T-cell response to unique and shared antigens and vaccination of cancer patients

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Abstract

Most vaccination studies of cancer patients find no clear association between clinical and immunological responses to the vaccine. We discuss the possible kinetics of the T cell response in melanoma patients against unique or shared tumor antigens. We hypothesize that a response against unique antigens prevails during primary melanoma growth, causing the selection of tumor cells lacking most of these antigens unless these are necessary to maintain the neoplastic state. After a subset of tumor cells metastasize to the lymph nodes, T cells are activated against previously ignored shared, differentiation-like antigens, owing to a new environment where pro-inflammatory cytokines can be present. The development of a T cell response to such normal epitopes then associates with tumor growth, but remains clinically inefficient. We predict that two immunologically different subsets of melanoma patients may exist, one that mounted an early immune response against melanoma antigens and one that did not. A paradox may emerge when vaccination is attempted in these two groups of subjects, with the second group being more prone to develop an effective immune response if the vaccine is potent enough to activate naive T cells, while the first has probably already eliminated most of the tumor antigens potentially recognizable by the host T cells owing to the previous selection made by the immune response developed early during tumor growth. Thus, it is likely that the subgroup of metastatic patients with a high frequency of anti-melanoma memory T cells may not show a clinical response to vaccination.

Introduction

During the last few years the results of clinical studies of vaccination with class I HLA-restricted tumor peptides recognized by T cells have been published, not only for metastatic melanoma patients \cite{1, 2, 3, 4, 5, 6, 7} but also for other types of cancer (e.g. pancreatic or prostate) \cite{8, 9}. A paradox is apparent in the majority of these
reports, namely that the vaccine-specific response of the patients' peripheral blood cytotoxic T lymphocytes was not associated with the clinical outcome, but rather that the occurrence of clinical responses tended to exclude anti-vaccine immune reactions and vice versa (1, 3, 4, 10, 11, 12, 13, 14). Even the use of dendritic cell-based vaccines fails to alter the overall picture. In fact, while DC-based immunization clearly increases the strength and frequency of anti-vaccine T-cell responses, no significant improvements were observed in the clinical response rate of patients having undergone DC-based vaccination as compared to that obtained in vaccination trials not involving DCs (2, 5, 15).

What we would like to propose is that such a lack of association between tumor-specific, alphabeta T lymphocyte-mediated responses to vaccine antigens and clinical outcome reflects the immune status of patients, as determined by their previous exposure to the cancer antigens subsequently used for vaccination. To explain our hypothesis, we will try to recapitulate the possible kinetics of interaction between alphabeta T cells and cancer antigens using human melanoma as paradigm.

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**Tumor-host relationship during primary tumor growth**

During primary melanoma growth, cutaneous lesions may present with infiltration of T lymphocytes most likely due to recognition of tumor antigens by T cells (16, 17) and often accompanied by an area of tumor regression. In fact, albeit in rare occasions (owing to the difficulty of isolating such cells), T lymphocytes deriving from primary lesions, including regressing ones, could be shown to destroy autologous melanoma cells *in vitro* and to encompass dominant clonotypes (18, 19). Moreover, spontaneous regression was associated with the presence of an infiltrate expressing a TH1 cytokine profile, as evaluated by PCR and *in situ* hybridization (20).

This suggests that a local, and perhaps systemic, sensitization against one or more melanoma antigens can occur during primary melanoma growth. This early antigen recognition by T cells could lead to either (i) an elimination of the most aggressive, metastasis competent, melanoma cells, which would explain the better prognosis of primary lesions characterized by brisk lymphocyte infiltration (21), or (ii) a selection in favor of tumor cells expressing antigen(s) not recognized by the infiltrating lymphocytes, or not expressing antigens at all, and thus endowed with an immunologically unrestricted metastatic potential (Figure 1). In fact, partial regression of primary melanoma is considered a bad prognostic factor at least by some authors (16). A side-effect of such a first selection could also be the elimination of tumor cells which express high levels of the given class I HLA allele, thus reducing the possibility that other peptide/HLA complexes could be seen by the host's T cells. In fact, the downregulation or absence of class I HLA has been reported even for primary melanoma lesions, although only in 10-16% of cases (22). The concept that the immune response can not only protect against the development of carcinogen-induced and spontaneous immunogenic tumors, but also select neoplastic cells with reduced immunogenicity, has been recently proven using wild-type and immunodeficient mice with targeted disruptions of either the recombination activating gene 2 (*RAG2*) or the gene encoding the transcription factor mediating signaling by the interferon-gamma and interferon alpha/beta receptors (23). It is of note that, in addition to alphabeta T lymphocytes, even gammadelta T and NK cells may participate in immune surveillance against tumors (24) although, in the case of human melanoma, such a process is likely to occur early in the process of UV carcinogenesis. In this phase of the innate type of antitumor reaction, NK and/or gammadelta T lymphocytes can recognize MIC/A/B or HLA-G antigens on tumor targets by the NKG2D receptor or KIR respectively (25, 26). Elimination of these tumor cells may go undetected until a macroscopic skin tumor, devoid of NK-activating ligands or expressing NK-inhibitory ligands like HLA-G (26), comes to the attention of the patient or doctor. It is likely that, at that time, HLA-A, -B and -C-restricted alphabeta T cells remain the main players in the immune recognition of HLA-restricted melanoma antigens. However, in association with the down-regulation of
or disappearance of class I HLA, a re-activation of the NKT, NKG2D+ infiltrating lymphocytes may also occur in metastatic lesions while expression of MIC is not modified (27).

Figure 1. Flow chart of the possible kinetics of interactions between melanoma and alphabeta T cells of tumor-bearing patients. Early T cell response may or not be activated (left, central and right upper panel). If activated (left and central panel), T cells can preferentially recognize strongly immunogenic unique antigens. T cell recognition may result in either complete or partial reduction of the most immunogenic and metastasis competent tumor cells. Patients whose immunogenic/metastasis competent cells are eliminated will then be cured by surgery (left panel), whereas partial elimination of such neoplastic cells will allow recurrences to occur in at least a fraction of the patients. Patients in whom no immune recognition in the primary lesion takes place (right panel) will recur with a high frequency. Vaccination of metastatic patients with antigens which have been selected against during tumor growth will be ineffective due to the lack or low expression of common differentiation antigens and the useless high frequency of CTLps directed to unique antigens, these unique antigens not being included in the vaccine. However, patients whose T cells failed to recognize unique and/or differentiation antigens during tumor growth could be prone to respond to vaccines containing differentiation antigens should the vaccine formulation result in priming of naive T cells (e.g. using DC) that can find their target (differentiation antigens) or the antigenically unselected tumor cells.

However, even alphabeta T cells may fail to recognize primary tumors despite their antigenic potential for a variety of reasons, including (i) the possible detrimental effect on antigen-presenting cell (APC) function and on T-cells accompanying the process of neoplastic transformation of melanocytes caused by UV irradiation (28, 29), and (ii) the production of a variety of cytokines (30) and the expression or release of FasL (31) by tumor cells that could locally suppress an otherwise effective immune response (tumor counterattacker), though the latter phenomenon remains controversial (32). In such cases, no effective local or systemic immunization will take place and, therefore, no tumor antigen selection will ensue.
As a consequence, the circulating tumor cells of these two clinically similar groups of patients whose primary melanoma has been surgically removed (i.e. those who mounted an immune response against the primary neoplasm and those who did not) should be antigenically different, the melanomas of the first group having lost one or more epitope/HLA complexes in a significant fraction of tumor cells whereas the melanomas of the second group maintain their original, unmodified antigenic profile. These two groups of patients should also show marked differences in terms of specific cytotoxic T lymphocyte precursors (CTLp) and effectors to different melanoma antigens, with patients who mounted an immune response during early tumor growth having a high frequency of these memory CTLs in their blood and/or tissues, whereas individuals in whom no immunization occurred and therefore bearing unselected neoplastic cells having negligible numbers of anti-melanoma CTLs (Figure 1).

### Tumor antigens recognized in early vs. metastatic neoplastic lesions

Which cells are eliminated in such an early phase and which is the molecular identity of the targeted antigens on primary tumors is unknown. We favor the idea that alphabetaT cells infiltrating primary melanomas probably predominantly recognize antigens forming an entirely new epitope in neoplastic, as compared to normal, cells (for a list of such antigens, refer to 33). In fact, a large number of somatic mutations accumulate in cancer cells in response to both environmental agents (such as the UV component of sunlight, ionizing radiation, chemicals) and products of normal cellular metabolism. Mouse models have clearly demonstrated that UV rays, the main culprit of human melanoma, can induce tumors bearing several unique, strong antigens (34). Some of these somatic mutations will result in proteins that provide novel immunogenic peptides (epitopes) recognized in the context of at least some HLA molecules. However, depending on the gene affected, the immune system may face two different situations, owing to the fact that some of these gene products are irrelevant to the tumorigenic process whereas others can have a crucial role in the development and progression of the tumor. In both cases the new epitope may elicit a strong immune T-cell response that may result in antigen-loss cancer cell variants. However, while antigen-loss variants are likely to be generated in tumor cells bearing the mutated, but growth-unrelated epitope, the immune response will have to cope with the inherent advantage offered by the altered protein to the malignant phenotype when a growth-indispensable gene is involved. Thus one may predict that the latter, but not the first, type of unique antigens should be found in metastatic lesions, a hypothesis which appears to be corroborated by the admittedly few available data on the molecular characterization of unique antigens in melanoma. In fact, while the only unique antigen described in primary melanoma is the result of a mutation in myosin (35), certainly not a growth-related protein, unique antigens of metastatic tumors derive from mutated carcinogenesis- and growth-related proteins or essential housekeeping genes like *N-RAS* (36), p16INK4a (37), *beta-catenin* (38), ribosomal-like proteins (39), caspase-8 (40) or MUM-2, a protein with an essential function in eukaryotic cells (41). Conversely, tumor antigens corresponding to differentially-expressed normal protein/peptides (differentiation antigens) or similar antigens expressed only in particular normal tissues, i.e. testis, but often expressed in primary tumors (e.g. MAGE, NY-ESO-1) should be less susceptible to immune selection due to their weak or null "spontaneous" immunogenicity that may result from several factors (42). A possible exception is Melan-A/MART-1, which is considered the immunodominant antigen both *in vitro* and *in vivo* as compared to other melanoma differentiation antigens (43, 44). In fact, it was recently found that primary melanoma regression is associated with an immune response to Melan-A/MART-1 which causes a selection for antigen-negative cell variants (45). This possibility was confirmed by an analysis of healthy controls and melanoma patients in different clinical conditions by HLA-A2/epitope tetramer staining. Such a study has revealed a low but detectable frequency of anti-Melan-A/MART-1 but no or appreciably fewer anti-gp100 or -tyrosinase T cells in stage I patients (primary tumor); however, the frequency of T cells recognizing all three antigens studied increases with increasing tumor burden in the advanced disease (46). Moreover, T lymphocytes of HLA-A2 metastatic (stage III) melanoma patients treated with surgery alone only seldom recognized unique
antigens on autologous (possibly immunoselected) metastatic melanoma, whereas T cells recognizing differentiation antigens were found in most of these patients (47). The possible anti-tumor activity of T cells directed to unique antigens is suggested by the detection of unique antigens endowed with pro-tumorigenic properties in metastatic melanoma and non-melanoma patients enjoying a favorable prognosis (39, 48, 49). In such patients, tetramer staining with soluble HLA/peptide complexes revealed a high frequency of CTLs, often clonally expanded, directed against these unique mutated peptides. These effectors persisted for a long time (48, 49) and were often accompanied by a loss of previously recognized tumor antigens (48). This suggests that, in these patients, a systemic immunization against unique tumor-specific antigens may occur during primary tumor growth and when antigen-bearing cells are not eliminated, such an immunization can effectively help to control the progression of the disease. Evidently, these mutations affect proteins which cannot be lost by tumor cells, as has recently been shown for ribosomal proteins serving as unique antigens in mice (50).

**Shaping of the tumor cell antigenic phenotype during progression**

In patients who mounted an immune response, the early difference in tumor antigen expression with patients who failed to do so could be quantitatively and qualitatively accentuated during the period of clinically silent metastatic growth (e.g. invasion of draining lymph nodes). Moreover, inflammatory-like reactions at the site of tumor growth or other favorable factors may help the host's immune system to break tolerance and to become primed against melanoma antigens formed by normal proteins (e.g. Melan-A/MART-1, MAGE) as well, thus giving rise to a second round of immunoselection driven by such antigens (refer to Figure 1).

Spontaneous immunoselection has been documented by several reports where different metastatic nodules of the same melanoma patient could be tested at different time points, showing a progressive loss of tumor antigen/HLA complex epitopes derived from normal proteins (51, 52, 53).

Thus, in advanced melanoma patients in whom immunoselection has occurred, the T cell repertoire will have a heterogeneous composition. In fact, it would include CTLs recognizing tumor-restricted (unique or shared) early epitopes maintained, in a minority of patients, on primary and metastatic cells owing to the growth advantage conferred by such epitopes to melanoma cells. An additional subset of CTLs will encompass those recognizing normal, shared antigens (like tyrosinase, MAGE, etc.) encountered in the subsequent metastatic phase of the disease, in addition to the anti-Melan-A/MART-1 effectors generated during primary tumor growth (45, 54, 55). In addition, it is also likely that priming against the latter melanoma antigens may occur more frequently in advanced disease owing to (i) a larger antigen burden, as suggested by the work of Dunbar (56) and shown in a large series of melanoma patients studied in our institute (46), and/or (ii) a higher level of gene expression possibly associated with increasing de-methylation occurring during tumor growth (57).

**Effect of vaccination**

Vaccinations have been and are being carried out in advanced metastatic patients that, for the reasons outlined above, may belong to two distinct immunological and clinical subsets (refer to Figure 1).
The first group includes patients whose metastases consist of cells which have gone through a CTL-mediated immune selection during primary and metastatic tumor growth. It should be pointed out that, to date, all vaccination protocols have involved immunization against shared tumor antigens, irrespective of the vaccination mode (peptide, protein or recombinant vaccine). The only exceptions may be represented by autologous tumor cell vaccines (58) or autologous heat shock protein gp96 vaccines (59) which may contain unique antigens, although not demonstrated in these clinical trials. Thus, at present, the role (if any) of unique antigens in human cancer vaccination is entirely unknown. As for shared antigens, a clinical response will fail in these patients if the vaccine includes peptide epitopes previously seen by their immune system but present only in a fraction of metastatic cells. This is likely to have occurred in many trials. In fact, only some clinical vaccination protocols required, among the eligibility criteria, an assessment of the expression in metastatic biopsies of the antigen used in the vaccine. Moreover, this assessment often consisted of only a non-quantitative PCR allowing large, but still partial, loss of antigen(s) by melanoma cells to go undetected. In other cancer patients the metastatic lesions affect visceral organs from which even fine-needle biopsies cannot be obtained. In such cases a functional dissociation may occur between the lack of local immune response at metastatic tumor sites and the systemic anti-tumor reaction (60). In fact, in these same patients, a strong anti-vaccine T cell immunity could be expected to develop if the vaccine includes tumor-specific antigens against which a high frequency of CTLps have been expanded during previous tumor growth.

The second group of patients, whose immune system has ignored tumor antigens corresponding to normal proteins throughout tumor growth, therefore has few or no CTLps against them. These subjects, who may include up to 50% of metastatic patients (61, 62), if appropriately immunized with multiepitope vaccines, may be those in whom the CTL response has the highest chance of recognizing epitopes expressed by the previously unselected tumor cells and therefore cause significant tumor destruction, ultimately leading to a clinically-relevant shrinkage of the tumor mass. In these patients the relatively low frequency of CTLps (due to the absence of or limited boosting during tumor growth) will reduce the possibility of detecting anti-melanoma specific CTLs in the blood, such effectors being concentrated in tumor lesions to function as killer cells. This group of metastatic patients will most likely need to be immunized using potent antigen-presenting cells (e.g. DCs) and T helper epitopes because priming is necessary to break/activate their tolerant/ignorant immune system. The number of vaccinations may also be relevant in determining immune and clinical responses as recently shown in an animal model (63). As suggested by the long time necessary for the clinical response to become evident, this may have occurred with patients who showed a durable clinical response in the vaccination trial with the MAGE-3.A1 peptide (4).

Evaluation of the expression of the tumor antigen(s) included in the vaccine, as well as of the frequency of CTLps directed against these in peripheral blood and, when possible, at the tumor site, will allow this hypothesis to be tested in future clinical trials.

A recent publication reported on a successful vaccination of follicular B lymphoma patients (64). In this instance, important drawbacks which occur in patients bearing metastatic solid tumors, such as the heterogeneity of tumor antigens, their selection by the immune system and the large tumor burden were overcome by the tumor system used. In fact, these lymphomas express an operationally homogeneous tumor antigen (the Ig idio type) on all neoplastic cells, thus representing an ideal target for the immune reactivity elicited by vaccination. Moreover, chemotherapy renders these patients clinically tumor-free, though molecularly detectable lymphoma cells can still be found in the majority of patients whose tumor burden is therefore very limited and unable to activate important tumor escape mechanism. These factors thus facilitate the destructive action of the tumor-specific immune cells induced by the vaccine. It should also be noted that until vaccination is initiated, lymphoma patients do not appear to have developed a spontaneous anti-tumor immunity, thus resembling the subgroup of immunologically tolerant melanoma patients discussed above.
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