

REVIEW ARTICLE

Clinical Implications of the New Biology in the Development of Melanoma Vaccines

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There has been a resurgence in clinical research of vaccine therapies, particularly for the treatment of melanoma. The renewed interest in this field is attributable to an increased understanding regarding the immune response to tumors and the immunobiology of melanoma. Molecular biology techniques have enabled investigators to develop genetically engineered tumor vaccines that are intended to favor the type 1 immune response over the type 2 response. Melanoma-associated antigens have been characterized at the molecular level and are currently being investigated in clinical trials. Dendritic cell biology has also provided a potent method to present antigens to the host for immunization. Lastly, vaccines are being explored as a method to generate immune T-cells for adoptive immunotherapy. These new areas of clinical investigation will be reviewed in the context of the historical developments that have laid the foundations of this field. *J. Surg. Oncol.* 1999;70:263-274. © 1999 Wiley-Liss, Inc.

KEY WORDS: melanoma; vaccine; gene therapy; immunotherapy; tumor antigens

INTRODUCTION

The concept of sensitizing the human immune system by exposing it to foreign antigenic material, in an attempt to protect prophylactically against a fatal infectious disease, was pioneered by Dr. Edward Jenner over 200 years ago. His work resulted in the advent of the smallpox vaccine and eventual eradication of this disease. Approximately 100 years later, Dr. William B. Coley (Fig. 1) extended the concept of vaccine therapy to neoplastic diseases when he observed tumor regression in a patient with a systemic bacterial infection. He went on to infect deliberately cancer patients with both live and heat-killed bacteria (Coley's toxins) in an attempt to stimulate the elimination of the tumor cells by the patient's own immune system. Complete tumor regression was seen in some individuals, but overall his results were inconsistent, and this approach was eventually discontinued [1,2].

Over the ensuing years, there has been increasing evidence that melanoma is one of the more immunogenic human solid tumors. It is well known by clinicians that cutaneous melanomas can demonstrate partial regression

of the primary lesion, thus giving rise to the classic diagnostic features of variation in color and irregular borders. These characteristics have been attributed to a spontaneous host antitumor phenomenon. Laboratory investigators have shown that blood from melanoma patients contain antibodies against tumor antigens [3] and that patients with localized melanoma or those who have undergone spontaneous regression of their primary melanoma have a significantly higher incidence of antimelanoma antibodies than those with advanced metastatic disease [4]. Cytotoxic T-lymphocytes that can kill tumor cells *in vitro* in an immunologically specific manner have been isolated from melanoma patients [5,6]. These tumor-reactive cytotoxic T-lymphocytes can produce tu-

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Fig. 1. William B. Coley, MD, was a general surgeon in New York City during the 1890s. (Photograph courtesy of Dr. Helen Coley Nauts.)

mor regression after expansion in vitro and reinjection into the same patient [7]. From other clinical studies, it is known that 3%–15% of cutaneous melanomas are first diagnosed as lymphatic or visceral metastases with no physical evidence of a primary tumor. The ability to cure a subgroup of these patients with surgical resection suggests that immunological mechanisms are capable of managing residual micrometastatic disease [8]. All these observations lend credence to the notion that melanoma tumors express antigens that can serve as targets for immunotherapy. This review article will focus on the development of vaccines for melanoma with specific references to basic immunological principles required for such vaccines, as well as the current clinical trials that are being performed.

DISCUSSION

Nonspecific Immunostimulants

The initial attempts at treating melanoma were with nonspecific immunostimulants to induce host responses against tumor without targeting specific antigens. Similar to Coley's early observations, Morton et al. [4] later dem-

TABLE I. Prospective, Randomized Trials of Adjuvant BCG in Melanoma

Study ^a	Year	Number of patients ^b	Stage	Outcome ^c
ECOG [9]	1979	443	II, III	N.S.
UCLA [10]	1981	91	III	N.S.
		137	I, II	N.S.
MSKCC [11]	1981	47	III	N.S.
NCI [12]	1981	83	II, III	N.S.
WHO [13]	1982	388	II, III	N.S.
U. Alberta [14]	1984	199	II, III	N.S.
RPMI [15]	1987	57	I, II, III	N.S.
NCI-Canada [16]	1991	273	I, II, III	N.S.
EORTC [17]	1993	327	II	N.S.

^aAll studies involved intradermal BCG vs. observation. ECOG = Eastern Cooperative Oncology Group; UCLA = University of California at Los Angeles; MSKCC = Memorial Sloan-Kettering Cancer Center; NCI = National Cancer Institute; WHO = World Health Organization; RPMI = Roswell Park Memorial Institute; EORTC = European Organization for the Research and Treatment of Cancer.

^bTotal = 2,045.

^cN.S. = not significant.

onstrated that intralesional injection of viable *Bacillus Calmette-Guerin* (BCG) organisms could lead to the regression of intradermal metastases of melanoma. More interesting was the fact that nearby uninjected lesions occasionally regressed as well. These findings provided the rationale to investigate BCG as an immune adjuvant after resection of melanomas in patients with a high risk of relapse. Table I summarizes all of the prospective randomized trials that evaluated BCG as an immune adjuvant [9–17]. Unfortunately, BCG did not prove to be effective in reducing relapses after resection. Other nonspecific immunostimulants have been evaluated. Dinitrochlorobenzene (DNCB) is a synthetic primary allergenic molecule that can be used to test the cellular immune competence of an individual for delayed-type hypersensitivity reactions. It has also been used topically and intralesionally for locoregional control of recurrent melanoma. Like BCG, DNCB has been associated with isolated cases of tumor regression in nontreated skin or lymph node metastasis, suggesting that a systemic immune reaction can be induced [18,19]. Additional nonspecific immunostimulants studied clinically include *Corynebacterium parvum*, levamisole, transfer factor, isoprinosine, and thymic factor, thymostimulin. However, in the adjuvant setting, none of these immunostimulant agents alone has proved to be effective in prolonging survival in melanoma patients [20]. Currently, nonspecific agents are usually administered as an adjuvant combined with tumor antigens to enhance the overall immune reactivity and augment a tumor-specific response.

Tumor Cell Vaccines

Unlike nonspecific immunostimulants, tumor-derived vaccine reagents are designed to elicit a host immune

TABLE II. Defined Melanoma Antigens

Antigen	Mechanism of recognition	
	Cell type	HLA restriction
Ganglioside carbohydrate epitopes		
GM ₂ [21]	B	Not restricted
GD ₂ [22]	B	Not restricted
9-0-acetyl GD ₃ [23]	B	Not restricted
Peptide epitopes		
MAGE-1 [24]	T	HLA-A1, -Cw*1601
MAGE-3 [25]	T	HLA-A3
BAGE [26]	T	HLA-Cw*1601
GAGE [27]	T	HLA-Cw6
MART-1 (Melan-A) [28]	T	HLA-A2
gp100 (pmel 117) [29,30]	T	HLA-A2
gp75 (TRP-1) [31]	T	HLA-A31
Tyrosinase [32–35]	T	HLA-A2, -A24, -B44, -DRB1*0401
CDK4 [36]	T	HLA-A2

response to known or as yet uncharacterized tumor-associated antigens. A list of tumor antigens associated with melanoma is summarized in Table II [21–36]. In this regard, vaccine therapy has also been termed “active specific immunotherapy.” The immune response to active specific immunotherapy is both humoral and cell-mediated. Humoral immunity consists of antibody production from mature B-lymphocytes and usually involves the presence of antigen-specific helper T-cells. Cellular immunity involves stimulation of CD4⁺ helper T-cells by processed tumor antigens present on the surface of a “professional” antigen-presenting cell (i.e., a dendritic cell as opposed to a tumor cell). This interaction is restricted to antigen-presenting cells and CD4⁺ T-cells that express the same major histocompatibility (MHC) class II molecules. Two different functional subsets of CD4⁺ helper T-cells can be generated through this interaction and are characterized by the profiles of cytokines that they secrete. Type 1 (Th1) cells principally secrete interleukin-2, interferon- γ , and tumor necrosis factor- α , while type 2 (Th2) cells primarily secrete interleukin-4, interleukin-5, interleukin-6, and interleukin-10. Th1 CD4⁺ helper T-cells can enhance the induction of CD8⁺ cellular cytotoxic T-lymphocyte response, whereas Th2 cells exert their effect on humoral immunity by inducing B-lymphocytes to differentiate into antibody-producing cells. Several studies suggest that type 2 responses of T-cells can have a negative impact on, or suppress, type 1 T-cell responses [37,38]. The sensitization or induction of CD8⁺T-cells to react to tumor antigen also requires the interaction of dendritic cells that have been previously activated by CD4⁺ cells [39]. Both the activation and effector phases of these interactions are summarized in Figure 2. An effective tumor vaccine requires activation of both CD4⁺ and CD8⁺ cell-mediated immune responses; namely, a class II-restricted Th1 helper response

followed by class I-restricted killer cell response, respectively.

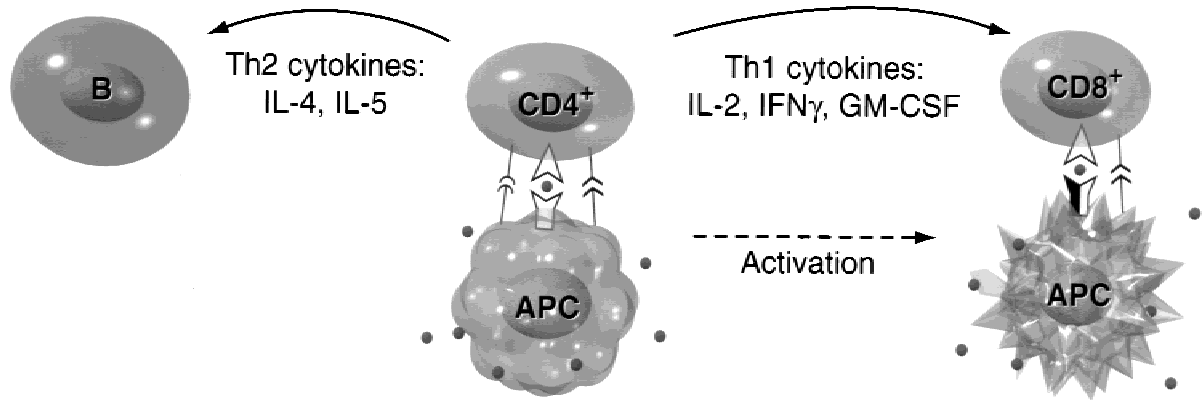
Autologous Whole-Cell Vaccines

Whole tumor cells inactivated by irradiation so they are not capable of growth can be effective vaccine agents in syngeneic animal tumor models. Autologous tumor cells used as vaccines have the theoretical advantage of ensuring that all biologically relevant antigens are presented to the immune system. However, this approach is limited to individuals with sufficient tumor available to prepare a vaccine. Such patients typically have bulky nodal or distant metastatic disease. Furthermore, patients usually have significant residual tumor burdens that can induce immunosuppressive effects, making them less than ideal candidates for an immunotherapeutic approach. Nevertheless, vaccine approaches in this group of patients have resulted in a subgroup of patients who have responded to therapy.

Berd et al. [40,41] have conducted clinical studies in patients with stage IV melanoma utilizing autologous tumor vaccines. In their protocols, autologous whole-cell tumor vaccine preparation involved taking freshly excised tumor, dissociating the tumor into a single cell suspension, and then cryopreserving the cells in liquid nitrogen. Three days prior to vaccination patients receive intravenous cyclophosphamide as an adjuvant. Low doses of cyclophosphamide, as used in this trial, are thought to reduce the tumor-induced suppression observed in experimental models. On the day of vaccination the melanoma cells were thawed, irradiated, and combined with BCG. The autologous vaccine was administered by intradermal injections in the extremities, with repetition of the cycle every 4 weeks. A nonrandomized trial of this autologous tumor vaccine demonstrated a 12.5% response rate [41]. Antitumor responses were associated with the development of delayed-type hypersensitivity to autologous melanoma cells assessed by skin testing. This suggested that an intact host immune response was required for vaccine-induced tumor regression.

It has been shown in a murine model that presentation of tumor cells conjugated to a strongly immunogenic hapten, such as trinitrophenyl, can induce the development of systemic T-cell immunity to unmodified tumor cells [42]. Berd et al. [40,41] have developed an autologous whole-cell melanoma vaccine approach that employs a hapten modification of the above preparation in an effort to induce a tumor inflammatory response in metastatic melanoma patients. Dinitrophenyl (DNP) is utilized as the hapten and is conjugated to the autologous melanoma cells during vaccine preparation. Patients are sensitized to DNP by topical application of dinitrofluorobenzene prior to vaccine administration. This treatment has been reported to induce inflammatory responses in

A. Activation Phase



B. Effector Phase

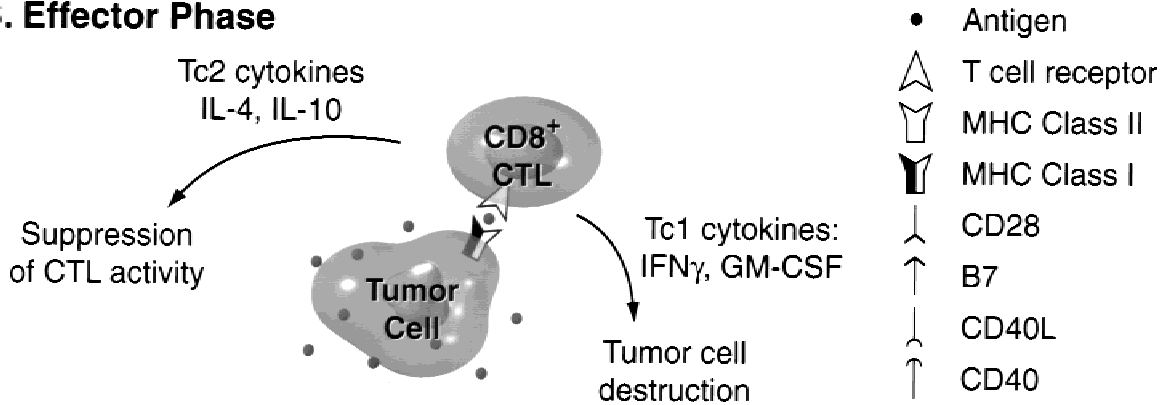


Fig. 2. Schematic diagram of cellular interactions involved in the activation of T-cells resulting in tumor recognition. (A) Activation phase of both CD4⁺ and CD8⁺ T-cells require priming by antigen-presenting cells (APC). CD4⁺ T-cells require MHC class II restriction and costimulation with CD28/B7 and CD40L/CD40 ligand-receptor interactions. This results in Th1 cytokine release to provide help for CD8⁺ T-cell activation as well as activating APC to prime CD8⁺ cells to antigen. Th2 cytokines released by CD4⁺ cells stimulate B-cells to produce antibodies. (B) The effector phase involves CD8⁺ T-cells primed to tumor antigen. Upon engagements with tumor cells presenting antigen via MHC class I molecules, a type 1 (Tc1) or type 2 (Tc2) cytokine response occurs, which can result in tumor destruction or suppression of the immune response, respectively. Note that not all of the costimulatory interactions between T-cells, APC, and tumor cells are depicted.

metastases, and also significantly increases disease-free survival and overall survival in patients with bulky resectable nodal metastasis when compared to surgery alone [43]. Interesting immunologic findings include marked infiltration of metastases with CD8⁺ T-lymphocytes, presence of m-RNA for interferon- γ , and the appearance of novel T-cell receptor V β structures in patients with metastatic melanomas in which inflammation was induced by the DNP-modified vaccine [43–45]. The concept of attaching an immunogenic molecule to the tumor cell to make it a more effective vaccine is utilized as a rationale for investigating genetically modified tumor cells as vaccines.

Allogeneic Polyvalent Vaccines

Polyvalent melanoma cell vaccine. The polyvalent “antigen-enriched” whole-cell melanoma vaccine (PMCV) described by Morton et al. [46] is a live-cell preparation of three allogeneic melanoma cell lines chosen for their high content of immunogenic tumor-

associated antigens and melanoma-associated antigens. The cell lines are grown separately, harvested, combined in equal parts, irradiated, and then cryopreserved in liquid nitrogen. Patients receive biweekly intradermal injections of PMCV during a 12-week induction phase. The first two vaccinations are given with BCG. The patient then receives monthly vaccinations during the first year followed by injections every 2–3 months thereafter. A phase II trial of PMCV for stage IV melanoma patients showed a higher median survival for vaccine recipients (23.1 months) vs. historical control patients receiving other therapies (7.5 months) [47,48]. Survival was significantly related to the development of delayed cutaneous hypersensitivity to the melanoma antigens and induction of antimelanoma IgM antibody titers. Another phase II trial of PMCV for stage III melanoma revealed a significantly higher median survival for patients receiving adjuvant therapy with PMCV following complete resection of regional metastases compared to a historical

control group that received other postoperative adjuvant therapies (>80 vs. 24 months, respectively) [48]. A phase III prospective randomized trial of adjuvant PMCV vs. interferon- α for stage III melanoma patients was begun last year at the John Wayne Cancer Institute.

This polyvalent melanoma cell vaccine induced IgM and IgG antibodies to a 90-kDa glycoprotein melanoma-associated antigen (TA90) [49]. In stage IV melanoma patients postoperative TA90 immune complex (TA90-IC) levels prior to adjuvant therapy with PMCV correlated with survival. Median and disease-free overall survival were significantly higher for patients with negative prevaccine TA90-IC levels than for those with positive TA90-IC levels. Thus prevaccine TA90-IC levels were felt to be a predictor of survival in melanoma patients receiving adjuvant immunotherapy with PMCV [50].

Viral lysates. Cell lysates of tumor cells have been utilized as a source of tumor antigens. One method of producing cell lysates are by infecting cells with lytic viruses. Viral-induced oncolysis can induce long-lasting antitumor immunity in a host by combining strong viral antigens with weak tumor-associated antigens that result in an enhanced immune response to the tumor antigens. An active specific immunotherapy approach using this methodology has been developed and evaluated by Wallack et al. [51] using vaccinia virus. A vaccinia melanoma oncolysate (VMO) vaccine was produced by infecting four established allogeneic melanoma cell lines, Mel-2, Mel-3, Mel-4, and Mel-B, with live vaccinia virus. The cells were incubated overnight and a nucleus-free cell lysate containing membrane fragments of virus-lysed cells extracted by repeated sonication and centrifugation. The lysates of all four cell lines were pooled in equal concentrations to produce a tetravalent VMO vaccine. This polyvalent vaccine contained a variety of melanoma-associated antigens such as nerve growth factor receptor, Ia antigen, melanoma transferrin, melanoma antigen-1, melanoma antigen-3, MART-1, gp100, fetal-associated antigen, urinary tumor-associated antigen, GD3, GD2, GM2, and high-molecular-weight glycoprotein antigen [52].

A phase III prospective randomized trial of VMO vs. vaccinia virus alone has been conducted in stage III melanoma patients after surgical resection of involved nodes [53]. In this trial, patients received a smallpox vaccine injection 1 week before the initiation of treatment. The VMO vaccine or vaccinia virus alone was administered intradermally near the sites of nodal basins weekly for 13 weeks and then biweekly for 1 year. Unfortunately, final results at a median follow-up time of 46.3 months showed no statistically significant increase in either disease-free interval or overall survival between the VMO vaccine group and the vaccinia virus-alone treatment groups. A retrospective subset analysis showed a statistically significant improvement in survival at 2-,

3-, and 5-year intervals for a subset of males between the ages of 44 and 57 years with one to five positive nodes, after treatment with VMO when compared to the vaccinia virus alone group. The cell lines used contained most of the HLA components; however, there were no CTL-producing peptide antigens associated with HLA-A2 because the VMO was prepared from cell lines that did not express HLA-A2. This has raised the concern that the VMO treatment may not have been able to induce an effective cytolytic T-cell immunity to HLA-A2-restricted tumor antigens in patients being treated [27]. Approximately 50% of individuals in the North American population are HLA-A2-positive.

Another vaccinia melanoma cell lysate (VMCL) vaccine has been developed by Hersey [54]. This vaccine utilizes only one allogeneic melanoma cell line, but is otherwise prepared by a method similar to that used by Wallack et al. A phase II trial of VMCL for stage III melanoma showed improved 5-year survival for those treated with VMCL alone (60%) vs. both a historical (34%) and a concurrent nonrandomized (35%) control group [55]. The addition of low-dose cyclophosphamide to VMCL treatment did not improve survival when compared to the VMCL alone treatment group. A 400-patient prospective, randomized study of VMCL vs. no immunotherapy has completed enrollment and awaits final analysis [56].

Mechanical lysates. Mechanical disruption of cell membranes represents an alternative method to prepare cell membrane-associated antigens. Utilizing a high-speed tissue homogenizer and three freeze-thaw cycles, a mixture of mechanical lysates of two melanoma cell lines was created by Mitchell et al. [57]. This allogeneic homogenate was used in combination along with a novel adjuvant, DETOX (a combination of detoxified bacterial endotoxin and mycobacterial cell wall skeletons; Ribi ImmunoChem Research, Hamilton, MT), as a vaccine (Melacine, Ribi ImmunoChem Research) for injection into metastatic melanoma patients. DETOX was selected as an adjuvant because it resembled Freund's adjuvant and was capable of augmenting both antibody induction and cell-mediated immunity. The Melacine vaccine was administered subcutaneously into the upper extremity and buttocks on days 1, 8, 15, 22, and 36 for one or two courses and then monthly in patients with clinical responses. Vaccine administration was associated with minimal pain and little to no systemic toxicity.

Combined results of both phase I and phase II trials in stage III and IV melanoma patients showed an overall response in 20 of 106 patients; 5 of 20 had complete responses and 15 partial responses. The median duration of a response was 21 months. Overall survival was not increased, but 12 of the responding patients have lived 2 or more years with a median survival of over 36 months [58]. The strongest correlate of clinical response was an

increase in precursors of cytotoxic T-lymphocytes; however, only 30% of those who generated these precursors had an objective long-term remission of more than 1-year duration. The failure to generate cytotoxic T-lymphocytes was invariably associated with failure of the vaccine to induce tumor shrinkage and only those patients in which a cell-mediated response was elicited were capable of rejecting their melanoma tumors [59]. Eighteen patients who failed to respond to at least one treatment cycle of the Melacine vaccine were then treated with interferon- α -2b at $5\text{--}6 \times 10^6$ U/m² three times per week subcutaneously for at least 2 months. Eight of the 18 patients (44%) had a major objective clinical response to the interferon- α -2b treatment [60]. A phase III trial of Melacine with interferon- α -2b vs. interferon- α -2b alone is underway in patients with stage IV disease [61]. In another trial, the Southwest Oncology Group (SWOG) has completed enrollment of 689 patients with node-negative, intermediate-thickness melanomas (1.5–4.0 mm) into a prospective, randomized trial evaluating Melacine vs. observation after resection (Dr. Vernon K. Sondak, personal communication). This represents the largest prospective randomized vaccine trial ever conducted. The final results of this trial await maturation with further follow-up.

Gene-Modified Tumor Cell Vaccines

The concept of genetically modifying tumor cells to be more immunogenic by introducing genes that encode immunomodulatory proteins (e.g., cytokines, costimulatory molecules, immunogenic proteins) continues to stimulate interest in autologous tumor vaccines. Many studies have shown that the transduction of murine melanoma tumor cells with a variety of cytokine genes leads to the rejection of the genetically modified cells by syngeneic hosts [62–66]. These studies indicate that cells expressing interferon- γ , interleukin-2, tumor necrosis factor- α , interleukin-4, interleukin-6, or interleukin-7 increase systemic immunity as well, because mice vaccinated with transduced cells rejected a subsequent challenge of non-transduced cells, and in some cases eliminated a preexisting tumor. GM-CSF was the most potent stimulator of systemic antitumor immunity of over 25 different cytokines and other immunomodulators tested in a murine melanoma model [65]. Both CD4⁺ and CD8⁺ T-lymphocytes were required for effective vaccination with GM-CSF, since depletion of either T-cell subset before or after vaccination abrogated the development of systemic immunity. Localized expression of GM-CSF may specifically enhance tumor antigen presentation by host dendritic cells, thus leading to antigen-specific T-cells. Dranoff et al. [67] have initiated a phase I trial in stage IV melanoma patients with a vaccination of autologous, irradiated melanoma cells engineered to secrete GM-

CSF. Preliminary results have shown the development of potent, specific, and long-lasting systemic immunity, with evidence of brisk T- and B-cell responses associated with dying tumor cells at sites of preexisting masses. Intradermal vaccine sites showed prominent dendritic cell accrual [68]. Cytotoxic T-lymphocytes reactive with autologous melanoma cells were detectable after vaccination, both in limiting-dilution analysis and in bulk culture without added cytokines. Analysis of the cytotoxic T-lymphocytes showed a conversion from a purely CD8⁺ response to a high proportion of CD4⁺ clones following vaccination, suggesting that vaccination induced MHC class I-restricted tumor-specific killing.

Two phase I clinical trials involving autologous melanoma tumor cells genetically modified to secrete interferon- γ have been reported [69,70]. Nemunaitis et al. [69] reported no toxicity with repeated subcutaneous injections every 2 weeks for a total of six injections. There were no measurable tumor responses observed in the five patients treated. Abdel-Wahab et al. [70] reported on 13 patients who completed vaccination and demonstrated 8 of them developing a humoral immunoglobulin IgG response against autologous and allogeneic melanoma cells. Two patients with significant increases in serum IgG had tumor responses and two additional patients with low serum IgG responses had transient shrinkage of nodular disease during therapy.

Defined Antigen Vaccines

A number of antigens present on melanoma cells that could potentially serve as targets for the host immune response have been identified (Table II). Although all of these antigens may not be present on every tumor cell, as the number of defined antigens increases, it becomes progressively more likely that at least one relevant antigenic target can be identified for every patient's melanoma. Melanoma antigens can be categorized into two broad groups: tumor-associated antigens and melanoma-associated antigens. Tumor-associated antigens are cell surface products in embryologic tissues, proto-oncogene products, or antigens associated with viral transformation common to melanoma and other tumor cells. Melanoma-associated antigens are primarily proteins or glycoproteins found predominantly in melanomas, but can also be expressed in normal melanocytes.

An example of a family of tumor-associated antigens that have been used for vaccine therapy of melanoma are the gangliosides (Table II). These are carbohydrate antigens expressed on several different tumors (i.e., sarcomas, lung cancers) and are known to be major cell surface constituents of melanoma cells. The gangliosides GM₂, GD₂, and 9-0-acetyl GD₃ are antigens known to be recognized by antibodies, but not by T-cells. Monoclonal antibodies with specificity to these gangliosides can be

found in patients with melanoma. Furthermore, melanoma tumor cells expressing gangliosides can be lysed by antiganglioside antibodies. Livingston et al. [71] have investigated the use of GM₂ ganglioside admixed with BCG as adjuvant therapy for patients with resected stage III nodal disease. In this study, patients were randomized to receive GM₂/BCG vs. BCG alone. Among the patients who were documented to develop IgM antibody responses to GM₂, there was significantly improved disease-free and overall survival compared with patients in the control group. This single-institution study is now being evaluated in a multi-institutional cooperative study in patients with resected stage III disease who are randomized to receive GM₂ in conjunction with keyhole limpet hemocyanin (KLH) and QS21 vs. interferon- α -2b. The KLH and QS21 are immune adjuvants documented to be more effective than BCG in the induction of antibody responses to GM₂ [71].

The ability to identify and grow T-cell clones that can specifically recognize melanoma tumors in an MHC class-restricted manner has allowed investigators to characterize peptide antigens [72]. cDNA libraries generated from tumor cells recognized by the T-cell clones are used to transfect target cells bearing the appropriate MHC class I or II restriction elements. The transfectants can then be screened for recognition by the T-cell clones, and the genes from those transfectants used to identify the amino acid sequence of peptide antigens. These peptide antigens generally represent fragments of protein consisting of eight or nine amino acids called "epitopes" and can be part of a larger protein antigen structure (i.e., tyrosinase). A summary of known melanoma-associated peptide antigens and their MHC restriction elements are listed in Table II. Rosenberg et al. [73] have utilized melanoma-associated peptides for vaccine therapy in patients with stage IV disease. A gp100 epitope, g209–217, was modified (g209-2M) and bound to the HLA-A2 molecule with greater affinity than the unmodified peptide. This peptide was shown to have an increased ability to generate melanoma-reactive cytotoxic T-lymphocytes *in vitro*. A clinical trial was conducted in HLA-A2⁺ patients with metastatic melanoma using an emulsification of the g209-2M peptide in incomplete Freund's adjuvant injected every 3 weeks. Immunologic assay of peripheral blood mononuclear cells from patients after treatment showed that 91% of the patients were successfully immunized with this synthetic peptide. In another cohort of patients who received the peptide vaccine plus adjuvant interleukin-2, 13 of 31 patients (42%) had objective clinical responses. It was interesting to note that only a minority of these latter patients demonstrated immunologic reactivity of their peripheral blood lymphocytes to the peptide despite manifesting tumor regression. A prospective, randomized trial to evaluate the efficacy of this peptide vaccine plus IL-2 vs. IL-2 alone is being con-

ducted by the Surgery Branch, NCI (Dr. Douglas Schwartzentruber, personal communication).

Dendritic Cell-Based Vaccines

CD8⁺ cytotoxic T-lymphocytes appear to be a critical component of the immune response to tumors [74]. These cytotoxic T-lymphocytes recognize peptide epitopes presented by class I major histocompatibility complex molecules that are expressed on the tumor cell surface [75]. Although the recognition of peptide class I complexes is sufficient to trigger target cell lysis, priming of cytotoxic T-lymphocyte responses requires the presentation of the relevant antigen by professional antigen-presenting cells capable of expressing costimulatory molecules [76]. The presentation of tumor antigen by antigen-presenting cells in concert with help provided by CD4⁺ cells to prime these antigen-presenting cells is crucial for the induction of a cytotoxic T-lymphocyte response (Fig. 2) [39].

Dendritic cells are rare leukocytes named for their stellate morphology and are considered to be the most potent of all antigen-presenting cells [77]. Dendritic cells are generated in the bone marrow, course through the blood stream, migrate into the tissues, and become strategically located at sites of potential antigen exposure. These cells are capable of responding to cytokines at a locus of inflammation, acquiring antigen, carrying it to the adjacent lymph nodes, sensitizing naive T-cells to defined antigen, and eliciting a primary T-lymphocyte response [78]. Dendritic cells are the only natural antigen-presenting cells that have been shown to prime naive T-lymphocytes both *in vitro* and *in vivo*, and they express high levels of costimulatory molecules (B7.1, B7.2, and CD40). Given these properties, dendritic cells have been proposed as the ideal candidates for the induction of antitumor immunity in a vaccine setting.

Autologous dendritic cells can be obtained in large numbers by differentiation of peripheral blood monocytes in the presence of GM-CSF and interleukin-4 [79]. These cultured dendritic cells maintain the antigen capturing and processing capacity characteristic of immature dendritic cells *in vivo*. An alternative approach involves the induced differentiation of bone marrow CD34⁺ progenitors to dendritic cells by G-CSF stimulated mobilization and subsequent culture with GM-CSF, TNF- α , stem cell factor, and FLT3 ligand [80,81]. CD34⁺-derived dendritic cells have been shown to be more efficient in the activation of Mart-1-specific CTLs from low-frequency precursors than dendritic cells derived from monocytes [81].

Bone marrow-derived dendritic cells generated by culture with GM-CSF and interleukin-4, then pulsed with major histocompatibility class I peptide antigen, are ca-

DENDRITIC CELL-BASED VACCINE

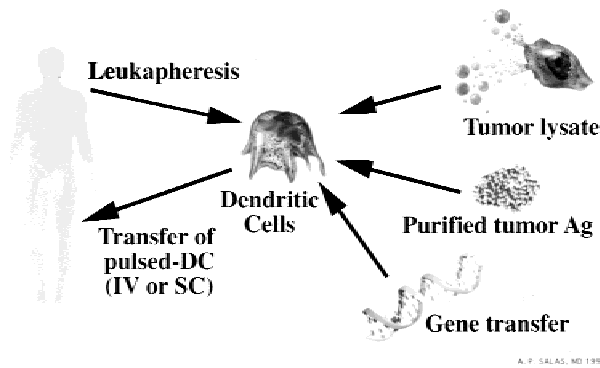


Fig. 3. Schematic diagram of dendritic cell protocols being evaluated in clinical trials.

pable of completely immunizing naive mice against a subsequent lethal tumor challenge by tumor transfected with the antigen gene in a murine M05 melanoma model [76]. In addition, these peptide-pulsed dendritic cells exhibit a strong peptide-specific cytotoxic T-lymphocyte response in vitro [82]. The immunity is antigen-specific, requiring expression of the antigen gene by the tumor target, and is eliminated by in vivo depletion of CD8⁺ T-cells.

In a recent phase I study reported by Nestle et al. [83], dendritic cells were generated from peripheral blood using GM-CSF and interleukin-4. These dendritic cells were pulsed with either melanoma tumor lysate or a cocktail of melanoma peptides known to be recognized by cytotoxic T-lymphocytes, depending on the patient's HLA haplotype. KLH was added as a helper antigen and immunological tracer molecule. Sixteen patients with stage IV disease were treated on an outpatient basis. Dendritic cell preparations were injected into an uninvolved inguinal lymph node at weekly and monthly intervals for up to 10 vaccinations, depending on clinical response. Vaccination was well tolerated in all patients. Objective responses were observed in 5 of 16 treated patients (two complete, three partial responses) with regression of metastasis in various organs (skin, soft tissue, lung, and pancreas). Besides pulsing dendritic cells with tumor lysates or known peptide antigens, investigators have reported that it is possible to introduce genes encoding tumor antigens into these cells, which result in their ability to prime T-cells [84,85]. These various approaches of pulsing dendritic cells are being evaluated in clinical trials at various institutions (Fig. 3).

Future Approaches to Tumor Vaccines

The combination of vaccination therapy in conjunction with exogenously administered cytokines will be evaluated more extensively in clinical trials in the near future. As noted above, peptide vaccines administered in con-

junction with IL-2 was associated with measurable tumor responses in patients with stage IV disease compared to peptide vaccine alone [73]. The immunopotentiating effect of certain cytokines appear to augment the antitumor response induced by vaccine therapies. This has been well-documented in preclinical animal models. Both IL-2 and IL-12 systemic administration have been shown to potentiate the effects of active specific immunotherapy in animal studies [86,87]. IL-2 is a growth factor for activated T-cells while IL-12 facilitates the induction of Th1 cells.

A novel molecular-based approach to autologous tumor cell vaccines is the in situ genetic modification of tumors with immunostimulatory genes. Using a plasmid encoding an allogeneic MHC class I gene that was complexed to liposomes, Plautz et al. [88] reported that the direct intratumor inoculation of this nonviral vector resulted in regression of established subcutaneous tumors. Analysis of this phenomenon revealed that the in vivo lipofection of the tumor with an allogeneic class I gene resulted in T-cell immunity to the allogeneic class I antigen as well as native tumor antigens. Presumably, the expression of foreign MHC class I molecules within the tumor resulted in a brisk inflammatory response that enhanced the induction of immunity to tumor antigen. Advantages of this technique to modify molecularly tumors in situ is the avoidance of viral vectors and bypassing the need to culture tumor cells ex vivo for gene transfer, which is labor-intensive and time-consuming. Gene expression was required in >10% of the tumor cells to achieve an immunologic response. Clinical studies investigating this approach have been carried out at the University of Michigan. These studies involved the treatment of stage IV melanoma patients who were administered a plasmid encoding for the HLA-B7 class I molecule inoculated intratumorally three times over the course of 1 month [89,90]. Patients were required to be HLA-B7-negative to be eligible for the study. Successful gene expression was documented in the majority of patients, and in isolated cases tumor regression of the injected lesions were observed. Of interest with these studies was the assessment of the immune reactivity of T-cells derived from tumor after gene injections compared to pretreatment tumor-associated T-cells. These tumor-infiltrating lymphocytes (TIL) were cultured and assayed for reactivity to autologous tumor by measuring cytokines released by the TIL in in vitro assays where irradiated autologous or allogeneic tumor cells were added as stimulator cells (Fig. 4). TIL were found to be more reactive to autologous tumor cells after HLA-B7 therapy compared to pretreatment; this response was immunologically specific. A clinical trial to treat patients with TIL derived from tumors inoculated with this gene is currently underway at the University of Michigan (Fig. 5).

Cytokine release by TIL before and after HLA-B7 transfection

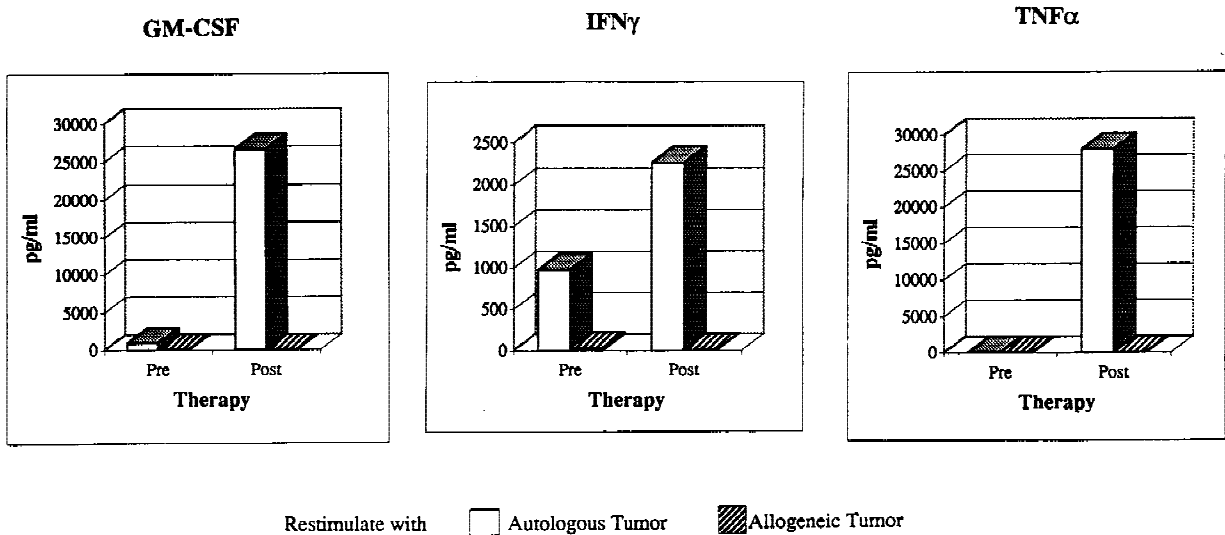


Fig. 4. Cytokine release profile of TIL cells derived from a melanoma patient before and after in situ HLA-B7 transfection. Enhanced interferon- γ , GM-CSF, and TNF α was released by the TIL after gene transfer compared to before treatment. This response was immunologically specific since cytokine release was stimulated by autologous tumor cells, and not allogeneic tumor cells.

The technology of delivering genes in situ has major implications to vaccine therapies. Besides lipofection techniques as described above, another nonviral gene transfer method that has higher efficiency rates is the use of the gene gun. The gene gun involves shooting gold particles complexed with gene onto target tissues such as tumor or skin. The gold particles are projected into the tissue by compressed gases, i.e., helium. The particles enter the cells and the gene of interest is transcribed in an episomal fashion. An illustration of how the apparatus appears and is utilized in an animal model is depicted in Figure 6. Genes encoding tumor antigens, immunostimulatory molecules (e.g., MHC antigens, costimulatory molecules, superantigens, heat shock proteins, etc.), or cytokines can be delivered using this methodology [91]. It has the advantages of employing nonviral vectors and, because of its localized delivery, may be useful intraoperatively after exposure of a tumor.

As described above, direct gene transfer into a progressively growing tumor is one method to derive enhanced tumor-reactive T-cells (i.e., TIL) for use in adoptive immunotherapy. Our laboratory has used vaccines to induce tumor-reactive T-cells in draining lymph nodes which we have documented can be expanded ex vivo and used in adoptive immunotherapy to mediate tumor regression [92–94]. Based on extensive preclinical studies, we have conducted clinical trials involving vaccines comprised of autologous tumor cells admixed with BCG to prime draining lymph nodes that can be harvested, ex vivo expanded, and used for adoptive immunotherapy [95]. More recently, we have examined the use of gene-

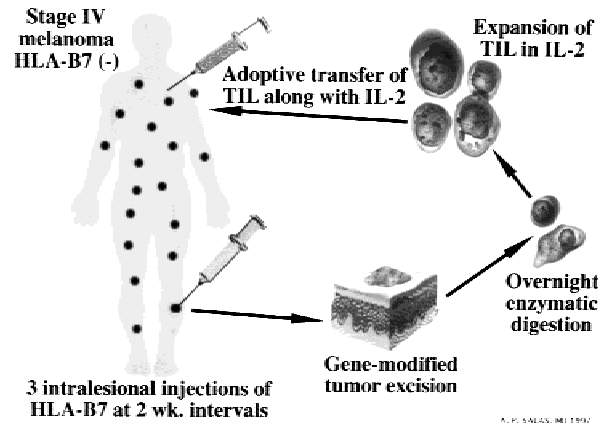


Fig. 5. Schematic diagram of a TIL trial being performed at the University of Michigan. TIL are primed in vivo by the intratumoral inoculation of HLA-B7 plasmid complexed with liposomes.

modified tumor cells as a method to prime draining lymph nodes for adoptive immunotherapy. In a murine melanoma model, we have found that tumor cells modified to secrete GM-CSF are more potent than tumor cells engineered to secrete IL-2, interferon- γ , or IL-4 to prime draining lymph node cells [96,97]. Moreover, GM-CSF secretion was more effective than admixing a bacterial adjuvant in generating “vaccine-primed” lymph nodes cells used in adoptive immunotherapy. We are currently conducting a clinical trial in melanoma patients to evaluate the utility of genetically engineered autologous tumor cells to prime draining lymph nodes for adoptive immunotherapy [98].

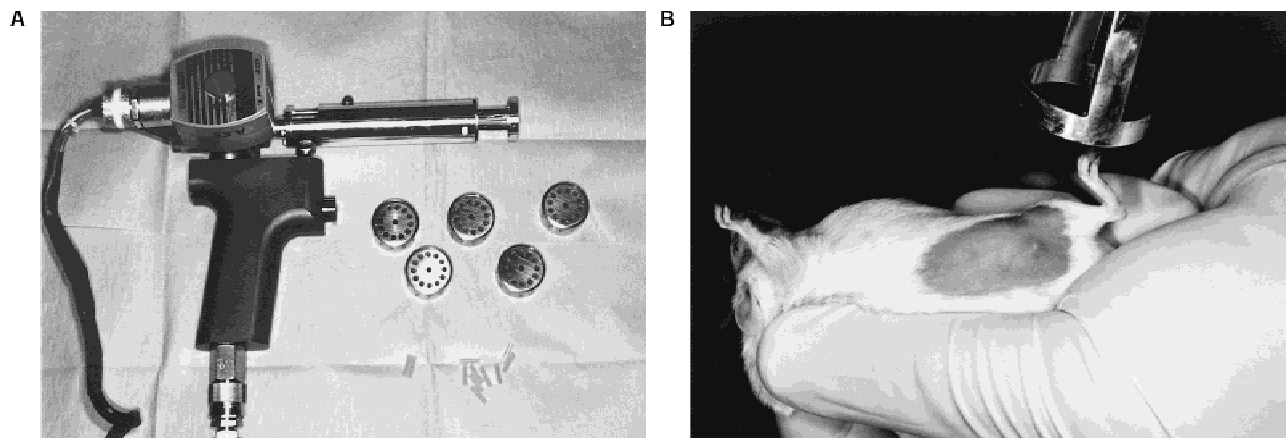


Fig. 6. (A) Gene gun apparatus for in vivo and in vitro gene transfer of plasmids attached to gold particles. The round cartridges are loaded with the gold particles. (B) The gene gun that is connected to a helium gas tank can be applied directly to a target organ or tumor as depicted in an animal model.

CONCLUSION

Melanoma represents one of the few solid malignancies where immunotherapy has become a standard mode of therapy. For resected stage III disease, interferon- α -2b has been identified to have a significant effect on prolonging both disease-free and overall survival [62]. Unfortunately, this therapy is associated with significant side effects, and it is hoped that vaccines may eventually replace interferon- α -2b as an adjuvant therapy. It is predicted that the process of vaccination with tumor antigens, namely active specific immunotherapy, will have its greatest efficacy in the setting of residual micrometastatic disease. There are several clinical trials currently underway to test these hypotheses. Other advances in vaccine development involve molecular engineering of tumor cells, the characterization of melanoma-associated peptide antigens, and the development of cellular reagents for use in immunotherapy (i.e., dendritic cells and primed T-cells). These investigations have led to encouraging clinical trials in melanoma patients with stage IV disease. As we continue to increase our knowledge regarding the interaction of the host immune response to vaccines and the effector mechanisms required to eradicate established tumor, active specific immunotherapy will eventually become a standard approach to treatment of melanoma as well as other malignancies.

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