

HUMAN TUMOR IMMUNOLOGY*

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In association with the Ludwig Institute :see *Analysis of T cell responses of vaccinated cancer patients* (research at LICR, Brussels)

Human tumor cells bear antigens that are not present on normal cells and that can be specifically recognized by autologous CD4 or CD8 T lymphocytes. We contributed to the identification of several tumor-specific antigens, present on melanoma or lung carcinoma cells (1-4). Tumor-specific antigens, such as those encoded by the MAGE genes, have been used to vaccinate melanoma patients with detectable disease. About 20 % of the vaccinated patients display a tumor regression, a frequency that appears well above the level reported for spontaneous melanoma regressions. Nevertheless, the treatment fails in most patients, and this can probably only be improved if a better understanding of the immune responses of the patients is acquired. Our group focuses on such analyses.

A new method for the detection of low level tumor-specific T cell responses

V. Karanikas and T. Aerts in collaboration with D. Colau, Brussels branch of the Ludwig Institute for Cancer Research

The failure of 80 % of the vaccinated patients to show tumor regression could be due to two major causes which are not mutually exclusive : a failure of the vaccine to induce an adequate T cell response or a resistance of the tumor to immune attack. To the extent that the limiting factor for success would be the level of the T cell response to the vaccine, one ought to find a correlation between the occurrences of T cell responses and those of tumoral regression. We focused our efforts on the detection of cytolytic T lymphocytes (CTL) recognizing a peptide encoded by gene *MAGE-3* and presented by HLA-A1. The group of Pierre van der Bruggen showed that the frequency of anti-MAGE-3.A1 CTL in the blood of an individual without cancer is about $4 \cdot 10^{-7}$ of the CD8 cells. To detect safely an increase of at least 10-fold

of this naive frequency of anti-MAGE-3.A1 T cells, we resorted to an approach based on an *in vitro* restimulation of blood lymphocytes with the antigenic peptide and T cell growth factors over two weeks, followed by labeling with A1/MAGE-3 "tetramers" : fluorescent soluble complexes of HLA-A1 molecules presenting the MAGE-3.A1 peptide. In order to evaluate precursor frequencies, these cultures are carried out in limiting dilution condition. Cells that are labeled with the tetramer are cloned, and their diversity analyzed by TCR sequencing.

This MLPC/tetramer/cloning approach was applied to a metastatic melanoma patient who responded clinically to vaccination with peptide MAGE-3.A1 administered without adjuvant (5). We found that the vaccination induced at least a 100-fold amplification of the anti-MAGE-3.A1 CTL present in the blood, with a postvaccination frequency of $5 \cdot 10^{-5}$ of the CD8 cells. Surprisingly, TCR analysis of anti-MAGE-3.A1 CTL clones derived from positive cultures indicated that this CTL response was monoclonal. This finding enabled us to analyze directly the frequencies of blood T cells expressing this TCR, using "clonotypic" PCR amplifications specific for

its V α or V β rearrangement. The frequencies of anti-MAGE-3.A1 CTL measured by this genetic approach closely matched those found by the MLPC/tetramer method. These results

demonstrate that some patients vaccinated with a MAGE antigen mount CTL responses, even after vaccination with an antigenic peptide without adjuvant.

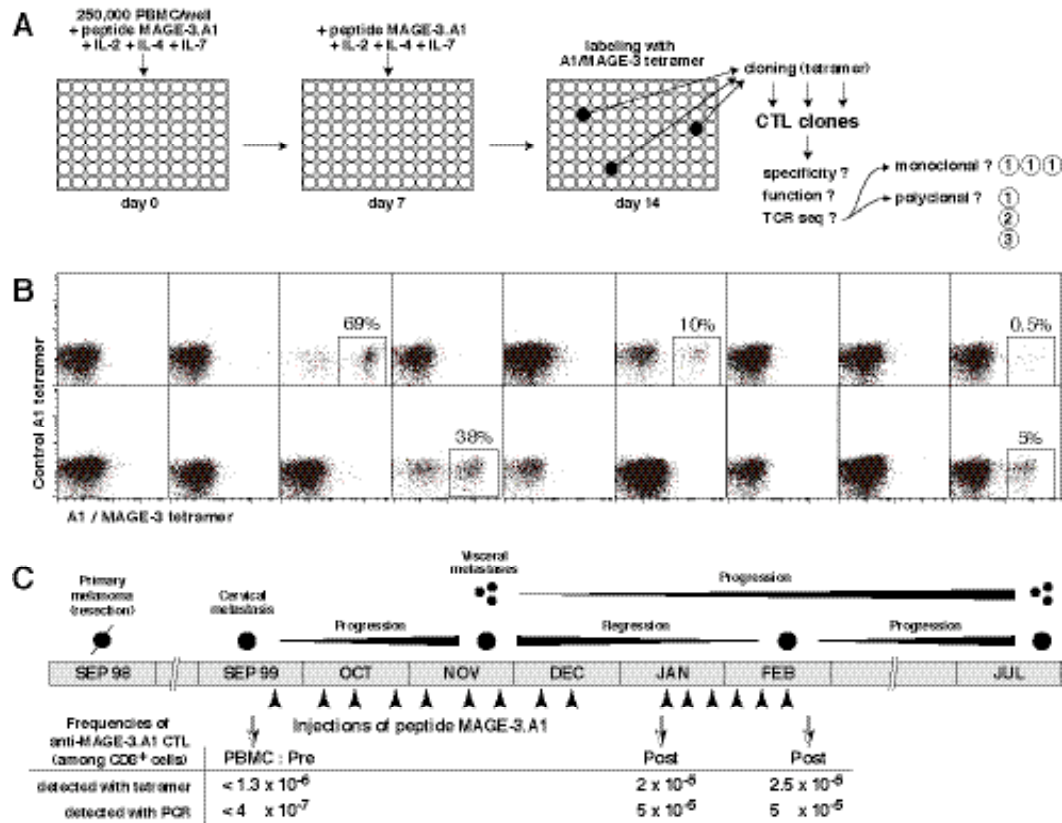


Fig. 1. A. Overview of the MLPC/tetramer/cloning procedure for the analysis of anti-MAGE-3.A1 T cells. B. Typical results of labeling with A1/MAGE-3 tetramer after two weeks of stimulation. Groups of 160,000 postvaccination PBMC from patient CP64 were stimulated as described in A. The lymphocytes were labeled on day 15 with tetramer and anti-CD3 and anti-CD8 antibodies. Only CD3⁺CD8⁺ lymphocytes are included in the plots. Two types of HLA-A1 tetramer were used, containing peptide MAGE-3.A1 or a control influenza nucleoprotein peptide. Clusters of lymphocytes specifically labeled with the A1/MAGE-3 tetramer are boxed, and their proportion among the CD3⁺CD8⁺ cells is indicated. C. Clinical evolution of patient CP64, and frequencies of blood anti-MAGE-3.A1 T cells.

Anti-MAGE CTL responses in vaccinated patients : Correlation with clinical responses ?

V. Karanikas, T. Connerotte, V. Corbière, C. Muller, C. Mondovits, T. Hanagiri in collaboration with C. Lurquin and D. Colau, Brussels branch of the Ludwig Institute for Cancer Research

We have analyzed the T cell responses of metastatic melanoma patients with detectable disease, following vaccination with a recombinant poxvirus of the ALVAC type, which bears a short MAGE-3 gene sequence coding the MAGE-3.A1 antigenic peptide. Three out of five patients who showed tumor regression had an anti-MAGE-3.A1 CTL response. For two patients, there was a clear increase in the frequency of anti-MAGE-3.A1 CTL after vaccination : from < 3.10⁻⁷ to 3.10⁻⁶

in one patient and from 8.10^{-7} to 3.10^{-3} in the other. These two CTL responses were monoclonal. In the third patient the anti-MAGE-3.A1 CTL frequency did not increase after vaccination, but the TCR analysis indicated that one CTL clone had been amplified. These results indicate that, like peptide immunization, ALVAC immunization usually produces a monoclonal response. Only two anti-MAGE CTL responses were observed among 12 patients who did not show tumor regression following ALVAC vaccination. These results suggest that there is a certain correlation between CTL response and tumor regression, but this will need confirmation with larger numbers (6, 7).

Analysis of regressing tumors

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If clinical responses correlate with immunological responses, anti-vaccine T cells probably initiate tumor rejection. One possibility is that they reach and destroy the tumor. This could be examined with a clonotypic PCR applied to a tumor specimen biopsied at the time of regression. Preliminary results obtained from two patients suggest that the anti-vaccine CTL clones found in the blood were not enriched in tumors.

Another possibility is the involvement of T cells recognizing tumor-specific antigens absent from the vaccine. Detection of such CTL requires the establishment of an autologous tumor cell line, and stimulation of blood lymphocytes with the tumor cells to derive tumor-specific CTL clones. The specificity and the TCR diversity of these CTL clones can then be analyzed. We have started this analysis with a melanoma patient who displayed tumor regression after vaccination with the ALVAC/MAGE construct. We measured the blood frequencies of CTL recognizing the vaccine antigen, MAGE-3.A1, and of CTL recognizing other, undefined, tumor antigens. We observed that the anti-vaccine CTL frequency rose from $< 5.10^{-7}$ to 3.10^{-6} of the CD8 after vaccination. On the other hand, the anti-tumor CTL frequencies were already about 10^{-3} of the CD8 before vaccination, and increased slightly after vaccination. TCR analysis of panels of anti-tumor CTL clones indicated that one clone had been amplified after vaccination. We identified the antigen recognized by this anti-tumor CTL : it is a peptide presented by HLA-A2 and encoded by gene *MAGE-C2*, which is

specifically expressed in tumors. These results suggest that other tumor-specific CTL than those that recognize the vaccine antigen can be stimulated after vaccination.

We then used TCR-specific PCR to detect the anti-vaccine (MAGE-3.A1) and the anti-MAGE-C2.A2 CTL in a tumor sample collected from this patient after vaccination. The results are very clear : the anti-vaccine CTL is present in the tumor at the same frequency than in the blood, whereas the anti-MAGE-C2.A2 CTL is considerably enriched (at least 100-fold) in the tumor.

These results suggest that tumor-specific CTL others than those that recognize the vaccine antigen may participate in the tumor rejection that follows vaccination. A likely scenario involves « antigen spreading » : an initial and low level CTL response against the vaccine peptide leads to the destruction of a small number of tumor cells, followed by the processing and presentation of other tumor antigens. New waves of CTL may then follow, that recognize these antigens. This would explain how tumors can be rejected with a very low frequency of anti-vaccine CTL. We are now trying to validate this model with other patients. We will also examine the functional properties of the anti-vaccine T cells. It is indeed surprising that a tumor rejection takes place after the activation of a few anti-vaccine lymphocytes, even though many other tumor-specific T cells were already present before vaccination. By comparing these two types of lymphocytes, we may understand why only the anti-vaccine lymphocytes appear to initiate tumor rejection.

A new lung tumor antigen

P.G. Coulie in collaboration with J.-F. Baurain, Department of Oncology, Cliniques Universitaires St Luc, Université de Louvain, Brussels, and H. Echchakir, Laboratoire Cytokines et Immunologie des tumeurs Humaines, Institut National de la Santé et de la Recherche Médicale, Institut Gustave Roussy, Villejuif, France)

We have identified an antigen recognized on a human large cell carcinoma by an autologous tumor-specific CTL clone that was derived from mononuclear cells infiltrating the primary tumor (8). The antigenic peptide is presented by HLA-A2 molecules and is encoded by the *α -actinin-4* gene, which is expressed ubiquitously. In the tumor cells, a point mutation generates an amino acid change that is essential for recognition by the CTL. The mutation was not found in *α -actinin-4* cDNA

sequences from about 50 lung carcinoma cell lines, suggesting that it is unique to this patient. Although he did not receive chemotherapy or radiotherapy, the patient has been without evidence of tumor since the resection of the primary lesion in 1996. Using tetramers of soluble HLA-A2 molecules loaded with the mutated antigenic peptide, anti- α -actinin-4 CTL could be derived from blood samples collected from the patient in 1998 and 2000. It is possible that these CTL, recognizing a truly tumor-specific antigen, play a role in the clinical evolution of this lung cancer patient.

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