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# Adjuvant Immunotherapy in Melanoma With Irradiated Autologous Tumor Cells and Low Dose Cyclophosphamide

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**Background:** Patients with metastatic melanoma to their regional lymph nodes have a poor prognosis despite lymphadenectomy. In an attempt to improve their survival, this feasibility study was undertaken.
**Methods:** Twenty-two melanoma patients, who presented with enlarged regional lymph nodes, underwent therapeutic lymphadenectomy. They were found to have $N_2$ nodal disease, with no evidence of distant metastases, i.e., advanced Stage III disease. One month after the lymphadenectomy, each patient received five autologous tumor vaccines. Each vaccine consisted of $20 \times 10^6$ irradiated autologous tumor cells (20,000 cGy) injected intradermally. The first two vaccines contained BCG and were given 1 week apart. The other three vaccines consisted of irradiated tumor cells only without BCG, administered over 2-, 4-, and 8- week intervals, respectively. Cyclophosphamide was administered intravenously as 300 mgm/m$^2$ 3 days prior to vaccines 1, 4, and 5 to reduce the population of T-suppressor cells. The patients were observed with no additional therapy. Three patients developed recurrences and these site were resected, and the patients were revaccinated in the same fashion utilizing the new tumor cells.
**Results:** After a follow-up of 4–6 years, 15 patients (including 3 who were revaccinated) of the initial 22 patients (68.2%) are alive free of disease.
**Conclusions:** Adjuvant immunotherapy with irradiated autologous melanoma cells and low dose cyclophosphamide seemed to yield better relapse-free survival than the historically reported 10–25%. J. Surg. Oncol. 64:17–22 

KEY WORDS: melanoma; adjuvant therapy; autologous vaccine; revaccination

## INTRODUCTION

Surgery remains the most effective therapeutic modality in the management of patients with melanoma, but its role had reached a plateau. The most recognized prognostic factor in these patients is the stage of the disease. The 10-year survival of those with stage I disease is ~85% and for those with stage II is 45% [1]. Patients with more advanced stages of melanoma have a much worse prognosis. Those with regional lymph node (LN) metastases, i.e., stage III disease, have an overall 10-year survival of 25%. However, patients with clinically palpable regional LNs, which were found to be pathologically positive for metastases have a 5-year survival of 10–24% [2]. Such patients have a survival ranging from 4–115 months with a median of 24 months postlymphadenectomy [3].

Patients with stage III and limited stage IV melanoma are managed by surgical resection. However, despite creating disease-free status in these patients, their prognosis remains guarded. Therefore, adjuvant therapy is sought

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in an attempt to improve their survival. Adjuvant therapy is being explored utilizing biologic response modifiers alone, or with chemotherapy with some success, but further exploration in the field of tumor-specific immunotherapy is needed [4–8].

We have previously reported some survival advantage for the use of mitomycin-c-treated allogenic melanoma cell vaccine as adjuvant therapy after surgical resections in advance stage III melanoma [9]. This was based on the evidence that melanoma cells share common tumor antigens. However, it has been shown that the actual cytotoxic activity of sensitized lymphocytes seems to depend on HLA-antigen [10]. It also has been shown that low dose cyclophosphamide can selectively reduce T-suppressor cells population and enhance the immune response [11]. Therefore, in this study, we have utilized autologous tumor cells for vaccination preceded by low dose cyclophosphamide.

## MATERIALS AND METHODS

### Protocol

This was a single arm prospective study, approved by the Institutional Review Board, to evaluate the potential benefit of an adjuvant tumor-specific active immunotherapeutic approach in patients with high risk of recurrence and death from melanoma. Patients with clinically enlarged regional LNs who were found postoperatively to have metastatic melanoma were the subjects for this study. They were surgically rendered disease-free. Those admitted to this study had no previous therapy for their disease other than surgery. They were not receiving any type of steroids or immunosuppressive therapy. They had no clinical or radiological evidence of distant metastases. All the patients presented with enlarged regional lymph nodes, underwent complete therapeutic regional lymph node dissection, and were found to have metastases to two or more LNs with at least one measuring 3 cm or more in size, or matted LNs, i.e., advanced stage III disease [12]. Also included were patients with regional recurrences after previous regional lymphadenectomy. Four weeks postregional lymphadenectomy, each patient received five autologous tumor vaccines. Each vaccine consisted of $20 \times 10^6$ irradiated (20,000 cGy) tumor cells, which were to be administered intradermally at several sites. The first two vaccines contained Bacillus Calmette-Guerin (BCG) and were given 1 week apart. The third vaccine was administered 2 weeks later, the fourth vaccine was given 4 weeks later, and the fifth vaccine was injected 8 weeks later. Vaccines number 3, 4, and 5 consisted of irradiated autologous tumor cells only, without BCG. Cyclophosphamide was administered intravenously as 300 mgm/m$^2$, 3 days prior to vaccines 1, 4, and 5 to reduce suppressor T-cell population. The patients were then observed without further treatment. In case of recurrence or metastasis, this was surgically resected, if feasible, and the patient was revaccinated in the same fashion using the new clones of cells obtained from the new metastases. The patients were observed again. Patients who develop unresectable or wide spread metastases received systemic chemotherapy.

The third, fourth, and fifth vaccines, which were administered without BCG, were utilized not only for immunization, but also to observe for delayed hypersensitivity skin reaction to the irradiated autologous tumor cells. Patients with metastases to one lymph node and those whose tumors did not yield enough melanoma cells for the vaccine were excluded from the study.

### Patients

Thirty-one patients who presented with clinically enlarged regional LNs underwent lymphadenectomy. The tumors of seven of them did not yield enough tumor cells of $100 \times 10^6$, and they were disqualified. Two other patients whose tumors yielded enough cells refused vaccination. The remaining 22 patients—12 men and 10 women—received the total vaccination program, and none of them withdrew from the study. Their ages ranged from 19 to 80 years with a median of 54 years. The sites of LN metastases were: 11 in the axillae, 7 in the inguinofemoral area (superficial groin), 2 in the pelvic LN (deep groin), and 2 in the neck. They all presented with clinically enlarged and palpable LNs. The pathological findings are shown in Table I. It should be noted that most of the patients had advanced stage III ($N_2$) or early stage IV melanoma when they were admitted to this study.

### Vaccine Preparation

Fresh tumor was obtained under sterile conditions from each patient. Part of this tissue was examined histologically to confirm the diagnosis of metastatic melanoma. The rest of the tumor tissue was utilized for the vaccine preparation. This part of the tumor was washed in Hank's buffered-saline solution (HBSS), mechanically dissociated, treated enzymatically by collagenase type IV and deoxyribonuclease, then passed through nytex filter, and collected as a single cell suspension in RPMI medium. The tumor cells were then washed three times in RPMI medium. After the last wash, the tumor cells were counted and suspended in autologous serum containing 10% DMSO and transferred to cryo vials. They were then frozen in a temperature controlled system (Bio-Cool II System) which lowered the temperature 1°/min to −40°C. The vials were transferred to a −80°C freezer overnight and to liquid nitrogen storage the next day.

On the day of the vaccination, the tumor cells were rapidly thawed in a 37°C water bath. The cells were washed three times in RPMI. Cell viability was determined by the trypan blue exclusion method. A viability of 80–90% was acceptable. The cells were then irradiated

**TABLE I. Characteristics of the Metanoma Patients Treated with Adjuvant Therapy**

| Patient | Age | Sex | Site | Pathological findings[a] | Comments |
|---|---|---|---|---|---|
| 1 | 54 | F | groin | 5 cm of matted LN& 4 positive LN | |
| 2 | 29 | M | iliac | 4.5 cm of matted LN→ | 1st vaccination |
| | | | jejunum | two large masses→ | revaccination |
| 3 | 49 | F | groin | 6 × 8.5 cm matted LN | |
| 4 | 46 | M | axilla | 5 cm mass | |
| 5 | 19 | M | groin | 4 matted LN | |
| 6 | 43 | F | groin | 5 × 4 cm mass & 2 positive LN→ | 1st vaccination |
| | | | iliac | matted LN + 9 positive LN→ | revaccinated |
| 7 | 38 | M | axilla | 2 masses 5 cm each & 8 positive LN | |
| 8 | 63 | M | axilla | 2 large LN & intransit metastases | |
| 9 | 80 | M | groin | 3 cm LN & another positive LN | |
| 10 | 67 | F | axilla | 3.5 × 2.5 cm of matted LN | |
| 11 | 37 | F | axilla | 3 × 2 cm LN & another positive LN | |
| 12 | 31 | M | axilla | 3 cm of matted LN | |
| 13 | 68 | M | neck | matted LN | |
| 14 | 40 | M | axilla | 3 cm LN & 2 other positive LN | |
| 15 | 60 | F | axilla (R) | 3.5 × 3 cm of matted LN→ | no vaccination |
| | | | axilla (L) | 7 positive LN→ | 1st vaccination |
| | | | axilla (L) | 5 cm of matted LN→ | revaccinated |
| 16 | 74 | F | groin | 5 cm of matted LN | |
| 17 | 42 | M | axilla | 6 cm mass & 2 positive LN | |
| 18 | 62 | F | axilla | 4 cm mass | |
| 19 | 37 | M | axilla | 3.5 × 4 cm mass | |
| 20 | 77 | F | groin | 3.5 × 3 × 2 cm mass | |
| 21 | 60 | M | neck | 4.5 cm mass | |
| 22 | 59 | F | groin | matted LN invading fat | |

[a]LN: lymph nodes.

in a Westinghouse Coronado 250KV 15m AMP cGy X-ray unit at 470 cGy per minute for a total dose of 20,000 cGy.

### Immunization

Four weeks after regional lymphadenectomy, each patient received five vaccinations. Each vaccination was administered intradermally at several sites. The first two vaccinations consisted of irradiated tumor cells and Bacillus Calmette-Guerin (BCG) 0.1 ml of $10^7$ organism/ml for a total volume of 0.3–0.4 ml per each vaccine. These first two vaccines were given 1 week apart. The third, fourth, and fifth vaccinations were administered without BCG. The third vaccination was given 2 weeks after the second vaccine, the fourth vaccination was administered 4 weeks later, and the fifth and final vaccination was injected 8 weeks later. Cyclophosphamide was given as 300 mg/m$^2$ intravenous push 3 days before vaccine #1,4, and 5.

### Follow-up

All the patients were then followed without further therapy. They were examined every 3 months and had complete blood count, fasting blood sugar, and blood urea nitrogen, serum creatinine, and liver function tests performed at each visit. Chest X-rays were obtained every 6 months. After the first 3 years of follow-up, the patients were examined every 6 months and had laboratory tests twice per year. Chest X-rays were performed annually. Computerized tomography (CT) and bone scan were not repeated unless recurrences or metastases were suspected clinically or radiologically. In case of recurrence or metastasis, the patient was re-evaluated and if possible, the metastasis was resected, and the patient was revaccinated in the same fashion.

## RESULTS

Twenty-two patients who were admitted on this study completed 4–6 years of follow-up, with a median of 62 months. Fifteen of them (68.2%) are alive and free of the disease. Three of these 15 patients developed other metastases after their first set of vaccinations. These metastases were resected and they were revaccinated. These three patients have been long-term survivals, and we present their cases here in some detail.

Patient 1 is a 29-year-old white male who was diagnosed to have cutaneous melanoma in the left thigh in January 1987, Clark IV, Breslow 1.0 mm, which was widely excised. In July 1988, he developed an enlarged LN in the left inguinofemoral (superficial groin) area, and he underwent superficial groin lymphadenectomy and one out of 15 LNs contained metastases. Four months later, he developed subcutaneous metastasis in the left subclavicular area, i.e., distant metastasis, and

this was excised. In August 1989, a CT of the abdomen and pelvis showed a 4.3 cm mass in the left iliac area. He was referred to us and the workup revealed no other metastases. Therefore, he underwent left deep groin dissection. The pathological examination showed matted LNs. His vaccination was initiated. However, he was noted to develop anemia during his third vaccination. His vaccination was continued and the workup, which included small bowel endoscopy, revealed metastatic melanoma in the jejunum. He underwent resection of a segment of his jejunum, which contained two large melanotic metastases. He continued to receive his original vaccines for a total of five. This was followed by a second set of five vaccinations utilizing the tumor cells from the small bowel. He is now living free of disease.

Patient 2 is a 43-year-old white female who underwent wide excision of cutaneous melanoma from the left thigh, Clark IV, Breslow 1.2 mm, in July 1990. In August 1991, she developed enlarged LNs in the left groin area, and she underwent superficial groin LN dissection. The pathology revealed a 4 × 5 cm mass and two out of 28 LNs contained metastases. She received five vaccinations. In July 1992, a CT of the abdomen and pelvis showed enlarged iliac LNs. She underwent deep groin lymphadenectomy. The pathology showed that 9 out of 23 LNs examined contained metastases. She was revaccinated utilizing her tumor cells from the deep groin (iliac) LNs. She is alive and free of disease.

Patient 3 is a 60-year-old white female who underwent wide excision of cutaneous malenoma, Clark IV, Breslow 1.25 mm, from her back in January 1990. That July, she developed enlarged LNs in the right axilla, and she underwent right axillary dissection. The pathology showed that one out of 13 LNs contained metastasis. In May 1991, she developed enlarged LNs in the left axilla and underwent left axillary dissection. Seven of 11 LNs had metastases. She was then referred to us, and we received the tumor (from her left axilla) with poor cell viability of 10%. However, we accepted her on the program, and she received the first four vaccines when she was noted to have recurrence in her left axilla. She underwent a second left axillary dissection, and the pathology showed that 14 out of 14 LNs had metastatic melanoma with 5 cm matted LNs. She was revaccinated utilizing tumor cells from the second left axillary dissection. She has been living free of disease.

The other 12 patients who received a single set of vaccinations are alive free of disease. Seven patients died of widespread metastases. Four of them developed metastases during the course of vaccination and succumbed to their disease within 4–12 months. The other three patients developed metastases and died 2 and 2½ years postvaccination.

The relapse-free survival and overall survival of this group of patients are expressed by product limit plot

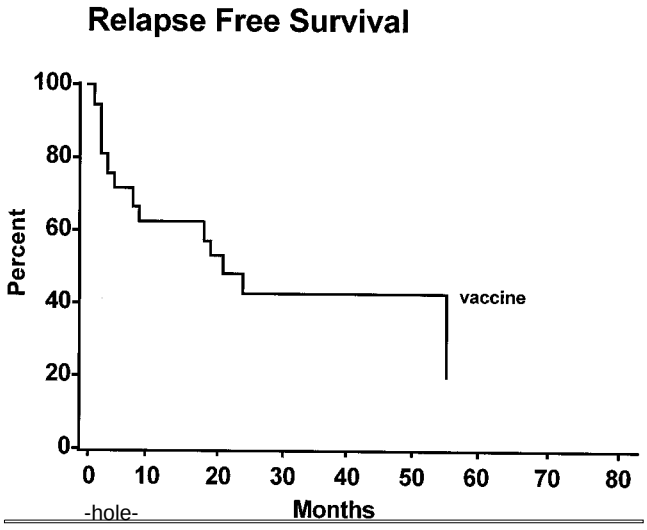


Fig. 1. Relapse-free survival expressed by product limit plot (Kaplan-Meier) of the 22 patients studied. Median = 22.5; Mean = 30.5 ± 5.34 months. Note: Patients who failed after the first vaccination and were revaccinated were considered failures at that point. This was despite the fact that they were revaccinated and are living disease-free.

(Kaplan Meier) in Figures 1 and 2, respectively. Delayed hypersensitivity skin reactions were observed in each patient to vaccines number 3, 4, and 5, where irradiated tumor cells were administered intradermally, without BCG. We categorized the reactions into three types: no reaction (0), erythema of 1 cm or less without induration (±), or erythema of 2 cm or more with induration of 1 cm or more (+). These results were called according to the strongest reaction of the three sets of injections. The results revealed that nine patients showed no reaction (0), four patients expressed (±) reaction, and nine patients expressed (+) reaction. When these results were correlated to the survival, four of the nine patients who had (0) reaction, three of the four with (±) reaction, and eight of the nine with (+) are alive and free of disease.

### Toxicity

Other than the skin erythema at the site of vaccination, none of the patients developed constitutional, neurological, hematological, renal, or hepatic toxicity. However, some of the skin reactions were more severe than others. None of the patients developed any autoimmune disease.

## DISCUSSION

Biological modifiers and cytokines have been used in the management of patients with melanoma with some success. Interferons and interleukins have been utilized in the treatment of patients with metastatic melanoma with some good responses. Recently, it has been reported that there is some beneficial effect of utilizing high dose interferon alpha-2b as a postoperative adjuvant therapy in melanoma patients with regional LN metastases, i.e.,

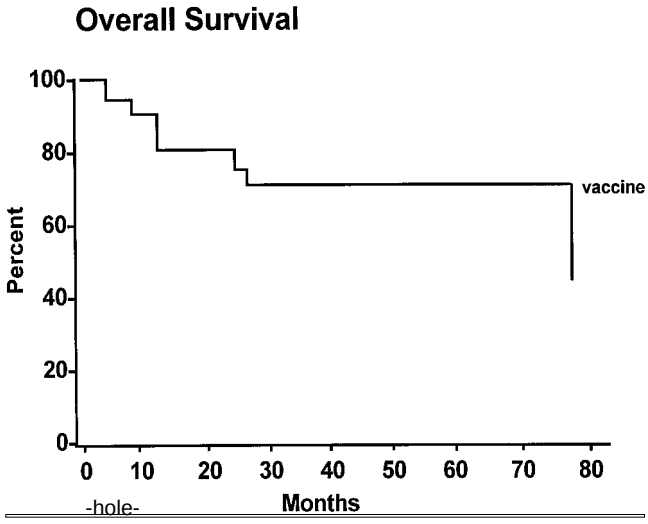


Fig. 2. Overall survival by product limit plot (Kaplan Meier) of the 22 patients studied. Median = 77, Mean = 59.909 ± 6.49 months.

stage III (mostly $N_1$ disease) [17]. Such highly toxic programs improved the disease-free survival from 26% in the untreated controls to 37% in those who received interferon. Therefore, there is no doubt that there is a large margin for further improvement.

The use of several different allogenic tumor cell preparations in combination with BCG or Detox (Ribi Vaccine) have yielded an antitumor response in 10–15% of patients [18,19]. Levington and his colleagues have demonstrated the difficulty in inducing immunization with gangliosides, which are located on the melanoma cell surface [20]. However, the identification of such surface antigens defined as gangliosides $GM_2$ and $GD_3$ with molecular cloning of these antigens recognized by the autologous T cells give a promising new avenue to specific immunotherapy.

Active specific immunotherapy is most effective when given to patients with low tumor load, i.e., grossly free of disease. It also requires an immunocompetent recipient capable of mounting an immune response. Therefore, it is ideally administered as adjuvant to potentially curative surgery in patients who are at high risk of recurrence or metastases. However, such an approach is not effective in patients with widespread metastases. Such patients are overwhelmed by their tumor, have excess tumor antigens and suppressor cells, and are incapable of mounting an immune response. Therefore, they are not candidates for such an approach of active immunization.

The patients in this study have had very advanced stage III melanoma and some had limited distant metastases, i.e., stage IV disease, considering those with small bowel metastases and those with deep groin (iliac) LN metastases. They underwent resection of all their gross tumor and received postoperative autologous tumor vaccine. Their overall survival is 68.2%, compared to the historical controls, who were treated by surgery only and who had 10–24% survival rates in various studies [2,3].

In this study, we noted that there were two patterns of failure. Early failures seemed to have fast progression of their disease after their regional lymphadenectomy. They failed during their vaccination and died within the first year. These patients might have undetected distant metastases that caused such early failure. This included the four patients who died within 4–12 months. The other group who failed later, at 2 years, represented true failure to the vaccine. The sites of failure in these three patients was characteristic of melanoma metastases which included soft tissue, viscera, and brain. These types of metastases were multicentric, overwhelming in character, and did not give us a chance to resect them and revaccinate these patients. As of today, there are no other failures after the 2½ years postvaccination.

The revaccination with new clones of tumor cells obtained from new metastatic sites is a new approach. This is based on the hypothesis that different clones of cells may be committed to different sites or tissues. Fidler and associates provided the first evidence of metastatic heterogenicity [13]. In an experimental model, they proved the role of organ selectivity of metastases [14]. Their results clearly demonstrated that the sites of metastases are determined by both the tumor cells and the specific environment of the host tissue [15]. Nicholson [16] had also shown that there is preferential organ invasion by tumor cells selected for different metastatic potential. Therefore, we felt that the new metastases might have constituted new clones of cells that were not presented in the first set of vaccinations rather than failure of the vaccination. The three long-term survival postrevaccination might confirm such hypothesis.

The use of autologous tumor cells in this study was based on the fact that the actual cytotoxic activity of sensitized lymphocytes seemed to depend on HLA-antigens [10]. We have also utilized low dose cyclophosphamide prior to vaccination in an attempt to reduce the population of T-suppressor cells with the hope of enhancing the cytotoxic response to the tumor cells [11]. Cyclophosphamide was administered prior to the first, fourth, and fifth vaccinations, but not before the second and third vaccines for several reasons: (1) the short duration between the first and second as well as between the second and third vaccines; to give cyclophosphamide prior to the second and third vaccines would result in delaying their administration, (2) close and frequent administration of cyclophosphamide could result in side effects that might interfere in the immune response to vaccination, and (3) we presumed that those patients who have no gross tumor would have low tumor load and equally lower suppressor cell count. However, the use of five vaccinations over a 15-week period was to induce and create hypersensitive cytotoxic T cells. The major

limiting factor in admitting patients to this study was the limited availability of sufficient numbers of autologous tumor cells. Our results seemed to be compatible to those reported by Berd and his colleagues [21] utilizing hapten as a conjugate with autologous tumor cells.

## CONCLUSIONS

In spite of the small numbers of patients who have been studied, it seemed that active immunization utilizing irradiated autologous tumor cells preceded by low dose cyclophosphamide in the adjuvant setup can improve the survival of patients with advanced stage III and limited stage IV melanoma. Such an approach had no significant toxicity other than the local skin reactions at the vaccination sites. The revaccination of the patients who developed limited resectable recurrences or metastases after the initial vaccination is a new approach that seemed to result in some survival advantage. Hypothetically, this can be explained by heterogenicity of the tumor cells obtained from different metastatic sites. Whereas a prospective controlled randomized study comparing such vaccination approach to high dose interferon may be in order, a combination approach of autologous tumor cell vaccine and immune modulator(s) may further improve the results.

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