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# Monitoring of Anti-Vaccine CD4 T Cell Frequencies in Melanoma Patients Vaccinated with a MAGE-3 Protein<sup>1</sup>

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Quantitative evaluation of T cell responses of patients receiving antitumoral vaccination with a protein is difficult because of the large number of possible HLA-peptide combinations that could be targeted by the response. To evaluate the responses of patients vaccinated with protein MAGE-3, we have developed an approach that involves overnight stimulation of blood T cells with autologous dendritic cells loaded with the protein, sorting by flow cytometry of the T cells that produce IFN- $\gamma$ , cloning of these cells, and evaluation of the number of T cell clones that secrete IFN- $\gamma$  upon stimulation with the Ag. An important criterion is that T cell clones must recognize not only stimulator cells loaded with the protein, but also stimulator cells transduced with the *MAGE-3* gene, so as to exclude the T cells that recognize contaminants generated by the protein production system. Using this approach it is possible to measure T cell frequencies as low as  $10^{-6}$ . We analyzed the frequencies of anti-vaccine CD4 T cells in five metastatic melanoma patients who had been injected with a MAGE-3 protein without adjuvant and showed evidence of tumor regression. Anti-MAGE-3 CD4 T cells were detected in one of the five patients. The frequency of the anti-MAGE-3 CD4 T cells was estimated at 1/60,000 of the CD4 T cells in postvaccination blood samples, representing at least an 80-fold increase in the frequency found before immunization. The frequencies of one anti-MAGE-3 CD4 T cell clonotype were confirmed by PCR analysis on blood lymphocytes. The 13 anti-MAGE-3 clones, which corresponded to five different TCR clonotypes, recognized the same peptide presented by HLA-DR1. *The Journal of Immunology*, 2005, 174: 2404–2411.

**G**ene *MAGE-A3*, hereafter called *MAGE-3*, encodes several antigenic peptides that bind to HLA class I or class II molecules and are recognized by T lymphocytes on tumor cells (1). *MAGE-3* is expressed in 76% of metastatic melanoma and in many other tumors of various histological types (2, 3). Because Ags encoded by *MAGE-3* are not expressed in normal tissues, they should represent safe targets for cancer immunotherapy. These Ags have therefore been used for small-scale therapeutic vaccination trials of cancer patients.

Vaccines designed to stimulate CD8 T lymphocytes against a MAGE-3 Ag consisted of a MAGE-3 peptide presented by HLA-A1, a pox family recombinant virus (ALVAC) carrying a *MAGE-3* sequence coding for the peptide, and dendritic cells pulsed with the peptide (N. van Baren, manuscript in preparation) (4–6). Evidence of tumor regression was observed in

~20% of the patients, but clinical benefit was limited to about half these patients. To understand why most patients failed to show any tumor regression, we analyzed the CTL responses of vaccinated patients using a sensitive approach based on in vitro restimulation of blood lymphocytes with the antigenic peptide over 2 wk, followed by labeling with HLA/peptide tetramers. To evaluate precursor frequencies, these mixed lymphocyte-peptide cultures were conducted under limiting dilution conditions. Cells that were labeled with the tetramer were cloned, the lytic specificity of the clones was verified, and their diversity was analyzed by TCR sequencing (7–10). In individuals not suffering from cancer, the frequency of the T cell precursors against Ag MAGE-3.A1 is  $\sim 4 \times 10^{-7}$  of CD8 T cells (11). The intensity of anti-vaccine MAGE-3.A1 responses in patients showing evidence of tumor regression varied widely, with frequencies ranging from  $10^{-6}$  to  $10^{-3}$  among blood CD8 cells, often not higher than  $10^{-5}$ , and sometimes undetectable (8, 9). We observed a certain correlation between tumor regression and anti-MAGE-3.A1 CTL responses in patients vaccinated with the ALVAC virus encoding the MAGE-3.A1 Ag; a CTL response was found in three of four patients who showed tumor regressions and in one of 11 patients who did not (N. van Baren, manuscript in preparation) (11).

Immunizing patients with a MAGE-3 recombinant protein should induce T cell responses against several MAGE-3 peptides, including peptides recognized by CD4 T cells, and this might result in a more effective antitumor response. Moreover, protein vaccination alleviates the need to select patients according to their HLA, because many peptides presented by various HLA alleles are expected to be presented. In a first study, patients were vaccinated with a recombinant MAGE-3 protein mixed with adjuvant ASO2B

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(12). The recombinant MAGE-3 protein was a fusion protein comprising the full-length MAGE-3 sequence fused with part of a bacterial protein, namely, protein D derived from *Haemophilus influenzae*. Among 33 metastatic melanoma patients, we observed two objective responses, two mixed responses, and one stabilization that lasted for >1 year. Interestingly, one of the two objective responses occurred in a patient vaccinated without the adjuvant, suggesting that the recombinant MAGE-3 protein alone is immunogenic, which might be due to immunological help from its protein D portion (12). This led us to perform another study in which protein D (ProtD)<sup>3</sup>-MAGE-3 was injected without adjuvant. One partial response and four mixed responses were observed among the 26 melanoma patients vaccinated (W. H. J. Kruit and M. Marchand, manuscript in preparation).

Monitoring the anti-vaccine T cell frequencies in patients vaccinated with a protein is complex. The responses could be directed against a large number of HLA-peptide combinations, including many that are presently unknown. Therefore, the approach that combined mixed lymphocyte-peptide cultures and analysis by tetramer and was used for the detection of CD8 responses is not appropriate. In a previous work we had stimulated CD4 T cells with autologous dendritic cells loaded with a MAGE protein, and the responder cells of each microculture were tested for their ability to secrete IFN- $\gamma$  upon stimulation with the MAGE protein. This approach was useful for identification of new MAGE antigenic peptides presented by different HLA class II molecules (13–16), but it was not suitable to estimate the frequency of anti-vaccine T cells due to poor reproducibility, nonspecific release of IFN- $\gamma$ , and rapid proliferation of large numbers of CD4 T cells, which possibly overwhelmed the anti-MAGE-3 T cells.

We have undertaken a systematic effort to develop a reproducible monitoring approach of high specificity and sensitivity. It combines sorting of living blood T cells producing IFN- $\gamma$  after a short antigenic stimulation and detailed functional analyses of the cells amplified under clonal conditions. Using this approach, we present in this study the analysis of the anti-vaccine CD4 T cell responses of the five patients who showed tumor regression after injection of ProtD-MAGE-3 without adjuvant.

## Materials and Methods

### Patients and vaccination

Twenty-six melanoma patients with detectable cutaneous and/or lymph node metastasis, but without visceral metastasis, were involved in clinical trial LUD 99-003 (W. H. J. Kruit and M. Marchand, manuscript in preparation). It was approved by the protocol review committee of Ludwig Institute for Cancer Research and by the ethics committee Commission d'Éthique Biomédicale Hospitalo-Facultaire de la Faculté de Médecine de l'Université de Louvain. Informed consent forms were signed by the patients. The patients were also required to have tumor expression of gene MAGE-3, as assessed by RT-PCR. They were intradermally or s.c. injected with a recombinant MAGE-3 protein, which was expressed in *Escherichia coli* as a fusion protein with ProtD derived from *H. influenzae* at the N terminus, and a sequence of several histidine residues at the C terminus of the protein (ProtD-MAGE-3/His) (12).

### Cell lines, media, reagents, and MAGE-3 proteins

An EBV-transformed B cell line was generated for each responding patient. The EBV-B cell lines were cultured in IMDM (Invitrogen Life Technologies) supplemented with 10% FCS (MP Biomedicals); 0.24 mM L-asparagine, 0.55 mM L-arginine, and 1.5 mM L-glutamine (AAG); 100 U/ml penicillin; and 100  $\mu$ g/ml streptomycin. Human rIL-2 was purchased from Eurocetus, IL-7 from Genzyme, and GM-CSF (Leukomax) from Schering Plough. Human rIL-4 was produced in our laboratory. Dendritic

cells were obtained by culturing monocytes in the presence of IL-4 (200 U/ml) and GM-CSF (70 ng/ml) in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with AAG and 1% autologous plasma. One-fourth of the medium was replaced by fresh medium and cytokines every 2 days. On day 5, the nonadherent cell population was used as a source of enriched dendritic cells, as described previously (17). Anti-HLA-DR Ab L243 was obtained from American Type Culture Collection and was used at a 1/5 dilution of a culture supernatant.

Three different MAGE-3 proteins were used. One was produced in our laboratory in *Spodoptera frugiperda* (Sf9) insect cells using a baculovirus expression system (BD Pharmingen) as described previously (15). It will be referred to hereafter as protein MAGE-3<sup>insect</sup>. A second protein, ProtD-MAGE-3/His, was provided by GlaxoSmithKline Biologicals. The recombinant MAGE-3 protein was expressed in *E. coli* as a fusion protein with a ProtD derived from *H. influenzae* at the N terminus and a sequence of several histidine residues at the C terminus of the protein. It was extensively purified to eliminate bacterial contaminants and was used for vaccination. The third MAGE-3 protein, His/MAGE-3, was also produced in bacteria and provided by GlaxoSmithKline Biologicals. His/MAGE-3 has no ProtD moiety, but contains a sequence of several histidine residues at the N terminus of the protein. It was extensively purified to eliminate bacterial contaminants and was used for delayed-type hypersensitivity tests. We have used as control protein the E7 protein of HPV16, which was produced in bacteria and purified by GlaxoSmithKline Biologicals.

### Construction of the retrovirus encoding invariant chain (Ii)-MAGE-3

For producing the retrovirus encoding Ii-MAGE-3, the sequence encoding a truncated form of low-affinity receptor of the nerve growth factor (LNGFR) was amplified from plasmid pUC19- $\Delta$ LNGFR (provided by Dr. C. Traversari, Istituto Scientifico H.S. Raffaele, Milan, Italy). Briefly, LNGFR was ligated into pCR2.1 to an internal ribosome entry site (IRES) sequence derived from the encephalomyocarditis virus. The IRES- $\Delta$ LNGFR sequence was then transferred into pMFG-Ii80, which encodes the first 80 aa of the human Ii (Ii80). A complete MAGE-3 cDNA was then ligated downstream Ii80 into pMFG-Ii80-IRES- $\Delta$ LNGFR, allowing simultaneous expression of the Ii-MAGE-3 fusion protein and the truncated LNGFR receptor. An EBV-B cell line expressing the Ii-MAGE-3 fusion protein was generated for each of the analyzed patients. The procedure for transducing EBV B cell lines has been described previously (18).

### Ex vivo sorting of cells producing IFN- $\gamma$

Autologous dendritic cells were loaded with 20  $\mu$ g/ml His/MAGE-3 in the presence of GM-CSF and IL-4 and 1 ng/ml TNF- $\alpha$ , then incubated for 24 h. PBMC were thawed and incubated at  $4 \times 10^6$  cells/ml in 48-microwell plates (1 ml/well) in IMDM supplemented with AAG and 1% autologous plasma at 37°C in the presence of DNase (5 U/ml) for 1 h. The nonadherent cells were removed, washed, and resuspended at  $4 \times 10^6$  cells/ml in IMDM/AAG/1% autologous plasma. These cells were seeded into 48-microwell plates (0.5 ml/well), and we added 0.5 ml/well MAGE-3-loaded dendritic cells ( $2 \times 10^5$  cells/ml). Alternatively, we used as stimulators autologous adherent cells pulsed for 4 h with 10  $\mu$ g/ml of the MAGE-3.DR1 peptide, ACYEFWGPRLVETS. Sixteen hours later, the cells were collected, washed, resuspended, labeled with IFN- $\gamma$  capture Ab (1/10 dilution; IFN- $\gamma$  Secretion Assay-Detection kits PE; Miltenyi Biotec), and incubated for 45 min at 37°C under slow rotation. The cells were subsequently labeled with the IFN- $\gamma$  detection Ab conjugated to PE (1/20), anti-CD3-allophycocyanin (1/40; clone UCHT1; BD Biosciences), and anti-CD8-FITC (1/40; BD Biosciences). Incubation continued for 15 min at 4°C. The cells were then washed, and CD3<sup>+</sup>CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells were sorted on a FACS Vantage flow cytometer (BD Biosciences).

### Culture of sorted cells

Sorted cells were stimulated with irradiated autologous EBV-B cells transduced with retro-Ii.MAGE-3 ( $1 \times 10^3$ – $2 \times 10^4$  cells). Irradiated LG2-EBV cells were added as feeder cells ( $1$ – $2 \times 10^4$  cells/well). Cells were cultured in IMDM/AAG/10% human serum, with the addition of IL-2 (50 U/ml), IL-4 (5 U/ml), IL-7 (10 ng/ml), and PHA (PHA HA16; Murex Biotech; 125 ng/ml).

### Specificity assay

Aliquots of each growing clone (~5,000 cells) were stimulated with ~20,000 autologous EBV-B cells either pulsed with 5  $\mu$ g/ml MAGE-3.DR1 peptide or loaded for 20 h with 20  $\mu$ g/ml MAGE-3 protein. After 20 h of coculture in round-bottom microwells and in 150  $\mu$ l of complete

<sup>3</sup> Abbreviations used in this paper: ProtD, protein D; AAG, 0.24 mM L-asparagine, 0.55 mM L-arginine, and 1.5 mM L-glutamine; Ii, invariant chain; IRES, internal ribosome entry site; LNGFR, low-affinity receptor of the nerve growth factor.

IMDM supplemented with IL-2 (25 U/ml), IFN- $\gamma$  released in the supernatant was measured by ELISA using reagents from Medgenix Diagnostics-Biosource.

#### TCR analysis and clonotypic PCR

For TCR analysis, RNA was extracted from  $3 \times 10^5$  cells with Tripure reagent (Roche) and converted to cDNA at 42°C for 90 min with 200 U of Maloney's murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). TCR V $\alpha$  and V $\beta$  usage was assessed by PCR amplification using a complete panel of V $\alpha$ - or V $\beta$ -specific sense primers and C $\alpha$  and C $\beta$  antisense primers, respectively (19). Primers were chosen on the basis of described panels of TCR V region oligonucleotides and with the alignments of TCR sequences available at the International Immunogenetics Database. Each PCR product was purified and sequenced to obtain complete identification of the CDR3 region.

A clonotypic detection procedure was designed for clonotype 1 of patient 101. PCR amplification uses nested pairs of primers, with the first external pair consisting of one upstream V primer and one downstream C primer (V $\alpha$ , upper, 24 nt, GGC CTG GTG GAC ATC CCG TTT TTT; C $\alpha$ , lower, labeled with NED in 5' end, 20 nt, CTC CAG GCC ACA GCA CTG TT; V $\beta$ , upper, 27 nt, GGA GAG AAA GTT TTT CTG GAA TGT GTC; C $\beta$ , lower, 24 nt, CGG GCT GCT CCT TGA GGG GCT GCG). A fraction of the amplified products (1/100) was used for the second amplification round with a second internal pair consisting of another upstream V primer and one downstream primer with its 3' end matching N nucleotides of the CDR3 region and the junction with the J sequence (V $\alpha$ , upper, 23 nt, TAG GAA CCT ACT TCT GTG CAG TT; J $\alpha$ , 21 nt, GGT GAA TAG GCA GAC AGA CTT; V $\beta$ , upper, 20 nt, ACC TCT GTG CCA GCA TAA GA; J $\beta$ , lower, 18nt, GAC CTC GGG TGG GAA CAC). To increase the specificity of the procedure, one-quarter of the product of the second PCR was engaged in a run-off reaction (20) consisting of an extension procedure on the V $\alpha$  and V $\beta$  PCR products using a single fluorescent-labeled primer matching the rest of the CDR3 nucleotides that was not covered by the downstream primers of the second PCR (V $\alpha$ , 23 nt, 5' FAM, ACC TAC TTC TGT GCA GTT ACC AA; V $\beta$ , lower, 17 nt, 5'HEX, AAG AGG GGG CGC GGG AA). Size determination of the run-off products was performed by capillary electrophoresis in acrylamide gel with the ABI PRISM 3100 Genetic Analyzer and GeneScan analysis software (Applied Biosystems). To assess the sensitivity of the global detection procedure, dilutions of cDNA of clonotype 1 in irrelevant cDNA extracted from PBMC were tested. The threshold of detection was the equivalence of 0.5 cell of clonotype 1 into  $10^7$  PBMC. Total RNA from groups of 80,000 PBMC taken after the sixth vaccination and from  $10^6$  PBMC taken before vaccination of patient 101 was extracted and converted to cDNA as described above.

#### IFN- $\gamma$ ELISPOT assay

ELISPOT analysis was performed as previously described (21). Briefly, Multiscreen HA plates (Millipore) were coated with 10  $\mu$ g/ml monoclonal anti-human IFN- $\gamma$  Ab (1-D1K; Mabtech). Effector cells were seeded in triplicate together with Ag-loaded APC after thawing and incubation overnight in the presence of autologous serum and low dose IL-2. For ex vivo analysis, the patient's CD4 cells were isolated using immunomagnetic beads according to the manufacturer's instructions (MACS; Miltenyi Biotec) and seeded with  $1 \times 10^5$  cells/well, whereas the anti-MAGE-3 control clone was seeded with 5000 cells/well. The remaining CD4-depleted cells of all samples of the patient were pooled, and  $1 \times 10^5$  cells/well were used as APC. Peptides were added to a final concentration of 50  $\mu$ g/ml, and unloaded APC served as negative controls. The cells were incubated at 37°C in 5% CO<sub>2</sub> in a final volume of 100  $\mu$ l/well X-Vivo 15 (BioWhittaker) for 20 h. Captured cytokine was labeled after incubation for 2 h at 37°C with biotinylated mAb anti-hIFN- $\gamma$  (7-B6-1; Mabtech) at 2  $\mu$ g/ml in PBS/0.5% BSA using an avidin-peroxidase complex (1/100; Vectastain Elite Kit; Vector Laboratories). Peroxidase staining was performed with 3-amino-9-ethyl-carbazole (Sigma-Aldrich) for 4 min and was stopped by rinsing the plates under running tap water. Spot numbers were automatically determined with the use of computer-assisted video image analysis. Hard- and software of the imaging system used in this study were developed by Zeiss-Kontron.

#### Tetramer labeling

PBMC were thawed in IMDM/10% human serum/AAG, containing 5 U/ml DNase, seeded at  $4 \times 10^6$  cells/ml/cm<sup>2</sup>, and kept overnight in medium containing 5 U/ml DNase. Cells were washed and resuspended at  $1 \times 10^7$  cells/ml in PBS plus 1 mM EDTA plus 1% human serum and labeled with 100 nM DPB1\*0401 multimer folded with peptide KKLLTKHFVQENY-LEY. After 1-h incubation with constant shaking at 300 rpm, CD8-PE-Cy5

(BD Biosciences) and CD4-FITC, clones RPA-T4 (BD Biosciences) were added at 1/50. The cells were allowed to incubate for an additional 10 min, washed once, resuspended in HCF, passed through a 40- $\mu$ m pore size nylon filter (BD Biosciences), then kept on ice until sorted by flow cytometry. All labeling were performed at room temperature and in the dark.

#### Reverse phase chromatography and recognition assay of the different fractions

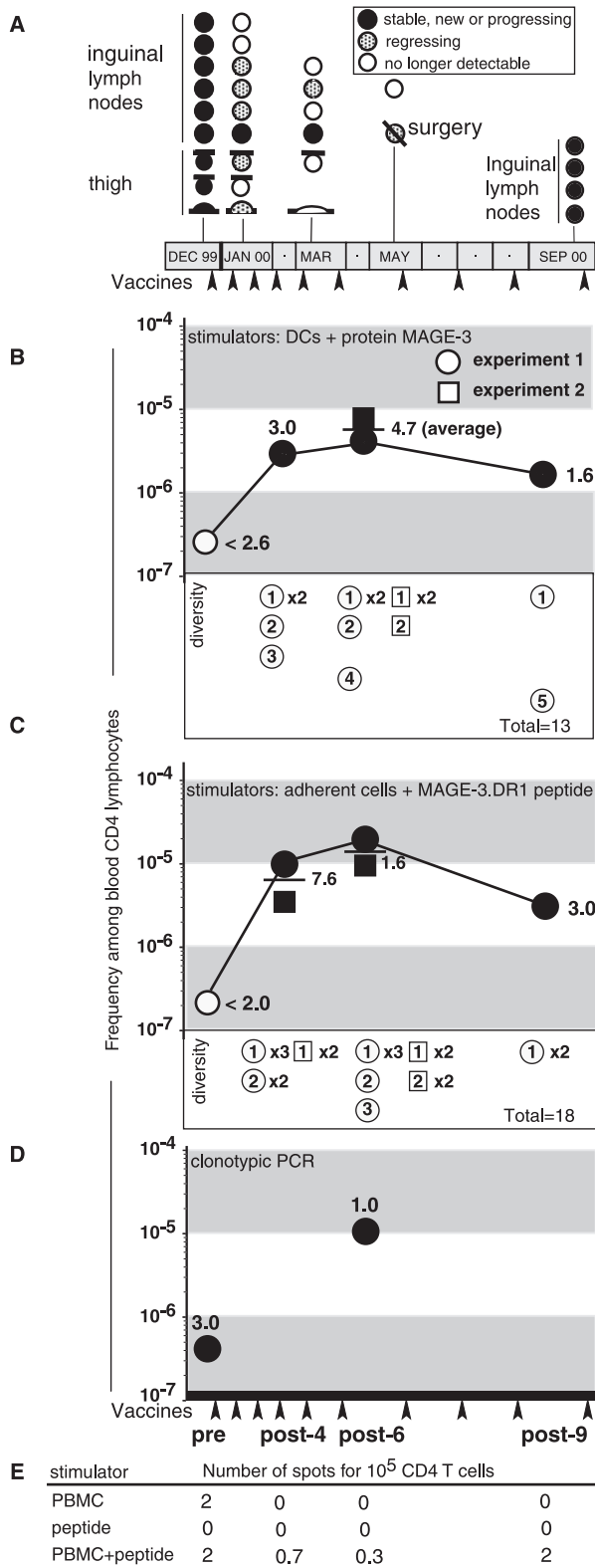
Prot.D-MAGE-3/His (120  $\mu$ g) was dissolved with PBS and fractionated by reverse phase HPLC. The protein was loaded onto a 4.6  $\times$  250-mm 214TP54 Vydac C4 column (The Separations Group) and eluted using a 30-min gradient of acetonitrile in water (5–80%) containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The column eluent was monitored with a UV detector at 210 nm. Fractions of 1 ml were collected and concentrated using vacuum centrifugation. Twenty thousand DDHK2 EBV-B cells were pulsed with 1/60 of each of the different fractions of protein MAGE-3 and tested with 5000 cells of each of the CD4 clones. After 20 h of coculture, IFN- $\gamma$  released in the supernatant was measured by ELISA using reagents from Medgenix Diagnostics-Biosource.

## Results

#### Stimulation of blood cells of patient 101 with protein and isolation of IFN- $\gamma$ -secreting CD4 clones

The course of the clinical response of melanoma patient 101, who received a ProtD-MAGE-3 vaccine, is described in Fig. 1A (W. H. J. Kruit and M. Marchand, manuscript in preparation and unpublished observations). PBMC were thawed and, after an adherence step, the nonadherent cells were stimulated overnight with dendritic cells loaded with a full-length MAGE-3 protein. We used a MAGE-3 protein without the ProtD moiety to take into account only the anti-vaccine T cell response directed against MAGE-3 peptides. IFN- $\gamma$  was retained on the secreting cells by a bispecific Ab molecule consisting of a conjugated pair of mAbs (IFN- $\gamma$  secretion assay; Miltenyi Biotec). One Ag-binding site binds to CD45, present on the surface of all leukocytes, whereas the other binding site recognizes IFN- $\gamma$ . The immobilized cytokines are revealed by adding a fluorescence-labeled anti-cytokine Ab, and living IFN- $\gamma$ -secreting cells can thus be sorted by flow cytometry (Fig. 2). A number of CD3<sup>+</sup>CD8<sup>-</sup>IFN- $\gamma$ <sup>+</sup> cells were selected, distributed at one cell per well, and stimulated with irradiated autologous EBV-B cells transduced with a retroviral construct encoding a truncated human Ii fused with the MAGE-3 protein. We did not select CD3<sup>+</sup>CD8<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells because we never observed a specific stimulation of established anti-MAGE-3 CD8 T cell clones by dendritic cells loaded with protein MAGE-3 (unpublished observation). Approximately 30% of the cloned T cells proliferated and were tested for their specific release of IFN- $\gamma$  upon contact with autologous EBV-B cells loaded with a MAGE-3 protein. Their anti-MAGE-3 specificity was also tested on autologous EBV-B cells expressing Ii-MAGE-3.

Only those T cell clones that recognized both the EBV-B cells loaded with the MAGE-3 protein and the cells expressing MAGE-3 were considered to be directed against anti-MAGE-3. Representative clones are shown in Fig. 3. We estimated the frequency by multiplying the fraction of IFN- $\gamma$ <sup>+</sup> CD4 T cells with the fraction of cloned cells that yielded anti-MAGE-3 CD4 clones (Fig. 2). No anti-MAGE-3 clone was found in the blood sample collected before vaccination. The frequency peaked at  $4.7 \times 10^{-6}$  of the CD4 blood T lymphocytes, after the sixth vaccination (Fig. 1B). The reproducibility of this estimation was indicated by the similar results obtained in two independent experiments. In experiment 1 the frequency was estimated at  $5 \times 10^{-6}$ , and in experiment 2 it was estimated at  $4 \times 10^{-6}$ , giving an average of  $4.7 \times 10^{-6}$  (Fig. 1B, post 6). To evaluate the diversity of the anti-vaccine response, we examined the TCR sequences of the anti-MAGE-3 clones and found five different TCR $\alpha\beta$  clonotypes, clonotype 1

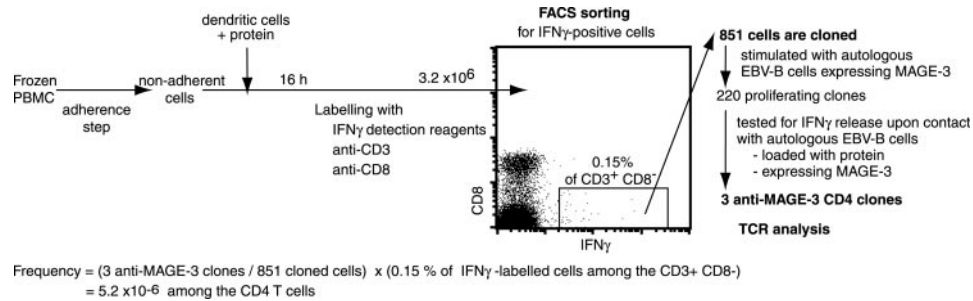


**FIGURE 1.** Clinical evolution and monitoring of the anti-MAGE-3 CD4 response in patient 101. *A*, The clinical evolution of patient 101 is partly described by W. H. J. Kruit and M. Marchand (manuscript in preparation). At study entry in December 1999, patient 101 had multiple in-transit metastases, the largest measuring 3.5 cm in diameter, and inguinal invaded lymph node of the left thigh (W. H. J. Kruit and M. Marchand, manuscript in preparation and unpublished observations). The patient received 300  $\mu$ g of MAGE-3 protein without any immunological adjuvant on six occasions at 3-wk intervals. In mid-April 2000, all but the largest lymph node metastasis had completely regressed. The clinical response was classified as a

was expressed by seven of the 13 clones (Figs. 1*B* and 3). Interestingly, all anti-MAGE-3 clones were found to recognize a MAGE-3.DR1 peptide previously identified with lymphocytes from this patient (16). The recognition of the peptide was abolished in the presence of an anti-DR Ab (data not shown). Other presenting cells, sharing only DR1 with patient 101, were also able to present the peptide to the anti-MAGE-3 clones, demonstrating that all the clones recognized the same HLA-peptide combination (data not shown).

We were surprised that the MAGE-3 CD4 T cell response of patient 101 seemed to be directed against only one peptide/HLA combination. The patient was typed HLA-DP4, and a MAGE-3 peptide presented by this HLA molecule has been found to be recognized by T cells of several melanoma patients (5, 15). We have first confirmed that dendritic cells of patient 101 that were loaded with protein MAGE-3 were able to present the antigenic peptide to an anti-MAGE-3.DP4 T cell clone (data not shown). We then searched for anti-MAGE-3.DP4 T cells with a very sensitive tool: a DP4 multimer folded with the MAGE-3 peptide. Blood cells from patient 101 collected after the sixth vaccination were labeled with the DP4/MAGE-3 multimer, and  $5 \times 10^6$  cells were passed through a flow cytometer. Two hundred and thirty-one cells were selected, distributed at one cell per well, and stimulated with irradiated DP4 cells loaded with the MAGE-3 peptide. No tetramer-positive clone was found among the proliferating cells. This

mixed response. The last lymph node metastasis was surgically removed in May 2000 and was shown to be 95% necrotic. Four additional immunizations with MAGE-3 protein were given at 6-wk intervals. In August and September 2000, new iliac lymph node metastases appeared. Additional MAGE-3 protein vaccinations associated with a MAGE-3.B35 peptide were initiated in October 2000, but the disease progressed. Despite additional treatments, the patient died of tumor progression in August 2001, 20 mo after the first vaccination with MAGE-3 protein. *B* and *C*, The *top panels* indicate the frequencies of anti-MAGE-3 CD4 T cell clones obtained after stimulation of PBMC with either autologous dendritic cells loaded with protein MAGE-3 (*B*) or autologous adherent cells pulsed with MAGE-3.DR1 peptide (*C*). Frequencies were calculated as described in Fig. 2. A line between a square and a circle indicates the average of the frequencies estimated in the two experiments. The average of the frequencies was weighed according to the number of cells engaged in the flow cytometer in each experiment. CD4 clones were considered anti-MAGE-3 clones if they recognized both cells loaded with peptide or protein and cells transduced with Ii-MAGE-3. IFN- $\gamma$  release had to exceed at least 2 times and by  $>150$  pg/ml the release of IFN- $\gamma$  upon contact with control EBV-B cells. The *bottom panels* indicate the TCR diversity. Each number represents a different TCR $\alpha\beta$  clonotype. The squares and circles represent clones obtained in different experiments. When several independent CD4 T cell clones share the same TCR $\alpha\beta$  sequence, they are illustrated by the same number. *D*, The blood frequency of clonotype 1 was evaluated directly by PCR on cDNAs extracted from blood cells and sequencing of the PCR products. Specific PCR amplification protocols were set up for the TCR $\alpha$  and - $\beta$  genes of clonotype 1 and used to test cDNAs derived from groups of freshly thawed PBMC. The PCR products were purified and sequenced. Four of 26 groups of  $8 \times 10^4$  PBMC tested positive. In the blood cells collected before vaccination, two of 24 groups of  $10^6$  PBMC tested positive. *E*, IFN- $\gamma$  ELISPOT assay:  $1 \times 10^5$  CD4 T cells were seeded in a plate precoated with anti-human IFN- $\gamma$  mAb. The remaining CD4-depleted cells ( $1 \times 10^5$  cells/well) were used as stimulators and were pulsed, or not, with 50  $\mu$ g/ml. Cells were incubated for 20 h, and the biotinylated mAb anti-IFN- $\gamma$  was added. Spot numbers were automatically determined with the use of computer-assisted video image analysis. Five thousand cells of the anti-MAGE-3 DR1 clonotype 1 were used as a positive control, and  $484 \pm 69$  spots were obtained in each microwell. The results shown represent the average of triplicate cocultures.



**FIGURE 2.** Overview of the procedure to obtain anti-MAGE-3 CD4 T cell clones. The nonadherent cells were stimulated with autologous dendritic cells loaded with 20  $\mu\text{g}/\text{ml}$  MAGE-3 protein without the ProtD portion. After overnight coculture, cells were labeled with an IFN- $\gamma$  capture reagent and incubated for 45 min at 37°C to allow cytokine secretion. They were subsequently labeled with an IFN- $\gamma$  detection Ab conjugated to PE, anti-CD3-allophycocyanin and anti-CD8-FITC. The cells represented on the FACS figure are the living CD3<sup>positive</sup> cells. The numbers illustrated on the figure were obtained in an experiment with PBMC collected from patient 101 after six vaccinations.

led us to estimate that their frequency was  $<8 \times 10^{-7}$  among CD4 blood T cells.

#### Confirmation of anti-MAGE3 CD4 frequencies with other methods

Based on the observation that patient 101 had several CD4 clones directed against the same peptide, we performed a series of experiments using this peptide as the stimulating Ag. The nonadherent cells were stimulated overnight with the adherent cells (or dendritic cells) loaded with the peptide. Stimulated cells were subsequently labeled, sorted, and amplified under clonal conditions as described above. Only the clones that recognized both autologous EBV-B cells loaded with the MAGE-3.DR1 peptide and autologous EBV-B cells expressing Ii-MAGE-3 were considered anti-MAGE-3 (Fig. 3). No anti-MAGE-3 clone was isolated from a blood sample collected before vaccination. The frequency peaked at  $1.6 \times 10^{-5}$  of CD4 blood T lymphocytes in samples collected after the sixth vaccination (Fig. 1C). This represents at least an 80-fold increase in the frequency found before immunization. Similar results were obtained in two independent experiments. In experiment 1 the frequency was estimated at  $2 \times 10^{-5}$ , and in experiment 2 it was estimated at  $9 \times 10^{-6}$ , giving an average of  $1.6 \times 10^{-5}$  (Fig. 1C, post 6). The frequencies estimated at different time points, using the peptide as the stimulating Ag, were in good agreement with those estimated with the protein. Because peptide-loaded cells are stronger stimulators than protein-loaded cells, it is not surprising that the frequencies were also higher. We examined the TCR sequences of the anti-MAGE-3.DR1 clones and found three TCR $\alpha\beta$  clonotypes. These three clonotypes were al-

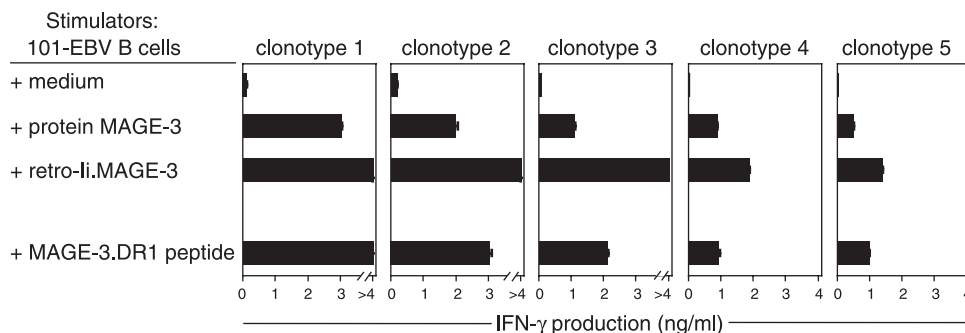
ready identified in the experiments with the protein, in particular clonotype 1, which was expressed by 12 of the 18 clones.

The blood frequencies of clonotype 1 were evaluated directly by clonotypic PCR and sequencing of the PCR products. Specific PCR amplification protocols were set up for the TCR $\alpha$  and - $\beta$  genes of clonotype 1 and used to test cDNA derived from groups of freshly thawed PBMC. Four of 26 groups of  $8 \times 10^4$  PBMC tested positive for both TCR $\alpha$  and - $\beta$ , leading to a frequency estimate for clonotype 1 at  $10^{-5}$  of CD4 T cells (Fig. 1D). This frequency is very close to that estimated by the cellular approach at  $9 \times 10^{-6}$ , indicating that our approach identified most, if not all, the anti-MAGE-3.DR1 clones and excluding that a number of anti-MAGE-3 T cells were not detected by our cellular approach, because of a lack of proliferation capacity of the specific cells. In the blood cells collected before vaccination, the frequency was estimated by the cellular approach to be  $<2.6 \times 10^{-7}$  of CD4 T cells. With the clonotypic PCR, two of 24 groups of  $10^6$  PBMC tested positive, leading to a frequency estimate at  $3 \times 10^{-7}$  of CD4 T cells.

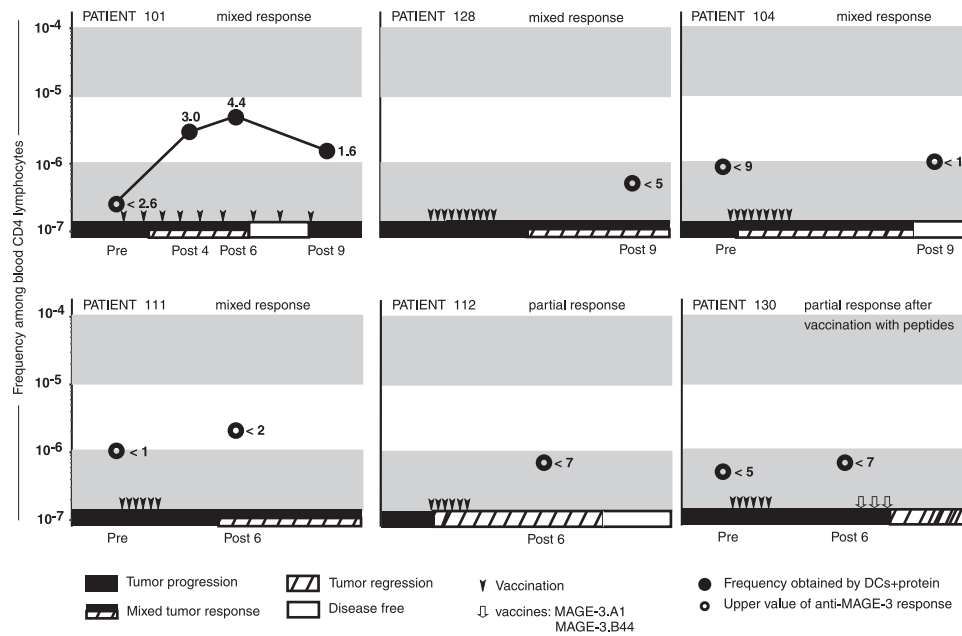
An IFN- $\gamma$  ELISPOT approach was also used to screen several blood samples for the presence of anti-MAGE-3.DR1 T cells. No anti-MAGE-3.DR1-specific T cell was detected either before or after vaccination (Fig. 1E).

#### Absence of detectable anti-vaccine response in the other vaccinated patients

Using the sorting approach described above that is based on IFN- $\gamma$  secretion after a short stimulation with autologous dendritic cells loaded with a MAGE-3 protein without the ProtD moiety, we



**FIGURE 3.** Recognition of a MAGE-3 Ag by some of the CD4 clones of patient 101. Five thousand CD4 T cells were cultured with 20,000 stimulator cells. Stimulators were the EBV-B cells from patient 101, either loaded with 20  $\mu\text{g}/\text{ml}$  of the ProtD-MAGE-3 protein, transduced with retro-Ii.MAGE-3, or pulsed with 5  $\mu\text{g}/\text{ml}$  MAGE-3.DR1 peptide. The presence of IFN- $\gamma$  in the supernatant was measured by ELISA after overnight coculture. The results shown represent an average of triplicate cocultures.



**FIGURE 4.** Monitoring of the anti-vaccine CD4 T cell response in patients immunized with ProtD-MAGE-3 without adjuvant. The patient number and the clinical staging data correspond to the information provided by W. H. J. Kruit and M. Marchand (manuscript in preparation). Tumor responses (mixed response or partial response) were assessed after the sixth vaccination according to the World Health Organization criteria. For each patient, the nonadherent fraction of PBMC was stimulated overnight with autologous dendritic cells loaded with MAGE-3 protein without the ProtD moiety. The CD4 T cells producing IFN- $\gamma$  were sorted by flow cytometry and cloned. The proliferating clones were tested for MAGE-3 specificity in an IFN- $\gamma$  release assay. Frequencies were calculated as described in Fig. 2. CD4 T cell clones were considered anti-MAGE-3 clones if they recognized both cells loaded with peptide or protein and cells transduced with Ii-MAGE-3. IFN- $\gamma$  release had to exceed at least 2 times and by  $>150$  pg/ml the release of IFN- $\gamma$  upon contact with control EBV-B cells.

screened blood cells from the four other clinical responders and those from a nonresponder (patient 130), who later showed a partial response after injections of another vaccine containing peptides. Only the clones that recognized both autologous EBV-B cells loaded with protein MAGE-3 and expressing Ii-MAGE-3 were considered anti-MAGE-3 clones. No anti-MAGE-3 clone was detected in any of these patients (Fig. 4).

#### *Anti-vaccine response in patient 101 that is not directed against MAGE-3*

In addition to the anti-MAGE-3 clones that recognized both EBV-B cells loaded with protein MAGE-3 and EBV-B cells transduced with retro-Ii-MAGE-3, in patient 101 we identified 40 clones that released IFN- $\gamma$  upon contact with EBV-B cells loaded with recombinant protein MAGE-3, but not with cells expressing Ii-MAGE-3, such as clones 1F6 and 1G9 (Fig. 5A). They were also tested for specificity and were found to recognize cells loaded with MAGE-3 proteins produced in bacteria, but not a MAGE-3 protein produced in insect cells. They did not recognize a control protein produced in the same type of bacteria as those used to produce the two MAGE-3 proteins (Fig. 5A). To further characterize the Ag recognized by clones 1F6 and 1G9, ProtD-MAGE-3 was loaded on a reverse phase HPLC column and separated into 12 fractions. Autologous EBV-B cells were loaded with each of these fractions and subsequently tested for recognition by clones 1F6 and 1G9 and an anti-MAGE-3 control clone. As expected, the anti-MAGE-3 clone was stimulated by several fractions corresponding to the major peak containing the MAGE-3 protein, whereas clones 1F6 and 1G9 recognized fractions clearly distinct from the main peak assigned to MAGE-3 (Fig. 5B). The Ag recognition of clone 1F6 was restricted by HLA-DQ, whereas recognition of clone 1G9 was restricted by HLA-DR (data not shown). We concluded that clones 1F6 and 1G9 recognized a contaminant in the batch of ProtD-

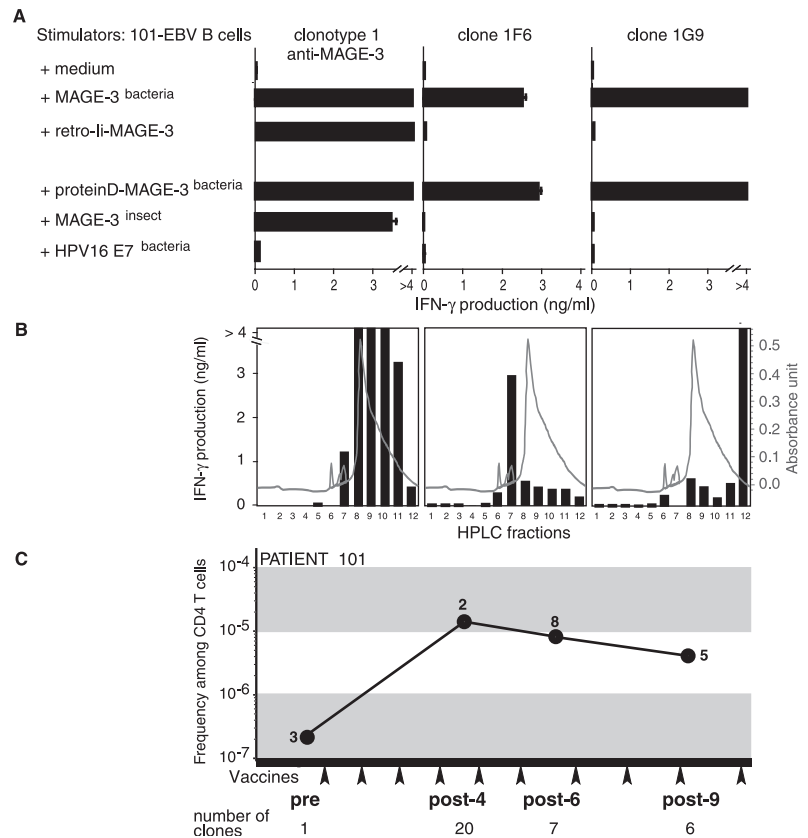
MAGE-3 protein used for vaccination. It is probably a product of bacterial origin that was copurified with MAGE-3. The anti-vaccine response in patient 101 that is directed against contaminants reached  $2 \times 10^5$  after the fourth vaccination (Fig. 5C). At the different time points that were analyzed, the frequencies of anti-contaminant T cells were similar to the frequencies of anti-MAGE-3.DR1 T cells (Fig. 5, B and C). Two anticontaminant clones were also detected in patient 128, leading to a frequency estimated at  $10^{-6}$  of CD4 T cells.

During screening of the proliferating clones for MAGE-3 specificity, we observed clones that were stimulated to produce IFN- $\gamma$  by EBV-B cells transduced with retro-Ii-MAGE-3 and not by EBV-B cells loaded with protein MAGE-3, or clones that were stimulated by peptide-pulsed EBV-B cells and not by EBV-B cells expressing Ii-MAGE-3. These clones were not considered anti-MAGE-3 clones. Taken together, these results indicate that establishing the MAGE-3 specificity and the ability to recognize naturally processed Ags necessitates testing with different Ag formats.

## Discussion

The monitoring procedure described in this study has the potential to detect the complete set of T lymphocytes that recognize the vaccine-derived peptides on various HLA molecules. Frequencies as low as one per million specific CD4 T cells can be measured with frozen samples corresponding to  $<50$  ml of blood, whereas other techniques, such as ex vivo ELISPOT assays, reach their limit of detection at one per 25,000 CD4 T cells. The availability of T cell clones ensures strict assessment of the specificity of T cells, including their ability to recognize both cells loaded with the protein and cells transduced with the protein-coding sequence. It also makes it possible to define the TCR sequence of the anti-vaccine T cells and, therefore, to analyze the TCR diversity and establish the presence of repeated clonotypes, an essential criterion

**FIGURE 5.** Characterization of the anticontaminant clones in blood cells from patient 101. Five thousand CD4 T cells were cocultured overnight with 20,000 EBV-B cells from patient 101. The concentration of IFN- $\gamma$  produced in the medium was measured by ELISA. The results shown represent an average of triplicate cocultures. **A**, CD4 clone 749/B1, corresponding to clonotype 1 and two other CD4 clones, 1F6 and 1G9, were tested for their ability to recognize cells pulsed with different MAGE-3 proteins and with a control protein, E7 from human papilloma virus, produced in bacteria under the same conditions as MAGE-3 protein. **B**, ProtD-MAGE-3 protein (120  $\mu$ g), produced in bacteria, was loaded on a reverse phase HPLC column and separated into 12 fractions that were pulsed (1/60 of each fraction/microwell) onto autologous 20,000 EBV-B cells, which were tested for recognition by 5,000 cells of each CD4 clone. Absorbing units were measured with a UV detector at 210 nm. **C**, Frequencies in the blood of patient 101 were calculated as described in Fig. 2. Clones were considered anticontaminants if they recognized cells loaded with the ProtD-MAGE-3 protein produced in bacteria, but not cells expressing Ii-MAGE-3, such as clones 1F6 and 1G9.



to assess the occurrence of a response when the frequency is low (11). In addition, a direct quantitative evaluation of the frequency of certain clonotypes can be obtained by PCR performed on RNA extracted from blood lymphocytes and tumor samples.

A limitation of our experiments is that only those anti-MAGE-3 CD4 T cells that produce IFN- $\gamma$  can be detected. Clearly, vaccination with MAGE-3 might induce T cells that do not produce IFN- $\gamma$ , including T cells that contribute to an inhibitory environment in the tumor. An interesting example of such cells is the anti-LAGE CD4 T cell clones that were generated from tumor-infiltrating lymphocytes of a cancer patient and possessed suppressive activity on the proliferative response of naive CD4 T cells (22). However, our approach could easily be extended by using a cytokine secretion assay for the detection of cells producing another cytokine, such as IL-10. It could also be extended to the detection of specific CD8 T cells, provided stimulator cells are used that are able to activate both CD8 and CD4 T cells. Various autologous cells, either electroporated with RNA constructs or infected with recombinant viral vectors, are currently being tested.

We observed in patient 101 similar frequencies for CD4 T cells directed against MAGE-3 and for CD4 T cells directed against contaminants in the vaccine preparation. Because the bacterial contaminants copurified with MAGE-3 and not with an HPV16 control protein, anticontaminant T cells would have been considered specific for MAGE-3 in assays based on cytokine secretion, such as the ELISPOT assay, even if another protein produced in bacteria had been used as a control. On the basis of our experience, we considered it likely that responses against contaminants will occur often. Moreover, modification of the protein during the production process, such as carboxymethylation used to avoid formation of disulfide bonds and aggregation of the protein, will produce neo-Ags. From a patient who was vaccinated with protein MAGE-3, we have isolated a CD4 clone that recognizes a

MAGE-3 peptide only when this peptide is carboxymethylated (our unpublished observations). Strict specificity can easily be assessed by testing T cell clones as described in this study, but as an alternative in ELISPOT assays, a set of different stimulator cells should be used, either loaded with different proteins, infected with viral vectors, or electroporated with RNA (23).

In one of five metastatic melanoma patients who were vaccinated with a MAGE-3 protein without adjuvant and who responded clinically, we measured a >80-fold increase in the frequency of anti-MAGE-3 CD4 T cells. The peak of the cellular response reached  $1.6 \times 10^{-5}$  of the blood CD4 T lymphocytes after the sixth vaccination. It is surprising that vaccination with a protein containing 314 aa led to presentation of only one peptide in the context of one HLA allele. In particular, patient 101 expressed the HLA-DP4 allele, and we would have expected a response against the MAGE-3.DP4 peptide that was shown to be antigenic in patients vaccinated with peptide-pulsed dendritic cells (24). The 31 anti-MAGE-3 clones isolated in patient 101, involving five different TCR clonotypes, were all directed against the same MAGE-3.DR1 peptide.

In other clinical trials, the MAGE-3 protein was injected with adjuvant AS02B (12, 25). In a group of melanoma patients, two clinically responding patients were reported to have significant frequency of anti-MAGE-3 CD4 T cells, but they were pre-existing to the vaccine (26). Among the lung cancer patients, about half were reported to have a CD4 T cell response against the MAGE-3.DP4 peptide (25).

The results of monitoring the anti-vaccine CD4 T cells described in this study lead us to the puzzling conclusion that patients with evidence of tumor regression after vaccination often have a low or undetectable frequency of anti-vaccine CD4 T cells in their blood. Anti-vaccine T cells might be undetectable because of the



lack of immunological adjuvant, or because the response was mediated by T cells that do not produce IFN- $\gamma$ . We cannot exclude that the ProtD part of the vaccine is a strong immunogen that either had an inhibitory effect on the induction of MAGE-3-specific CD4 T cell responses or deviated the anti-MAGE-3 response to a Th2-type response. However, vaccination with ProtD fused to a mutated E7 protein from human papillomavirus 16 induced an increase in anti-vaccine CD8 T cells producing IFN- $\gamma$  and both E7-specific and ProtD-specific IgG responses (27).

Patients with evidence of tumor regression after vaccination with a class I-restricted peptide also often have a low or undetectable frequency of anti-vaccine CD8 T cells in their blood (8). This observation appears to extend to the tumor site (C. Lurquin, P. Coulie, and T. Boon, manuscript in preparation). In one patient regressing after vaccination with a recombinant poxvirus containing a minigene encoding a MAGE-3 Ag presented by HLA-A1, followed by peptide boosts, the frequency of anti-MAGE-3.A1 T cells was  $2.5 \times 10^{-6}$  of CD8 T cells in the blood, and it was 6-fold higher in an invaded lymph node. An antitumor CTL recognizing an Ag encoded by *MAGE-C2* showed a considerably greater enrichment. Whereas in the blood, the frequency of this CTL was  $9 \times 10^{-5}$ , it was  $\sim 1000$  times higher in the invaded lymph node. Several other antitumor T cell clonotypes also had frequencies  $>1\%$  and appeared to constitute the majority of the T cells present at this site. Similar findings were made on a regressing cutaneous metastasis. These results suggest that the anti-vaccine T cells may not be the principal effectors that kill the bulk of the tumor cells. They may exert their effect mainly by creating at the tumor site conditions enabling the stimulation of large numbers of T cells that are directed against other tumor Ags than those contained in the vaccine and then proceed to destroy the tumor cells.

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