

Polyvalent Melanoma Cell Vaccine Induces Delayed-Type Hypersensitivity and *In Vitro* Cellular Immune Response¹

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Abstract

Patients with melanoma metastatic to distant sites or at high risk for recurrent melanoma have been treated with a polyvalent melanoma cell vaccine (MCV) in phase II protocols. We assessed *in vivo* and *in vitro* cell-mediated responses to MCV in 163 patients who had undergone surgical resection of American Joint Committee on Cancer stage III melanoma. During the first 4 months of vaccine immunotherapy, 135 patients (83%) responded by developing a positive delayed-type hypersensitivity reaction ≥ 6 mm to MCV. In a mixed lymphocyte tumor cell reaction using peripheral blood lymphocytes, 35 of 42 patients (83%) showed a recall proliferative response to one or more of the three cell lines of MCV. There was a significant correlation between delayed-type hypersensitivity reaction and mixed lymphocyte tumor cell reaction ($P = 0.013$). After 4 months of MCV therapy, 8 of 11 patients had an increased mixed lymphocyte tumor cell reaction to autologous melanoma cells. During the first 4 months of vaccine therapy, 16 of 33 patients developed more than a 50% increase in cytotoxic T-cell activity against one of the cell lines of MCV. Overall survival was significantly prolonged in patients with a positive delayed-type hypersensitivity reaction ($P = 0.0054$) and/or increased cytotoxic T-cell activity ($P = 0.02$). These findings suggest that MCV induces specific T-cell responses which are correlated with clinical course; the data also suggest that some of these responses are directed against autologous melanomas and may play a major role in controlling the progression of melanoma.

Introduction

Melanoma patients with regional soft tissue or lymph node metastases (AJCC³ stage III disease) have a high risk of recurrence and a 10-year survival rate of 15–40%, depending on the extent of nodal involvement (1). In the absence of effective adjuvant chemotherapy protocols for melanoma, many investigators are attempting to induce or augment specific T-cell responses that may control disease progression (2–8). Our recent phase II study showed that patients whose advanced melanoma was treated with repeated intradermal injections of polyvalent allogeneic MCV had a significantly prolonged overall survival compared to historic controls (5). The present report analyzes the magnitude of the T-cell response to MCV in AJCC stage III melanoma patients.

Materials and Methods

Patients. We developed MCV in 1984 and initiated our phase II trial of MCV immunotherapy on September 25, 1984. In the present report, the study

population of 163 patients treated between January 1, 1985, and July 1, 1989, represented all patients who had undergone surgical resection of AJCC stage III melanoma, and were followed at least 3.5 years after beginning postoperative MCV immunotherapy. There were 99 males and 64 females; their median age was 41 years (range, 16–79). The majority (63%) of the primary lesions were on the head and neck or the trunk; the remaining 37% were on the extremities or of unknown primary site. Only 33% of the primaries were thin lesions (<1.5 mm). Before surgery, 21% of the patients had in-transit metastases and 79% had lymph node metastases (24% had 1 positive lymph node, 30% had 2–4 positive nodes, and 25% had ≥ 5 positive nodes). All patients were clinically free of disease after surgery, as determined by physical examination and radiographic imaging of brain, chest, abdomen, and pelvis. All had a life expectancy of more than 6 months and normal blood count, liver enzymes, and creatinine. None of the female patients was pregnant. No patient had received immuno-, chemo-, or radiation therapy within the past 30 days. Written consent for MCV immunotherapy was obtained from all patients, and the MCV protocol was approved by the Human Subjects Protection Committees of the John Wayne Cancer Institute and Saint John's Hospital and Health Center, and the UCLA Jonsson Comprehensive Cancer Center.

MCV Cell Lines and Preparation. MCV comprises three well-characterized allogeneic melanoma cell lines (M10, M24, and M101). These cell lines were established in our laboratory and selected for their content of immunogenic MAA (5). Their HLA class I types are as follows: M10 (A24,33; B35,38), M24 (A11,33; B35,62), and M101 (A2,29; B44). Their surface expression of HLA-DR antigen is $<2\%$.

The preparation of MCV is described elsewhere (5). Briefly, cells from each line are grown in serum-free medium, harvested, and pooled (8×10^6 cells/line; 24×10^6 total cells per vaccine treatment). Individual batches of MCV are analyzed for antigen expression and screened for infectious disease contaminants. MCV is irradiated at 100–150 Gy and then cryopreserved. Immediately before administration, the vaccine is thawed and washed 3 times in sterile physiological phosphate-buffered saline.

MCV Administration. MCV therapy was initiated within 3 months after surgical removal of regional lymph node or soft tissue disease (9), using a previously described protocol (5). The vaccine was injected intradermally in axillary and inguinal regions every 2 weeks $\times 3$, and then monthly for a year. After 1 year the interval was increased to every 3 months $\times 4$, and then every 6 months. For the first two treatments MCV was mixed with the Tice strain of *Bacillus Calmette-Guerin* (Organon Teknika Corp., Durham, NC) (8×10^6 organisms). Some patients received immunomodulators such as cimetidine (SmithKline, Philadelphia, PA), indomethacin (Lederle, Wayne, NJ), or cyclophosphamide (Mead Johnson, Princeton, NJ) (5). Clinical and laboratory evaluations were performed at each vaccine administration; chest X-rays were repeated every 2 months for the first year and then with each vaccine administration.

DTH. Equal amounts of each MCV cell line were pooled to a total dose of 2.4×10^6 cells and administered intradermally at a remote site on the forearm. DTH was defined as the average diameter of induration after 48 h. A positive response was ≥ 6 mm of induration, independent of accompanying erythema. The greatest DTH during the first 4 months was used for survival analysis. Since autologous cell lines were not available during the first 16 weeks of MCV therapy, autologous DTH could not be performed.

MLTR. Cryopreserved PBL from weeks 0, 4, and 16 of MCV immunotherapy were available for testing from 42 AJCC stage III patients. These PBL were simultaneously thawed, washed, and resuspended in AIM-V culture

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³ The abbreviations used are: AJCC, American Joint Committee on Cancer; MAA, melanoma-associated antigens; MCV, melanoma cell vaccine; DTH, delayed-type hypersensitivity reaction; PBL, peripheral blood lymphocytes; MLTR, mixed lymphocyte tumor cell reaction; PHA, phytohemagglutinin; CTL, cytotoxic T-lymphocytes; LU, lytic units.

medium (GIBCO, Grand Island, NY) containing 10% human AB heat-inactivated serum (Irvine Scientific, Santa Ana, CA), and stimulated at a 5:1 ratio (PBL:melanoma) with each MCV line. In eight cases, a melanoma cell line developed from the patient's biopsy specimen was available for autologous MLTR (10). Autologous MLTR was also performed by using melanoma cell lines developed from biopsy specimens of three additional patients receiving MCV after surgical resection of distant metastases (AJCC stage IV disease).

The MLTR assay was performed as described elsewhere (11). Briefly, PBL were cocultured in triplicate 96-well microplates containing 200 μ l of culture medium supplemented with 20 units/ml of recombinant interleukin 2 (Cetus, Emeryville, CA). Cells were then incubated for 6 days at 37°C. Respective control cultures of PBL were assayed in medium alone and with PHA (Burroughs-Wellcome, Triangle Park, NC) at a suboptimal concentration of 0.1 μ g/ml. During the last 18 h of the 6-day assay, cells were pulsed with [³H]thymidine (New England Nuclear, Boston, MA) and harvested. Data were analyzed as mean cpm for triplicate measurements (SD < 15%) at each time point.

Correlation of MLTR with DTH. Since DTH represents a combined reaction against all three MCV cell lines, MLTR responses to each line were combined to create a MLTR index. To determine this index, the cpm of each cell line at week 0, 4, or 16 was divided by its cpm at week 0. The MLTR index for a specific week was the sum of these values; thus, the MLTR index was always 3 at week 0. The correlation between DTH and MLTR index was determined by linear regression analysis.

Cytotoxic T-Cells. Cryopreserved PBL obtained before treatment (week 0) and after 4 and 16 weeks of MCV therapy were simultaneously thawed, washed, and grown for 4 days in AIM-V culture medium containing 10% human AB heat-inactivated serum, 10 units/ml interleukin 2, and 0.1 μ g/ml PHA. CTL-mediated lysis of MCV cell lines was assessed in a standard 4-h ⁵¹Cr-release assay using three effector:tumor target cell ratios (11). Results were expressed in LU according to the formula described by Pross and Maroun (12). One lytic unit was defined as the number of effector cells required to lyse 33% (LU₃₃) of 5 × 10³ target cells. CTL assays were performed early during MCV treatment, without further knowledge of the patient's clinical status. Analysis of representative stimulated lymphocyte subpopulations by flow cytometry with specific T-cell monoclonal antibodies revealed 88% CD3+ and 50% CD8+.

Survival Analysis. The overall survival curves were estimated by the Kaplan-Meier method. The log-rank test was used to determine survival differences among patient subgroups defined by DTH or cytotoxicity. All tests were two-sided. Survival time was defined as the interval between the initiation of MCV therapy and the patient's death.

Results

DTH. The mean DTH of the 163 patients increased significantly from a base line of 3.2 ± 0.4 mm (mean ± SEM) pretreatment to a maximum of 13.5 ± 1.1 mm at week 4 ($P < 0.01$), and dropped slightly to about 10 mm for the following 3 months. One hundred thirty-five patients (83%) had a maximum DTH ≥ 6 mm within 4 months of beginning MCV therapy, while the remaining 28 patients (17%) exhibited no DTH response to MCV (<6 mm). Maximum DTH was 15.3 ± 1.2 mm for responders (week 4; Fig. 1A) and 3.6 ± 1.1 mm for nonresponders (week 16; Fig. 1B). The distribution of positive lymph nodes was similar between the two groups ($P = 0.8$), as was the thickness of the primary lesion. After a median follow-up of 60 months (range, 43–93), patients with a positive DTH had a median overall survival of 52 months, compared to only 22 months in patients with no DTH response ($P = 0.0054$) (Fig. 2). Patients with a DTH ≥ 6 mm and involvement of 1, 2–4, or ≥ 5 nodes had 5-year survival rates of 59, 46, and 34%, respectively.

Autologous MLTR. Autologous tumor cell lines were available for 11 patients. Week 16 was selected as the evaluation point based upon the number of vaccinations ($n = 5$) and the plateau phase for DTH. After 16 weeks of active specific immunotherapy with MCV, 5 of the 11 patients demonstrated more than a 3-fold increase in proliferative response to their own melanoma (Fig. 3); three other patients showed more than a 1.5-fold increase. Of the three remaining patients

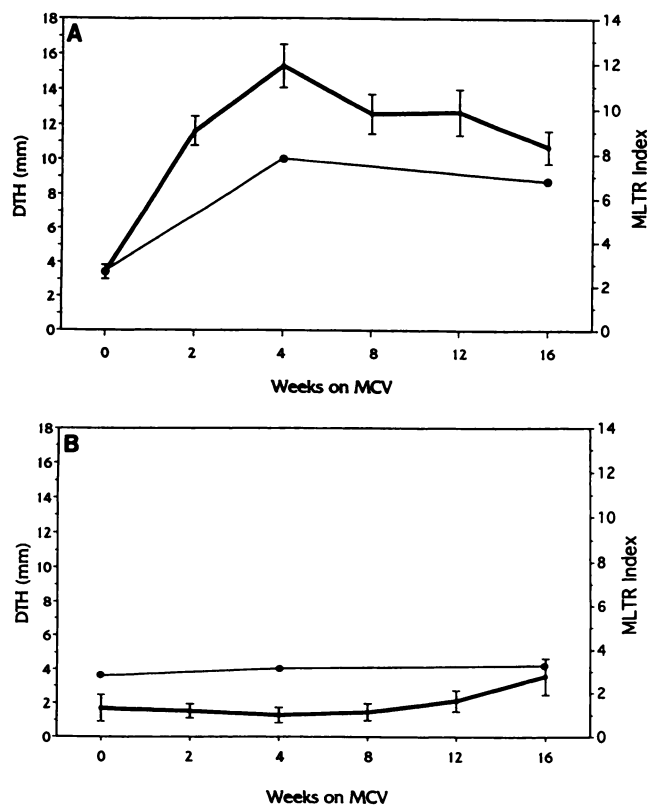


Fig. 1. *In vivo* and *in vitro* T-cell responses to MCV. —, DTH (mean ± SEM); —, MLTR index. A, DTH of 135 patients with a maximum DTH ≥ 6 mm and MLTR index of 35 responders. B, DTH of 28 patients with maximum DTH < 6 mm and MLTR index of 7 nonresponders. Maximum MLTR was 7.8 for responders and 3.3 for nonresponders. The magnitude of the response was independent of the number of positive lymph nodes ($P = 0.8$).

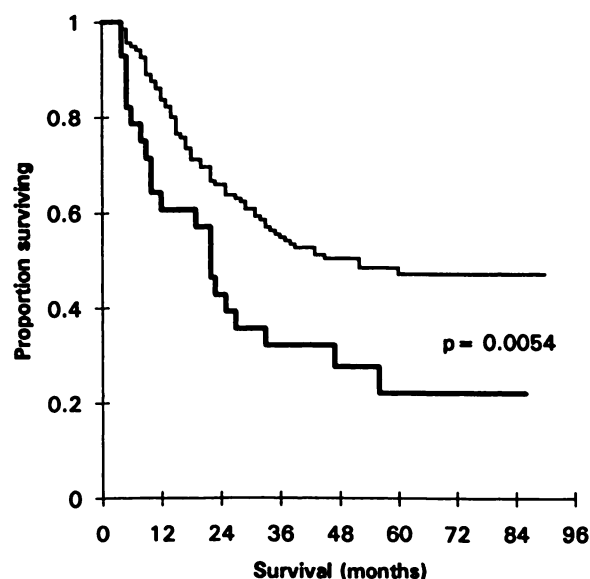


Fig. 2. Overall survival of 163 AJCC stage III melanoma patients undergoing MCV adjuvant immunotherapy initiated within 3 months after surgical removal of regional lymph nodes or soft-tissue disease. After a median follow-up of 60 months, the 135 patients with a DTH ≥ 6 mm (—) had a significantly longer overall survival than the 28 patients with DTH < 6 mm (---): median, 52 versus 22 months ($P = 0.0054$).

with no increase, two had AJCC stage IV melanoma. The eight patients with an enhanced response also showed increased reactivity to at least one of the cell lines of MCV. The overall MLTR response of the 11 patients was 28,100 ± 8,500 cpm at week 0, significantly

increasing to $50,000 \pm 12,600$ cpm at week 16 ($P < 0.01$). Stimulation of PBL with interleukin 2 alone (20 units/ml) produced a response in the 11 patients of $27,300 \pm 8,200$ cpm at week 0, and $32,700 \pm 10,100$ cpm at week 16. Similarly, stimulation with PHA alone (0.1 $\mu\text{g/ml}$) produced a response in the 11 patients of $142,500 \pm 44,900$ cpm at week 0, and $165,700 \pm 52,400$ cpm at week 16. Responses produced by stimulating PBL with either PHA alone or interleukin 2 alone failed to reach statistical significance between weeks 0 and 16.

Correlation between DTH and MLTR. PBL from weeks 0, 4, and 16 were available for 42 of the 163 patients. In 35 of 42 patients (83%), MLTR demonstrated a recall proliferative T-cell response to one or more of the three cell lines of MCV. The MLTR index of the 35 responders peaked at week 4 and then decreased slightly to a plateau (Fig. 1A); the 7 nonresponders demonstrated no change in MLTR index during the observed period (Fig. 1B). Linear regression analysis of data for the 42 patients confirmed a significant correlation ($P = 0.013$) between DTH and MLTR index at weeks 4 and 16.

Cytotoxic T-Cells. During the first 16 weeks, CTL-mediated lysis of one or more MCV cell lines increased in 16 of 33 patients by more than 50% above pre-MCV values, to at least $5 \text{ LU}_{33}/10^6$ effector cells (range, 5–51). CTL activity was HLA class I restricted, as demonstrated by blocking with W6/32 (anti-HLA class I) monoclonal antibody, using respective controls. Individual patients showed no significant changes (weeks 0–16) in natural killer-type activity (K562 cell killing). Fourteen of the 16 responders (88%) shared HLA-A alleles with MCV cell lines (13). Responders demonstrated a significant increase in cytotoxicity against HLA-A-matched targets from week 0 to weeks 4 and 16 ($P < 0.01$), whereas cytotoxicity of nonresponders against HLA-A-matched targets decreased slightly (Fig. 4). As demonstrated in our earlier studies (10, 11), a parallel increase in cytotoxicity against HLA-A-matched melanoma and autologous melanoma cells could be observed (Fig. 5). The overall survival of patients who had an increase in CTL activity after MCV treatment was significantly longer than that of nonresponders ($P = 0.02$), in spite of an approximately equal balance of prognostic factors (Fig. 6).

Discussion

This brief report describes the induction and prognostic significance of *in vivo* and *in vitro* T-cell immunity in AJCC stage III melanoma patients receiving adjuvant MCV immunotherapy. Of 163

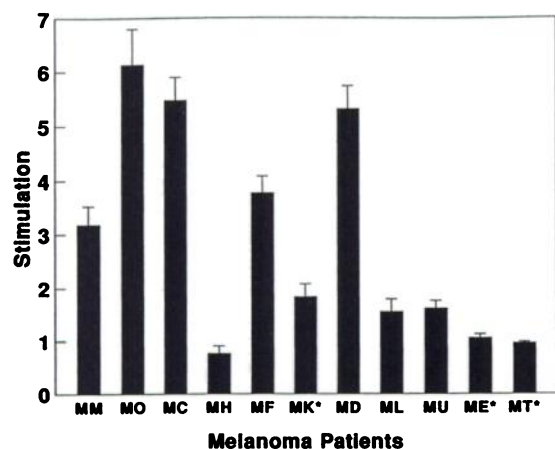


Fig. 3. Autologous MLTR (mean \pm SD) of 11 patients from whose tumor a cell line was established. Results are expressed as ratios (mean cpm week 16/mean cpm week 0). By week 16 of MCV therapy, the recall proliferative response to autologous tumor had increased more than 3-fold in five patients and more than 1.5-fold in three patients. *, AJCC stage IV melanoma patient.

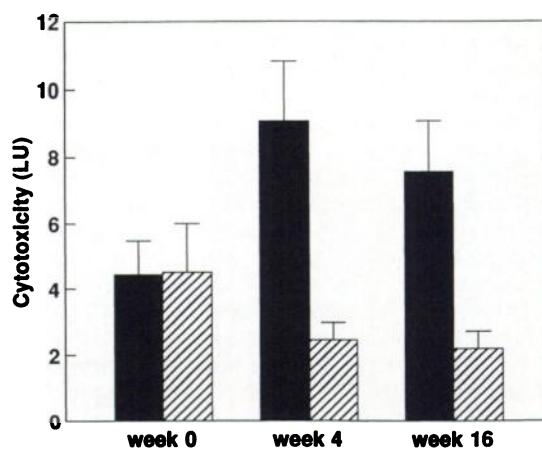


Fig. 4. Cytotoxicity of PBL (mean \pm SEM) from responders (■, $n = 14$) and nonresponders (▨, $n = 11$) against allogeneic HLA-A-matched melanoma targets. Responders demonstrated a significant increase in cytotoxicity against HLA-A-matched melanoma from week 0 to weeks 4 and 16 ($P < 0.01$), whereas cytotoxicity of nonresponders against HLA-A-matched targets decreased slightly.

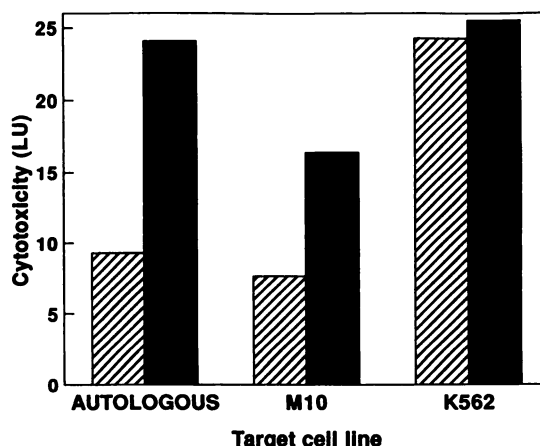


Fig. 5. Cytotoxicity of PBL (A24, 32; B7, 44) against autologous melanoma, allogeneic HLA-A-matched melanoma (M10), and K562. From week 0 (▨) to week 4 (■) there was a parallel increase in cytotoxicity against allogeneic HLA-A-matched and autologous melanoma; there was no change in cytotoxicity against K562.

patients, 135 (83%) developed a DTH ≥ 6 mm following vaccine treatment. A similar degree of sensitization was demonstrated by MLTR *in vitro*. Positive DTH to MCV correlated with improved survival; by contrast, in a separate study of 148 AJCC stage IV melanoma patients (14) we were unable to demonstrate a correlation between survival and DTH to common recall antigens (mumps, *Candida albicans*, and purified protein derivative). A significant association between survival and DTH to a tumor vaccine has also been reported by Berd *et al.* (7) and Bystryń *et al.* (6) for high-risk melanoma patients, and by McCune *et al.* (15) for patients receiving active specific immunotherapy against metastatic renal cancer. Bloemena *et al.* (16) recently noted a positive correlation between DTH and *in vitro* proliferative T-cell responses to a mixture of colon tumor-associated antigens. These studies suggest that strong DTH responses during cancer vaccine therapy indicate successful activation of cell-mediated immunity.

MCV may augment T-cell responses in several ways. The enhanced T-cell response to autologous melanoma cells after MCV immunotherapy may result from direct recognition by host T-cells of MAA presented by shared or cross-reactive HLA molecules on MCV lines, as demonstrated *in vitro* (10, 13). Alternatively, activation may occur through antigen processing and presentation of MCV's MAA to host

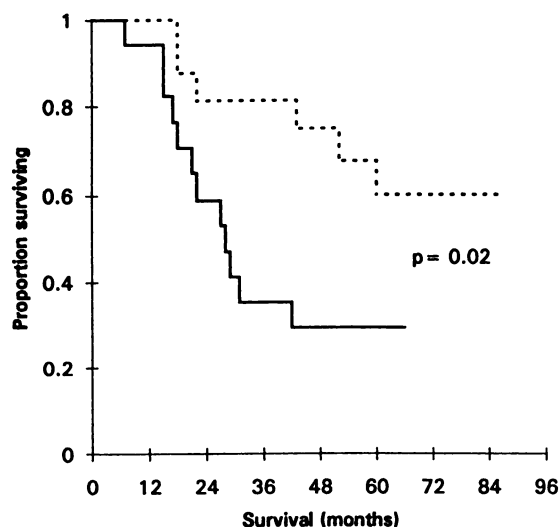


Fig. 6. Overall survival of 33 AJCC stage III melanoma patients undergoing MCV adjuvant immunotherapy initiated within 3 months after surgical removal of regional lymph nodes. After a median follow-up of 61 months, the 16 patients with more than a 50% increase in CTL activity (---) had a significantly longer overall survival (median, >60 months) than the 17 patients with no increase in CTL activity (median, 28 months) (—); $P = 0.02$.

T-cells by antigen-presenting cells. The allogeneic HLA antigens on the melanoma cells may stimulate alloreactive T-cells that migrate to the intradermal site of MCV injection; this induces cytokine release and attracts antigen-presenting cells in the microenvironment, which can present common MAA from vaccine cells to MAA-specific T-cells (5, 11, 17–19). This concept was recently suggested by a clinical study showing that *in vivo* transfection of the gene for an allogeneic HLA class I antigen into a patient's melanoma induced specific systemic T-cell immunity (8).

Approximately 20% of our patients did not show a T-cell response to MCV. This may reflect T-cell anergy due to continual exposure of T-cells to MAA and/or cytokine(s) (20). Alternatively, the T-cell response may have been suppressed by factors such as prostaglandins and/or suppressor T-cells (21). Specific biological modifiers administered with MCV may enhance T-cell immune responses; there is evidence that suppressor cells can be inhibited by low doses of cyclophosphamide and indomethacin, thereby up-regulating effector or helper T-cell responses (5, 21, 22).

Augmentation of the T-cell effector response to common MAA may be an important factor in the control of melanoma. In our study, enhanced CTL activity against one or more of the lines of MCV correlated with better survival. In general, CTL kill cancer cells through recognition of MAA as short peptide sequences within HLA molecules expressed on the surface of the cancer cell (23). We and others have demonstrated that CTL can kill allogeneic melanomas expressing the HLA-A antigens of CTL (11, 17, 18). We have also shown that CTL induced by sensitization with allogeneic melanoma cells expressing HLA-A antigens shared by the CTL can recognize and kill autologous melanoma cells (10). These *in vitro* studies suggest recognition of common MAA and support the potential *in vivo* mechanism of MCV cell recognition by MAA-specific T-cells. A defined polyvalent whole-cell MCV immunizes patients with multiple common MAA, thereby inducing an antigen-specific immune response that is effective against different melanoma lesions from the same patient or from different patients. Theoretically, this appears to be one of the most practical approaches to the problem of inducing active specific immunotherapy against tumors that are antigenically heterogeneous. We are currently attempting to characterize MCV antigens that are recognized by T-cells.

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