rapidly progressive disease prior to the fourth immunization (one in the cyclophosphamide arm and two in the vaccine-only arm).

<table>
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* The abbreviations used are: MoAb, monoclonal antibody; DTH, delayed-type hypersensitivity.

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and one patient in each arm was ineligible for reasons of noncompliance.

Toxicity. There was no vaccine-related toxicity in any patient, other than transient urticaria at the site of vaccine injection. An asymptomatic granuloma (<4 mm in size) persisted for several months, in most instances, at the site of injection of vaccine bound to alum. Sites of skin test injection (of vaccine not bound to alum) had no granuloma formation. Cyclophosphamide-related toxicity was limited to grade 1 nausea in 3 of 24 (12.5%) treated patients and was easily controlled with oral prochlorperazine. One patient had grade 3 nausea/vomiting, despite aggressive antiemetic pretreatment, and cyclophosphamide was discontinued following the third immunization. No hematological toxicity was seen with this dose and schedule of cyclophosphamide, and no significant alopecia was noted.

Stimulation of DTH Response by Melanoma Vaccine. DTH responses were evaluable in 40 of the patients entered into the study. DTH responses were characterized as strong (≥15-mm induration), intermediate (1–14-mm induration), or absent (no induration). Prior to treatment, a strong baseline DTH response to melanoma was present in 3 of 22 (14%) patients pretreated with cyclophosphamide and in 3 of 18 (17%) patients treated with vaccine alone. A similar proportion of patients in each group had intermediate and absent baseline DTH responses (Table 2).

Following the fourth vaccine immunization, strong DTH responses were present in 11 of 22 (50%) of cyclophosphamide-pretreated patients and in 11 of 18 (61%) of those treated with vaccine alone. An additional 5 of 22 (23%) and 4 of 18 (22%) patients in the cyclophosphamide and vaccine-only groups developed intermediate DTH responses. There was no significant difference in the mean size of DTH responses after treatment in the two groups (Table 2).

We also compared the increase in the size of the DTH response induced by vaccine treatment (post-vaccine treatment—baseline). A strong increase in DTH response (≥15-mm increase in induration over baseline measurement in the same patient) was induced in 7 of 22 (32%) of the cyclophosphamide-pretreated patients, compared to 6 of 18 (33%) of those treated with vaccine alone. The average size of the increase was 9.5 mm and 9.9 mm of induration in the two groups, respectively.

Stimulation of Antibody Responses by Melanoma Vaccine. Antibody responses to melanoma were evaluated by an immunoprecipitation sodium dodecyl sulfate-polyacrylamide gel electrophoresis assay in an unselected subset of 24 of the patients entered into the study, 12 of whom were treated with vaccine plus cyclophosphamide and 12 with vaccine alone. Prior to treatment, antibodies to melanoma were present in three patients, all of whom were in the group treated with vaccine alone. One week following the fourth vaccine immunization, antibodies to melanoma were present in 8 of 12 (66%) of the cyclophosphamide-pretreated patients and in 9 of 12 (75%) of those treated with vaccine alone (Table 3).

Clinical Follow-up. Clinical follow-up is included for all 45 patients entered into the study, including the five patients not evaluable for DTH response. Median follow-up for all patients is 23 months. The nonevaluable patients are included in the arms to which they were randomized.

The median overall survival has not yet been reached for either group but approaches 22.3 months for the cyclophosphamide pretreatment group and 18.5 months for the vaccine-alone group. This difference is not statistically significant (Fig. 1).

The median disease-free interval has not yet been reached but is greater than 14 months for both groups. This difference does not appear to be statistically different (Fig. 2).

In another report (12), we have indicated that the size of DTH response induced by this allogeneic melanoma vaccine may correlate with clinical outcome. In this study, a total of 43 evaluable patients were treated. As shown in Table 2, 14 patients had no vaccine-induced increase in DTH response, 13 patients had DTH augmentations of 1–14 mm, and 13 patients had posttreatment DTH responses measuring ≥15 mm. The curves for disease-free survival versus size of DTH response are shown in Fig. 3. The median has not yet been reached for any group. Given the small numbers of patients and relatively short follow-up, no statistically significant differences were seen between these groups.

**DISCUSSION**

The most important finding of this study is that pretreatment with an immunomodulatory dose of cyclophosphamide in this
dose and schedule did not potentiate the immunogenicity of a melanoma antigen vaccine in patients with surgically resected malignant melanoma.

We have previously demonstrated that immunization with a polyvalent, allogenic, melanoma antigen vaccine significantly augmented both humoral and cellular immune responses to melanoma in patients treated in an adjuvant setting following complete surgical resection of regional metastases. The median disease-free survival of those patients who developed a strong DTH response to vaccine immunization was >4 years longer than that of patients who failed to respond (12). Others have similarly observed a correlation between the ability of melanoma vaccines to induce antibody or cellular immune response and improved clinical outcome. Unfortunately, the melanoma vaccines in current use are poorly immunogenic and fail to augment immunity to melanoma in many patients. The development of methods of potentiating the activity of vaccine is thus a critical element in the development of effective vaccines for melanoma.

One strategy to potentiate the immunogenicity of vaccines is to inactivate suppressor mechanisms that down-regulate host responses to immunization. Immunization to antigen triggers complex cellular processes, including suppressor responses. As a result, what could be a potent immune response to a vaccine may be prevented by this normal regulatory system. Immuno-modulators which inhibit the generation of suppressor cells can enhance immune responses to antigen (28, 29). Cyclophosphamide is an immunomodulator which may be particularly promising from this perspective.

Cyclophosphamide selectively inactivates some subsets of suppressor cells (30, 31), leading to augmented humoral and cellular immune responses to non-tumor- (32-34) and to tumor-associated antigens (19, 28, 35). Small doses of this drug administered 2–4 days prior to vaccine immunization have been reported to potentiate immune responses to immunization (16, 32, 36). In animals, cyclophosphamide pretreatment has been reported to enhance humoral and cellular immune responses to vaccines (19, 20, 32, 36, 37), including tumor vaccines (21), and to potentiate their antitumor effect. In humans, pretreatment with cyclophosphamide has been reported to enhance DTH responses to keyhole limpet hemocyanin and to melanoma vaccines in patients with advanced disease (11, 18, 19). Berd et al. (11) reported that cyclophosphamide pretreatment increased the frequency of acquisition of DTH to autologous melanoma vaccines in patients with advanced metastatic disease.

In contrast to these reports, we did not find in this randomized study that an immunomodulatory dose of cyclophosphamide increased the immunogenicity of a melanoma antigen vaccine in patients with regional, stage II, surgically resected melanoma. The incidence and magnitude of DTH and antibody responses induced by vaccine treatment were similar in both groups.

The reason for the difference between our results and those of prior studies does not appear to be due to differences in the dose or schedule of cyclophosphamide used. Cyclophosphamide was used at a dose of 300 mg/m², administered i.v. 3 days prior to each vaccine immunization. This is the standard immunomodulatory dose schedule which has been used in other studies. Rather, we suspect the difference is due to differences in the stage of disease being treated and in the level of baseline suppressor cell activity.

Livingston et al. (38) studied the effect of cyclophosphamide pretreatment on a variety of in vivo and in vitro immune parameters in 20 patients with malignant melanoma, 16 of whom had metastatic disease and four of whom had completely resected malignancy and were without evidence of the disease at the time of study. They found that cyclophosphamide significantly enhanced the ability of patients' lymphocytes to produce antibodies against sheep RBC antigens in vitro and enhanced the proliferation of lymphocytes by concanavalin A. This effect was largely restricted to patients who had endogenously increased suppressor cell activity and was observed in the patients with advanced disease. Similarly, in other studies the immunomodulatory effect of cyclophosphamide was most evident when suppressor cell activity was augmented (29, 39–40). Of particular interest in Livingston's studies was the clear lack of immunomodulatory activity of low-dose cyclophosphamide in patients who had normal rather than elevated baseline levels of suppressor cell activity. Lymphocytes from those patients with low tumor burden showed no improvement in immune reactivity after exposure to cyclophosphamide.

In this context, it is significant that the patients treated in our study had early disease, whereas those treated by Berd et al. (11) and Livingston et al. (38) had advanced metastatic disease and presumably greater suppressor cell activity. However, other explanations may account for the lack of enhanced
immunogenicity in the cyclophosphamide-pretreated group. The cyclophosphamide effect in preclinical models depends upon the particular type of antigen, prior immunization, schedule, and adjuvants used. The studies by Berd et al. (11) utilized autologous tumor antigens rather than allogeneic soluble antigens, and alum was not present in their vaccine as it was in ours. The process of immunization to antigens on whole autologous melanoma cells may be different from that to soluble allogeneic antigens mixed with other adjuvants. Furthermore, the timing and schedule of cyclophosphamide administration are known to affect both immunoinhibitory and immunosuppressive T cell subsets. In our study, cyclophosphamide was given every 3 weeks, as opposed to every 4 weeks, as in the study of Berd et al.

In summary, the results of this study demonstrate that pretreatment with a low dose of cyclophosphamide does not potentiate vaccine immunogenicity, as measured by increased DTH reactivity or antibody titers detected by immunoprecipitation, in patients with early resected malignant melanoma. The previously described enhancing effect of cyclophosphamide on vaccine immunogenicity may be restricted to patients with advanced melanoma, in whom there is greater disturbance in suppressor cell function, or related to differences in the nature of immunizing tumor antigens, adjuvants, and schedule of cyclophosphamide used.

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