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ABSTRACT

After vaccination of melanoma patients with MAGE antigens, we observed that even in the few patients showing tumor regression, the frequency of anti-vaccine T cells in the blood was often either undetectable or <10(-5) of CD8 T cells. This frequency being arguably too low for these cells to be sole effectors of rejection, we reexamined the contribution of T cells recognizing other tumor antigens. The presence of such antitumor T cells in melanoma patients has been widely reported. To begin assessing their contribution to vaccine-induced rejection, we evaluated their blood frequency in five vaccinated patients. The antitumor cytotoxic T lymphocyte (CTL) precursors ranged from 10(-4) to 3 x 10(-3), which is 10-10,000 times higher than the anti-vaccine CTL in the same patient. High frequencies were also observed before vaccination. In a patient showing nearly complete regression after vaccination with a MAGE-3 antigen, we observed a remarkably focused antitumoral response. A majority of CTL precursors (CTLp's) recognized antigens encoded by MAGE-C2, another cancer-germline gene. Others recognized gp100 antigens. CTLp's recognizing MAGE-C2 and gp100 antigens were already present before vaccination, but new clonotypes appeared afterwards. These results suggest that a spontaneous antitumor T cell response, which has become ineffective, can be reawakened by vaccination and contribute to tumor rejection. This notion is reinforced by the frequencies of anti-vaccine and antitumor CTLs observed inside metastases, as presented by Lurquin et al. (Lurquin, C., B. Lethe, V. Corbiere, L...)

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High frequency of antitumor T cells in the blood of melanoma patients before and after vaccination with tumor antigens

Catherine Germeau,1 Wenbin Ma,1,2 Francesca Schiavetti,1 Christophe Lurquin,1,2 Emmanuelle Henry,1 Nathalie Vigneron,1,2 Francis Brasseur,1,2 Bernard Lethé,1,2 Etienne De Plaen,1,2 Thierry Velu,3 Thierry Boon,1,2 and Pierre G. Coulie1

1Cellular Genetics Unit, Université de Louvain, B-1200 Brussels, Belgium
2Ludwig Institute for Cancer Research, Brussels Branch, B-1200 Brussels, Belgium
3Medical Oncology, Erasme Hospital, B-1070 Brussels, Belgium

After vaccination of melanoma patients with MAGE antigens, we observed that even in the few patients showing tumor regression, the frequency of anti-vaccine T cells in the blood was often either undetectable or \( < 10^{-5} \) of CD8 T cells. This frequency being arguably too low for these cells to be sole effectors of rejection, we reexamined the contribution of T cells recognizing other tumor antigens. The presence of such antitumor T cells in melanoma patients has been widely reported. To begin assessing their contribution to vaccine-induced rejection, we evaluated their blood frequency in five vaccinated patients. The antitumor cytotoxic T lymphocyte (CTL) precursors ranged from \( 10^{-4} \) to \( 3 \times 10^{-3} \), which is \( 10^{-10} \) times higher than the anti-vaccine CTL in the same patient. High frequencies were also observed before vaccination. In a patient showing nearly complete regression after vaccination with a MAGE-3 antigen, we observed a remarkably focused antitumoral response. A majority of CTL precursors (CTLp's) recognized antigens encoded by \( MAGE-C2 \), another cancer-germline gene. Others recognized gp100 antigens. CTLp's recognizing MAGE-C2 and gp100 antigens were already present before vaccination, but new clonotypes appeared afterwards. These results suggest that a spontaneous antitumor T cell response, which has become ineffective, can be reawakened by vaccination and contribute to tumor rejection. This notion is reinforced by the frequencies of anti-vaccine and antitumor CTLs observed inside metastases, as presented by Lurquin et al. (Lurquin, C., B. Lethé, V. Corbière, I. Théate, N. van Baren, P.G. Coulie, and T. Boon. 2004. J. Exp. Med. 201:249–257).

Antitumor CTL clones derived from the blood or from metastases of melanoma patients by in vitro stimulation with autologous tumor cells have been found to recognize several classes of antigens. First, there are those encoded by the “cancer-germline” genes, such as the \( MAGE, BAGE, GAGE, \) and \( LAGE \) families (1–5). These genes are expressed in a large fraction of tumors of various histological types and therefore code for shared antigens. These antigens are strictly tumor specific because the cancer-germline genes are silent in normal adult tissues with the exception of male germ cells, which do not carry HLA molecules and therefore cannot present antigens to T cells. Three subfamilies of \( MAGE \) genes are expressed in tumors: \( MAGE-A \), which codes for most of the antigens identified so far, \( MAGE-B \), and \( MAGE-C \). A second major class of tumor antigens is encoded by genes that are mutated in tumor cells. These antigens are also strictly tumor specific, but in most cases they were observed only in a single tumor with notable exceptions, such as one encoded by a mutated \( CDK4 \) (6). In a third class are antigens encoded by melanocytic differentiation genes, such as tyrosinase, \( Melan-A^{MART-1} \), or gp100\(^{Pm117} \) (7–10).

Antigens encoded by \( MAGE \) genes have been used for small-scale therapeutic vaccination trials of metastatic melanoma patients with
detectable disease. The vaccines consisted of either antigenic peptides, a protein, a recombinant poxvirus of the ALVAC-type encoding two antigenic peptides, or dendritic cells pulsed with an antigenic peptide (11–14). In the peptide, protein, and ALVAC-MAGE trials, some evidence of tumor regression was observed in ~20% of the patients. In the trials involving dendritic cells, the rates of tumor regression were not clearly different from those observed with other vaccine modalities (13).

Our initial work suggested that most vaccinated patients, including those who displayed tumor regression, did not have anti-vaccine T cells in the blood at frequencies \(>10^{-4}\) of CD8 T cells. Therefore, we developed a sensitive approach aimed at detecting low CD8 T cell responses. It is based on in vitro restimulation of PBMCs with the antigenic peptide followed by labeling with tetramers. To evaluate CTL precursor (CTLp) frequencies, these mixed lymphocyte–peptide cultures (MLPCs) are performed under limiting dilution conditions. The tetramer-stained cells of the positive microcultures are cloned, the lytic specificity of the clones is verified, and their diversity is analyzed by TCR sequencing (15). This approach enables us to detect CTL responses as low as \(8 \times 10^{-7}\) of the CD8 T cells (16).

We have focused our efforts on the detection of CTLs recognizing the antigenic peptide MAGE-3_{168-176}, which is encoded by the gene MAGE-A3 and presented by HLA-A1. In patients vaccinated with a recombinant canarypox virus of the ALVAC-type coding for the MAGE-3.A1 peptide, anti-MAGE-3.A1 CTL responses were found in 3 out of 4 patients who showed tumor regression and in 1 out of 11 who did not (15–17). Among seven patients vaccinated with MAGE-3.A1 peptide who showed evidence of tumor regression, only one had a detectable CTL response (16). Even among those vaccinated patients who showed a CTL response, most had a low frequency of anti-MAGE-3.A1 CTLs in the blood, ranging between \(10^{-6}\) and \(10^{-5}\) of CD8 T cells (15, 17).

These results clearly contradicted our initial view on antitumoral vaccination, namely that massive anti-vaccine CTL responses would be required for tumor rejection. Moreover, they raised the possibility that the low frequency of anti-vaccine CTLs would be insufficient to provide all the specific effector cells that lyse the tumor cells. Therefore, we set out to examine whether CTLs directed against other antigens present on the tumor might provide additional effectors for the tumor rejections observed after vaccination. As a first step, we show here the presence of high levels of such antitumor CTLs in the blood of vaccinated patients, and we describe the antigens recognized by these CTLs in one patient. In Lurquin et al. (18), which appears in this issue of JEM, we describe the distribution of anti-vaccine and other antitumor CTLs inside the metastases of this patient.

RESULTS
High frequencies of antitumor CTLp’s in the blood of melanoma patients

We selected five metastatic melanoma patients who had been vaccinated with MAGE antigens (Table I). They were selected because it had been possible to derive a permanent cell line from their tumor cells. We set up mixed lymphocyte–tumor cell cultures (MLTCs) to estimate the blood frequencies of CTLp’s directed specifically against the tumor. Purified CD8 blood T cells were stimulated with irradiated autologous tumor cells in medium containing IL-2, IL-4, and IL-7. These stimulations were performed in limiting dilution conditions with 300–4,000 lymphocytes per microculture (19). After three weekly stimulations, the lytic activity of the microcultures was tested against the autologous tumor culture (19). After three weekly stimulations, the lytic activity of the microcultures was tested against the autologous tumor cell line. The microcultures were then expanded with IL-2 and IL-7. These stimulations were performed in limiting dilution conditions with 300–4,000 lymphocytes per microculture (19). After three weekly stimulations, the lytic activity of the microcultures was tested against the autologous tumor culture (19).

### Table I. Frequencies of antitumor CTLp’s in vaccinated melanoma patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Vaccine antigens</th>
<th>Tumor regression</th>
<th>Anti-vaccine CTLp frequency</th>
<th>Antitumor CTLp frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>CP64</td>
<td>MAGE-3.A1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>(5 \times 10^{-5})</td>
<td>(6 \times 10^{-5})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3 \times 10^{-6})</td>
<td>(3 \times 10^{-4})</td>
</tr>
<tr>
<td>EB81</td>
<td>MAGE-3.A1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+</td>
<td>(&lt;3 \times 10^{-7})</td>
<td>(5 \times 10^{-4})</td>
</tr>
<tr>
<td></td>
<td>MAGE-1.A1</td>
<td></td>
<td>(&lt;3 \times 10^{-7})</td>
<td>(10^{-4})</td>
</tr>
<tr>
<td>CP67</td>
<td>MAGE-3.A1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+</td>
<td>(3 \times 10^{-7})</td>
<td>(2 \times 10^{-3})</td>
</tr>
<tr>
<td></td>
<td>MAGE-1.A1</td>
<td></td>
<td>(&lt;3 \times 10^{-7})</td>
<td>(2 \times 10^{-3})</td>
</tr>
<tr>
<td>BB132</td>
<td>MAGE-3.A1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>(10^{-7})</td>
<td>(6 \times 10^{-4})</td>
</tr>
<tr>
<td></td>
<td>MAGE-1.A1</td>
<td></td>
<td>(&lt;3 \times 10^{-7})</td>
<td>(6 \times 10^{-4})</td>
</tr>
<tr>
<td>LB2269</td>
<td>MAGE-4.A2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-</td>
<td>(&lt;10^{-7})</td>
<td>(6 \times 10^{-4})</td>
</tr>
<tr>
<td></td>
<td>MAGE-10.A2</td>
<td></td>
<td>(5 \times 10^{-15})</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Some evidence of tumor regression observed after vaccination as detailed in Materials and Methods.
<sup>b</sup>Among blood CD8 lymphocytes. The anti-vaccine CTLp frequencies were measured by in vitro restimulation of PBMCs with the antigenic peptide in limiting dilution conditions followed by labeling with tetramers. Before and After stands for before and after vaccination.
<sup>c</sup>15 injections of 300 µg of peptide without adjuvant.
<sup>c</sup>3 injections of ALVAC-MAGE followed by 3 injections of peptides without adjuvant.
<sup>c</sup>3 injections of 300 µg of each peptide with rIL-12.
<sup>c</sup>A similar frequency was already present before vaccination.
a cancer-germline gene, and differentiation gene (20, 21). These antigens were encoded by only two genes: MAGE-2, a melanocytic differentiation gene, and gp100, a melanocytic differentiation gene (20, 21).

Precise criteria and representative results are shown in Fig. 1.

For all five patients, antitumor CTLp’s were found at high frequencies, i.e., from $10^{-4}$ to $3 \times 10^{-3}$ of the CD8 T cells in the blood after vaccination (Table I). These frequencies were considerably higher than that of the anti-vaccine CTLs, ranging from 12- to 20,000-fold higher (Table I and Fig. 2). Antitumor CTLp’s were already present at similar high frequencies before vaccination (Table I). In two patients who showed evidence of tumor regression, including patient EB81 (Fig. 2), the frequencies of antitumor CTLp’s were $\sim$10-fold higher after vaccination. But this was not observed in the other regressor patient and therefore may not be significant. The level of antitumor CTLp’s found either before or after vaccination was not higher in the three regressors than in the two progressor patients.

Antigens recognized by antitumor CTLs of patient EB81

To establish the relevance of the antitumor T cells observed at such high frequencies, we set out to identify their target antigens. We focused our efforts on patient EB81, who had shown complete regression of a large number of cutaneous metastases. Stable CTL clones were derived from microcultures of postvaccination lymphocytes that displayed a high level of antitumor lytic activity and did not lyse EBV-B cells (EB81-EBV), autologous EBV-transformed B cells (EB81-EBV), and K562. On day 20 only, effector cells and $^{51}$Chromium-labeled targets were incubated in the presence of a 50-fold excess of unlabeled K562 to quench the lytic activity of NK-like effectors. Microcultures were considered to contain antitumor CTLs if they satisfied the following criteria: lysis of tumor $>10\%$ and lysis of EBV-B and K562 lower than one third of the lysis of tumor.

The detailed identification of these antigens is described elsewhere (22). Finally, one CTL clone recognized a third peptide, MAGE-C2/50 ASSTLYLVF, on HLA-B57. All of the clones recognized peptide-pulsed targets with half-maximal lysis at peptide concentrations between 20 and 100 nM, the usual range for such CTLs. Thus, five different clonotypes recognizing three different MAGE-C2 antigenic peptides constituted the majority of the antitumor CTL clones derived from the blood of patient EB81. Two additional clonotypes were found, using HLA-A2 tetramers, in blood collected 7 mo later (Fig. 2). 3 of the 15 antitumor CTL clones recognized an antigenic peptide encoded by gp100, namely gp100243–251 ITDQVPFSV, which is presented by HLA-A2 (21). Two had the same TCR.

These results show that the massive antitumor CTL response of patient EB81 is directed against antigens that belong to the main known classes of tumor antigens. The antitumor CTL response of this patient was remarkably focused on MAGE-C2 antigens, targeting three different peptides with seven different clonotypes, whereas no CTL directed against other antigens encoded by cancer-germline genes was observed. This is not due to the lack of expression of other cancer-germline genes. Although MAGE-C2 appeared to have the highest expression, several other cancer-germline genes, such as MAGE-A3, MAGE-A6, and LAGE-I, were expressed at similar levels in tumor samples and in the tumor cell line (Table II). It is worth noting in Table II that the gene expression profile of the melanoma cell line, which was used to derive all of the antitumor CTLs of patient EB81, shows a good match with those of the autologous tumor samples.

**Presence of anti–MAGE-C2 and anti-gp100 CTLs before vaccination**

We observed that a spontaneous CTL response against MAGE-C2 had already occurred in patient EB81 before vaccination. Eight independent antitumor CTL clones were each having a different TCR. The detailed identification of these antigens is described elsewhere (22).
obtained from the prevaccination lymphocytes (Fig. 2). Two recognized the MAGE-C2191–200 peptide (Fig. 2). Both carried the same TCR. Another CTL clone recognized gp100154–162.

Considering the presence of CTLs directed against MAGE-C2 and gp100 both before and after vaccination, we wondered whether individual CTL clonotypes also had a constant presence. Some clonotypes were clearly present in the blood before and after vaccination. An example is anti–MAGE-C2191–200 CTL clone 11. As shown in Lurquin et al. (18), its frequency among blood CD8 T lymphocytes was evaluated with a clonotypic PCR. It was $9 \times 10^{-6}$ before
A more complete description of the frequencies of various clonotypes before and after vaccination, not only in the blood but also inside metastases, is provided in Lurquin et al. (18).

A high frequency of antitumor CTLs was already present in all five patients before vaccination. In patient EB81, antigens encoded by MAGE-C2 and gp100 were targets of antitumor T cells found both before and after vaccination. Several anti-MAGE-C2 clonotypes were found at frequencies equal or greater than $10^{-5}$ in the blood before vaccination (see Lurquin et al. [18]). Their cumulative frequency was $\sim 7 \times 10^{-5}$. It is noteworthy that in face of such a high prevaccination T cell response, the tumor had not lost the expression of the MAGE-C2 target antigens.

In conclusion, it appears that the melanoma patients who are being vaccinated have already mounted a strong spontaneous T cell response against the types of antigens used in the vaccines. At the time of vaccination, these highly frequent T cells are clearly ineffective in halting tumor progression. This is not due to a lack of presence of these cells inside the tumors, but rather to a lack of expression of the relevant genes in the tumor cells.
as shown in Lurquin et al. (18). We also show in this report that after vaccination, antitumor CTLs are highly concentrated in the tumors. This reinforces the possibility that they constitute the main component of the effectors that cause tumor rejection.

One might regard the unexpected observation of tumor rejection in the face of a low anti-vaccine CTL response as a minor issue, fading away with the use of better vaccines. In our view, this opinion neglects the uncomfortable fact that up to now no vaccination modality has stood out as producing a superior frequency of tumor rejection, even though higher frequencies of anti-vaccine CTLs are observed in regressing patients with certain modalities, such as dendritic cell vaccination. Recruitment of other antitumoral T cells by the anti-vaccine T cell may therefore be a general feature of any successful vaccination procedure. A possible consequence might be that the functional properties enabling anti-vaccine T cells to do so matter more than their number. Elucidating what these functional properties are and how to generate them might be key to improving anti-tumoral vaccination strategies.

**MATERIALS AND METHODS**

**Patients.** The clinical study protocol was approved by the Ethics Review Committee of the Faculty of Medicine of the Catholic University of Louvain. Five metastatic melanoma patients with measurable disease at the time of vaccination were included in this study. Patient CP67 received 15 injections of 300 μg of peptide MAGE-3.A1 without adjuvant. He mounted a monoclonal CTL response to the vaccine antigen (15) and showed an about threefold higher frequency of tumor rejection, even though higher frequencies of anti-vaccine CTLs are observed in regressing patients with certain modalities, such as dendritic cell vaccination. Recruitment of other antitumoral T cells by the anti-vaccine T cell may therefore be a general feature of any successful vaccination procedure. A possible consequence might be that the functional properties enabling anti-vaccine T cells to do so matter more than their number. Elucidating what these functional properties are and how to generate them might be key to improving anti-tumoral vaccination strategies.

**Cells.** Melanoma cell lines, EBV-transformed B cells, and K562 were cultured in Iscove’s medium (Life Technologies) supplemented with 10% FCS, 1% L-glutamine, 1% L-arginine, 36 mg/L L-asparagine, and 216 mg/L L-glutamine.

**Analysis of antitumor CTLs with MLTC.** PBMCs isolated by Lymphoprep (Nycomed) density gradient centrifugation were cryopreserved in Iscove’s medium supplemented with 10% human serum and 10% DMSO. Blood CD8+ lymphocytes were purified from thawed PBMCs by sorting on a FACS Vantage (Becton Dickinson) or with magnetic beads (Miltenyi Biotec). Lymphocytes were seeded at 200–2,000 cells/well with 48 or 96 wells/cell number in V-bottom microwells (Nunc) and stimulated by the addition of irradiated (100 Gy) autologous tumor cells (3,000 cells/well) pretreated with 50 U/ml IFN-γ (Roche Diagnostics) in a final volume of 100 μl Iscove’s medium supplemented with 10% human serum, 100 μM 1-methyl-L-tryptophan, 20 U/ml IL-2, 10 ng/ml IL-4, and 5 ng/ml IL-7. The microcultures were restimulated each week by the addition of irradiated tumor cells and the same cytokine cocktail. Around day 21, aliquots collected from all microcultures were used as effector cells in a lyss assay.

**MLPCs.** MLPCs were performed as described previously (17). In brief, PBMCs from patient EB81 were incubated for 60 min at room temperature
with antigenic peptide MAGE-C2191 or MAGE-C2336 at 20 μM, washed, and distributed at 2 × 10⁶ cells /microwell in 0.2 ml of medium containing 20 U/ml IL-2, 10 ng/ml IL-4, and 10 ng/ml IL-7. On day 7, 50% of the medium was replaced by fresh medium containing 20 μM of peptide, IL-2, IL-4, and IL-7. Tetramer labeling was performed on day 14 using a control tetramer as described previously (17).

**TCR analysis.** Total RNA from CTL clones was extracted with the TriPure reagent (Roche Diagnostics). Reverse transcription was performed as described previously (17). cDNA served as template for PCR amplifications using panels of Vα- or Vβ-specific upstream primers and one downstream Cα or Cβ primer. PCR products were purified and sequenced to obtain a complete identification of the CDR3 region. Frequency estimations using clonotypic PCR on PBMCs were based on limiting dilution analysis as described in Lurquin et al. (18).

**Gene expression analysis.** Expression of cancer-germline and melanocyte differentiation genes was assessed by reverse transcription and conventional PCR amplification. Gene expression levels were evaluated semiquantitatively by the intensity of the bands obtained by separating PCR products in agarose gel. Expression of all of these genes in the vaccination metastasis of patient EB81 was also measured by real-time quantitative PCR based on TaqMan methodology using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Sequences of primers and probes are available from the authors and will be described elsewhere.

We thank C. Muller, T. Aerts, and M.-C. Letellier-Prayudi for excellent technical assistance; S. Depechon for editorial assistance; and B. Van den Eynde, P. van der Bruggen, and A. Van Pet for comments on the manuscript.

This work was supported by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister’s Office, Science Policy Bruggen, and A. Van Pet for comments on the manuscript.

The authors have no conflicting financial interests.

Submitted: 9 July 2004
Accepted: 7 December 2004

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