A MAGE-3 Peptide Presented by HLA-DR1 to CD4$^+$ T Cells That Were Isolated from a Melanoma Patient Vaccinated with a MAGE-3 Protein[1]

Yi Zhang,* Pascal Chaux,[2]* Vincent Stroobant,* Alexander M. M. Eggermont,† Jurgen Corthals,‡ Bernard Maillère,§ Kris Thielemans,‡ Marie Marchand,* Thierry Boon,* and Pierre van der Bruggen[3]*

"Cancer-germline" genes such as those of the *MAGE* family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They encode shared tumor-specific Ags, which have been used in therapeutic vaccination trials of cancer patients. *MAGE-3* is expressed in 74% of metastatic melanoma and in 50% of carcinomas of esophagus, head and neck, bladder, and lung. We report here the identification of a new MAGE-3 peptide, which is recognized by three different CD4$^+$ T cell clones isolated from a melanoma patient vaccinated with a MAGE-3 protein. These clones, which express different TCRs, recognize on HLA-DR1 peptide ACYEFLWGPRALVETS, which corresponds to the MAGE-3$_{267–282}$ and the MAGE-12$_{267–282}$ protein sequences. One of the T cell clones, which expresses LFA-1 at a high level, lysed tumor cells expressing DR1 and MAGE-3. Another of these DR1-restricted CD4$^+$ clones recognized not only the MAGE-3/12 peptide but also homologous peptides encoded by genes *MAGE-1, 2, 4, 6, 10,* and *11*. *The Journal of Immunology,* 2003, 171: 219–225.

Human tumors bear Ags that are recognized by autologous T lymphocytes and that are highly specific for tumors. Some of these Ags are shared by many tumors of various histological types (1). Shared tumor-specific Ags, such as those encoded by gene *MAGE-3*,[4] are attractive candidates for therapeutic vaccination aimed at eliciting antitumoral T cell responses in cancer patients (2). First, the strict tumoral specificity of these Ags ought to ensure the absence of damage on normal tissues after immunization. Second, the presence of these Ags on many tumors makes it possible to apply the same vaccine to a large set of patients. Therefore, these Ags have been used for small-scale therapeutic vaccination trials of melanoma patients with detectable disease. The vaccines consisted of an antigenic peptide, a protein, a pox family recombinant virus carrying a MAGE-3 sequence, and dendritic cells (DCs) pulsed with an antigenic peptide (3–8). No significant toxicity was observed. Complete or partial clinical responses have been observed in only a small proportion of the patients, but the rate of tumor regressions appears to be well above the rates of spontaneous regressions that have been reported (9).

The use of defined antigenic peptides greatly facilitates the immunological monitoring because the presumed target of the antivaccine T cells is completely defined. It allows, for instance, the use of HLA-peptide tetramers, which are very sensitive tools for the detection of T cell responses in patients vaccinated with tumor-specific peptide (10, 11). Alternatively, a protein-based vaccine offers the advantage that antigenic peptides binding to a broad set of HLA molecules can be processed from the vaccine, so that the patients do not have to be selected according to their HLA. In a recently completed trial, patients with a measurable tumor expressing *MAGE-3* were vaccinated with escalating doses of a recombinant MAGE-3 protein combined for most of the patients with adjuvant SBAS-2 (8). The immunization schedule included four i.m. injections at 3-wk intervals of a fusion protein containing the MAGE-3 portion, a lipidated protein D derived from *Haemophilus influenzae* at its N terminus, and a sequence of several histidine residues at its C terminus (Prot.D/MAGE-3/His). Among the 33 melanoma patients who were evaluable for tumor response, we observed two partial responses, two mixed responses, and one stabilization. Interestingly, one of the partial responses occurred in a melanoma patient who did not receive the adjuvant. Thus, the Prot.D/MAGE-3/His protein given alone, without adjuvant, could also have the capacity to immunize, which might be due to the properties of its lipidated protein D.

This is presently investigated in a new trial in which the protein is given intradermally and subcutaneously without adjuvant. In this study, a partial response occurred upon vaccination: most of the in-transit and lymph node metastases of patient DDHK2 completely regressed after six injections of the MAGE-3 protein. Three different anti-MAGE-3 CD4$^+$ T cell clones were isolated from the blood cells of this patient. Here we are describing the identification of a new MAGE-3 antigenic peptide that is presented to these three T cell clones by HLA-DR1 molecules.

*Ludwig Institute for Cancer Research and Cellular Genetics Unit, Université de Louvain, Brussels, Belgium; †Rotterdam Cancer Institute and University Hospital, Rotterdam, The Netherlands; ‡Laboratory of Physiology, Medical School, Vrije Universiteit Brussel, Brussels, Belgium; and §Département d'Ingénierie et d'Etudes des Protéines, Commissariat à l'Energie Atomique-Saclay, Gif-sur-Yvette, France

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[2] Current address: Oncodesign S.A., Biotechnology, Parc Technologique Toison d'Or, 28 rue Louis de Broglie, F-21000 Dijon, France.

[3] Address correspondence and reprint requests to Dr. Pierre van der Bruggen, Ludwig Institute for Cancer Research, 74 avenue Hippocrate, UCL 74.59, B-1200 Brussels, Belgium. E-mail address: pierre.vanderbruggen@bru.licr.org

[4] Abbreviations used in this paper: DC, dendritic cell; EBV-B cell, EBV-transformed B cell; Ii, invariant chain; LNGFR, low-affinity nerve growth factor receptor; IRES, internal ribosome entry site.

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Materials and Methods

Melanoma patient DDHK2

Patient DDHK2 was included in clinical trial LUD 99–003. It was approved by the Protocol Review Committee of the Ludwig Institute for Cancer Research and by the ethics committee Commission d'Ethique Biomédicale Hospitalo-Facultaire de la Faculté de Médecine de l'Université de Louvain. The informed consent form was signed by the patient. Patient DDHK2 was vaccinated with recombinant protein Prot.D/MAGE-3/His, which was produced in *Escherichia coli* as a fusion protein with lipidated protein D derived from *H. influenzae* at the N terminus and a sequence of several histidine residues at the C terminus of the MAGE-3 protein. Injections were given without adjuvant at 3-wk intervals, intradermally and s.c. Blood cells were collected 2 wk after the fourth injection of the MAGE-3 protein.

Cell lines, media, and reagents

The EBV-transformed B (EBV-B) cell lines and the tumor cell lines were cultured in IMDM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Life Technologies), 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human rIL-2 was purchased from Eurocetus (Amsterdam, The Netherlands), IL-7 from Genzyme (Cambridge, MA), GM-CSF from Schering Plough (Brinny, Ireland), and TNF-α from R&D Systems (Abingdon, U.K.). Human recombinant IL-4, IL-6, and IL-12 were produced in our laboratory. Anti-HLA-DR Ab L243 was obtained from the American Type Culture Collection (Manassas, VA). Anti-HLA-DP Ab B7/21 and anti-HLA-DQ Ab SPV-L3 were kindly provided by A. Mulder (Department of Immunohematology, University of Leiden, Leiden, The Netherlands). The Abs were used at a 1/5 dilution of the culture supernatant.

MAGE-3 proteins

Two 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, San Diego, CA), as described previously (12). It will be referred to hereafter as protein MAGE-3^{insect}. The other MAGE-3 protein, hereafter referred to as protein MAGE-$3^{bacteria}$, was produced in *E. coli* by GlaxoSmithKline Biologicals (Rixensart, Belgium), as previously reported (13). It contains a sequence of several histidine residues at the N terminus of the protein. The recombinant MAGE-3 protein used in the vaccine is a fusion protein with a lipidated protein D derived from *H. influenzae* at its N terminus and a sequence of several histidine residues at the C terminus of the protein (Prot.D/MAGE-3/His). The inclusion of the first 109 residues of the protein D as a fusion partner was expected to improve the immunogenicity and to provide the vaccine protein with additional bystander help properties, whereas the inclusion of a His affinity tail facilitated the purification of the fusion protein. The protein was produced in *E. coli* and extensively purified to eliminate bacterial contaminants.

Construction of the retrovirus encoding MAGE-3, MAGE-1, MAGE-4, and invariant chain (Ii)-MAGE-3

The retroviral vectors encoding MAGE-1, 3, and 4 were derived from the LXSN backbone, an expression vector derived from Moloney murine leukemia virus (Clontech, Palo Alto, CA). Respectively, they encode the full-length MAGE-1, 3, and 4 under the control of the long terminal repeat and the truncated form of the human low-affinity nerve growth factor receptor (LNGFR) driven by the SV40 promoter. For transduction, EBV-B cell lines were cocultivated with irradiated Am12 vector-producing cells in the presence of polybrene (0.8 mg/ml) for 72 h. A pure population of transduced cells was obtained by immunoselection with anti-LNGFR mAb 20.4 (American Type Culture Collection) and goat anti-mouse IgG FITC (BD Biosciences, San Jose, CA). For producing the retrovirus encoding Ii-MAGE-3, the sequence encoding a truncated form of LNGFR was amplified from plasmid pUC19-ΔLNGFR, which was kindly provided by C. Traversari (Istituto Scientifico H.S. Raffaele, Milano, Italy). Briefly, LNGFR was ligated into pCR2.1 to an internal ribosome entry site (IRES) sequence, derived from the encephalomyocarditis virus. The IRES-ΔLNGFR sequence was then transferred into pMFG-Ii80, which encodes the first 80 aa of the human invariant chain (Ii80). A complete MAGE-3 cDNA was then ligated downstream Ii80 into pMFG-Ii80-IRES-ΔLNGFR, allowing the simultaneous expression of the Ii-MAGE-3 fusion protein and the truncated LNGFR receptor. The procedure for transducing cell lines has been described previously (14).

DCs and CD4$^+$ responder T cells

DCs were obtained by culturing monocytes in the presence of IL-4 (200 U/ml) and GM-CSF (70 ng/ml) in RPMI 1640 medium supplemented with asparagine-arginine-glutamine and 1% autologous plasma. One-fourth of the medium was replaced by fresh medium and cytokines every 2 days. On day 7, the nonadherent cell population was used as a source of enriched DCs, as described previously (15). Rosetted T cells were treated with NH_4Cl (160 mM) to lyse the sheep erythrocytes, and they were washed. CD4$^+$ T lymphocytes were isolated from rosetted T cells by positive selection using magnetic microbeads coupled to an anti-CD4 mAb (Miltenyi Biotech, Bergisch Gladbach, Germany) and by sorting through a MACS, as recommended by the manufacturer (Miltenyi Biotech).

Mixed lymphocyte/DC culture

DCs (5×10^5/ml) were incubated at 37°C, 5% CO_2, for 20 h in complete RPMI 1640 medium supplemented with IL-4, GM-CSF, and TNF-α (1 ng/ml) in the presence of MAGE-$3^{bacteria}$ (20 μg/ml). The small amount of TNF does not induce the maturation of DCs, as measured by CD83 expression. However, we cannot exclude that additional TNF produced by T cells or activation through CD40-CD40 ligand during the coculture led to DC maturation. Cells were washed and added at 10^4 per round-bottom microwell to 10^5 autologous CD4$^+$ T lymphocytes in 200 μl of IMDM supplemented with asparagine-arginine-glutamine and 1% autologous plasma in the presence of IL-6 (1000 U/ml) and IL-12 (10 ng/ml). The CD4$^+$ T lymphocytes were restimulated on days 7, 14, 21, and 28 with autologous DCs freshly loaded with MAGE-$3^{bacteria}$ and were grown in IMDM supplemented with asparagine-arginine-glutamine and 1% autologous plasma (hereafter referred to as complete IMDM) supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml). Aliquots of each microculture (~5,000 cells) were stimulated on day 42 with ~20,000 autologous EBV-B cells loaded for 20 h with 20 μg/ml MAGE-$3^{bacteria}$, MAGE-3^{insect}, or OVA. After 20 h of coculture in round-bottom microwells and in 100 μl of complete IMDM supplemented with IL-2 (25 U/ml), IFN-γ released in the supernatant was measured by ELISA using reagents from Medgenix Diagnostics-Biosource (Fleurus, Belgium).

CD4$^+$ T cell clones

Cells from positive microcultures were cloned by limiting dilution, using irradiated autologous EBV-B cells transduced with retro-Ii.MAGE-3 (5×10^3–2×10^4 cells) as stimulator cells. Irradiated allogeneic LG2-EBV cells (5×10^3–10^4) were used as feeder cells. CD4$^+$ T cell clones were supplemented once a week with fresh culture medium in the presence of IL-2 (50 U/ml), IL-7 (5 ng/ml), and IL-4 (5 U/ml). The cytokines produced by the CD4$^+$ T cell clones upon contact with the Ag were measured after overnight coculture using the Cytometric Bead Array kit from BD PharMingen.

TCR analysis

For TCR analysis, 3×10^5 cells from each clone were used for extracting RNA with the Tripure reagent (Boehringer Mannheim, Mannheim, Germany) and were converted to cDNA at 42°C for 90 min with 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies). TCR Vα and Vβ usage was assessed by PCR amplification by using a complete panel of Vα- or Vβ-specific sense primers and Cα and Cβ antisense primers, respectively (16). Primers were chosen on the basis of described panels of TCR V region oligonucleotides and with alignments of TCR sequences available at the International Immunogenetics Database (http://imgt.cines.fr). Each PCR product was purified and sequenced to obtain a complete identification of the CDR3 region.

Recognition assays with peptides

Peptides were synthesized on solid phase using 9-fluorenylmethoxycarbonyl chemistry for transient NH_2-terminal protection and were characterized using mass spectrometry. All peptides were >90% pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved at 5 mg/ml in 10 mM acetic acid and 10% DMSO and were stored at −20°C. EBV-B cells were distributed at 20,000 cells per round-bottom microwell and were incubated for 2 h at 37°C in the presence of the different peptides, the indicated concentrations representing their concentrations during the incubation step. CD4$^+$ T lymphocytes (5000) were added in 100 μl of complete IMDM (Life Technologies) supplemented with IL-2 (25 U/ml). Supernatants were harvested after 20 h of coculture, and IFN-γ production was measured by ELISA. The peptides used in Fig. 4*A* correspond to the MAGE-$1_{260–275}$, MAGE-$2_{267–282}$, MAGE-$3_{267–282}$, MAGE-$4_{268–283}$, MAGE-$6_{267–282}$, MAGE-$10_{292–307}$, MAGE-$11_{270–285}$, and MAGE-$12_{267–282}$ protein sequences.

Recognition assays with cell lysates

EBV-B cells (5×10^4) and tumor cells (5×10^4) were lysed in 50 μl of complete RPMI 1640 by three cycles of rapid freeze-thawing. HLA-DR1 monocyte-derived DCs (2.5×10^4) were then added to the lysates in 150 μl of complete RPMI 1640 supplemented with IL-4 (100 U/ml) and GM-CSF (70 ng/ml) and were kept at 37°C for 24 h. DCs were washed and 5000 $CD4^+$ lymphocytes were added in 150 μl of complete IMDM supplemented with IL-2 (25 U/ml). Supernatants were harvested after 20 h of coculture, and IFN-γ production was measured by ELISA. Experiments with lysates of tumor cells were performed with autologous DCs, and the experiments with lysates of EBV-B cells were performed with DR1-matched DCs.

Recognition of tumor cells

Tumor cells were distributed at 20,000 cells per round-bottom microwell together with 5,000 $CD4^+$ T lymphocytes in 100 μl of complete IMDM in the presence of IL-2 (25 U/ml). Supernatants were harvested after 20 h of coculture, and IFN-γ production was measured by ELISA. For measuring lytic activity, cells were labeled with 100 μCi of $Na^{51}CrO_4$, and 1000 targets were added to the T cells at different E:T ratios. Chromium release was measured after 4 h of incubation at 37°C.

HLA-DR peptide binding assay

Purification of HLA-DR molecules and peptide binding assays was performed as previously described (17, 18). Briefly, HLA-DR molecules were purified from EBV-B homozygous cell lines by affinity chromatography. They were incubated with different concentrations of competitor peptide and an appropriate biotinylated peptide. The biotinylated peptides were the following: HA 306–318 (PKYVKQNTLKLAT) for DRB1*0101 (1 nM, pH 6), DRB1*0401 (30 nM, pH 6), DRB1*1101 (20 nM, pH 5), and DRB5*0101 (5 nM, pH 5.5); YKL (AAYAAAKAAALAA) for DRB1*0701 (10 nM, pH 5); A3 152–166 (EAEQLRAYLDGTGVE) for DRB1*1501 (10 nM, pH 4.5); MT 2–16 (AK TIAYDEEARRGLE) for DRB1*0301 (100 nM, pH 4.5); B1 21–36 (TERVR LVTRHIYNREE) for DRB1*1301 (200 nM, pH 4.5); and LOL 191–210 (ES WGAVWRIDTPDKLTGPFT) for DRB3*0101 (5 nM, pH 5.5). The binding was evaluated in a fluorescence assay. Data were expressed as the peptide concentration that prevented binding of 50% of the labeled peptide (IC_{50}). Averages were obtained from at least two independent experiments. Unlabeled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment.

Results

Derivation of anti-MAGE-3 $CD4^+$ T cell clones

Monocyte-derived DCs of patient DDHK2 were loaded overnight with a MAGE-3 protein produced in bacteria ($MAGE\text{-}3^{bacteria}$). In two independent experiments, a total of 192 microcultures of 10^5 $CD4^+$ T cells and 10^4 stimulator DCs were set up. To favor the activation of Th1 lymphocytes, the culture medium was supplemented with IL-12. After four weekly restimulations with protein-loaded DCs, the responder cells were tested for their ability to secrete IFN-γ upon stimulation with the Ag. Considering that a large proportion of the $CD4^+$ T cells obtained in our initial experiments appeared to be directed against bacterial contaminants, we used a protein produced in insect cells ($MAGE\text{-}3^{insect}$) for this test. Seven microcultures that specifically produced IFN-γ were cloned and restimulated with autologous EBV-B cells transduced with a retroviral construct encoding a truncated human invariant chain (Ii) fused with the MAGE-3 protein (retro-Ii.MAGE-3) (13, 19). In this chimeric protein, signals within the Ii should target the MAGE-3 protein to the class II Ag-processing compartments (19).

Anti-MAGE-3 $CD4^+$ T cell clones were obtained from only three of the seven microcultures that were cloned. These clones recognized autologous EBV-B cells either loaded with 20 μg/ml of protein $MAGE\text{-}3^{bacteria}$ or $MAGE\text{-}3^{insect}$ or transduced with retro-Ii.MAGE-3 (Fig. 1*A*). Each of these three clones was able to lyse EBV-B cells expressing the Ii-MAGE-3 fusion protein (Fig. 1*B*). They produced high amounts of IFN-γ and TNF upon contact with the Ag and smaller amounts of IL-2, IL-4, and IL-10 (Fig. 1*C*). Each of these three clones had a different TCR (Table I).

Identification of the antigenic peptides

We tested for recognition by each of the three clones autologous EBV-B cells pulsed with a set of peptides of 16 aa, overlapping by 12 residues and covering the complete MAGE-3 sequence. Two overlapping peptides, GSDPACYEFLWGPRAL ($MAGE\text{-}3_{263–278}$) and

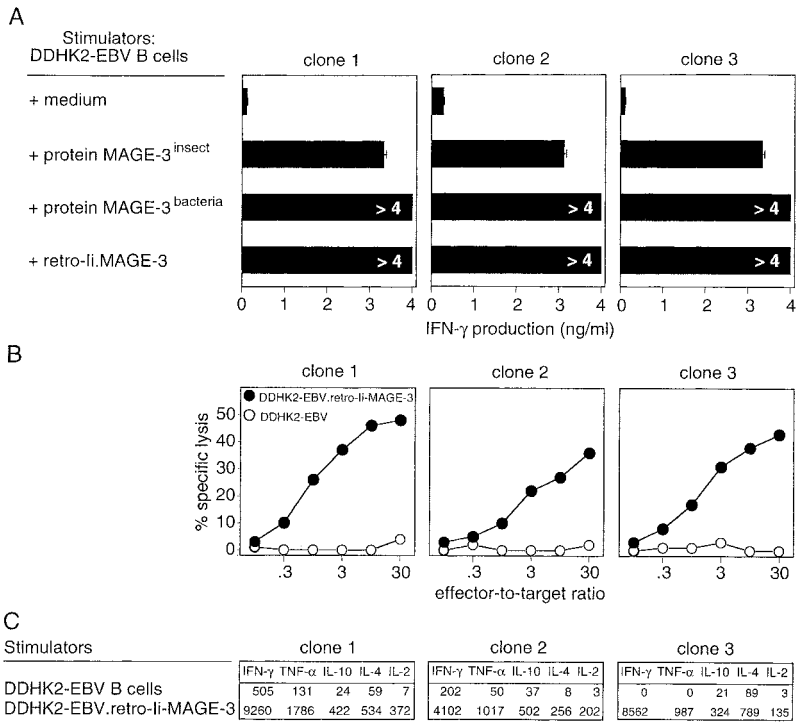

FIGURE 1. Recognition of a MAGE-3-derived Ag by three $CD4^+$ T cell clones. *A*, Stimulator cells were the autologous EBV-B cells either loaded with 20 μg/ml protein $MAGE\text{-}3^{bacteria}$ or $MAGE\text{-}3^{insect}$ or transduced with a retroviral construct encoding a truncated human Ii fused with the MAGE-3 protein (retro-Ii.MAGE-3). Stimulator cells (20,000) were cocultured overnight with 5,000 $CD4^+$ T cells. The concentration of IFN-γ produced in the medium was measured by ELISA. The results shown represent an average of triplicate cocultures. *B*, HLA-DR1 DDHK2-EBV B cells are autologous to the $CD4^+$ T cells. DDHK2-EBV.retro-Ii.MAGE-3 were obtained by transduction of DDHK2-EBV with a retroviral construct encoding a truncated human Ii fused with the MAGE-3 protein (retro-Ii.MAGE-3). Targets were chromium labeled for 1 h and incubated for 4 h with the CTL at indicated E:T ratios. *C*, Stimulator cells (20,000) were cocultured overnight with 5,000 $CD4^+$ T cells in a total volume of 150 μl. The concentration of cytokines produced in the medium was measured by cytometric bead array analysis. Numbers represent pg/ml.

Table I. *TCR sequences of the three anti-MAGE-3 CD4$^+$ T cell clones*[a]

Clone	TCRα V	CDR3	J
α chain			
Clone 1	V9-2*01-J49*01	TGTGCTCTTGAGAACACCGGTAACCAGTTCTATTTTGGG C A L E N T G N Q F Y F G	ACAGGGACAAGTTTGACGGTCATT T G T S L T V I
Clone 2	V36/DV7*04-J37*01	TGTGCTGTGGTGTCTGGCAACACAGGCAAACTAATCTTTGGG C A V V S G N T G K L I F G	CAAGGGACAACTTTACAAGTAAAA Q G T T L Q G I
Clone 3	V27*04-J52*01	TGTGCAGGAAGGGGAAGAGGTACTAGCTATGGAAAGCTGACATTTGGA C A G R G E G T S Y G G K L T F G	CAAGGGACCATCTTGACTGTCCAT Q G T I L T V H
β chain	TCRβ V	CDR3	J
Clone 1	V28*01-J2-2*01	TGTGCCAGCAGACCCTTTCCGGGGAGCTGTTTTTGGA C A S R F F P G E L F F G	GAAGGCTCTAGGCTGACCGTACTG E G S R L T V L
Clone 2	V9*01-J2-1*01	TGTGCCAGCAGCGTGTACTCCAATGAGCAGTTCTTCGGG C A S S V Y S N E Q F F G	CCAGGGACACGGCTCACCGTGCTA F G T R L T V L
Clone 3	V6-3*01-J1-2*01	TGTGCCAGCAGTCTGACAGGGACCAACTATGGCTACACCTTCGGT C A S S L T G T N Y G Y T F G	TCGGGGACCAGGTTAACCGTTGTA S G T R L T V G

[a] V and J rearrangements were attributed according to the nomenclature available at http://imgt.cines.fr.

FIGURE 2. Recognition of peptide ACYEFLWGPRALVETS by each of the three CD4$^+$ clones. DDHK2 EBV-B cells were distributed in microwells (2×10^4 cells) and incubated for 2 h with the indicated concentrations of peptides. A total of 5×10^3 cells from each autologous CD4$^+$ T cell clone was added, and the presence of IFN-γ in the supernatant was measured by ELISA after overnight coculture. The results shown represent an average of triplicate cocultures.

ACYEFLWGPRALVETS (MAGE-$3_{267–282}$), stimulated the production of IFN-γ by all three clones (Fig. 2). The latter peptide is also encoded by MAGE-12 (MAGE-$12_{267–282}$). Testing shorter peptides indicated that the most efficiently recognized was slightly different for each clone: ACYEFLWGPRALVETS for clone 1, ACYEFLWG PRALVE for clone 2, and both ACYEFLWGPRALVET and ACY EFLWGPRALVETS for clone 3 (Fig. 2).

The antigenic peptide is presented to T cells by HLA-DR1 molecules

For each of the three clones, the recognition of autologous EBV-B cells loaded with peptide MAGE-$3_{267–282}$ was abolished by an anti-HLA-DR Ab, but not by Abs against HLA-DP or HLA-DQ (data not shown). Patient DDHK2 was typed HLA-DR1, DR15, and DR51. Peptide ACYEFLWGPRALVETS was loaded on several EBV-B cell lines sharing HLA-DR molecules with patient

Table II. *MAGE-3 peptide ACYEFLWGPRALVETS is presented by HLA-DR1*[a]

EBV-B Cell Line	Serological Specificity	IFN-γ Production (pg/ml) Clone 1	Clone 2	Clone 3
DR1 positive				
DDHK2	DR1 DR15 DR51	>4000	>4000	>4000
LB1158	DR1 DR13 DR52	2439	2029	1698
LB831	DR1 DR7 DR53	2037	1418	1371
LB2138	DR1 DR13	1810	2046	2053
DR1 negative				
LB650	DR7 DR15 DR51 DR53	83	160	150
LB1870	DR15 DR53 DR7	88	104	119
LB1856	DR15	49	187	164
LB2095	DR13 DR15 DR51 DR52	22	172	125

[a] EBV-B cells were incubated for 2 h with 5 μg/ml peptide ACYEFLWGPRAL VETS, washed, and incubated (20,000 cells) with 5,000 cells of clone 1, 2, or 3. IFN-γ production was measured by ELISA after overnight coculture. The results shown represent the average of triplicate cocultures.

Table III. *Binding of peptide MAGE-3$_{267–282}$ to multiple HLA-DR molecules*[a]

HLA-DR Alleles	IC_{50} (nM) of Reference Peptides[b]	IC_{50} (nM) of MAGE-3$_{267–282}$	Ratios (IC_{50} MAGE-3/ IC_{50} Reference)
DR1	2 (±1)	0.4 (±0.2)	0.2
DR3	350 (±0)	>10,000	
DR4	40 (±4)	730 (±4)	18
DR7	10 (±3)	1,200 (±350)	120
DR11	25 (±0)	150 (±0)	6
DR13	390 (±140)	>10,000	
DR15	15 (±0)	220 (±35)	15
DRB3	20 (±0)	>10,000	
DRB4	40 (±19)	2,800 (±1,400)	70
DRB5	15 (±6)	170 (±140)	11

[a] The capacity of the peptide MAGE-3$_{267–282}$ to bind multiple HLA-DR molecules was investigated on purified DR molecules in competition assays using fluorescent reference peptides (±SD). To facilitate the comparison, data are also presented as the ratio between the IC_{50} of the MAGE peptide and that of the reference peptide. A ratio <10 indicates peptides with a high affinity for a given HLA class II molecule, whereas a ratio >10 corresponds to intermediate binders.

[b] Reference peptides are described in *Materials and Methods*.

DDHK2. All and only those expressing DR1 were recognized by the three clones when loaded with the peptide (Table II).

The binding of peptide MAGE-3$_{267–289}$ to purified HLA-DR molecules of various allotypes was tested in competition assays using HLA-DR-specific biotinylated peptides (Table III). The MAGE-3 peptide displayed a very high affinity for DR1, binding more efficiently than the reference peptide (20). It also bound to the following molecules, listed by decreasing affinity: DR11, DRB5, DR15, DR4, DRB4, and DR7. No binding was observed on DR3, DR13, and DRB3.

Recognition of tumor cells

Proteins that carry an endosomal targeting sequence are directed to the endosomes, enabling the cell to present on class II molecule peptides derived from internal proteins. For instance, some melanocytic proteins are specifically targeted to melanosomes, which are organelles derived from the endocytic compartment (21). MAGE proteins do not contain signal sequences or endosomal targeting sequences. Therefore, class II-restricted MAGE peptides are not expected to be presented by the tumors expressing *MAGE*.

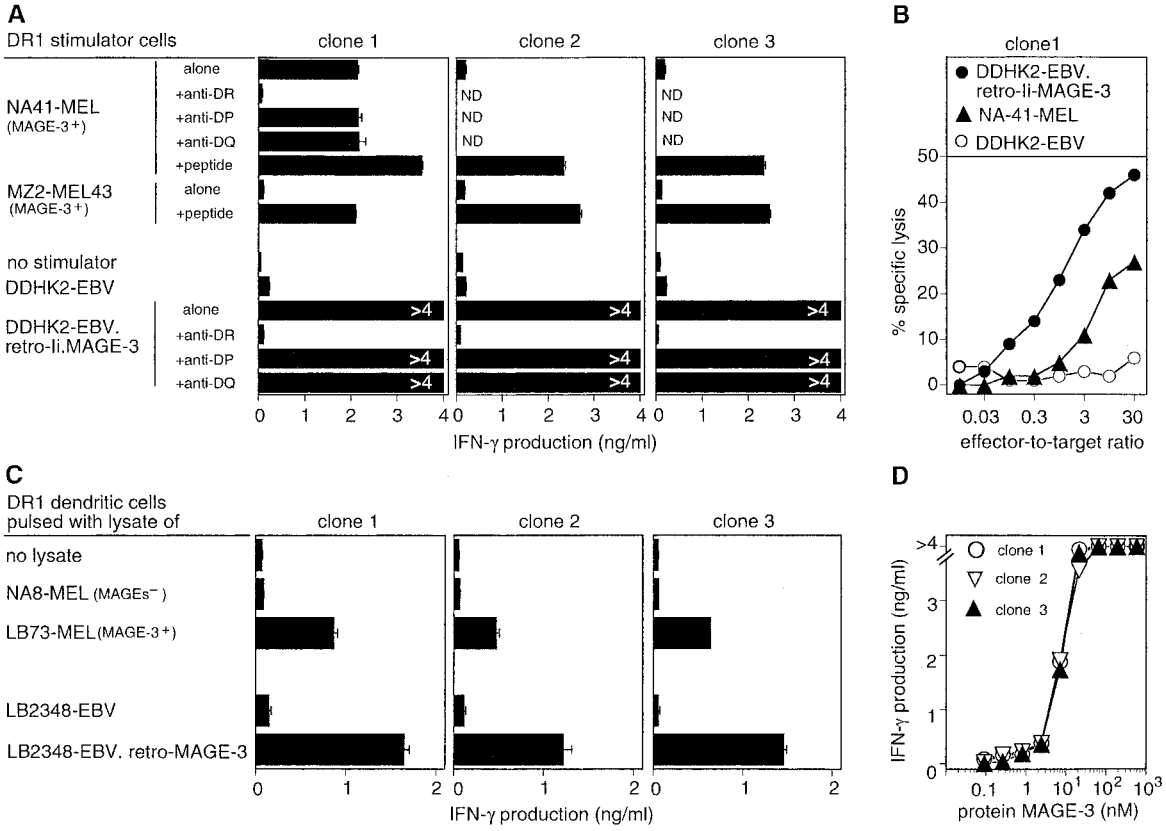

FIGURE 3. Recognition of tumor cell lines and of DCs loaded with tumor-derived MAGE-3. HLA-DR1 melanoma lines NA41-MEL and MZ2-MEL.43 express *MAGE-3*. HLA-DR1 DDHK2-EBV B cells are autologous to the $CD4^+$ T cells. DDHK2-EBV.retro-Ii.MAGE-3 were obtained by transduction of DDHK2-EBV with a retroviral construct encoding a truncated human Ii fused with the MAGE-3 protein (retro-Ii.MAGE-3). *A*, Direct recognition of tumor cell lines. Cells were distributed in flat-bottom microwells (2×10^4 cells per well) and, if indicated, pulsed for 2 h with 5 μg/ml peptide ACYEFLWGPRALVETS and washed. If indicated, mAbs directed against HLA-DR, DP, or DQ were added in the medium. Five thousand $CD4^+$ T cells were added to the stimulator cells. IFN-γ production was measured by ELISA after 20 h of coculture. The results shown represent an average and SD of triplicate cocultures. ND, No data. *B*, Lysis of a tumor cell line by clone 1. Targets were chromium labeled for 1 h and incubated for 4 h with the CTL at indicated E:T ratios. The results shown represent an average of triplicates. *C*, Recognition of DCs loaded with tumor-derived MAGE-3. The tumor cells and EBV-B cells do not express DR1 and were lysed by freeze-thawing. Melanoma cell line LB73-MEL expresses *MAGE-3*, whereas NA8-MEL does not express *MAGE-3* or other MAGE-A genes. HLA-DR1 DCs (2.5×10^4 cells per well) were cultured for 24 h with lysates at the equivalent of two cells per DC. After washing, the DCs were incubated with 5×10^3 T cells per well. IFN-γ production was measured after 20 h by ELISA. The results shown represent an average of triplicate cocultures. *D*, Titration of the MAGE-3 protein. HLA-DR1 DCs (2×10^4 cells per flat-bottom microwell) were cultured for 24 h with different concentrations of the MAGE-$3^{bacteria}$ protein, washed, and incubated with 5×10^3 cells per well from each $CD4^+$ T cell clone. IFN-γ production was measured by ELISA after 20 h of coculture. The results shown represent an average and SD of triplicate cocultures.

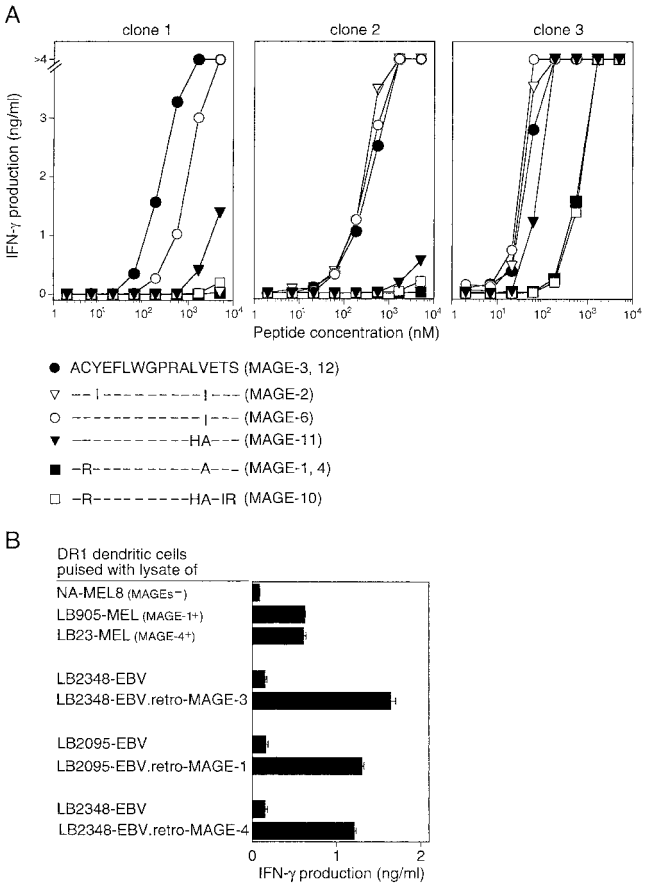

FIGURE 4. Recognition of different MAGE peptides by the three CD4$^+$ T cell clones. *A*, DDHK2-EBV B cells (2×10^4) were incubated for 2 h with the indicated peptides. A total of 5×10^3 cells from each autologous CD4$^+$ T cell clone was added, and IFN-γ production in the supernatant was measured by ELISA after overnight coculture. *B*, Recognition of DCs loaded with tumor-derived MAGE proteins. The tumor cells and EBV-B cells do not express DR1 and were lysed by freeze-thawing. Melanoma cell line NA8-MEL does not express *MAGE-3* or other MAGE-A genes. LB905-MEL expresses *MAGE-1* but not other MAGE-A genes; LB23-MEL expresses *MAGE-4* but not other MAGE-A genes. HLA-DR1 DCs (2.5×10^4 cells per well) were cultured for 24 h with lysates at the equivalent of two cells per DC. After washing, the DCs were incubated with 5×10^3 T cells per well. IFN-γ production was measured after 20 h by ELISA. The results shown represent an average of triplicate cocultures.

In accordance with this, we described two DR13-restricted MAGE-3 peptides and one DR15-restricted MAGE-1 peptide that were not recognized by CD4$^+$ T cell clones at the surface of tumor cells (13, 22). However, we and others have identified MAGE-3 peptides presented by DR11 and DP4 that were recognized on tumor cells expressing MAGE-3 (12, 23). How these peptides are processed and presented is unclear.

Here, we tested the production of IFN-γ by the three CD4$^+$ clones stimulated by the melanoma lines NA41-MEL and MZ2-MEL.43, which express the *DR1* and *MAGE-3* genes. Clone 1 recognized the NA41-MEL cells, indicating that MAGE-3 antigenic peptide can be naturally processed and presented by melanoma cells (Fig. 3*A*). Recognition of NA41-MEL cells was abolished by an anti-HLA-DR Ab, but not by Abs against HLA-DP or HLA-DQ. This CD4$^+$ clone also lysed NA41-MEL cells and the autologous EBV-B cells that were transduced with retro-Ii. MAGE-3 (Fig. 3*B*). The absence of recognition of MZ2-MEL.43 cannot be explained by a lower level of expression of *MAGE-3*, because expression was equivalent in NA41-MEL and MZ2-MEL.43, as measured by semiquantitative RT-PCR (data not shown). The two other CD4$^+$ clones were not stimulated by the melanoma lines, although the avidity of the three clones was equivalent. We compared the three clones by testing their ability to be stimulated by DCs loaded with decreasing concentrations of protein MAGE-3, and we observed that cells incubated with <10 nM protein stimulated the three clones to produce 2 ng/ml IFN-γ (Fig. 3*D*). We tentatively concluded that the direct recognition of tumors is not only dependent on the adequate expression of MAGE-3 or a high avidity of the CD4$^+$ T cell clone.

Stimulation of CD4$^+$ T cells can also occur via presentation of tumor debris by APCs. Here, we have pulsed DR1 DCs with lysates of DR1^{NEG} cells expressing MAGE-3. Each of the three CD4$^+$ T cell clones released IFN-γ in response to tumor-derived MAGE-3 processed by the DR1 DCs (Fig. 3*C*). They also specifically recognized the DCs pulsed with a lysate of cells transduced with a retrovirus construct containing the coding sequence of MAGE-3.

Recognition of the homologous MAGE peptides

Peptide ACYEFLWGPRALVETS is encoded by *MAGE-3* and *MAGE-12*. Homologous peptides encoded by other *MAGE* genes are only slightly different from the MAGE-3/12 peptide (Fig. 4*A*). All of the peptides share the central part EFLWGPRA. They were tested for their ability to stimulate the CD4 clones. The MAGE-2 and MAGE-6 peptides were recognized by clones 2 and 3 as efficiently as was the MAGE-3 peptide, despite the replacement of a tyrosine or a valine by isoleucines (Fig. 4*A*). The MAGE-11 peptide was recognized by clone 3, despite the replacement of 2 aa. The MAGE-1, MAGE-4, and MAGE-10 peptides were also recognized by clone 3, but only when 20 times higher peptide concentrations were used. DR1 DCs were incubated with lysates of EBV-B cells expressing *MAGE-1*, *3*, or *4* and were tested for their ability to stimulate clone 3 to produce IFN-γ (Fig. 4*B*). As expected, clone 3 was stimulated with cells pulsed with lysates of *MAGE-3*-expressing cells. Importantly, it was also stimulated with cells pulsed with lysates of MAGE-1- or MAGE-4-expressing cells, demonstrating that clone 3 is able to recognize naturally processed MAGE-1 or MAGE-4 proteins and not only synthetic peptides.

Discussion

The new MAGE-3 antigenic peptide described here, ACYEFL WGPRALYETS (MAGE-$3_{267-282}$), was presented to three CD4$^+$ T cell clones by HLA-DR1 molecules, which are expressed in ~18% of Caucasians, ~11% of Africans, and ~6% of Asians. This peptide is also encoded by *MAGE-12*. It can also bind efficiently to HLA-DR11 and to a lesser extent to DRB5, DR15, DR4, DRB4, and DR7 molecules. Interestingly, the homologous peptides encoded by *MAGE-1*, *2*, *4*, *6*, *10*, or *11* are only slightly different from the MAGE-3 peptide. Because these homologous peptides are also recognized by one of the CD4$^+$ T cell clones, we can suppose that such T cells may also be activated in patients injected with the MAGE-3 peptide or a MAGE-3 protein. These T cells will be able to recognize not only the *MAGE-3*-expressing cells, but also other tumor cells that do not express *MAGE-3* or that have lost the expression of *MAGE-3*, provided that they express one of the other *MAGE* genes. The activation of these T cells should reduce the emergence of Ag-loss variants in vaccinated patients.

Stimulation of the CD4$^+$ antitumoral T cells could result from the recognition of tumor Ags on macrophages or DCs that have endocytosed tumor cell debris. This recognition could stimulate the CD4$^+$ T cells to provide "help" that would magnify the CTL

response (24). We have shown here that anti-MAGE-3.DR1 $CD4^+$ T cells can recognize DCs loaded with lysates. Another mode of action of $CD4^+$ T cells could result from a direct recognition of Ags presented by class II molecules on the surface of the tumor cells, resulting in the destruction of these cells. Tumor cells of many types have been shown to bear class II molecules (25, 26). We have also shown here that some anti-MAGE-3.DR1 $CD4^+$ T cells can recognize and lyse NA41-MEL cells, which express *MAGE-3*.

The three $CD4^+$ T cell clones were unable to recognize MZ2-MEL.43 cells, which also express *MAGE-3*. Preliminary experiments have shown that adhesion molecule ICAM-1 was less expressed on MZ2-MEL.43 than on NA41-MEL, which was recognized by clone 1 (data not shown). Moreover, LFA-1, the ligand of ICAM-1, was more highly expressed on clone 1, the only clone that directly recognized NA41-MEL (data not shown). This suggests that the direct recognition of tumor cells is not only dependent on an adequate expression of MAGE-3. A high expression of adhesion molecules seems to also be required, on both the T cells (LFA-1) and the tumor cells (ICAM-1).

The first clinical trial with HLA-class II-restricted MAGE peptides was reported recently. Mature monocyte-derived DCs were loaded with keyhole limpet hemocyanin and MAGE-3.DP4, MAGE-3.DR13, tyrosinase.DR4, or gp100.DR4 peptide. Autologous Ag-loaded DCs were injected in metastatic cancer patients (7). Eight of 16 evaluable patients exhibited stable disease, and one patient experienced a complete regression of metastases in lung and pancreas. IFN-γ-producing cells were detected by ELISPOT analyses in most patients, after in vitro stimulation of the blood cells with the MAGE-3 peptides, but not when cells were stimulated with the tyrosinase or the gp100 peptide.

It is interesting to note here that patient DDHK2 was injected with the entire MAGE-3 protein and that we isolated $CD4^+$ T cells that were specific for only one MAGE-3 peptide, despite the use of DCs loaded with the entire MAGE-3 protein as stimulators. Therefore, this peptide might be a immunodominant peptide in HLA-DR1 patients. This hypothesis could be tested by the analysis of the immune response of several other DR1 patients vaccinated with a MAGE-3 protein.

A possibility for this monitoring is the use of the peptide that can be used either to select and amplify peptide-specific T cells in vitro or to directly label TCRs with soluble HLA class II tetramers presenting the relevant peptide (27). A reliable and quantitative monitoring of the specific $CD4^+$ T cell response will be important for the improvement of vaccination strategies. This is difficult because these responses do not appear to be massive, as suggested by a quantitative monitoring of the $CD8^+$ T cell response directed against a MAGE-3.A1 peptide (11).

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