

VACCINATION OF CANCER PATIENTS WITH TUMOR ANTIGENS RECOGNIZED BY T LYMPHOCYTES

THIERRY BOON, BERNARD LETHÉ, CHRISTOPHE LURQUIN,
PIERRE VAN DER BRUGGEN, FRANCIS BRASSEUR, PIERRE COULIE

It is now generally recognized that human tumors bear antigens that can be recognized by T lymphocytes of cancer patients. The approach that led to the identification of these antigens was based on *in vitro* stimulation of T lymphocytes of cancer patients with irradiated cells of the autologous tumor. This provided cytolytic T lymphocytes (CTL) clones that were capable of lysing with great specificity the autologous tumor cell line. Gene transfection experiments combined with recognition of the transfected cells by these CTL clones then led to the identification of the genes coding for the antigens.

When these approaches were applied to human melanoma, four classes of antigens were identified, according to the genetic or epigenetic processes that produced them (Figure 1). First, there were antigens encoded by cancer-germline genes. These genes are not expressed on normal adult cells except on male germline cells. They are expressed in a fraction of tumors of many histological types. As major histocompatibility complex molecules are not expressed on male germline cells, this implies that the antigens recognized by T lymphocytes are present only on tumor cells. These antigens are therefore common to many tumors and strictly tumor-specific. A second class of antigens was found to be encoded by genes that are expressed in normal cells but are overexpressed in a number of tumors. A third class results from point mutations found in a wide array of genes. Interestingly many of these mutations have clear oncogenic potential, such as one found in cyclin-dependent kinase 4 and another found in caspase 8. A fourth class was unexpected: many T lymphocytes of melanoma patients recognized melanocyte differentiation antigens, such as tyrosinase. One would have expected that natural tolerance would have eliminated these T

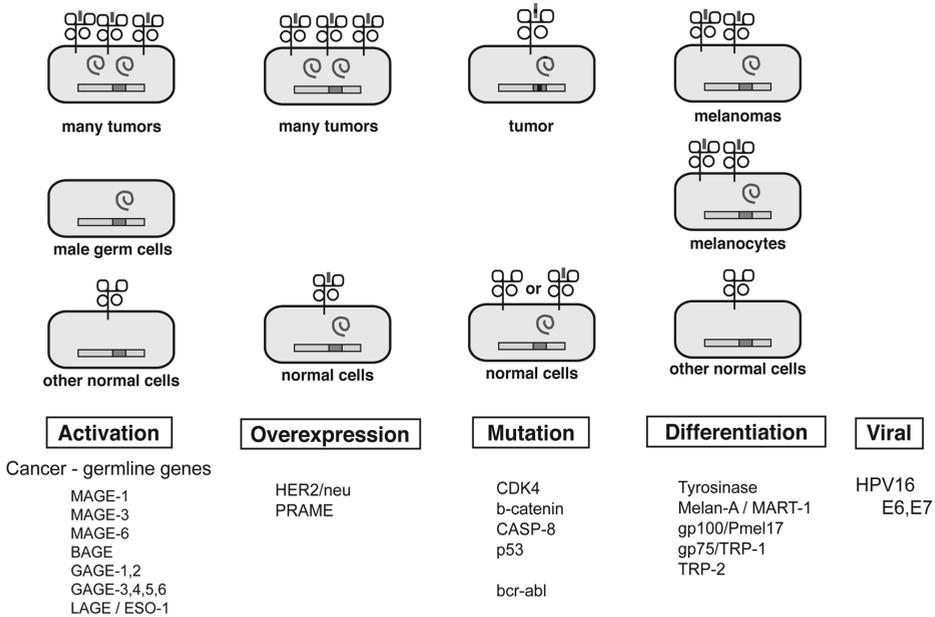


Figure 1. Main categories of tumor antigens.

lymphocytes. We believe that these four classes of antigens will also be found with other tumors. A fifth class should be mentioned: oncoviral antigens. For instance, uterine cervix cancer is caused by human papilloma virus (HPV16) and viral genes E6 and E7 code for antigens that are recognized by T cells.

The antigens encoded by cancer-germline genes ought to be good candidates for the therapeutic vaccination of cancer patients as they are strictly tumor-specific and present on many tumors, unlike the mutational antigens which are highly specific for each individual tumor. The first cancer-germline genes that were identified belong to the MAGE-A family, which comprises 12 genes. By now, about 10 families of cancer-germline genes have been identified. Most of these genes are located on the X chromosome. Some of these genes are expressed in many tumors (Table 1). For instance MAGE-3 is expressed in 74% of metastatic melanomas and in 47% of non-small cell lung cancer. These genes are activated in tumors as a result of the demethylation of their promoter.

PERCENTAGE OF TUMORS EXPRESSING GENES		
	MAGE-1	MAGE-3
Melanoma	46	74
Esophagus	53	63
NSCLC	46	47
Head & Neck	31	51
Bladder	32	57
Advanced myeloma	32	32

Table 1.

Much of our effort on therapeutic vaccination of cancer patients has focused on gene MAGE-3. A large number of MAGE-3 antigens presented by class I and class II major histocompatibility complex molecules have been identified. A MAGE-3 encoded peptide presented by HLA-A1 (MAGE-3.A1 antigen) has been used in several clinical trials. A first trial involved three subcutaneous and intradermal injections of this peptide, in the absence of adjuvant. The patients were metastatic melanoma patients with detectable disease. Tumor regressions were observed in 7 out of the 26 patients who completed the trial. We then examined whether more frequent injections or the addition of adjuvant improved the results. No improvement was observed. Immunization with MAGE-3 protein mixed with an adjuvant did not produce a higher rate of tumor regression. Neither did vaccination with recombinant poxvirus ALVAC harboring a minigene coding for MAGE-3.A1.

After vaccinating a total of about 200 metastatic melanoma patients with detectable disease, our results can be summarized as follows. No significant toxicity has been observed, implying that this form of therapy

ought to be applicable to earlier stages of the disease and to patients with better prognosis. Tumor regressions are observed in about 20% of the patients, but only in 10% of the patients are these tumor regressions medically significant (complete or partial responses). When tumor regressions occur, they proceed slowly and in the absence of noticeable inflammation.

Originally, our notion was that high-level anti-vaccine responses, i.e. anti-MAGE-3.A1 CTL, would be necessary, but perhaps not sufficient, in order to cause tumor regressions. We were therefore very surprised to find little or no CTL responses in the blood of most patients who showed tumor regressions. Much of our present effort is aimed at the analysis of low-level CTL responses in vaccinated patients. For MAGE-3.A1, the level of CTL precursors found in the blood of non-cancerous individuals is 4.10^{-7} of CD8 T cells. The diversity of this anti-MAGE-3.A1 T cell repertoire is high: our observations lead to an estimate of at least 100 different T cell receptors (clonotypes). As humans have a total of 4.10^{10} CD8 T cells, this implies that each of the 100 clonotypes is present at a frequency of 4.10^{-9} and comprises about 160 T cells.

For the evaluation of the frequency of anti-MAGE-3.A1 CTL, we restimulate *in vitro* blood T lymphocytes with peptide tetramers for two weeks, in limiting dilution conditions. The microcultures are then assessed with HLA-peptide for the presence of positive cells. The positive cultures are cloned and the specific lytic ability of the clones on tumor cells expressing the MAGE-3.A1 antigen is evaluated. This approach enables us to detect responses with frequencies as low as 10^{-6} of CD8 T cells. This approach has been applied to a number of patients vaccinated with the MAGE-3.A1 peptide, with recombinant ALVAC virus carrying the MAGE-3.A1 minigenes. The anti-vaccine CTL responses that are identified are usually low: mostly between 10^{-6} and 10^{-5} , even though one response as high as 10^{-3} has been observed (an example is shown in Figure 2). Strikingly, these responses are monoclonal. This enables us to evaluate the responses directly in the blood with clonotypic PCR, with results that have been in good agreement with those obtained after restimulation *in vitro*. For the ALVAC trial, there is clearly a correlation between anti-MAGE-3.A1 CTL responses and tumor regressions. Out of 5 patients who showed tumor regressions, 4 showed a CTL response. Out of 14 patients who did not, only 2 showed anti-MAGE-3.A1 CTL and one of them already had elevated CTL before vaccination. This correlation suggests, but does not rigorously prove, that the occasional tumor regressions that are observed following vaccination, are caused by the vaccination.

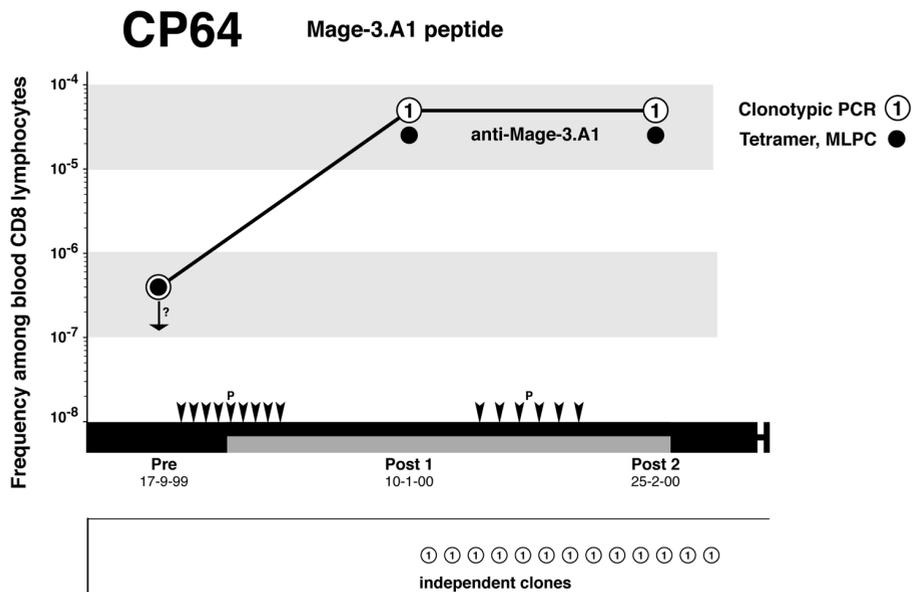


Figure 2. EXAMPLE OF ASSESSMENT OF FREQUENCY OF ANTI-VACCINE CTL CLONES.

The patient was vaccinated with the Mage-3.A1 peptide. The blood frequency of anti-Mage-3.A1 CTL is shown with a black dot. The response comprised a single CTL clone-type labelled 1. The frequency obtained by clonotypic PCR is shown by the symbol '1'. Peptide injections are indicated with symbols labelled 'P'. The patient showed a mixed tumor response.

A similar analysis has been carried out on patients vaccinated with autologous dendritic cells pulsed with peptide MAGE-3.A1. In contrast with the results obtained with peptide clone or with ALVAC, polyclonal responses were observed.

In most of the patients who show evidence of tumor regression following vaccination, the anti-vaccine cytolytic T cell (CTL) response is either undetectable or present at a low level, which might be deemed insufficient to produce tumor rejection. We therefore set out to examine whether T cells recognizing other tumor antigens might participate in the tumor regression process. As a first step, we estimated in 6 patients the blood frequencies of anti-tumor CTL, namely lytic effectors that recognized the autologous

melanoma cells but not autologous B cells nor NK target K562. After vaccination, frequencies of anti-tumor CTL in the blood ranged from 10^{-4} to 3×10^{-3} of the CD8 T cells, i.e. 10 to 10,000 times more than the anti-vaccine CTL in the same patient. Similar anti-tumor CTL frequencies were already present in the blood prior to vaccination. From a patient who had shown nearly complete tumor regression following vaccination, we derived 15 anti-tumor CTL clones. Ten CTL clones recognized antigens encoded by cancer-germline gene *MAGE-C2*, and 3 recognized antigens encoded by melanocyte differentiation gene *gp100*. These antigens were also recognized by CTL present at high frequency in the blood of this patient before vaccination. These results suggest that melanoma patients carry very high frequencies of anti-tumor CTL, which are directed against the main categories of tumor antigens defined previously.

As a second step in assessing the respective contribution of anti-vaccine and anti-tumor CTL to tumor regression, we investigated the presence of anti-tumor and anti-vaccine CTL inside metastases in the same patient who showed tumor regression after vaccination against antigen MAGE-3.A1. The frequency of anti-MAGE-3.A1 CTL was 2.5×10^{-6} of CD8 T cells in the blood and it was 6-fold higher in an invaded lymph node. An anti-tumor CTL recognizing an antigen encoded by MAGE-C2 showed a considerably higher enrichment. Whereas in the blood the frequency of this CTL was 9×10^{-5} , in the invaded lymph node it was about 400 times higher. Several other anti-tumor T cell clonotypes had frequencies of above 1% inside metastases. These results suggest that the anti-vaccine CTL may not be the principal effectors that kill the bulk of the tumor cells. They may exert their effect mainly by an interaction with the tumor, which creates conditions that enable the stimulation of large numbers of other anti-tumor CTL, which then proceed to destroy the tumor cells. Consistent with this model, we observed that new anti-tumor CTL clonotypes appeared following vaccination and were present in the tumor at a very high frequency.

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