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Monoclonal Anti-MAGE-3 CTL Responses in Melanoma Patients Displaying Tumor Regression after Vaccination with a Recombinant Canarypox Virus

Vaios Karanikas,* Christophe Lurquin,‡ Didier Colau,† Nicolas van Baren,† Charles De Smet,† Bernard Lethé,‡ Thierry Connerotte,* Véronique Corbière,* Marie-Ange Demoitie,*, Danielle Liénard,‡ Brigitte Dréno,§ Thierry Velu,¶ Thierry Boon,*,† and Pierre G. Coulie,*‡

We have analyzed the T cell responses of HLA-A1 metastatic melanoma patients with detectable disease, following vaccination with a recombinant ALVAC virus, which bears short MAGE-1 and MAGE-3 sequences coding for antigenic peptides presented by HLA-A1. To evaluate the anti-MAGE CTL responses, we resorted to antigenic stimulation of blood lymphocytes under limiting dilution conditions, followed by tetramer analysis and cloning of the tetramer-positive cells. The clones were tested for their specific lytic ability and their TCR sequences were obtained. Four patients who showed tumor regression were analyzed, and an anti-MAGE-3.A1 CTL response was observed in three of these patients. Postvaccination frequencies of anti-MAGE-3.A1 CTL were \(3 \times 10^{-6}\), \(3 \times 10^{-5}\), and \(3 \times 10^{-7}\) of the blood CD8 T cells, respectively. These three responses were monoclonal. No anti-MAGE-LA1 CTL response was observed. These results indicate that, like peptide immunization, ALVAC immunization produces monoclonal responses. They also suggest that low-level antivaccine CTL responses can initiate a tumor regression process. Taken together, our analysis of anti-MAGE-3.A1 T cell responses following peptide or ALVAC vaccination shows a degree of correlation between CTL response and tumor regression, but firm conclusions will require larger numbers.


The “cancer-germline” genes, such as the MAGE gene family, are expressed in male germline cells and not in normal tissues. They are also expressed in many tumors of various histological types (1–3). These genes code for Ags that can be recognized on tumor cells by T lymphocytes, and these Ags ought to be strictly tumor specific because the only normal cells that express the encoding genes, the spermatogonia, do not bear HLA molecules on their surface. A large number of MAGE antigenic peptides have been identified, that are recognized on human tumors by HLA class I- or class II-restricted T cells (4–7).

Ags encoded by gene MAGE-3 have been used for small-scale therapeutic vaccination trials of melanoma patients with detectable disease. The vaccines consisted of either an antigenic peptide, a protein, or dendritic cells pulsed with an antigenic peptide (8–11). In the peptide and protein trials, tumor regressions were observed in ~20% of the patients (Refs. 9 and 10 and our unpublished data), a proportion which appears to be well above the rates of spontaneous regressions that have been reported (12). In the absence of a randomized study including a placebo control arm, which could demonstrate vaccine effectiveness, our working hypothesis is that the regressions are caused by the vaccine.

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. If a limiting factor for success is 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 CTL recognizing the antigenic peptide MAGE-3/168–176.A1, which is encoded by gene MAGE-A3 and presented by HLA-A1. To detect the anti-MAGE-3.A1 CTL, our approach is based on an in vitro restimulation of PBMC with the antigenic peptide over 2 wk, followed by labeling with A1/MAGE-3 tetramers. To evaluate precursor frequencies, these mixed lymphocyte-peptide cultures (MLPC)3 are conducted under limiting dilution conditions. Cells that are labeled with the tetramer are cloned and their diversity is analyzed by TCR sequencing (13).

To strengthen the basis of our evaluation of low-level CTL responses, we have recently considered the size of diversity of the anti-MAGE-3.A1 TCR repertoire (Refs. 13 and 14 and C. Louchay, S. Lucas, T. Boon, and P. Van Der Bruggen, manuscript in preparation). Our latest estimate of the frequency of naïve anti-MAGE-3.A1 T cells found in the blood of individuals without cancer is ~4 \(\times 10^{-7}\) of the CD8 cells. In a noncancerous individual, we obtained a series of 14 independent anti-MAGE-3.A1

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1 This work was supported by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister’s Office, Science Policy Programming and by grants from the Fonds J. Maisin (Belgium), the Fédération Belge contre le Cancer (Belgium), the Fonds National de la Recherche Scientifique (Belgium), and the Fortis Banque Assurances and VIVA (Belgium). T.C. is a research fellow with the Fonds National de la Recherche Scientifique (Belgium).

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3 Abbreviations used in this paper: MLPC, mixed lymphocyte-peptide culture; HS, human serum; CTLp, CTL precursor.
HLA-A1 patients received the four ALVAC-MAGE injections, 1.A1 and MAGE-3.A1 without adjuvant. All vaccines were ad-

Moingeon, manuscript in preparation). This vector will be referred

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the MAGE-1.A1 and MAGE-3.A1 peptides (Refs. 16 and 17 and

narypox virus of the ALVAC type containing a minigene encoding

monoclonal (13).

The PCR product was digested with

resuspended at 107 cells/ml in Iscove

PBMC isolated by Lymphoprep (Nyomed, Oslo, Norway) density gradi-

washed in culture medium with IL-2 (100 U/ml), IL-4 (10 ng/ml), and IL-7

performed on day 14.

Improved protocol for the detection of tetramer-positive cells

We have observed in many MLPC/tetramer experiments that the propor-

tions of cells labeled by a tetramer were <0.03% of the CD8 cells. As the

background labeling by a tetramer is in the same range, we use two criteria
to distinguish the cells that are specifically labeled by a tetramer from those
that are labeled nonspecifically or that appear to be labeled because they

display autofluorescence (see Fig. 1). We gate out the cells that display

autofluorescence and those that are equally labeled by the relevant A1/

MAGE tetramer and by a control HLA-A1 tetramer containing an influenza

peptide. Briefly, MLPC were washed, resuspended in PBS with 1% HS,

and incubated for 30 min at 37°C with the two HLA-A1 tetramers. Anti-

CD8 Abs coupled to FITC (SK1; BD Biosciences, Mountain View, CA)

were then added and after a further incubation for 30 min at 37°C, the cells

were washed, fixed with 0.5% formaldehyde, and analyzed on a FACS-

Calibur flow cytometer (BD Biosciences). Typical results are shown in

Fig. 1 for four MLPC set up with PBMC from patient EB81 (A-C) or LB2196 (D).

The CD8+ cells were incubated with peptide MAGE-3.A1. The classical CD8+tetramer plots

are shown in column 2 for lymphocytes gated on their light scattering

properties, as shown in column 1. Column 3 shows that the specificity

detection is improved by gating out cells that emit a higher than

background fluorescence at 670 nm when excited at 488 nm. Considering that

no dye emitting at 670 nm is present during labeling, the positive events in

this “empty channel” are probably cells with a high autofluorescence. This

autofluorescence is also apparent in the PE detection channel, leading to

false positives. The usefulness of gating out these events is clearly apparent

for MLPC D, which contains cells that appear to be CD8+ and tetramer

positive. With the autofluorescence correction, this culture can safely be

considered negative. Column 4 shows the results when the labeling with

the tetramer into account. In MLPC A and B, there are clearly CD8 cells that are labeled with the two tetramers, artificially

increasing the sizes of the A1/MAGE-3+ clusters detected with a single

tetramer.

Typical labeling data of a MLPC/tetramer experiment are shown in

Fig. 2.

Materials and Methods

**Tetramer production**

An HLA-A*0101 cDNA clone served as template to amplify the sequence

coding for the extracellular domains (residues 1–276) of the HLA-A1 H

chain with primers A1M8 (5′-AAGAAGGAGATACATTACGGGGTCACA
cagTTGAgcatITTttatatcactggtccg) and A257 (5′-ATATGACGAGG

ATCCTTTAATTTCTGACATGTCGACACCAACCCACAGGCT

CATCTCAGGGTGG). A1M8 contains several base changes (small letters) de-

signated to optimize protein expression in

Escherichia coli

(13).

An HLA-A*0101 cDNA clone served as template to amplify the sequence

containing a minigene encoding the MAGE-1.A1 and MAGE-3.A1 peptides (Refs. 16 and 17 and

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Lienard, B. Escudier, T. Boon, P. Coulie, M.-C. Bonnet, and P.

Moingeon, manuscript in preparation). This vector will be referred to as

ALVAC-MAGE. Vaccination included four injections of AL-

VAC-MAGE followed by three injections of peptides MAGE-

1.A1 and MAGE-3.A1 without adjuvant. All vaccines were ad-

ministered every 3 wk by intradermal and s.c. routes. Twenty-three

HLA-A1 patients received the four ALVAC-MAGE injections, and five of them showed tumor regression.

**MLPC**

PBMC isolated by Lymphoprep (Nycomed, Oslo, Norway) density gradi-

tent centrifugation were cryopreserved in Iscove’s medium supplemented

with 10% human serum (HS) and 10% DMSO. After thawing, PBMC were

resuspended at 107 cells/ml in Iscove’s medium supplemented with 1% HS

divided into two groups. One group was incubated for 60 min at room

temperature with peptide MAGE-1.A1 (20 μM). The other was incubated

with MAGE-3.A1. These pulsed PBMC were washed, pooled, and distrib-

uted at 2.5 × 105 cells/0.2 ml in round-bottom microwells in Iscove’s

medium with HS (10%), L-arginine (116 mg/l), L-aspartagine (36 mg/l),

-t-glutamine (216 mg/L), IL-2 (20 U/ml), IL-4 (10 ng/ml), and IL-7 (10

ng/ml). On day 7, 50% of the medium was replaced by fresh medium

containing IL-2, IL-4, and IL-7. Peptide MAGE-3.A1 (20 μM) was added to

cultures. Peptide MAGE-1.A1 was added to all cultures 1 day later.

During the second week of stimulation, the cultures were divided according

to proliferation in medium containing IL-2 alone. Tetramer labeling was

performed on day 14.

**T cell clones and TCR analysis**

To derive stable CTL clones from the populations of tetramer-positive

cells, which are usually clonal populations, cells stained by tetramer were

seeded at one cell per well in round-bottom microplates using flow cyto-

metry and stimulated by the addition of irradiated (100 Gy) allogenic PBMC

(8 × 104/well) as feeder cells and irradiated allogenic HLA-A1 EBV-B

cells (2 × 105/well) incubated with the MAGE-3.A1 peptide (20 μM) and

washed in culture medium with IL-2 (100 U/ml), IL-4 (10 ng/ml), and IL-7

(10 ng/ml). The CTL clones were restimulated weekly by the addition of

feeder cells and peptide-pulsed EBV-B cells in medium with growth fac-

tors. After ~3 wk, they were transferred into 2 ml wells and maintained by

weekly restimulations with allogenic LG2-EBV-B cells and peptide-pulsed

HLA-A1 tumor cells.

Total RNA from PBMC or tumor material was extracted with the Tri-

pure reagent (Roche Diagnostics, Brussels, Belgium). Reverse transcrip-

tion was performed at 42°C for 90 min with 200 U of Moloney murine

leukemia virus reverse transcriptase mixed with 4 μl of 5′ First Strand

Buffer (Life Technologies, Merelbeke, Belgium), 2 μl of 20 μM oli-

gos(dT12-18) 20 U of RNasin (Promega, Madison, WI), 2 μl of 100 mM DTT

(Life Technologies). 1 μl of each dNTP at 10 mM each (Takara, Shiga,

Japan), and diethyl pyrocarbonate-treated water in a total volume of 20 μl.

cDNA served as template for PCR amplifications using panels of Vα- or

Vβ-specific upstream primers and one downstream Cα or Cβ primer chosen

on the basis of described panels of TCR V region oligonucleotides (20) and

the sequences available at the International Immunogenetics Data-

base of M.-P. Lefranc (http://imgt.cines.fr). PCR products were purified and

sequenced to obtain a complete identification of the CDR3 region. The

anti-MAGE-3.A1 CTL clones expressed the following rearranged TCR

genes: CTL 35 of patient EB81: Vα12-13-Jα41 and Vβ24-1-Jβ2.7; CTL 103 of

patient EB81: Vα21-1a-Jα28 and Vβ5-5-Jβ2.7; CTL 1 of patient LAU147:

Vα12-1a-Jα43, Vβ24-1-Jβ2.7; and CTL 2 of patient NAP33: Vα29-Jα49 and

Vβ15-Jβ1.2.

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Clonotypic PCR

Frequency estimations using clonotypic PCR were based on limiting dilution analysis: PBMC were aliquoted in groups of 10^7 to 10^8 cells, and clonotypic RT-PCR was applied to all groups for the TCR α- and β-chains. The proportions of CD8 cells in the PBMC and of positive groups for the clonotypic PCR were used to calculate a frequency for a given CTL clone. Here follows the procedure for the anti-MAGE-3.A1 CTL clone of patient EB81. It involves three nested PCR amplifications. The rearranged TCR sequences were Vβ12–1/Jβ11 (5'-aggagcAGAAGAaaattc..) and Vβ24/ Jβ1–2 (5'-aggtagGTCGGgGACgCAATatgect..), with nucleotides that are not templated by V or J genes in capital letters and in lowercase letters the 3’ and 5’ ends of the V and J sequences, respectively. RNA was extracted from groups of PBMC using TriPure and converted to cDNA using the SuperScript II reverse transcriptase (Life Technologies) and an anchored oligo(dt)21 primer, which contains a T7 promoter sequence at its 5’ end. All of the cDNA was used as template in a PCR amplification with forward primers corresponding to Vβ24 (LUR353: 5’-cacaagacgaggaaggg) and Va12 (LUR351: 5’-tcagggagggccagctg), and a reverse primer (5’-gcaggaggaattacacgt) that are not templated by V or J genes in capital letters and in lowercase letters the 3’ and 5’ ends of the V and J sequences, respectively. The T7 promoter sequence at its 5’ end. All of the cDNA was used as template in a PCR amplification with forward primers corresponding to Vβ24 (LUR353: 5’-cacaagacgaggaaggg) and Va12 (LUR351: 5’-tcagggagggccagctg), and a reverse primer (5’-gcaggaggaattacacgt). The T7 promoter sequence at its 5’ end. All of the cDNA was used as template in a PCR amplification with forward primers corresponding to Vβ24 (LUR353: 5’-cacaagacgaggaaggg) and Va12 (LUR351: 5’-tcagggagggccagctg), and a reverse primer (5’-gcaggaggaattacacgt). The cDNA was used as template in a PCR amplification with forward primers corresponding to Vβ24 (LUR353: 5’-cacaagacgaggaaggg) and Va12 (LUR351: 5’-tcagggagggccagctg), and a reverse primer (5’-gcaggaggaattacacgt).

Results

To analyze the anti-MAGE-1.A1 and anti-MAGE-3.A1 CTL responses of four patients who showed tumor regression after ALVAC-MAGE vaccination, we used in vitro restimulation of PBMC with the antigenic peptide, and Methods.

Figure 1. Procedure for the analysis of lymphocytes labeled by anti-CD8 Abs and A1/MAGE-3 tetramer. The indicated proportions (percent) correspond to CD8 cells that are labeled with the A1/MAGE-3 tetramer and satisfy the selection criteria mentioned above the columns. Results are shown for four independent anti-MAGE-3.A1 MLPC set up with PBMC from patient EB81 (A–C) or LB2196 (D) as described in Materials and Methods. After 14 days of restimulation with the antigenic peptide, cells were incubated with an A1/MAGE-3 tetramer labeled with PE, a control A1/influenza tetramer labeled with allophycocyanin, and anti-CD8 Abs labeled with fluorescein. In column 3, autofluorescence refers to fluorescence at 670 nm. Columns 1–3 show the definition of three gates, indicated with dotted lines, and columns 2–4 show the influences of these gates on the identification of clusters of cells labeled with the A1/MAGE-3 tetramer, indicated with squares. WALS, Wide angle light scatter; FALS, forward angle light scatter.

The logic of the selection of tetramer-labeled cells is shown in Fig. 1. A typical result is shown in Fig. 2. It should be noted that, as a result of the limiting dilution, the tetramer-labeled clusters in the positive microcultures (<10% of the microcultures) each represent a single clone. The tetramer-positive CD8 T cells were nevertheless cloned to eliminate contaminating lymphocytes. The stable CTL clones that were obtained were checked for their ability to lyse targets expressing Ag MAGE-3.A1. Only those microcultures that produced lytic clones were taken into account for the frequency evaluation. The TCR diversity was analyzed by sequencing. With this MLPC/tetramer/cloning approach, we observed a CTL response against Ag MAGE-3.A1 in three of the four patients. No CTL response directed against Ag MAGE-1.A1 was observed.

Regenator patient EB81

This patient had ~70 cutaneous metastases when she received the ALVAC-MAGE vaccine. About one-third of the metastases started to regress during these vaccinations, while the others regressed later during vaccination with the MAGE peptides (Fig. 3). All metastases eventually became undetectable, except for a new enlarged inguinal lymph node that was resected and found to contain tumor cells expressing MAGE-1 and MAGE-3. Subsequently, the other nodes of the same region were removed, but none of them contained melanoma cells. Three years after the onset of vaccination, the patient was still free of detectable melanoma.
named TCR 35. Three CTL expressed another receptor, TCR 103, and three expressed unidentified TCR, but different from TCR 35. As shown in Fig. 4, CTL clones 35 and 103 lysed specifically not only autologous EBV-B cells pulsed with the MAGE-3.A1 peptide but also the autologous melanoma cells, which express MAGE-3.

PCR amplifications specific for the TCR 35 Vα and Vβ rearrangements were applied directly to cDNA obtained from groups of PBMC. The observed frequencies closely matched those found by the MLPC/tetramer approach (Fig. 3). We conclude that the blood frequency of anti-MAGE-3.A1 CTL increased from $10^{-7}$ to $3 \times 10^{-6}$ of the CD8, and clonotypic PCR indicated that all of these CTL expressed TCR 35.

Lymph nodes were analyzed to find out whether they contained anti-MAGE-3.A1 CTL other than those found in blood. In a MLPC/tetramer experiment conducted with cells of the metastatic lymph node resected in April 2000, we found CTL 35 and CTL 103 and no other anti-MAGE-3.A1 CTL. In the surrounding lymph nodes, which did not contain tumor cells, MLPC/tetramer experiments indicated an anti-MAGE-3.A1 CTLp frequency of $2 \times 10^{-6}$ of the CD8, and clonotypic PCR indicated that all of these CTL expressed TCR 35.

We explored whether a set of vaccinations given at short intervals could boost the anti-MAGE-3.A1 CTL response. From February 2001, the patient received five weekly injections of peptide MAGE-3.A1. Anti-MAGE-3.A1 CTLp frequencies were $10^{-6}$ of the CD8 before the boost (Fig. 3, post 6) and $1.7 \times 10^{-6}$ one week after the fifth peptide injection (post 7). Six months later, the patient received 4 weekly injections of ALVAC-MAGE. The anti-
MAGE-3.A1 CTLp frequency increased almost 10-fold to 10^{-5} of the CD8 one week after the fourth injection (post 12). Clonotypic PCR for TCR 35 indicated a frequency of 1.3 \times 10^{-5} in this blood sample, confirming the increased CTL frequency and indicating that it corresponded to CTL 35 and not to a new CTL. New series of peptide and virus injections were administered in 2002. Here again, the blood frequency of CTL 35 did not increase significantly after the injections of peptide, but increased 3-fold after a single injection of ALVAC-MAGE (Fig. 3). It did not increase further after three additional ALVAC injections.

Regressor patient LAU147
This patient had one subcutaneous metastasis at study entry. It regressed completely after the fourth ALVAC-MAGE injection. But the detection of a brain metastasis resulted in patient withdrawal from the study before the injections of peptides. Before

FIGURE 4. Lytic activity of anti-MAGE-3.A1 CTL clones 35 and 103 from patient EB81. 51Cr-labeled targets included autologous melanoma cells EB81-MEL, NK target cells K562, and autologous EBV-transformed B cells incubated with peptides MAGE-1.A1 (EADPTGHSY) or MAGE-3.A1 (EVDPIGHLY) at 2.5 \mu M. For peptide titration (right panels), CTL clones were added at an E/T cell ratio of 10.
vaccination, the blood frequency of anti-MAGE-3.A1 CTLp was 4 × 10⁻⁷ of the CD8 cells (Fig. 5). At the time of the third ALVAC injection, this frequency was 2.5 × 10⁻⁴ of the CD8, and it increased up to 3 × 10⁻³ after the fourth injection. Five months later, it had decreased to 8 × 10⁻⁵ of the CD8. Nine anti-MAGE-3.A1 CTL clones were derived. All expressed the same TCR.

**Regressor patient NAP33**

This patient had three subcutaneous metastases at the onset of vaccination. These tumors progressed and a fourth metastasis appeared. Approximately 6 mo after the first vaccination, all metastases regressed slowly until complete clinical disappearance. Before vaccination, the frequency of anti-MAGE-3.A1 CTLp was 3.8 × 10⁻⁷ of blood CD8 cells and it did not increase after vaccination (Fig. 5). However, TCR analysis of 18 anti-MAGE-3.A1 CTL clones obtained after vaccination indicated that one clonotype, TCR 2, was repeated 6 times, whereas the 11 others were different. We conclude that CTL 2 expanded following vaccination.

Patient NAP33 also received series of weekly booster injections of peptides and ALVAC-MAGE (Fig. 5). The frequency of anti-MAGE-3.A1 CTLp did not increase after these vaccinations.

**Regressor patient CP67**

This patient showed regression of subcutaneous and lymph node metastases after the vaccinations with ALVAC-MAGE. No anti-MAGE-3.A1 or anti-MAGE-1.A1 CTLp could be found among 18 × 10⁶ postvaccination PBMC, indicating frequencies below 3.7 × 10⁻⁷ of CD8 cells.

**Patients without tumor regression**

Twelve of 16 HLA-A1-vaccinated patients who did not display tumor regression were analyzed (Table I). An anti-MAGE-3.A1 CTL response was observed in two patients. In patient NAP36, the pre- and postvaccination CTLp frequencies were <1.4 × 10⁻⁷ and 2.5 × 10⁻⁶ of the CD8, respectively. Six of seven postvaccination CTL clones shared the same TCR, indicating that this CTL expanded after vaccination. For patient VUB39, pre- and postvaccination frequencies were 1.1 × 10⁻⁶ and 9.3 × 10⁻⁶ of the CD8. One CTL clone was already repeated before vaccination, suggesting that this patient had mounted a spontaneous anti-MAGE-3.A1 CTL response.

No evidence for an anti-MAGE-3.A1 CTL response could be found in the other patients. For nine patients, no anti-MAGE-3.A1 T cells could be detected in MLPC/tetramer experiments, resulting in frequency estimations below 3–9 × 10⁻⁷ of the CD8. For patient BB132, one microculture contained tetramer-positive cells, resulting in a frequency of 10⁻⁷ of the CD8.

**Discussion**

Our results demonstrate that vaccination with ALVAC-MAGE induced a CTL response in patients EB81 and LAU147, as a CTL response was observed before the peptide boost. This is the first demonstration that recombinant ALVAC can induce CTL responses against a tumor-specific Ag in cancer patients with a detectable tumor bearing this Ag. We also observed that booster injections of ALVAC-MAGE increased the blood frequency of previously activated CTL clones (see posts 12 and 18 in patient EB81 and post 2 in patient LAU147). Considering that in mice vaccinated with ALVAC encoding the P1A Ag of mastocytoma P815 (our unpublished observations) and in humans receiving the smallpox vaccine (21) the intensity of the T cell responses correlated with the dose of virus, it will be of great interest to examine the efficacy of a higher dose of ALVAC-MAGE.

The CTL responses that we have observed appear stable: when a responding CTL clone was detected, its blood frequency always remained detectable. But the intensities of the observed anti-MAGE-3.A1 CTL responses varied widely from one patient to another. In patients EB81, LAU147, and NAP33, the blood frequencies of the amplified CTL clone were 3 × 10⁻⁴, 3 × 10⁻³, and 10⁻² of the CD8 cells, respectively. It is worth noting that with a total number of ~4 × 10¹⁰ CD8 lymphocytes in a human individual, a frequency of 10⁻⁷ corresponds to a population of 4000 cells or 12 divisions after the activation of a naive precursor. Assuming that antivaccine T cells initiate the tumor regression process observed after peptide or ALVAC-MAGE vaccinations and that the CTL clones that we detected represent all of these antivaccine T cells, our results indicate that the initiation of the tumor regressions did not require a large number of antivaccine T cells.

The CTL responses observed after ALVAC-MAGE vaccination were monoclonal for patients LAU147 and NAP33. In patient EB81, one clone represented at least 95% of the response. Incidentally, the TCR sequences of the CTL found in all of these patients were completely different. Monoclonality has also been observed after vaccination with peptide MAGE-3.A1 without adjuvant (13, 15). It is, of course, impossible to exclude that other anti-MAGE-3.A1 T cells were activated in vivo, but failed to proliferate in vitro under our restimulation conditions. However, it is worth noting that when we applied the same MLPC/tetramer/cloning approach to metastatic melanoma patients receiving mature dendritic cells pulsed with peptide MAGE-3.A1, we observed polyclonal CTL responses (22). Presumably, the capacity of a peptide or ALVAC-MAGE injection to activate specific T cells is very limited in time and space, leading to at best a single hit. Improved vaccination modalities are expected to activate several T cell clones, and this is indeed the case in patients vaccinated with peptide-pulsed dendritic cells. In patients vaccinated with peptide or ALVAC-MAGE, the monoclonality of the response provides the opportunity to track down all of the antivaccine CTL directly in the blood using clonotypic PCR, to analyze their phenotype or function (15), and to assess their presence in regressing and nonregressing tumors.

In this study, we have analyzed four patients who showed tumor regression and we have observed three CTL responses. Assuming that a CTL response nevertheless occurred in the other patient, there are several plausible explanations for our inability to detect it. First, the patient could produce T cells against peptide MAGE-1.A1 or MAGE-3.A1 presented by a HLA molecule other than A1. Such T cells would escape detection by the tetramer method, which is locked on one presenting HLA molecule. In line with this...
Another possibility is that a monoclonal CTL response escaped detection by the MLPC/tetramer approach due to the inability of these T cells to grow under our restimulation conditions. Finally, mice were shown to reject tumors of ~0.5 cm³ with ~20,000 tumor-specific CTL. In humans, with 4 × 10¹⁰ CD8 T cells, this number of CTL corresponds to a blood frequency of 5 × 10⁻⁷ (15). It is therefore possible that a tumor regression process could be initiated by antivaccine CTL present in the blood at a frequency of about or below 10⁻⁷ of the CD8, rendering their detection almost impossible with present methods.

The evolution of the patients of the ALVAC-MAGE vaccination study mentioned in this report will be described in detail elsewhere (N. van Baren, M. Marchand, B. Dreno, T. Dorval, S. Piperno, D. Lienard, B. Escuder, T. Boon, P. Coulie, M.-C. Bonnet, and P. Moingeon, manuscript in preparation). Thirty melanoma patients received at least four ALVAC-MAGE vaccinations and were considered evaluable for tumor response. Of these, 21 were HLA-A1. The other patients expressed HLA-B35, which also presents the MAGE-1.A1 and MAGE-3.A1 peptides (23, 24). Tumor regressions were observed in 5 of the 21 HLA-A1 patients and CTL responses in 3 of the 4 who were tested. For the 16 HLA-A1 regressor patients, CTL responses were observed in 2 of the 12 that were tested. Adding these results to those obtained in peptide vaccination studies (9, 13) and our unpublished results), regressions of melanoma metastases were observed in 8 of 91 patients and anti-MAGE-3.A1 CTL responses were detected in 5 of 10 regressor patients and 2 of 18 progressors. These numbers suggest that there is a correlation between the occurrence of these CTL responses and the tumor regressions, but this needs to be confirmed with larger numbers.

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