A monoclonal cytolytic T-lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene *MAGE-3*

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Vaccination of melanoma patients with tumor-specific antigens recognized by cytolytic T lymphocytes (CTL) produces significant tumor regressions in a minority of patients. These regressions appear to occur in the absence of massive CTL responses. To detect low-level responses, we resorted to antigenic stimulation of blood lymphocyte cultures in limiting dilution conditions, followed by tetramer analysis, cloning of the tetramer-positive cells, and T-cell receptor (TCR) sequence analysis of the CTL clones that showed strict specificity for the tumor antigen. A monoclonal CTL response against a MAGE-3 antigen was observed in a melanoma patient, who showed partial rejection of a large metastasis after treatment with a vaccine containing only the tumor-specific antigenic peptide. Tetramer analysis after *in vitro* restimulation indicated that about 1/40,000 postimmunization $CD8^+$ blood lymphocytes were directed against the antigen. The same TCR was present in all of the positive microcultures. TCR evaluation carried out directly on blood lymphocytes by PCR amplification led to a similar frequency estimate after immunization, whereas the TCR was not found among 2.5×10^6 $CD8^+$ lymphocytes collected before immunization. Our results prove unambiguously that vaccines containing only a tumor-specific antigenic peptide can elicit a CTL response. Even though they provide no information about the effector mechanisms responsible for the observed reduction in tumor mass in this patient, they would suggest that low-level CTL responses can initiate tumor rejection.

The identification of tumor-specific antigens recognized on human tumors by autologous cytolytic T cells has led to the use of defined antigens for the therapeutic vaccination of cancer patients (1, 2). A number of clinical trials have involved antigens encoded by genes of the *MAGE* family, notably *MAGE-3* (3). Many tumors of various histological types express this gene as a result of an overall demethylation process that occurs during tumor progression and causes the expression of the *MAGE* genes (4, 5). For instance, *MAGE-3* is expressed in 76% of metastatic melanoma patients (6). These genes are not expressed at all in normal cells, with the exception of the male germline cells, which do not express HLA molecules on their surface. The antigens encoded by these tumor-germline genes therefore provide strictly tumor-specific targets for the T cells of cancer patients.

Several clinical trials, involving vaccination with MAGE peptides, have been applied to metastatic melanoma patients. In a trial involving three monthly vaccinations with a MAGE-3 peptide presented by HLA-A1 administered in the absence of any adjuvant, we observed that 7 of the 25 patients who completed the trial showed significant tumor regressions, of which three were complete (7, 8). Two of these patients, who had regional disease, have remained disease-free for over 3 years. Several of the regressions observed started only several months after the first injection and took several additional months to become complete. The rejection of cutaneous nodules occurred in the absence of strong inflammation. Other trials involving vaccination with MAGE antigenic peptides have confirmed the occurrence of tumor regressions in a minority of patients (unpublished observations). Advanced melanoma patients have also been immunized with dendritic cells pulsed with MAGE peptides. In a first study, a partial regression was reported for 1 of 4 vaccinated patients (9). In a second study involving patients with very advanced melanoma, all had overall progression, but 6 patients of 11 showed regression of some metastases (10).

Even though the frequency of regressions observed after MAGE vaccination appears to be well above the frequency of the spontaneous regressions that have been observed in metastatic melanoma patients (11), the possibility of obtaining antitumoral responses by vaccinating with peptides alone has met with justifiable skepticism, because it has been generally observed that peptides delivered without adjuvants do not produce cytolytic T lymphocyte (CTL) responses in mice and can even tolerize in some circumstances (12, 13). To consolidate the interpretation of the clinical results and to improve the efficacy of the antitumoral vaccinations, it is essential to evaluate precisely the anti-MAGE CTL responses elicited by the vaccinations and determine to what extent these CTL responses correlate with the clinical responses. This analysis is difficult, because these CTL responses do not appear to be massive. In one peptide vaccination trial, two patients who showed a complete clinical response were evaluated for their anti-MAGE-3.A1 CTL precursor frequency in the blood. In postimmunization lymphocytes, no evidence was found for a frequency above 3×10^{-7} of $CD8^+$, the level observed in a number of noncancerous individuals (8, 14). For a trial involving dendritic cells, it was reported that some patients showed postimmunization frequencies of the order of 10^{-4} among $CD8^+$ cells, but there was no clear correlation with clinical responses (10).

The evaluation of low-level anti-MAGE CTL responses is subject to many constraints. Direct measurement of frequency in blood lymphocytes by tetramer analysis is not sufficiently sensitive. Tests carried out with cells that are pulsed with the peptide used for vaccination may lead to false positives because of CTL that are directed against impurities present in the vaccine preparation. Restimulation *in vitro* under limiting dilution can be applied, but it is difficult to assess the exact specificity of the

Abbreviations: TCR, T-cell receptor; CTL, cytolytic T lymphocyte; CTLp, CTL precursors; APC, allophycocyanin; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; EBV, Epstein–Barr virus.

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effectors present in the microcultures, unless these are cloned and tested for their lytic activity on *MAGE*-expressing target cells. We have examined at the clonal level the CTL response of a melanoma patient vaccinated with a MAGE-3 peptide presented by HLA-A1. We observed a 100-fold expansion of a single CTL clonal population.

Methods

Tetramer Production and Flow Cytometry. Recombinant HLA-A1 molecules were folded *in vitro* with β2-microglobulin and peptides EVDPIGHLY (from MAGE-3) or VSDGGPNLY (from the influenza basic polymerase 1) purified by gel filtration, biotinylated, and mixed with extravidin-phycoerythrin (PE) (Sigma) or streptavidin–allophycocyanin (APC) (Molecular Probes), as described (15).

For surface antigen staining, thawed peripheral blood mononuclear cells (PBMC) or lymphocytes collected after *in vitro* restimulation were washed, resuspended in PBS with 1% human serum, and incubated for 15 min at room temperature with HLA-A1 tetramers loaded with MAGE-3.A1 or influenza peptides and coupled to PE and APC, respectively. Anti-CD3 antibodies coupled to FITC (SK7, Becton Dickinson) and anti-CD8 antibodies coupled to peridinin chlorophyll protein (SK1, Becton Dickinson) were then added and after a further incubation for 15 min at 4°C, the cells were washed, fixed, and analyzed. For the experiment shown on Fig. 2, PBMC were incubated with anti-CD45RA antibodies coupled to PE (HI100, BD PharMingen) and anti-CD45RO antibodies coupled to FITC (UCHL1, BD PharMingen), or with an anti-CCR7 IgM antibody (2H4, Becton Dickinson) followed by biotinylated anti-IgM antibodies and streptavidin–PE. Sorted cells were divided in groups, and clonotypic PCR for TCR 48 α and β chains were applied. The number of groups that were positive for both PCR were 21/29 for $CD45RA^{dim/-}$, 5/10 for $CD45RA^{+}$, 13/20 for $CCR7^{-}$, and 0/6 for $CCR7^{+}$.

***In Vitro* Restimulation of PBMC.** PBMC were thawed, incubated for 60 min at room temperature at 10^7 cells/ml in Iscove's medium with 1% human serum (HS) and 20 μM of MAGE-3.A1 peptide, washed, and distributed at 3×10^6 cells/2 ml, 5×10^5 cells/1 ml, or 1.6×10^5 cells/0.2 ml in Iscove's with 10% HS, arginine, asparagine, and glutamine (16), IL-2 (20 units/ml), IL-4 (10 ng/ml), and IL-7 (10 ng/ml). On day 7, the lymphocytes were incubated with peptide as on day 0, washed, and resuspended in fresh culture medium. For the cultures in microwells, 50% of the medium was replaced by fresh medium containing peptide.

T Cell Clones. Cells stained by tetramer were seeded at one cell/well by using flow cytometry and stimulated by the addition of irradiated (100 Gray) allogeneic PBMC (8×10^4/well) as feeder cells, irradiated allogeneic HLA-A1 Epstein–Barr virus (EBV)-B cells (2×10^4/well) incubated with the MAGE-3.A1 peptide (20 μM), and washed in medium with IL-2 (100 units/ml) and IL-4 (10 ng/ml). The CTL clones were restimulated each week by the addition of feeder cells and peptide-pulsed EBV-B cells.

TCR Analysis. Total RNA from PBMC or tumor material was extracted with the TriPure reagent (Roche Diagnostics), or by the guanidine-isothiocyanate/cesium chloride procedure, and converted to cDNA with M-murine leukemia virus reverse transcriptase (Life Technologies, Merelbeke, Belgium). TCR Vα and Vβ usage was assessed by PCR amplification by using panels of Vα- or Vβ-specific primers (ref. 17 and http://imgt.cines.fr). The rearranged TCR sequences of CTL clone 48 were as follows: Vα12–1/Jα16 (5′-tgtgtggtgaGGGGTGTCGGAtcagatggccagaagctg-3′) and Vβ27/Jβ1–2 (5′-tgtgccagcagtttaGTACCCCTCCCctatggctacaccttcggt-3′), with nucleotides that are not templated by V or J genes in capital letters, and V and J sequences in lowercase. Clonotypic PCR amplifications used seminested pairs of primers consisting of one upstream V primer and two downstream primers, one straddling the JC junction, and one with its 3′-end matching N nucleotides of the CDR3 region. A 10^{-5} or 10^{-6} fraction of the amplified product of the first PCR was used as template for the second round of amplification.

Immunochemistry on Tumor Cells. For detection of the CD8 cells, frozen sections (7 μm) were fixed in acetone and incubated with the anti-CD8β rat monoclonal antibody 1A3.3 (D. Olive, INSERM U119, Marseille), with biotinylated goat anti-rat Ig, and with streptavidin conjugated to peroxidase. For the detection of HLA class I molecules, frozen sections were fixed in formalin and incubated with antibodies W6/32 (anti-class I) or BM-63 (anti-β2-microglobulin), which were visualized by using the mouse ENVISION+ System Peroxidase kit (Dako).

Results

Patient CP64 underwent in September 1998 the surgical excision of a cutaneous melanoma on the left ear. In March 1999, a suspect cervical lymph node was removed and found to contain melanoma cells expressing *MAGE-3*. The patient received IFN-α as adjuvant treatment but soon presented new regional metastases that were removed. In September 1999, a cutaneous tumor 10 cm in diameter was present in the left cervical region. Because the patient was typed HLA-A1, vaccination was initiated with 300 μg of the MAGE-3.A1 peptide, injected s.c. and intradermally at sites distant from the tumor. Between September 1999 and February 2000, the patient received 15 injections of the peptide (Fig. 1*A*). The size of the cervical tumor increased until November, and then clearly decreased to reach a volume reduction of about 3-fold by the end of February. However, two small cutaneous metastases located nearby appeared in October and failed to regress. Visceral metastases appeared in November and progressed steadily. Because of this, the injections were stopped in February. The patient died from tumor progression in July 2000. PBMC were prepared from blood collected before vaccination in September 1999 (Pre) and after vaccination in January (Post I) and February 2000 (Post II) and frozen.

Detection of Anti-MAGE-3.A1 CTL in Postimmunization Lymphocytes After *in Vitro* Restimulation. We set out to detect anti-MAGE-3.A1 CTL in postimmunization PBMC by using multimeric HLA-A1 peptide complexes (15). For this purpose, the HLA-A1 molecule was produced in *Escherichia coli*, after the introduction of several modifications to the HLA-A1 nucleotide sequence. The soluble recombinant HLA-A1 molecules were folded with the MAGE-3 peptide, biotinylated, and multimerized with avidin conjugated to phycoerythrin. One million postimmunization PBMC (Post II) were incubated with the A1/MAGE-3 tetramer and with anti-CD8 and anti-CD3 antibodies. They were then analyzed by flow cytometry. Among the $CD3^{+}CD8^{+}$ cells, no cluster of cells that were stained with the tetramer could be distinguished from the bulk of the negative cells. We concluded that less than 0.05% of the $CD8^{+}$ cells were tetramer-positive as, under our conditions, the control anti-MAGE-3.A1 CTL clone formed a distinguishable cluster when diluted in PBMC at this level.

We then examined whether anti-MAGE-3.A1 $CD8^{+}$ T cells could be identified among postimmunization lymphocytes after *in vitro* restimulation with the antigen. Six groups of 3.5×10^6 PBMC (Post II) were incubated with the MAGE-3.A1 peptide, washed, and cultured in the presence of IL-2, -4, and -7. On day 7, it was still impossible to identify cells labeled by the A1/MAGE-3 tetramer in these cultures (Fig. 1*B*). On day 13, after a second stimulation with the antigen under the same conditions, cells labeled with the A1/MAGE-3 tetramer were clearly iden-

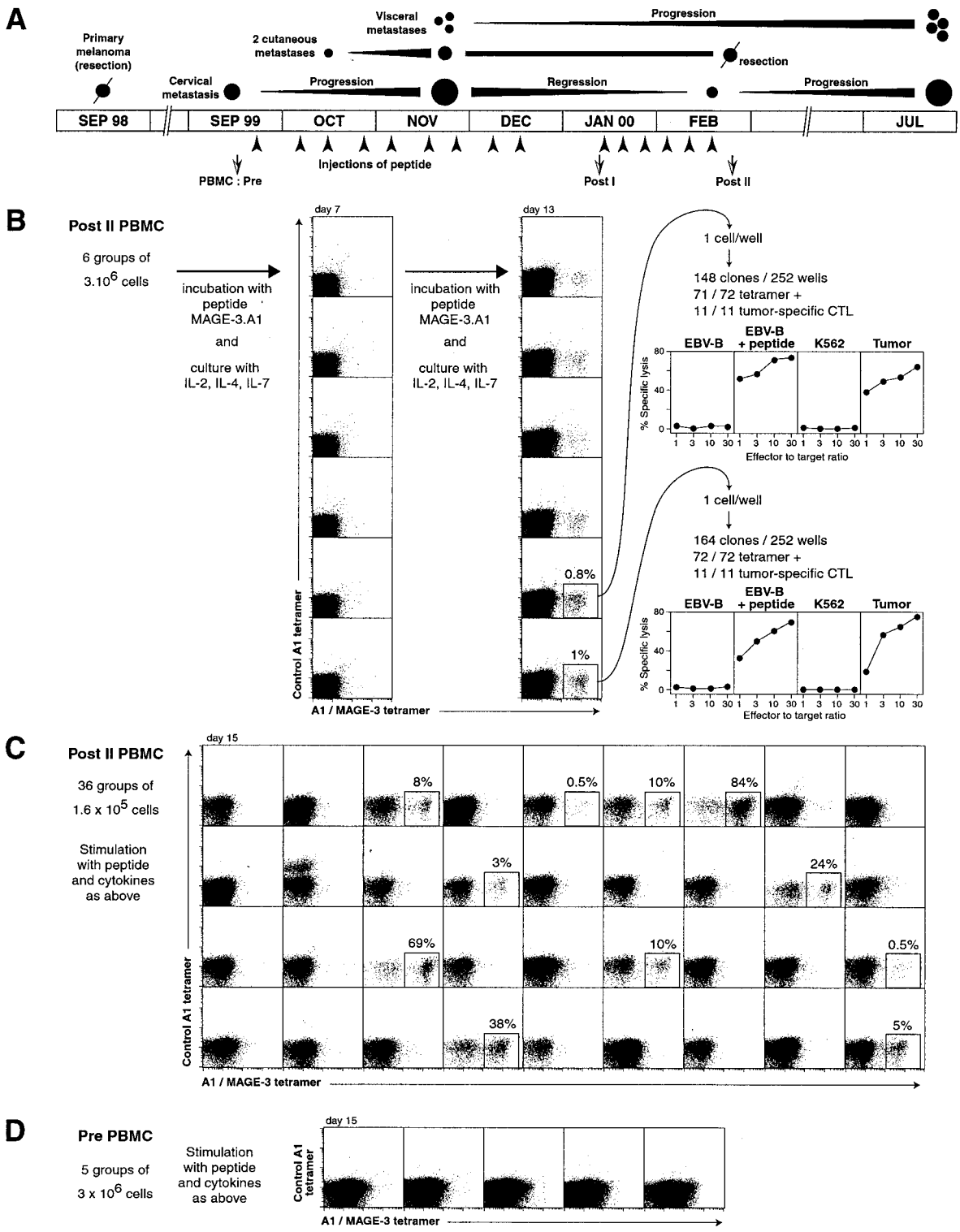

Fig. 1. Detection of anti-MAGE-3.A1 CTLp. (*A*) Clinical evolution of patient CP64. (*B*) Detection of anti-MAGE-3.A1 CTL in postimmunization PBMC. PBMC were thawed, incubated with peptide MAGE-3.A1, washed, and distributed at 3.10^6 cells/well in the presence of IL-2, -4, and -7. On day 7, the cultures were restimulated with peptide and cytokines. On days 7 and 13, the cells were incubated with the A1/MAGE-3 tetramer coupled to PE and with a control HLA-A1 tetramer containing an influenza peptide and coupled to APC. Anti-CD3 antibodies coupled to FITC and anti-CD8 antibodies coupled to peridinin chlorophyll protein were then added. Cells were washed, fixed, and analyzed by flow cytometry. One million events were acquired, but the plots include only the $CD3^+CD8^+$ lymphocytes. The lytic activity of CTL clones, derived from two populations of cells labeled with tetramer, was tested on HLA-A1 EBV-B cells incubated or not with the MAGE-3.A1 peptide (2 μM), natural killer target cells K562, and allogenic melanoma cells MZ2-MEL, which naturally express the MAGE-3.A1 antigen. The pattern of lysis of two representative clones is shown. (*C*) Estimation of the frequency of anti-MAGE-3.A1 CTLp in postimmunization PBMC. Thirty-six cultures were set up with 160,000 Post II PBMC stimulated with the MAGE-3.A1 peptide and IL-2, -4, and -7. The lymphocytes were restimulated on day 7 by the addition of peptide and cytokines and labeled on day 15 with tetramers and anti-CD3 and anti-CD8 antibodies. Only $CD3^+CD8^+$ lymphocytes are included in the plots. Clusters of lymphocytes specifically labeled with the A1/MAGE-3 tetramer are boxed, and their proportion among the $CD3^+CD8^+$ cells is indicated. (*D*) Analysis of prevaccination PBMC. Five cultures of 3 million PBMC were set up and analyzed as in *C*.

Table 1. Frequencies of anti-MAGE-3.A1 CTL 48

PBMCs	Proportion of $CD8^+$	Detection with tetramers after *in vitro* restimulation of groups of PBMCs: Number of PBMCs/group	Positive groups/ tested groups	Frequency (among $CD8^+$)	Detection of TCR 48 by PCR in groups of PBMCs: Number of PBMCs/group	Positive groups/ tested groups	Frequency (among $CD8^+$)
Preimmunization	5%	3×10^6	0/5	$<1.3 \times 10^{-6}$	10^7	0/5	$<4 \times 10^{-7}$
Postimmunization I	6%	5×10^5	7/16	1.9×10^{-5}	7.5×10^4	4/20	5×10^{-5}
Postimmunization II	9%	5×10^5	9/10				
		1.6×10^5	11/36	2.5×10^{-5}	10^5	11/30	5×10^{-5}

tifiable in the $CD3^+CD8^+$ fraction of each of the six cultures (Fig. 1*B*). The frequency of the putative anti-MAGE-3.A1 T cells in the $CD8^+$ populations ranged from 0.2 to 1%.

To assess the specificity of the tetramer staining, the positive cells from two populations of responding cells (groups 5 and 6) were sorted and seeded at one cell per well. They were restimulated with irradiated HLA-A1 EBV-transformed B cells that had been incubated with the MAGE-3 antigenic peptide, in the presence of irradiated allogeneic PBMC as feeder cells, IL-2 and -4. After 2 weeks, proliferation was observed in about 60% of the wells. Almost all these clones were stained by the A1/MAGE-3 tetramer but not by the A1 tetramer containing the influenza peptide (data not shown). The clones lysed HLA-A1 EBV-B cells incubated with the MAGE-3.A1 peptide but did not lyse these cells in the absence of peptide (Fig. 1*B Right*). They did not lyse HLA-A1 EBV-B cells incubated with peptide EADPTGHSY, which is encoded by gene *MAGE-1*, presented by HLA-A1, and shares six amino acids with the MAGE-3.A1 peptide (EVDPIGHLY) (data not shown). Finally, the CTL clones lysed melanoma cells that expressed the *HLA-A1* and *MAGE-3* genes, excluding the possibility that they recognized contaminants present in the MAGE-3.A1 peptide preparations. We concluded that the Post II blood sample contained precursors of CTL (CTLp) directed against the MAGE-3.A1 antigen.

Frequency of Anti-MAGE-3.A1 CTL Cells in Pre- and Postimmunization Lymphocytes. To estimate the frequency of anti-MAGE-3.A1 CTLp among Post II lymphocytes, groups of 160,000 PBMC containing 14,000 $CD8^+$ T cells were stimulated with the antigenic peptide as described above. Fifteen days later, 11 of the 36 groups contained tetramer-stained cells, with frequencies ranging from 0.5 to 84% of the $CD3^+CD8^+$ lymphocytes (Fig. 1*C*), indicating a frequency of anti-MAGE-3.A1 CTLp of 2.5×10^{-5} among the $CD8^+$ T cells in Post II blood (Table 1).

We carried out a similar experiment with Post I lymphocytes. Of 16 groups of 5.10^5 PBMC, 7 showed a cluster of lymphocytes stained by the A1/MAGE-3 tetramer after 2 weeks of *in vitro* stimulation, corresponding to a frequency of anti-MAGE-3.A1 CTL of about 1.9×10^{-5} of CD8 (Table 1). Considering that the Post I lymphocytes were collected 24 days after the last boost, whereas the Post II lymphocytes were collected 8 days after the last boost, this result suggested that the anti-MAGE-3.A1 CTLp frequency does not vary considerably in function of the number of days elapsed after the last immunization.

Five groups of 3×10^6 preimmunization PBMC were stimulated on day 0 and on day 7 with peptide and growth factors as above. On day 14, the proliferation of these lymphocytes was similar to that observed with the Post I or II PBMC, namely an overall amplification of 2- to 7-fold. However, no cluster of cells stained with the tetramer could be detected (Fig. 1*D*), suggesting that the frequency of anti-MAGE-3 CTLp among the $CD8^+$ cells was lower than 10^{-6} (Table 1). It is of course impossible to exclude that preimmunization anti-MAGE-3 CTLp were present at a higher level but did not proliferate *in vitro* under our conditions.

The Postimmunization Anti-MAGE-3 CTL Population Is Monoclonal. We analyzed the TCR Vβ gene expression of the two sets of 11 CTL clones, derived from the two Post II PBMC groups shown in Fig. 1*B*. cDNA was prepared and used as template for PCR amplifications with a panel of 30 sense primers, each specific for one Vβ sequence, and an antisense primer located in the Cβ region. All of the clones expressed a Vβ27 gene product. We sequenced the PCR product from four CTL clones of each group. All sequences proved identical, corresponding to a Vβ27-Jβ1.2 rearrangement. A similar analysis performed for the TCRα chain indicated that all clones shared the same functional Vα12-Jα16 rearranged product. The TCR of these anti-MAGE-3.A1 clones was named TCR 48.

We set up "clonotypic" PCR amplifications specific for the Vα and Vβ rearrangements of TCR 48. These PCR amplifications were sensitive enough to detect one CTL expressing TCR 48 mixed with 3×10^7 PBMC of a normal donor, and they were highly specific for TCR 48 insofar as no amplified product was obtained with cDNA prepared from groups of 3×10^7 PBMC from 5 different donors.

The 36 cultures obtained at day 15 by stimulating groups of 160,000 Post II PBMC (Fig. 1*C*) were tested with the TCR 48 β clonotypic PCR. All of the 11 cultures that contained T cells labeled with the A1/MAGE-3 tetramer scored positive, and none of the others did (Fig. 1*C*). The TCR 48 α clonotypic PCR was positive for the same groups. We concluded that the anti-MAGE-3.A1 CTL response detected by our *in vitro* approach was monoclonal.

Direct Evaluation of the Frequency of TCR 48 Among Blood Lymphocytes by PCR. To analyze directly the frequency of blood T cells expressing TCR 48 after immunization, 30 groups of 10^5 Post II PBMC were tested with the TCR 48 β clonotypic PCR. Twelve were positive, and eleven of these were also positive for the α chain PCR. Considering that $CD8^+$ cells amounted to 9% of the PBMC, these results indicated a frequency of T cells expressing TCR 48 of 5×10^{-5} among $CD8^+$ cells in Post II blood, about 2-fold higher than the frequency measured by tetramer-staining after *in vitro* restimulation (Table 1).

For the Post I PBMC, 20 groups of 75,000 cells were tested. Five groups were positive for the β chain PCR. Four of these groups, and only those, were also positive for the amplification of the α sequence. These results indicated a frequency of T cells expressing TCR 48 of 5×10^{-5} among $CD8^+$ cells, identical to that found in Post II blood (Table 1). This frequency was also about 2-fold higher than that observed with tetramer after *in vitro* restimulation, suggesting that for the anti-MAGE-3.A1 CTL clone bearing TCR 48, the efficiency of our restimulation conditions is $\approx$0.5.

For the preimmunization PBMC, RNA was extracted from 5 groups of 10^7 cells, and 3/4 of this material was converted to cDNA. It served as template for PCR amplifications of the TCR 48 α and β sequences. All five groups were negative. To verify that these results were not caused by an inhibitor of the TCR 48 PCR amplifications present in the cDNA preparations, the

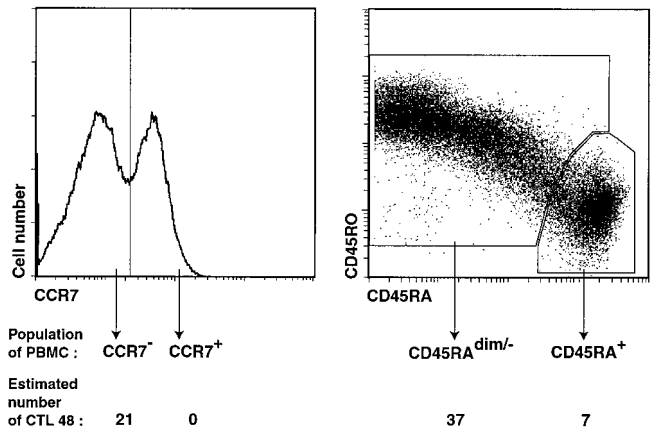

Fig. 2. Phenotype of PBMC expressing TCR 48. Postimmunization (Post II) PBMC were thawed and labeled either with anti-CD45RO antibodies coupled to FITC and anti-CD45RA antibodies coupled to PE, or with an anti-CCR7 IgM antibody followed by biotinylated anti-IgM antibodies and streptavidin coupled to PE. The cells were immediately sorted by flow cytometry. Sorted populations were divided in groups and cDNA obtained from each group was tested for the TCRα and β clonotypic PCR. Numbers of CTL 48 were calculated with the Poisson distribution.

remaining 1/4 of the RNA was mixed with an amount of RNA from postimmunization PBMC calculated to contain 1/4 of the RNA of one TCR 48 CTL. The five reconstituted control groups were positive for both the TCR 48 α and β PCR reactions. The frequency of cells expressing TCR 48 was therefore lower than 4×10^{-7} among the CD8 T cells from blood collected before vaccination with the MAGE-3.A1 peptide (Table 1). We concluded that the vaccination induced at least a 100-fold amplification of anti-MAGE-3.A1 CTL clone 48.

Phenotype of CTL Clone 48 in the Blood. When blood $CD8^+$ T lymphocytes are stained with antibodies against chemokine receptor CCR7 and against CD45RA, four populations of cells can be identified. Even though the correlation between phenotype and function of these populations is not entirely clear, several analyses of $CD8^+$ T cells suggest the following (18–20). $CD45RA^+$ $CCR7^+$ cells are naïve lymphocytes. $CD45RA^{dim/-}$ $CCR7^+$ are "central memory" lymphocytes that have contacted their antigen, contribute in maintaining a pool of memory cells and home to lymph nodes. $CD45RA^{dim/-}$ $CCR7^-$ are "effector memory" cells that home predominantly to peripheral tissues and may have some lytic ability. Finally, $CD45RA^+$ $CCR7^-$ cells are terminally differentiated highly lytic effectors.

To analyze directly the presence of CD45RA and CCR7 on CTL 48 present in postimmunization blood, we used the clonotypic PCR to locate CTL 48 among PBMC that had been thawed, immediately labeled with the relevant antibodies, and separated by fluorescence activated cell sorting (Fig. 2). The sorted populations were divided into groups that were sized to permit the evaluation of the number of CTL 48 that were present. Among the Post II PBMC, CTL 48 cells were exclusively found in the $CCR7^-$ population, indicating that none corresponded to the naïve or central memory types. Sorting on the basis of the CD45RA marker indicated that 84% (37/44) of the CTL 48 cells present in the blood corresponded to the $CD45RA^{dim/-}$ $CCR7^-$ "effector memory" type and that 16% (7/44) corresponded to the $CD45RA^+$ $CCR7^-$ terminally differentiated highly lytic type.

Analysis of a Nonregressing Metastasis. At the time when the main metastasis of patient CP64 was regressing, two smaller metastases located in the vicinity failed to show any sign of regression (Fig. 1*A*). They were excised, and one of them was examined for the presence of CTL 48. Sections that were 7-μm thick and $\approx$1 cm in diameter were divided in 12 regions, the mRNA was extracted, and the clonotypic reverse transcription–PCR amplification of TCR 48 was applied. An average of 3/12 positive areas was obtained. The analysis of immediately adjacent sections showed that there was no correlation between the location of the positive areas in different sections, suggesting that the positives represented single cells. By using CD8β immunostaining on adjacent sections to evaluate the number of $CD8^+$ T cells, we obtained a frequency of $\approx$1/5,000 CD8 for CTL 48 in the sections. This number may represent a 4-fold enrichment relative to blood frequency, but in our view this modest enrichment cannot be considered significant.

The melanoma cells of this metastasis were stained by the W6/32 conformational antibody, indicating that they expressed functional MHC class I molecules. Analysis with reverse transcription–PCR (RT-PCR) of small groups of tumor cells excised from sections with laser microdissection indicated expression of the *HLA-A1* gene. RT-PCR amplifications on mRNA extracted from whole sections indicated that the tumor cells expressed gene *MAGE-3*. Taken together, these results suggest that the absence of immune attack on this metastasis is not because of a lack of expression of the MAGE-3.A1 antigen by the tumor cells.

Discussion

We have observed a more than 100-fold increase in the frequency of an anti-MAGE-3.A1 CTL after 9 vaccinations of a melanoma patient with the antigenic peptide. This finding provides unambiguous evidence that vaccination with a strictly tumor-specific antigenic peptide, even without adjuvant, can produce a CTL response in cancer patients with detectable disease. But it remains to be seen whether vaccination with peptide alone induces a CTL response against tumor antigens regularly or only rarely.

The level of the CTL response observed in patient CP 64, who rejected a large volume of tumor, was only 5×10^{-5} of the $CD8^+$ T cells. Is it plausible that tumor regressions could be initiated by such low number of CTL? We believe that such responses might be effective on the basis of the following considerations. Mice have been observed to completely reject fast-growing tumors of about 0.5 cm^3 in less than 1 week, and these mice appear to display in their blood a level of antitumoral CTLp that is $\approx 10^{-3}$ of $CD8^+$ T cells (21). Considering that a mouse has a total of about 2×10^7 $CD8^+$ T lymphocytes in the blood, lymph nodes, and spleen, and assuming a uniform distribution of the antitumoral CTLp in these locations, a frequency of 10^{-3} in the blood corresponds to a total of 2×10^4 antitumoral $CD8^+$ T cells in the body. On the same basis, humans, who have a total of about 10^{11} $CD8^+$ T lymphocytes, have a total of 5×10^6 anti-MAGE-3.A1 $CD8^+$ when their blood frequency is equal to 5×10^{-5}. If these lymphocytes home to the tumor and attack it as efficiently as observed in the mouse, 5×10^6 $CD8^+$ T cells could bring about the elimination of a large volume of tumor, particularly in view of the fact that human tumors progress much more slowly than experimental mouse tumors. It is therefore conceivable that melanoma patients who eliminate small cutaneous metastases over a period of more than 1 month may do so with $CD8^+$ T cells that are one or two orders of magnitude lower than those observed here. This low frequency would be in line with our inability to detect in other patients who have shown clinical responses, frequencies of anti-MAGE CTL that are significantly above the level observed in normal individuals. A recent analysis of another clinically responding patient indicated an anti-MAGE-3.A1 CTL response at a level of 5×10^{-6} of $CD8^+$ (V.K., D.C., C.L., N.V.B., M.D., T.B., and P.C., unpublished work). The ability of a small number of CTL to cause the destruction of a rather large number of tumor cells may critically

depend on the involvement of other, possibly nonspecific, effector cells, once the lysis of some tumor cells has been effectuated by the CTL.

The anti-MAGE-3.A1 response observed with patient CP64 appears to be monoclonal. It is, of course, impossible to exclude that other CTL responded to the antigen *in vivo* but failed to proliferate *in vitro* under our restimulation conditions. Nevertheless, the results described here strongly suggest that CTL responses obtained after immunization with *MAGE* antigens may be monoclonal or oligoclonal, as were higher-level responses observed against other strictly tumor-specific antigens (16, 22), or against viral epitopes (23–26). This finding is in sharp contrast with the CTL directed against the Melan-A differentiation antigen that are found in many melanoma patients. There, a very large diversity of TCR was found in the blood (ref. 27 and unpublished observations). However the Melan-A situation is very different from the MAGE situation, as high frequencies of anti-Melan-A CTLp have been reported in patients who have not been vaccinated and even in normal individuals (28, 29).

At present, antitumoral vaccination with defined antigens fails to produce tumor regressions in most patients. Hopefully, the approach described here will contribute to assessing whether the predominant cause of failure is tumor resistance to immune attack or whether it is the lack of an adequate T-cell response. The occurrence of monoclonal or oligoclonal CTL responses ought to facilitate their detection, even when they are low, because of the possibility of tracing rare CTLp in the blood by clonotypic PCR. The induction of circulating CTL is clearly not sufficient for tumor regression. A second critical step must be the penetration and activation of the CTL into individual metastases, a process that should also be detectable by PCR. We feel that this process is very poorly understood. For instance, why were the small cutaneous metastases of patient CP64 not eliminated by the lymphocytes that attacked the main tumor, even though they seemed to present the MAGE antigen? Similar observations were made with several patients who showed tumor regression. Finally, the CTL response must be sustained until tumor rejection is complete. In patient CP64, the regression of the main tumor halted when vaccinations had to be discontinued because of progression of the visceral metastases. Does this imply that vaccination must be pursued to keep the CTL in an activated state? Possibly, our ability to understand this complex situation will be helped by the analysis of the functional markers of the CTL that we can trace by clonotypic PCR.

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