Intratumoral Therapy II: *In Vitro* and *In Vivo* Immunologic Testing and Therapy Options

Max Cohen1,2*, Alfred Ketcham2 and Ronald Herberman2

1George Washington University School of Medicine & Health Sciences, Washington, USA
2Fmr National Institutes of Health, Bethesda, USA

*Corresponding author: Max Cohen, George Washington University School of Medicine & Health Sciences, Washington, USA, Tel: 301 983-1840; Fax: 301-983-8884; E-mail: robyn8812@aol.com

**Abstract**

In a prospective randomized trial in patients with metastatic melanoma we compared two agents that had been used for metastatic melanoma intratumoral injections. Each patient had progressive metastatic disease no longer surgically controllable. Multiple metastases included satellitosis in the form of progressive nodules around the previously excised original melanoma site, and/or in-transit metastases in the form of observable tumor nodules progressing in a linear fashion toward a lymph node bearing area. As patients were receiving intratumoral injections, serially collected blood samples were tested for general immunologic reactivity and anti-melanoma reactivity. Specificity controls included breast cancer and lung cancer extracts. Additionally, as a measure of cell-mediated immunity, the patients were serially skin tested against antigens to measure general and melanoma-specific immunity. Depending on the patients’ clinical courses, we have divided the patients retrospectively into groups whose clinical courses were either better or worse than their cohorts, and determined the relationship between the immune testing and the clinical courses that the patients were experiencing as the serial testing was being conducted.

Additionally, in a group of similar patients that were ‘cured for life’, we analyze their treatment in light of therapeutic attempts made by others to similarly haptenize melanoma antigens. We describe potential synergies between newly discovered melanoma therapies and intratumoral injection treatments and point out potential combination therapies that may offer the potential for enhancement of antitumor effects without increased systemic toxicity, a desirable goal now that combinations of recently acceptable immunotherapies have been associated with severe potential toxicities including death.

**Keywords**

Metastatic melanoma; Intraleosional; Immunotherapy; Immune testing; Checkpoint inhibitor therapy

**Introduction**

In a previous randomized prospective study we showed that intraleosionally (intratumorally) injected dinitrochlorobenzene (DNCB) was as effective as intraleosionally injected Bacillus Calmette-Guerin (BCG) in the treatment of melanoma patients with progressive, ordinarily lethal melanoma satellitosis and/or in-transit metastases [1]. The patients did not have demonstrable metastasis past the regional lymph nodes at the time of randomization. We observed that the intraleosional DNCB-treated patients experienced fewer side effects but with comparable efficacy compared to the patients treated with intraleosional BCG, and recommended that the use of intraleosional BCG be abandoned in this setting. We now address the question of whether *in vitro* and *in vivo* serially obtained immunologic measurements during intraleosional treatments were correlated with the patients’ clinical courses during intraleosional treatment.

Additionally, in a selected non-randomized group of three similar patients, with similar distribution of disease, also treated with intraleosional DNCB and who became lifetime cures of their metastatic melanomas, we describe characteristics that coincided with their successful outcomes and offer reasons why intratumoral treatment of the type we have described may be synergistic with use of other immune therapies, including checkpoint inhibitor therapy.

**Methods**

In the randomized prospective study, each of the 18 randomized patients had multiple cutaneous local recurrences of melanoma, either in the form of satellitosis and/or, if visibly tracking up the lymphatic pathways, in the form of in-transit metastases. The metastatic disease in each patient was felt to be no longer controllable by surgical means. No patient had received prior immunotherapy, chemotherapy or radiation therapy. No patient had clinical or radiologic evidence of spread past the regional lymph nodes.

Each patient had been randomly assigned to receive injections of either intraleosional (IL) BCG (9 patients) or IL DNCB (9 patients) into the satellitosis or in-transit cutaneous and/or subcutaneous metastases. Serial immunological testing consisted of *in vitro* assays and *in vivo* skin tests. The *three in vitro* assays consisted of a) phytohemagglutinin (PHA) lymphocyte stimulation testing, b) measurement of the percentage of circulating lymphocytes that formed rosettes with sheep erythrocytes in a 29 degree centigrade assay, and c) determination of direct leukocyte migration inhibition in response to 3 M KCl-soluble extracts of different fresh melanomas, with breast cancer extracts and lung cancer extracts as specificity controls. The *serial in vivo* skin testing, as possible, was with a) Purified Protein Derivative antigen (PPD), b) Mumps antigen, c) Streptokinase/Streptodornase (SKSD), and d) inactivated melanoma extracts, using coded syringes [1]. We have retrospectively grouped the patients so as to correlate serial immunologic data, with whether the patients had better or worse clinical outcomes during intraleosional treatments.

As mentioned, patients had been randomized to repeatedly receive either IL BCG (9 patients), or IL DNCB (9 patients) into their progressive cutaneous, subcutaneous and lymphatic channel in-transit metastatic nodules. Retrospectively, and after conclusion of the study, a ‘BCG worse responder group’ was designated, consisting of 6 patients in whom distant metastasis was detected within 2 to 20 months of beginning intraleosional injections, and who died within 9 to 24 months from the beginning of such injections. A ‘BCG better responder group’ was also designated, consisting of 3 patients who were alive and in whom no distant metastases were detected for 12 to 39 months after beginning injections. These were retrospective designations as we...
sought, after conclusion of the study, a method of group designation and partition based on clinical outcome that might show differences in the in vitro and/or in vivo test results carried out during intralesional treatments, as we had not been able to identify test results obtained before intralesional treatment that predicted the subsequent clinical course associated with the intralesional treatments.

Similar to what was done with the 9 IL BCG patients, the patients that had been randomized to IL DNCB were also retrospectively divided into two groups after the conclusion of the study: A 'DNCB worse responder group' consisted of 6 patients in whom distant metastasis was detected within 5 to 11 months from the beginning of intralesional injections, and who died within 6 to 12 months from the beginning of injections. A 'DNCB better responder group' consisted of 3 patients who were alive and in whom no distant metastases were detected after 8 to 20 months from the beginning of injections. We then combined the two ‘worse responder groups’ into a single ‘worse responder group’ and the two better responder groups into a single ‘better responder group’. Again, these were retrospective designations, since after the conclusion of the study we attempted to investigate groups with better or worse clinical courses in order to see if any of the immune tests would show particular tests to be discriminatory with regard to the clinical courses of the tested patients.

Separately from the patients in the randomized prospective study, we studied three selected patients with the same clinical characteristics as the patients in the randomized trial, also treated with intralesional DNCB, who, in contrast to the patients in the randomized trial, became permanent lifetime tumor free survivors. Each of the patients had been treated with intralesional DNCB. However, unlike in the randomized study, in the three selected patients described, there was no preimmunization with DNCB two weeks before beginning intralesional injections, as there had been in the randomized patients.

Results
Among the three in vitro assays compared, PHA stimulation index levels during intralesional injections appeared to correlate best with clinical courses in patients undergoing BCG or DNCB intralesional treatments. PHA stimulation test results appeared to be higher in the retrospectively created 'better responder' groups compared to the retrospectively created 'worse responder' counterparts.

With regard to in vivo testing, in comparing the retrospectively created 'better responder' groups with the 'worse responder' groups, patients who during intralesional treatment had positive reactions to skin testing with melanoma extracts appeared to fare better than those who did not. All patient groups tended to react positively to SKSD, a detection

Discussion
As mentioned, regarding the serial in vitro assays that were compared, and after retrospectively designating better and worse responder groups related to clinical outcomes, positive results with serial testing with PHA lymphocyte stimulation assays appeared to correlate best with better clinical courses. Regarding the skin tests that were compared, and after retrospectively designating the same better and worse responder groups related to clinical outcomes, positive testing with melanoma extract (using breast cancer extracts as controls) appeared to correlate best with better clinical courses. Intralesional injections were generally more effective against superficial indermal metastases, rather than deeper and subcutaneous metastases.

Unlike our approach, other investigators have surgically harvested metastatic melanoma masses, then minced the tissue to obtain cells, which were then dinitrohalogenated and presumably haptenized in vitro with dinitrofluorobenzene instead of with the dinitrochlorobenzene compound that we had utilized. They then administered the dinitrofluorinated melanoma cells back to the individual patients from whom the tumors were harvested, in attempts to immunize the affected patients against their melanomas [2,3].

Their effort to immunize against melanoma therefore differed conceptually in several ways from the intralesional DNCB methodology we had previously described, and which was utilized in the currently reported patients. There in vitro dinitrohalogenation of metastatic melanoma cells followed by readministration of those cells had the potential benefit of being applicable in a wider range of metastatic melanoma patients, including in patients whose gross metastatic disease was located, for example, in harvestable lymph node, lung or liver tissue and whose therapeutic targets might be inside the body. However it may also be noted that patients with bulky nodal, lung or liver metastases from whom a significant and adequate amount of tumor could be harvested for in vitro dinitrohalogenation, may have more bulk disease and a worse prognosis than patients with clinical evidence of only cutaneous metastases. In comparing the two techniques it should also be noted that the surgical harvesting and in vitro handling of metastatic cells necessarily results in a degree of damage and death of tumor cells during the processes of harvesting, mincing, dinitrohalogenation and storage of the potential melanoma cell vaccines. However, although significant cell death occurred as a result of the harvesting and preparation of the dinitrohalogenated cells, there is some evidence in the aforementioned dinitrohalogenated cell system that dead cells retain some immunogenic potency [4].

The harvesting and in vitro haptenization of tumor cells is a more labor intensive process compared to injection of intact melanoma nodules on and below the skin. Also, while it is perhaps uncertain what concentration or concentrations of dinitrohalogenated compound should be added in vitro to harvested melanoma cells, there is, on the other hand, an automatically produced range of high and low concentration dinitrohalogenation in the in vivo melanoma nodules that we subjected to in vivo dinitrohalogenated administration. It should also be recognized that whereas it is relatively technically easier to subject a patient to a series of in vivo intralesional injections, it would be comparatively more difficult to repeatedly harvest bulk metastatic disease to prepare a series of in vitro haptenized cells. Indeed such serial harvesting of tumor for in vitro haptenization may not be feasible depending on the locations of the metastatic lesions, and/or the condition of the patient. Indeed a decline in the patient's condition might contraindicate a required surgical harvesting
Such a general increase in toxic immune reactivity can result in autoimmune complications in multiple organ systems. A combination of antibodies that inhibit cytotoxic T-lymphocyte checkpoint inhibitor treatments. On the one hand intralesional DNCB anesthesia. Nevertheless, it is of interest that there are other methods of immune capability. Neither does the repeat administration of the treatment with pembrolizumab, for example, has been associated with potential haptens for tumor cells, indicates that the dinitrohalogenation of tumor cells can result in an increase in the immunogenicity of the malignant cells, resulting in a halt and/or a reversal of otherwise progressive melanoma metastasis in some patients. Indeed the clinically observed halts and reversals in injected and un.injected sites represent perhaps the best indication of an increased antitumor immunologic capability. Thus, regarding the combination of intralesional and checkpoint inhibitor treatment, not only could the use of intralesional DNCB benefit the anti-melanoma activity of another anti-tumor treatment, such as a checkpoint inhibitor, but the checkpoint inhibitor could synergistically increase the immunologic capability of the injected dinitrohalogenated haptenized tumor nodule. The creation of haptenized tumor nodules potentially serves to create multiple vaccine-like sites on a patient who may be receiving simultaneously a checkpoint inhibitor or other method of immunotherapy. Such combination of intralesional haptenization with checkpoint inhibitor treatment may be delayed until the patient experiences tumor progression on checkpoint inhibitor treatment. Adding a second checkpoint inhibitor at that point is a consideration, at the risk however of a substantial increase in the possibility of serious side effects. On the other hand, adding intralesional haptenization to a single checkpoint inhibitor would instead add an agent characterized by an absence of systemic side effects, ease of administration, and decreased expense.

Historically, intraregional treatment has been essentially confined to use on surfaces, such as skin and bladder lining. However, with the development of precision radiological imaging and injection techniques, potential metastatic melanoma targets within the body may become more feasible and accessible injection targets, although the consequences of a possibly intense local inflammatory reaction at the injection site must be taken into account. Other cancer types, beside melanoma, may also be amenable to attempted cell haptenization in accordance with the techniques or principles discussed.

Conclusion

In summary and conclusion, patients undergoing intralesional treatments for progressive melanoma satellitosis or in-transit metastases were retrospectively placed in groups with better or worse outcomes. These patients had sequential tests for humoral and cell mediated immunity while receiving intratumoral injections. Among sequential humoral (in vitro) assays in these patients, sequential PHA stimulation test results appeared to be higher in the retrospectively grouped patients with better outcomes, compared to sequential PHA stimulation results in patients with worse outcomes.

With regard to cell mediated (in vivo) skin testing, in comparing the same two retrospectively created better and worse responder groups, patients who during intralesional treatment had positive reactions to procedure, which might also require the administration of general anesthesia. Nevertheless, it is of interest that there are other methods of potential melanoma immunization using a haptenizing dinitrohalogenation compound, in addition to the method we utilized. Also the multiplicity of these approaches indicates that dinitrohalogenation of melanoma cells can be associated with apparent clinical benefit through an apparently immune mechanism.

We showed that DNCB when topically applied to the skin can enhance general immune capability in multiple species, including humans [5]. However it is our belief that immunologic benefit derived following intratumoral injections of the type described above is the result of the attachment of the haptenizing chemical to tumor cells with enhancement of melanoma specific immune capability. On the other hand, immunotherapeutic agents such as checkpoint inhibitors produce a substantial and potentially toxic general increase in immune capability in association with the antitumor effect of the treatment. Such a general increase in toxic immune reactivity can result in autoimmune complications in multiple organ systems. The fact that the beneficial effects of intralesional injections described herein and previously [1] have occurred without systemic immune side effects indicates that the antimalanoma effects are not associated with the toxic side effects associated with a more general enhancement of immune capability. Neither does the repeat administration of the DNCB organic chemical cause the side effects [6-11] and death [12,13] associated with previously utilized intratumoral agents.

Therefore, to the extent that intralesional treatment is associated with specific antimalanoma activity, it may be useful to combine such tumor specific antitumor activity with the somewhat more general immunologic toxicity producing capabilities that are enhanced by treatments with immune checkpoint inhibitors such as, for example, the anti-CTLA-4 monoclonal antibody ipilimumab, or the anti-PD-1 monoclonal antibody pembrolizumab.

Regarding the potential combination of intralesional treatment and treatment with checkpoint inhibitors it has been observed that treatment with pembrolizumab, for example, has been associated with immune-mediated systemic complications such as colitis, pneumonitis, hepatitis, hypophysitis, thyroid function disorders, etc. [14]. A combination of antibodies that inhibit cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) has become a mainstay in the treatment of advanced melanoma because of improved survival and objective response rates. However the incidence of grade 3 and 4 treatment-related adverse events has been 59%, including death from multiple organ failure, which occurred in a series of patients treated with nivolumab and Ipilimumab. As mentioned, in contrast to the complications associated with these immune checkpoint inhibitors, we have noted essentially no systemic adverse reactions to intralesional DNCB [14]. The patients in the current report likewise experienced no adverse systemic manifestations. Thus intralesional DNCB presents a potentially valuable safety profile as an agent that may be considered for combination with agents such as immune checkpoint inhibitors, which are associated with potentially serious systemic side effects, especially when used in combination with each other. It should also be noted that potential synergy may exist between intralesional and checkpoint inhibitor treatments. On the one hand intralesional DNCB could potentially increase the specificity of the antimalanoma effect of an immune checkpoint inhibitor. On the other hand the potential unmasking of increased immunologic potency associated with administration of such checkpoint inhibitors may synergistically increase a specific antimalanoma effect of direct intralesional tumor antigen haptenization.

With regard to the haptenization capability of DNCB in the tumor cell environment, it may be noticed that DNCB, when dissolved in acetone and applied topically onto normal skin, produces a localized allergic reaction characterized by edema, erythema and pruritus, similar to that observed following repeat poison ivy exposure. Products derived from poison ivy oil affix to skin proteins to form highly immunogenic haptenized antigens. Our experience with DNCB and the work of others using dinitrohalogenated compounds as potential haptens for tumor cells, indicates that the dinitrohalogenation of tumor cells can result in an increase in the immunogenicity of the malignant cells, resulting in a halt and/or a reversal of otherwise progressive melanoma metastasis in some patients. Indeed the clinically observed halts and reversals in injected and un injected sites represent perhaps the best indication of an increased antitumor immunologic capability. Thus, regarding the combination of intralesional and checkpoint inhibitor treatment, not only could the use of intralesional DNCB benefit the anti-melanoma activity of another anti-tumor treatment, such as a checkpoint inhibitor, but the checkpoint inhibitor could synergistically increase the immunologic capability of the injected dinitrohalogenated haptenized tumor nodule. The creation of haptenized tumor nodules potentially serves to create multiple vaccine-like sites on a patient who may be receiving simultaneously a checkpoint inhibitor or other method of immunotherapy. Such combination of intralesional haptenization with checkpoint inhibitor treatment may be delayed until the patient experiences tumor progression on checkpoint inhibitor treatment. Adding a second checkpoint inhibitor at that point is a consideration, at the risk however of a substantial increase in the possibility of serious side effects. On the other hand, adding intralesional haptenization to a single checkpoint inhibitor would instead add an agent characterized by an absence of systemic side effects, ease of administration, and decreased expense.

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skin testing with inactivated melanoma extracts appeared to fare better than those who did not. All patient groups tended to react positively to skin testing with SKSD, a general sign of immune capability.

Intraregional DNCB treatment, without associated systemic toxicity, but with apparent antitumor immune enhancement, could be potentially synergistic with the use of monoclonal checkpoint inhibitor treatment, or other immunotherapy antitumor strategies that are associated with serious autoimmune side effects. This could be a useful consideration in patients who have failed checkpoint inhibitor therapy and are being considered for addition of a second checkpoint inhibitor agent, with its significantly increased side effect risks and costs.

References