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Polyclonal CTL Responses Observed in Melanoma Patients Vaccinated with Dendritic Cells Pulsed with a MAGE-3.A1 Peptide

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Vaccination with mature, monocyte-derived dendritic cells (DC) pulsed with the MAGE-3_168–176 peptide, which is presented by HLA-A1, has been reported to induce tumor regressions and CTL in some advanced stage IV melanoma patients. We present here a precise evaluation of the level of some of these anti-MAGE-3.A1 CTL responses and an analysis of their clonal diversity. Blood lymphocytes were stimulated with the MAGE-3.A1 peptide under limiting dilution conditions and assayed with an A1/MAGE-3 tetramer. This was followed by the cloning of the tetramer-positive cells and by TCR sequence analysis of the CTL clones that lysed targets expressing MAGE-3.A1. We also used direct ex vivo tetramer staining of CD8 cells, sorting, and cloning of the positive cells. In three patients who showed regression of some of their metastases after vaccination, CTL responses were observed with frequencies ranging from $7 \times 10^{-6}$ to $9 \times 10^{-4}$ of CD8+ blood T lymphocytes, representing an increase of 20- to 400-fold of the frequencies found before immunization. A fourth patient showed neither tumor regression nor an anti-MAGE-3.A1 CTL response. In each of the responses, several CTL clones were amplified. This polyclonality contrasts with the monoclonality of the CTL responses observed in patients vaccinated with MAGE-3.A1 peptide or with an ALVAC recombinant virus coding for this antigenic peptide. The Journal of Immunology, 2003, 171: 4893–4897.

Following the identification of human tumor-specific Ags recognized on human tumors by autologous T cells, a large number of small therapeutic vaccination trials have been performed (1). A number of metastatic melanoma patients with detectable disease have been vaccinated with Ag MAGE-3_168–176, which is encoded by MAGE-3, a gene with a cancer-germline expression profile (2). This Ag is presented by HLA-A1 and HLA-B35 and will be referred to as Ag MAGE-3.A1 (3).

Patients have been vaccinated with the MAGE-3.A1 peptide alone or with a recombinant canarypox virus (ALVAC) bearing short sequences coding for this MAGE-3.A1 Ag and for the homologous MAGE-1.A1 Ag (Ref. 4 and N. van Baren, M. Marchand, B. Dréno, T. Dorval, S. Piperno, D. Lienard, B. Escudier, T. Boon, P. Coulie, M.-C. Bonnet, and P. Moingeon, manuscript in preparation). Evidence of tumor regression has been observed in 18 of 91 patients who were vaccinated with the peptide or with ALVAC. The CTL responses to the vaccine were studied in most of the HLA-A1 patients who showed evidence of tumor regression and in a number of patients who did not. We resorted to an approach based on stimulation of blood lymphocyte microcultures with the antigenic peptide, followed by labeling of the microcultures with an A1/MAGE-3 tetramer (5). Among 10 regressing HLA-A1 patients who were evaluated, 5 showed an anti-MAGE-3.A1 CTL response (5, 6). The intensity of these responses varied widely, with CTL precursor (CTLp) frequencies ranging from $4 \times 10^{-7}$ to $3 \times 10^{-3}$ among blood CD8 cells. TCR sequence analysis indicated that all of these T cell responses were monoclonal. Among eight patients who did not show tumor regression, two had a CTL response. These numbers suggest that the occurrence of a detectable CTL response shows some degree of correlation with tumor regressions, opening the possibility that a limiting factor for the antitumor effect of the vaccine is the intensity of the CTL response to the vaccine. It is therefore of considerable importance to find out whether other vaccination modalities produce better CTL responses in cancer patients and, if they do, whether a higher rate of tumor regression ensues.

Dendritic cells are widely accepted to be particularly effective in presenting Ags to T cells (7). In a recent study, advanced melanoma patients with distant metastases were vaccinated with monocyte-derived dendritic cells matured by autologous monocyte-conditioned medium and pulsed with the MAGE-3.A1 peptide and with one of the recall Ags tetanus toxoid or tuberculin. Six patients of 11 showed evidence of tumor regression (8). Anti-MAGE-3.A1 CTL responses were observed in a number of patients. They were evaluated by in vitro restimulation of PBMC with peptide under limiting dilution conditions followed by a specific lysis assay. We have perfected and extended the analysis of the CTL responses of

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some of these patients with a more sensitive approach involving tetramer sorting and TCR analysis. Our results indicate that these CTL responses are polyclonal.

Materials and Methods

**In vitro peptide stimulation of PBMC (mixed lymphocyte-peptide culture (MLPC))**

Our method was very similar to that described by Karanikas et al. (6). PBMC were thawed and incubated at 10^5 cells/ml in Iscove’s medium containing 10% AB human serum (HS) at 37°C in the presence of DNase (5 U/ml) for 1 h to get rid of most dead cells. They were then washed and an aliquot was taken to measure the percentage of CD8+ cells by labeling with an anti-CD8-FITC (SK1; BD Biosciences, Mountain View, CA) Ab. PBMC were incubated at 5 × 10^5 cells/ml in Iscove’s medium with 1% HS and were pulsed with 20 μM peptide MAGE-3: A1 (EVDPIGHLY) for 60 min at room temperature. After washing, they were distributed at 1 × 10^5–3 × 10^5 cells/0.2 ml in microwells containing Iscove’s medium with 10% HS, IL-2 (20 U/ml), IL-7 (10 ng/ml; R&D Systems, Minneapolis, MN). At variance with Karanikas et al. (6), MAGE-3: A1 peptide at 1 μM was added to the microculture. In some experiments, IL-4 was added at 10 U/ml. But when we compared cultures performed with or without IL-4, no difference was observed in the number of wells positive for the tetramer. On day 7, 50% of the medium was replaced by fresh medium containing IL-2 (50 U/ml), IL-7 (10 ng/ml), and PHA-A (PHA-HA16, 125 ng/ml; Murex Biotech, bioTRADING, Bierbeek, Belgium). TCR Vα and Vβ were assessed by PCR amplification by using panels of Vα and Vβ primers that were designed in two multiplex amplifications, one for each clonotype. Each reaction used a Vα and a Vβ primer, downstream to those used in the first amplification, and a Jα and a Jβ primer, each with its 3’ end matching the 5’ portion of the CDR3 region. A fraction (¼) of the amplified product was used in a run-off reaction (11) starting from antisense fluorescent (5’-6-chlorofluorescein for the CDR3α and 5’-hexachlorofluorescein for the CDR3β) primers whose 3’ ends complement the 5’ portion of the CDR3 region. Size determination of the run-off products was performed with the ABI PRISM 3100 Genetic Analyzer and the GeneScan software (Applied Biosystems, Foster City, CA). The sensitivity of the procedure, one CTL among 10^6 PBMC, was established with dilutions of cDNA from CTL 1 and 5 in cDNA from irrelevant PBMC.

**Results and Discussion**

We evaluated the CTL response of HLA-A1 metastatic melanoma patients vaccinated with dendritic cells pulsed with the MAGE-3: A1 peptide (8). Two approaches were followed, as outlined in Fig. 1.

In the first approach, which was described previously (5), blood mononuclear cells were thawed and pulsed with the MAGE-3: A1 peptide (Fig. 1, left panel). They were distributed into microcultures of ~10^6 cells, 10–20% being CD8+ T cells, with the aim of obtaining limiting dilution conditions (<20% positive wells) for anti-MAGE-3: A1 CTLp. After two 7-day cycles of stimulation with the peptide, each of these MLPC was tested for the presence of CD8+ cells labeled by an A1/MAGE-3 tetramer. When a cluster of tetramer-labeled cells was found, the cells were sorted. Even though these cells represent a single T cell clone as a result of the limiting dilution conditions, these cells were cloned to eliminate contaminating lymphocytes. The clones that proliferated were tested for their ability to lyse cells presenting the MAGE-3: A1 Ag, including tumor cells expressing the MAGE-3 and HLA-A1 genes. In this “MLPC-tetramer-cloning” approach, we estimated the CTLp frequency by limiting dilution evaluation, counting as positive those microcultures that contained A1/MAGE-3 tetramer+ cells from which lytic anti-MAGE-3: A1 CTL clones were obtained.

In the second approach, thawed PBMC were assayed immediately with the A1/MAGE-3 tetramer, and the labeled cells were cloned (Fig. 1, right panel). Proliferating clones were tested for tetramer binding and lytic ability. In this “ex vivo-tetramer” approach, we estimated the CTLp frequency by multiplying the fraction of the CD8+ cells that bound the tetramer with the fraction of cloned cells that multiplied and lysed HLA-A1 target cells expressing gene MAGE-3.

To evaluate the diversity of the responses, the TCRβ chain of all the lytic CTL clones was sequenced.

We analyzed the responses of four advanced metastatic patients. Patients 04, 06, and 09 showed clear evidence of tumor regression (6), and different inclusions in the microwells led to two results.

**Regressor patient 04**

This advanced melanoma patient had visceral metastases when he received three s.c. and intradermal injections of pulsed dendritic cells followed by two i.v. injections, all at 14-day intervals. All but
one of a large number of lung metastases regressed following the vaccination, while metastases present in other locations progressed (8).

The preimmunization lymphocytes were tested by the MLPC-tetramer approach. No positive was observed among $6 \times 10^6$ CD8 T cells (Fig. 2). With the ex vivo-tetramer approach, two lytic clones were obtained from $10^6$ CD8 T cells. The weighed average of these two estimations, $2.8 \times 10^{-7}$, is within the range of what we observe in noncancerous individuals (Ref. 12 and C. Lonchay, S. Lucas, T. Boon, and P. van der Bruggen, manuscript in preparation).

After three dendritic cell vaccinations, the frequency of anti-MAGE-3.A1 CTLp was evaluated by the MLPC-tetramer approach to be $4 \times 10^{-6}$ of the CD8 T cells. After the fourth vaccination, a frequency of $5.5 \times 10^{-5}$ was observed. After the fifth, a frequency of $1.4 \times 10^{-4}$ was observed and the same frequency was obtained with the ex vivo-tetramer approach. Thus, the frequency of anti-MAGE-3.A1 CTLp rose steadily with successive vaccinations. This is at variance with the previous evaluation of this CTL response, where the CTLp frequency was reported to drop after the i.v. vaccination steps (8).

The analysis of the TCR$\beta$ sequences revealed a considerable diversity. Among the 71 CTL clones derived from lymphocytes collected after the third and fifth immunizations, 11 different TCR were observed. The pattern of different clonotypes obtained with the MLPC-tetramer approach was the same as that obtained with the ex vivo-tetramer approach. Since the frequency of the CTLp was more than 100 times the preimmunization level, $1\%$ of the characterized CTL clones ought to represent the preimmunization repertoire. Accordingly, at least 10 of the 11 different CTL clones observed in the postimmunization samples represent CTL that multiplied in response to the vaccine. A subset of clonotypes appears to dominate the response: 47 CTL clones had TCR 1 and 18 had TCR 5 (Fig. 2). We verified that these two groups were homogeneous: nine independently isolated clones of each were shown to have identical TCR$\alpha$ chains. Thus, the CTL response of this patient was highly polyclonal.
The blood frequency of clonotypes 1 and 5 was also evaluated directly by PCR. Clonotypic PCR for the TCRβ/H9251 and H9252 genes were set up and used to test cDNAs derived from freshly thawed groups of lymphocytes. No positive was found in preimmunization blood for CTL 1 and 5, leading for both to a frequency estimate below $4 \times 10^{-7}$ of CD8 T cells. For the lymphocytes collected after the fifth vaccination, a frequency of $6 \times 10^{-5}$ was observed for both clonotypes. These frequencies are in line with the frequency of $1.4 \times 10^{-4}$ obtained by the MLPC-tetramer and the ex vivo-tetramer approaches, considering that CTL clonotypes 1 and 5 constitute $\approx 50\%$ of the clones that were isolated. This indicates that these two clonotypes were not counterselected in the course of their in vitro expansion.

Regressor patient 06

This patient showed a complete regression of one lung and four subcutaneous metastases. However, there was overall progression (8). The preimmunization lymphocytes were tested by the MLPC-tetramer and ex vivo-tetramer approaches. No positive was observed among $2.2 \times 10^6$ and $4.4 \times 10^5$ CD8 T cells, respectively, leading to a frequency estimate of $<1.5 \times 10^{-7}$ of CD8. After the fifth vaccination, the anti-MAGE-3.A1 CTLp frequency was $3.6 \times 10^{-6}$ of CD8. After the sixth vaccination, frequencies of $1.4 \times 10^{-5}$ and $4.2 \times 10^{-6}$ were observed with the ex vivo-tetramer and MLPC-tetramer approaches, respectively, leading to a weighed average of $7.7 \times 10^{-6}$. The TCR of a total of 22 CTL clones were analyzed. Ten different clonotypes were found, one being repeated eight times (Fig. 2).

We conclude that this patient made a CTL response to the vaccine, because the frequency of anti-MAGE-3.A1 CTLp was $>50$ times the frequency observed before vaccination. This frequency implies that $>19$ of the 20 clonotypes obtained after the sixth vaccination represent CTL that expanded following vaccination. This CTL response is therefore clearly polyclonal.

Regressor patient 09

This patient showed partial regression of one lung metastasis but overall progression of others after the fifth vaccination with MAGE-3.A1-pulsed dendritic cells. This patient was then included in another protocol and received IFN-α, IL-2, and dendritic cells pulsed with MAGE-3.A1 and tyrosinase.A1 peptides. Since the samples from the blood collected after the fifth vaccination were no longer available, we analyzed the anti-MAGE-3.A1 response with the blood collected after eight vaccinations (Fig. 2). A frequency of $9 \times 10^{-4}$ was obtained by the MLPC-tetramer approach with six different clonotypes, three of them being repeated several times. The preimmunization sample gave a frequency of $2.6 \times 10^{-6}$, and the two CTL clones that were obtained were different from those of the postimmunization sample.
This patient completed the vaccination schedule without objective tumor regression. Lymphocytes collected after the eighth vaccination were tested by the MLPC-tetramer approach. No tetramer-positive CTL clone was obtained, leading to a frequency estimate below $5 \times 10^{-7}$ of CD8 T cells.

The results shown here indicate that the level of anti-MAGE-3.A1 CTL responses obtained upon dendritic cell vaccination varies greatly, as observed with peptide and ALVAC vaccinations. But the polyclonality of the T cell responses elicited by the dendritic cell vaccination is in sharp contrast with the monoclonality or quasi-monoclonality of the T cell responses elicited with antigenic peptide or ALVAC constructs (5). This polyclonality was observed even when the level of the T cell response elicited by the dendritic cell vaccination was lower than that observed in some patients immunized with peptide or ALVAC. This suggests that upon dendritic cell vaccination more precursors are activated, but that they do not necessarily enjoy a higher amplification. It will be important to establish definitively whether the average level of the CTL responses observed upon dendritic cell vaccination is higher than that obtained after peptide or ALVAC vaccination.

It is noteworthy that, whereas three patients activated many CTL clones, one patient showed no CTL response at all. At the present time, we do not have any understanding as to the reasons why some patients make a CTL response to tumor Ag vaccines while others do not. When the responses are monoclonal, it is possible to attribute the difference to a stochastic limiting event, which may be difficult to identify, such as the encounter between an initial CTLp and the Ag. On the other hand, when some patients make a polyclonal response whereas others do not make any response, as observed here, different explanations must be found. One possible explanation is that the prevaccination state of the immune system of the patients is different. For instance, some patients might have acquired immune tolerance for their tumor Ag. Another possible explanation is that the sensitivity of the tumors to immune attack varies widely among patients and that detectable CTL expansions can only be recorded following a successful interaction of responder CTL with the tumor.

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