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Identification of a Promiscuous T-Cell Epitope Encoded by Multiple Members of the *MAGE* Family[1]

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ABSTRACT

One of the major limitations of tumor-specific vaccination is the generation of antigen-loss variants that are able to escape the immune response elicited by a monoantigenic peptide epitope. Here, we report the identification of a new HLA-B*3701-restricted epitope shared by four different members of the *MAGE* family. Peripheral blood lymphocytes isolated from a melanoma patient were stimulated *in vitro* with the autologous HLA-negative melanoma line transfected with autologous HLA B*3701 molecule. This protocol led to the induction of tumor-specific, B*3701-restricted CTLs specific for a peptide epitope encoded by codons 127–136 of the gene *MAGE-1*. The same epitope is also encoded by the homologous region of three other members of the *MAGE* family, *MAGE-2*, *-3*, and *-6*. Consistent with the notion that the peptide encoded by *MAGE-1* codons 127–136 is, indeed, processed from the proteins encoded by all four *MAGE* family members, the CTLs were able to specifically recognize Cos-7 cells cotransfected with HLA-B*3701 and any of these *MAGE* genes. Moreover, the CTLs also recognized a *MAGE-6*-positive melanoma line transfected with the B*3701 molecule. These findings allow the inclusion of a new set of tumor patients into clinical cancer vaccination trials. Furthermore, they suggest that some promiscuous peptide epitopes shared by different members of the *MAGE* family might be less prone to escape the immune response by generation of *MAGE* antigen loss variants.

INTRODUCTION

A number of studies have demonstrated that CTLs recognizing human tumor cells can be isolated from PBLs[3] and tumor-infiltrating lymphocytes of cancer patients (1). An *in vivo* role of such tumor-specific effectors is suggested by the results of several immunotherapy trials, mainly performed in melanoma patients (2–5).

Over the last few years, the use of such tumor-specific effectors has allowed the isolation of several genes encoding tumor antigens (6). According to the pattern of expression in neoplastic and normal tissues, these antigens can be classified into four classes, which have different degrees of tumor specificity and clinical relevance. The first class comprises antigens encoded by genes expressed in various tumors of different histotypes but not in normal tissues, other than testis and placenta, such as *MAGE*, *GAGE*, and *BAGE* (7–9). The second class represents differentiation antigens that are only expressed in melanoma and melanocytes, such as *tyrosinase*, *Melan-A/MART-1*, *gp100*, *TRP-1*, and *TRP-2* (10–15). The antigens belonging to the third class are generated by point mutations in genes that are ubiquitously expressed (16–18). The fourth class of antigens, which has been defined only recently, is represented by *TRP-2-INT2*, an antigen shared between melanomas but not expressed in normal cells of the melanocytic lineage (19).

Tumor antigens belonging to the *MAGE* family have especially elicited considerable interest because six of them, *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12*, are expressed selectively by a significant proportion of primary and metastatic tumors, including melanomas, lung, bladder, ovarian, and breast carcinomas (7, 20–23).

Clinical trials of vaccination, based on the use of antigenic peptides encoded by *MAGE-1* and *-3*, are in progress in patients affected by melanoma and other neoplastic diseases (3). Nevertheless, the use of *MAGE-2*, *-4*, and *-6* proteins as targets for tumor-specific immunotherapy has been hampered by the uncertainty as to whether these proteins can be recognized by specific CTLs of the immune system.

Potential limitations to the wide application of this therapeutic approach are the limited number of characterized CTL epitopes (*i.e.*, tumor antigen peptides and appropriate HLA class I alleles) and the *in vivo* generation of antigen loss variants that are able to escape the immune response elicited by a monoantigenic vaccine (24). Indeed, such clinical protocols apply only to patients carrying a tumor expressing a well-known tumor antigen and a defined HLA allele. Unfortunately, a large majority of cancer patients do not fulfill these including criteria. Therefore, identification of new antigenic determinants is a priority because it would increase the number of patients that could benefit from antitumor vaccination protocols.

Here, we report the identification of a new HLA-B*3701-restricted epitope, encoded by homologous regions of the *MAGE-1*, *-2*, *-3*, and *-6* genes. The use of identical antigenic peptides derived from different highly homologous proteins, in protocols of vaccination, may represent a useful tool to avoid the generation of antigen loss variants. Moreover, this study presents, for the first time, evidence for the existence of human CTL recognizing peptides derived from MAGE-2 and -6 proteins, which now can be included in the list of possible antigens for targeted immunotherapy of neoplastic disorders.

MATERIALS AND METHODS

Cell Lines. The melanoma cell line MSR3-mel was established in our laboratory from a metastatic lesion of patient MSR3 and cultured in IMDM supplemented with 10% FCS. PBLs of this patient were serologically typed as: HLA-A1, A11, B37, B5, Cw6. The melanoma line MZ2-MEL.2.2 ET.1 (HLA-A1, B*3701, Cw6), hereafter referred to as ET1, and the Cos-7 cell line were kindly provided by Prof. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium) and maintained in DMEM supplemented with 10% FCS. Me14932 (HLA-A2/A3, B7/Bw50, Cw6/Cw7) was a kind gift of Dr. G. Parmiani (Istituto dei Tumori, Milano, Italy). The B-lymphoblastoid cell line LG2-EBV was kindly provided by Prof. T. Boon, whereas the MSR3-EBV was derived by transformation of peripheral blood B lymphocytes from patient MSR3 with the B95-8 strain of EBV.

Synthetic Peptides. Synthetic peptides were purchased from Primm (Milano, Italy). Peptides were: $MAGE_{127–136}$ (REPVTKAEML), encoded by codons 127–136 of *MAGE-1*, *-2*, *-3*, and *-6* genes; and $M4_{127–136}$ (KELVTKAEML) and $M12_{127–136}$ (REPFTKAEML), corresponding to amino acids 127–136 encoded by genes *MAGE-4* and *-12*, respectively. Peptides were dissolved to 10 mM in DMSO and diluted further in 0.9% NaCl.

Subcloning of the *HLA-B*3701* Allele. Total RNA was prepared from MSR3 PBLs by the RNeasy Total RNA Kit (Qiagen, Hilden, Germany). cDNA corresponding to 300 ng of total RNA was amplified by PCR using a primer pair suitable for specific amplification and directional cloning of the full-length

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[3] The abbreviations used are: PBL, peripheral blood lymphocyte; IMDM, Iscove's modified Dulbecco's medium; mAb, monoclonal antibody; IL, interleukin; LNGFR, low-affinity nerve growth factor receptor; β2-m, β2-microglobulin.

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coding region of *HLA-B* alleles (generous gift of Dr. Soo Young Yang, Memorial Sloan-Kettering Cancer Center, New York, NY). The 1.1-kb PCR product was subcloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen Corp., Oxon, United Kingdom). Plasmid clones encoding HLA-B*3701 and -B*52011 (the *HLA-B37* and *-B5* alleles of patient MSR3) were identified using diagnostic restriction enzymes. The *HLA-B*3701* gene was then sequenced to verify the correspondence to the published DNA sequence. This plasmid was called pcDNA3.1/B*3701.

Transfection of Melanoma Cell Lines. Melanoma cell lines were transfected by the calcium phosphate precipitation technique with pcDNA3.1/HLA-B*3701 and selected in G418. Expression of the transfected HLA-B*3701 molecule in stable transfectants was verified by flow cytometry with the HLA-A-, HLA-B-, and HLA-C-specific mAb W6/32.

***In Vitro* Induction of CTL Line 337.** CTL line 337 was obtained using a protocol previously described by others (25), with slight modifications. Briefly, PBLs from patient MSR3 were separated by Ficoll gradient and cultivated (1×10^6–2×10^6 cells/well) with the autologous, irradiated MSR3-B37 melanoma cells (0.5×10^5–1×10^5 cells/well) in 2 ml of IMDM supplemented with 10% human serum, glutamine, and antibiotics. After 3 days of culture, 10 units/ml IL-2 (Chiron, Milan, Italy) and 5 ng/ml IL-7 (Genzyme Corp., Cambridge, MA) were added. Lymphocytes were restimulated weekly with 0.5×10^5 irradiated MSR3-B37 cells and tested in a cytotoxicity assay after three stimulations. After the fifth restimulation, 2×10^6 irradiated LG2-EBV cells were added as feeder cells, and IL-2 was increased to 50 units/ml.

Assay for Cytolytic Activity and Peptide Binding Studies. Lytic activity of the cytotoxic T-cell lines was tested in a chromium release assay as described previously (26). Peptides were tested in chromium release assays: ^{51}Cr-labeled target cells were incubated for 1 h at room temperature in 96-well microplates with various concentration of the peptide before addition of effector cells at a fixed E:T ratio. Binding of peptides $M4_{127–136}$ and $M12_{127–136}$ to the HLA-B*3701 molecule was studied in a competition assay, as described previously (27). As standard peptide, we used peptide $MAGE_{127–136}$ (300 nM), recognized by CTL 337. CTLs were used at an E:T ratio of 30:1.

Production of Subfragments of *MAGE-1*. Subfragments of *MAGE-1* gene (495- and 1072-bp fragments) were obtained by digestion of *MAGE-1* cDNA with *Bgl*II and *Eco*RI. After purification on agarose gel, the fragments were cloned into the pcDNA3.1 plasmid. Clones were isolated, plasmid DNA was extracted and transfected into Cos-7 cells along with the *HLA-B*3701* gene.

Transfection of Cos-7 Cells and IFN-γ Release Assay. Transfection of Cos-7 cells was performed by the DEAE-dextran-chloroquine method (12). Briefly, 1.5×10^4 Cos-7 cells were transfected with 100 ng of plasmid pcDNA3.1/B*3701 and 100 ng of expression vectors containing the cDNA of one of the following genes: *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12*. Transfected Cos-7 cells were tested in a IFN-γ assay after 48 h: 5000 responder CTLs, at day 5 after stimulation, were added in 150 μl of IMDM-10% human serum supplemented with 25 units/ml IL-2. After 24 h at 37°C, 100 μl of supernatant were harvested, and the IFN-γ concentration was measured using a IFN-γ release kit (Genzyme Corp.) according to the manufacturer's recommendations.

Retroviral Vector-mediated Gene Transfer of *HLA-B*3701* into Me14932. The retroviral vector B37-CSM, coding for the HLA-B*3701 molecule of patient MSR3, was constructed as described previously (28). Briefly, the full-length cDNAs coding for the HLA-B*3701 molecule was cloned under the control of the viral long terminal repeat, whereas the truncated form of the human LNGFR (ΔLNGFR) was driven by the SV40 promoter. The ecotropic murine fibroblast cell line GP+E86 was transiently transfected with 30 μg of retroviral construct by standard calcium-phosphate method. Infection of the amphotropic murine packaging cell line GP+env Am 12, by supernatant of 48 h cultures of transfected GP+E86 cells, was performed for 4 h in the presence of 8 mg/ml polybrene. Infected packaging cells were immunoselected for ΔLNGFR expression by magnetic beads (Dynabeads M-450; Dynal A.S., Oslo, Norway) coated with the LNGFR-specific mAb 20.4 (American Type Culture Collection, Rockville, MD). Transduction of Me14932 was performed by cultivation with retrovirus-containing supernatant in the presence of polybrene (8 mg/ml). Five or six rounds of infection of at least 4 h were performed. Efficiency of infection was evaluated by immunofluorescence analysis with the LNGFR-specific mAb 20.4 and with a HLA-Bw4-specific mAb.

RT-PCR Assays. *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12* and *β2-m* cDNAs were detected by PCR amplification. Reaction mixture contained 5 μl of cDNA suspension, 4 μl of a 10 mM dNTPs mixture (containing each dNTP at 2.5 mM), 5 μl of 10× DNA polymerase buffer (Finnzymes Oy, Espoo, Finland), 2 units of DynaZyme DNA polymerase (Finnzymes Oy), and sterile distilled water up to a 50-μl total reaction volume. For oligonucleotide primer sequences and PCR amplification programs, see Weynants *et al.* (Ref. 20; MAGE-1, -2, and -3) and De Plaen *et al.* (Ref. 29; MAGE-4, -6, and -12). β-2m cDNA was amplified using the sense primer β 5′ (5′-AAC CAC GTG ACT TTG TCA CAG C-3′) and antisense primer β 5′ (5′-CTG CTC AGA TAC ATC AAA CAT G-3′). PCR amplification was performed for 30 cycles (1 min at 94°C, 30 s at 56°C, and 2 min at 72°C); the expected length of β-2m amplification product was 230 bp. RNA integrity was tested by PCR with β-actin-specific oligonucleotide primers (30). Samples scored positive when a band of the appropriate size was visible on a agarose gel in the presence of ethidium bromide.

RESULTS

MSR3-B37 Induces an Antigen-specific Immune Response. The melanoma line MSR3 was established from a cutaneous metastasis resected from patient MSR3. Expression of HLA class I alleles by the tumor cells was barely detectable (Fig. 1) and appeared to be inadequate to allow antigen presentation to immune effectors. Indeed, the MSR3 melanoma line failed to induce a cytotoxic response from autologous PBLs (data not shown). The lack of class I cell surface expression by MSR3-mel was not caused by impaired β-2m synthesis because a β-2m-specific mRNA was detected by RT-PCR analysis (data not shown).

To determine whether HLA class I antigen expression could be restored, MSR3-mel cells were stably transfected with cDNA encoding the autologous HLA-B*3701 molecule. After G418 selection flow cytometric analysis showed staining of the transfected MSR3-B37 cell line by the W6/32 mAb (Fig. 1).

To evaluate the presence on the surface of MSR3-B37 line of tumor-specific antigens, the melanoma cells were tested for their ability to induce tumor-specific cytotoxic effectors and for their susceptibility to lysis by these CTLs. Patient's PBLs were *in vitro* stimulated by MSR3-B37 as described in "Materials and Methods." After three rounds of stimulation, the polyclonal cytotoxic T cell line 337 (CTL 337) specifically lysed the MSR3-B37 cell line but not the untransfected MSR3-mel (Fig. 2). Autologous MSR3-EBV cells and PHA-activated T blasts were not recognized (data not shown), suggesting that the epitopes recognized by these CTLs are melanoma-/melanocyte-specific. Indeed, in addition to the autologous melanoma cells, CTL 337 also lysed the HLA-B*3701-positive melanoma line ET1 (Fig. 2), suggesting that one or more shared melanoma antigens are recognized.

These data indicate that HLA class I expression can be restored by transfection of MSR3 melanoma cells and that the melanoma line transfected with the HLA-B*3701 molecule is able to induce a tumor-specific cytotoxic T-cell response.

Identification of the Antigenic Epitope Recognized by CTL 337. To identify the antigen recognized by CTL 337, we evaluated the IFN-γ release of CTL 337 in the presence of Cos-7 cells transfected with plasmid pcDNA3.1/B*3701, along with cDNA encoding six members of the *MAGE* family (*i.e.*, *MAGE-1*, *-2*, *-3*, *-4*, *-6*, and *-12*), some of which are expressed by both MSR3-mel and ET1. CTL 337 specifically recognized Cos-7 cells transfected with *MAGE-1*, *-2*, *-3*, and *-6* (Fig. 3), suggesting that the epitope target of CTL 337 was shared among the four different antigens or that distinct components of the oligoclonal T-cell line were recognizing peptides derived from the four *MAGE* gene products. A low level of IFN-γ was detected in

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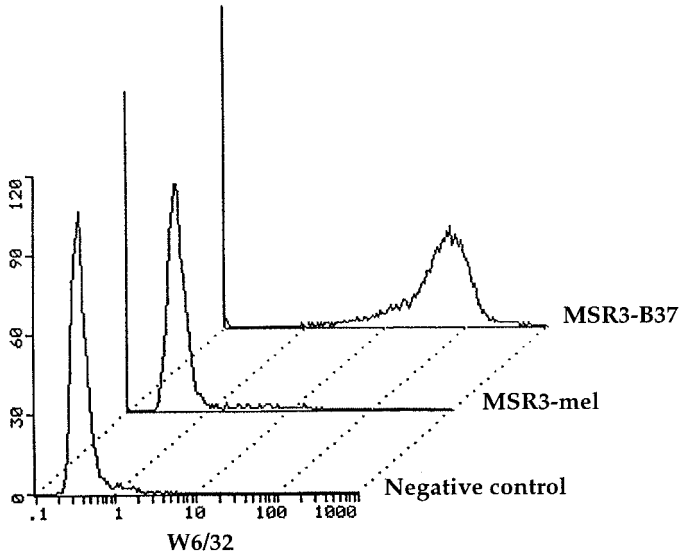

Fig. 1. Expression of HLA-class I molecules by MSR3-mel and MSR3-B37. Tumor cells were incubated with mAb W6/32 (anti-HLA-class I) or with an isotype control, washed, and labeled with goat antimouse immunoglobulin antibodies coupled to fluorescein. The analysis was performed before and after HLA-B*3701 transfection of MSR3-mel.

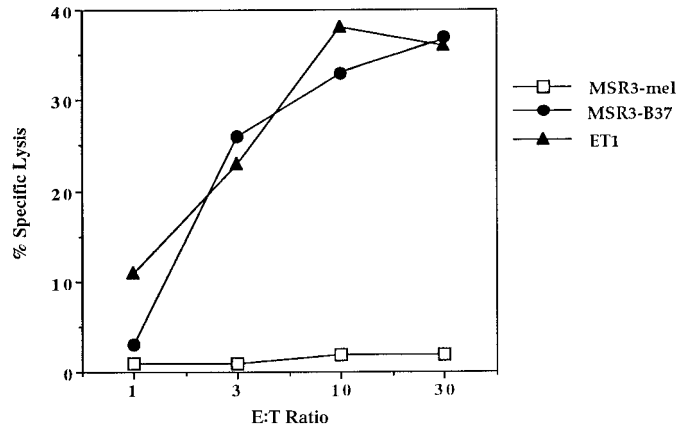

Fig. 2. Recognition of a shared HLA-B*3701-restricted antigen by CTL 337. The cytotoxic activity of CTL 337 cells was assessed against the autologous melanomas MSR3-mel and MSR3-B37 and against the allogenic melanoma ET1 at various E:T ratios.

the presence of *MAGE-4*- and *MAGE-12*-transfected Cos-7 cells (Fig. 3).

To identify the sequence coding for the antigenic peptide(s) recognized by CTL 337, we digested cDNA encoding *MAGE-1* with *Bgl*II and *Eco*RI obtaining two subfragments of ~495 and 1072 bp (Fig. 4). They were cloned into plasmid pcDNA 3.1 and transfected into Cos-7 cells along with HLA-B*3701 molecule. The presence of an in-frame start codon at 202 and 707 bp in the 495- and 1072-bp fragments, respectively, assured the expression of the two subfragments in the transfected cells. The level of IFN-γ released by CTL 337 cells in the presence of Cos-7 cells transfected with the 495-bp fragment was comparable to that conferred by the entire *MAGE-1* gene (Fig. 4), indicating that the antigenic peptide was encoded within this region. The amino acid sequence encoded by the 495-bp fragment (Fig. 5) was screened for peptides carrying the binding motif for HLA-B*3701 (31). Five peptides carrying aspartate or glutamate in position 2 and isoleucine or leucine in position 9/10 were identified (Fig. 5). One of these peptides, REPVTKAEML, was present also in the amino acid sequences encoded by *MAGE-2*, *MAGE-3*, and *MAGE-6*. This peptide, denominated MAGE.127–136, was used to sensitize the MSR3-EBV line to lysis by CTL 337 cells in a titration assay (Fig. 6*A*). The half-maximal lysis was reached with 90 nM peptide. No lysis of MSR3-EBV pulsed with an unrelated peptide that was able to bind to HLA-B*3701 was observed (Fig. 6*B* and data not shown).

Low levels of IFN-γ were released by the CTL 337 cells in the presence of Cos-7 cells expressing *MAGE-4* and *-12* (Fig. 3). To verify whether this release could be ascribed to recognition of peptides encoded by codons 127–136 within *MAGE-4* and *MAGE-12*, a peptide-binding study was performed, using MSR3-EBV cells pulsed with the two peptides as targets. Peptide $M4_{127–136}$, KELVTKAEML, differs by two amino acids (lysine *versus* arginine in position 1 and leucine *versus* proline in position 3) from peptide REPVTKAEML, whereas peptide $M12_{127–136}$, REPFTKAEML, differs by only one amino acid (phenyalanine *versus* valine in position 4). The results revealed that the two peptides can bind to HLA-B*3701 because increasing amounts of both were able to inhibit the lysis of MSR3-EBV pulsed with peptide REPVTKAEML but not with an unrelated HLA-A1-binding peptide (*i.e.*, M3.271–279; Fig. 6*B*). However, no recognition of EBV cells pulsed with peptides $M4_{127–136}$ and $M12_{127–136}$ was observed (data not shown).

Taken together, these data indicate that CTL 337 cells are able to recognize a peptide endogenously processed from *MAGE-1*, *-2*, *-3*, and *-6* products. The two peptides, encoded by the same region of *MAGE-4* and *-12*, respectively, are able to bind to HLA-B*3701, but they are not recognized by CTL 337 cells.

CTL 337 Cells Specifically Recognize *MAGE-2* and *-6* Gene Products. Until now, there was no evidence of the immunogenicity of *MAGE-2*- and *MAGE-6*-encoded proteins in humans. Indeed, peptides encoded by *MAGE-1*, *-3*, *-4*, and *-12* have been found to bind to various class I molecules to form antigens recognized by different CTLs, whereas no peptides encoded by the genes *MAGE-2* or *MAGE-6* have thus far been identified.

To demonstrate that peptide REPVTKAEML could also be processed from *MAGE-2* and *-6* and presented to CTL 337 cells, we attempted to look for melanoma cell lines expressing *MAGE-2* or *-6* but none of the other *MAGE* genes. Unfortunately, expression of the *MAGE* genes in melanomas is strictly correlated: most of the melanomas that express one member of the *MAGE* gene family also express the others. Indeed, we were unable to find a melanoma line

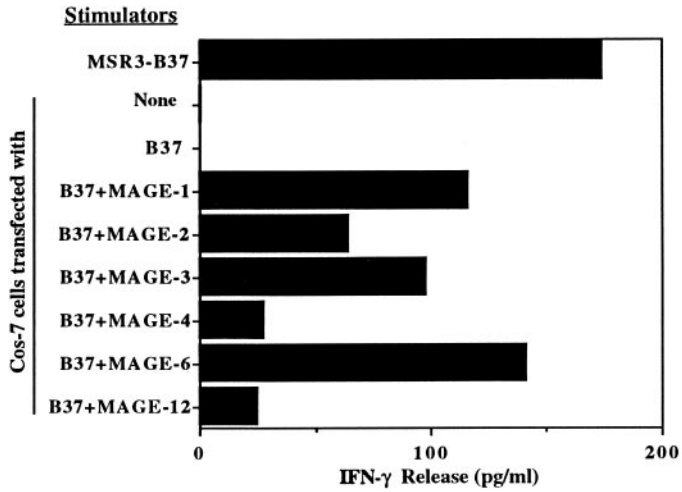

Fig. 3. Identification of the tumor-antigens recognized by CTL 337. Cos-7 cells were cotransfected with HLA-B*3701, alone or together with cDNAs encoding genes *MAGE-1*, *-2*, *-3*, *-6*, and *-12*. After 48 h, CTL 337 cells were added, and the IFN-γ released was measured 24 h later, as described in "Materials and Methods." MSR3-B37 was included as positive control.

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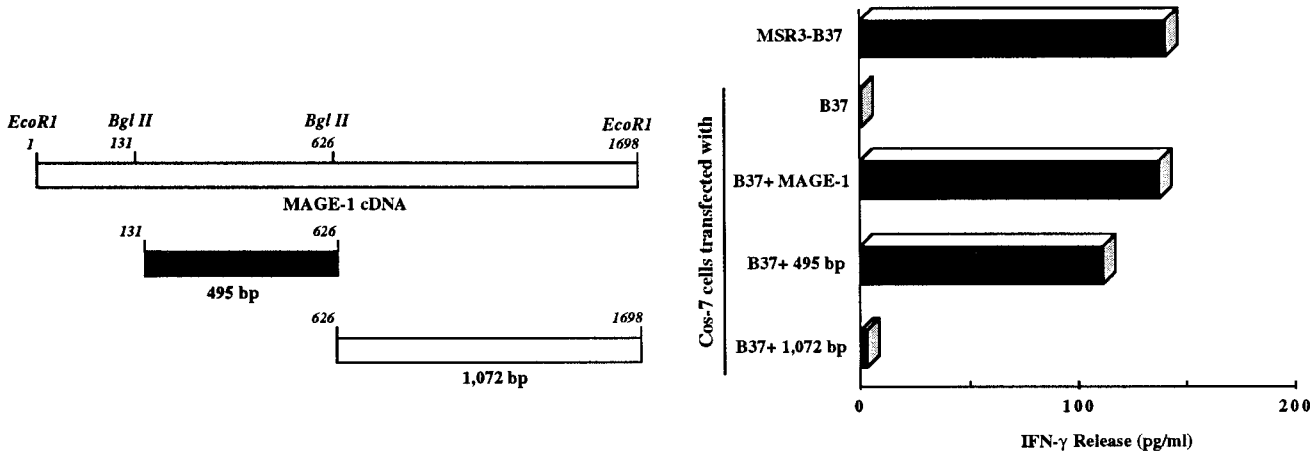

Fig. 4. Identification of the sequence coding for the antigenic peptide recognized by CTL 337. Cos-7 cells were cotransfected with two subfragments of *MAGE-1* cDNA together with HLA-B*3701. After 48 h, CTL 337 cells were added, and production of IFN-γ was measured 24 h later, as described in "Materials and Methods." As control, Cos-7 cells were transfected with HLA-B*3701, alone or together with the full-length *MAGE-1* cDNA.

that selectively expresses *MAGE-2*, but we succeeded in finding a single melanoma line, Me14932, that selectively expresses *MAGE-6* at low level (data not shown).

To verify whether peptide REPVTKAEML is endogenously processed from *MAGE-6* products and presented by HLA-B*3701, Me14932 was transduced by a retroviral vector encoding the HLA-B*3701 molecule. As indicated by immunofluorescence staining with a HLA-Bw4-specific mAb, cell surface expression of HLA-B*3701 on a pure population of transduced Me14932 cells was at least 2-fold lower than that of MSR3-B37 melanoma cells (data not shown). CTL 337 cells were able to recognize the Me14932-LB37 line in a cytotoxicity assay, and the level of lysis was increased by the exogenous addition of peptide REPVTKAEML, whereas there was no recognition of the pulsed and unpulsed Me14932 lines (Fig. 7). The low levels of lysis of the melanoma Me14932-LB37 might be explained either by weak expression of gene *MAGE-6* and by the weak surface expression of HLA-B*3701 molecules.

To evaluate whether the inclusion of *MAGE-2* and *-6* in the list of possible target antigens for specific immunotherapy could increase the proportion of eligible patients, we analyzed the expression of *MAGE-1*, *-2*, *-3*, and *-6* in fresh tumor samples of various histotypes. Melanomas were not analyzed because expression of the different *MAGE* genes was clearly correlated (32). The results indicate that 12% of the ovarian carcinomas and 5% of colon and breast carcinomas express *MAGE-2* and/or *-6* in the absence of *MAGE-1* and *-3* (Table 1). On the other hand, in all bladder and lung carcinomas studied the four genes were always coexpressed.

In conclusion, the data reported in this study indicate that *MAGE-2* and *-6* can be included in the list of possible target antigens for

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gaagatctgc ctgtgggtct tcattgccca gctcctgccc acactcctgc ctgctgccct gacgagagtc atcatgtctc ttgagcagag 221

                              L   E A Q   Q E A L   G L
gagtctgcac tgcaagcctg aggaagccct tgaggcccaa caagaggccc tgggcctggt gtgtgtgcag gctgccacct cctcctcctc 311


tcctctggtc ctgggcaccc tggaggaggt gcccactgct gggtcaacag atcctcccca gagtcctcag ggagcctccg cctttcccac 401

                                                          E E   G P S   T S C I / L E S
taccatcaac ttcactcgac agaggcaacc cagtgagggt tccagcagcc gtgaagagga ggggccaagc acctcttgta tcctggagtc 491

 L F R   A V I                 A D L   V G F L   L L                 R E P V   T K A
cttgttccga gcagtaatca ctaagaaggt ggctgatttg gttggttttc tgctcctcaa atatcgagcc agggagccag tcacaaaggc 581

 E M L
agaaatgctg gagagtgtca tcaaaaatta caagcactgt tttcc                                              671
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Mage-1	REPVTKAEML
Mage-2	REPVTKAEML
Mage-3	REPVTKAEML
Mage-4	KELVTKAEML
Mage-6	REPVTKAEML
Mage-12	REPFTKAEML

Fig. 5. *Top*, sequence of the 495-bp subfragment of *MAGE-1* cDNA. Peptides carrying the binding motifs for HLA-B*3701 are listed above their respective nucleotide sequences. *Bottom*, comparison of peptide REPVTKAEML (*underlined*), encoded by *MAGE-1*, with the peptides encoded by the homologous regions of other genes of the *MAGE* family.

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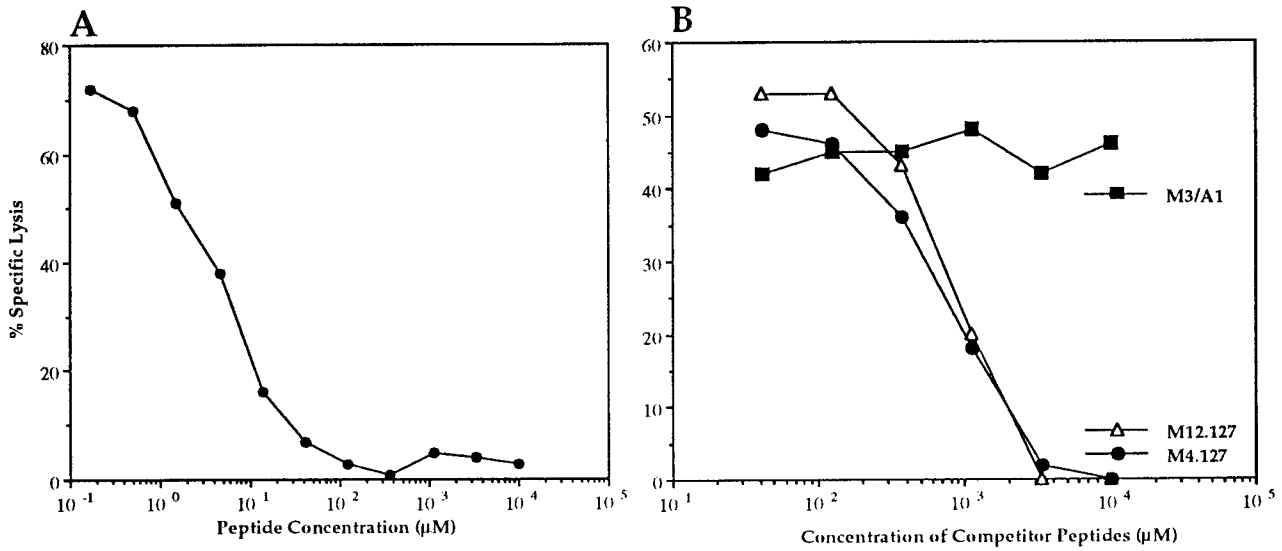

Fig. 6. *A*, recognition of the peptide $MAGE_{127–136}$ by CTL 337 cells. MSR3-EBV cells were incubated with 3-fold dilutions of peptide $MAGE_{127–136}$, starting from 10 mM and used as target cells in a standard cytotoxicity assay. The E:T ratio was fixed at 10:1. *B*, binding of peptides $M4_{127–136}$ and $M12_{127–136}$ to HLA-B*3701, evaluated in a competition assay. Competitor peptides included the $M4_{127–136}$ peptide KELVTKAEML and the $M12_{127–136}$ peptide REPFTKAEML. The M3.A1 (*i.e.*, $M3_{271–279}$) peptide, which was unable to bind to the HLA-B*3701 molecule, was used as negative control. Percentage lysis without competitor peptides was 52%.

tumor-specific immunotherapy, increasing the number of patients that could benefit from this therapy.

DISCUSSION

In the last few years, there has been a considerable effort to characterize antigenic peptides encoded by tumor-associated antigens and the HLA molecules responsible for their presentation (6). Several immunotherapy clinical trials of cancer vaccinations based on the use of these peptides are in progress, with quite positive preliminary results. Indeed, some objective cancer responses have been observed, consisting of both tumor regression (3, 5) and a few long-term complete responses (4).

One major limitation outlined by those studies is the development, in a significant proportion of the treated patients, of tumor variants that fail to express the antigen recognized by tumor-reactive lymphocytes (24). Those variants can be generated either by loss of the nominal antigen (referred to as antigen loss variants; Refs. 33 and 34) or by molecular defects affecting different steps of the antigen presentation pathway (referred to as presentation loss variants; Refs. 35–37). An active intervention of the immune system in the selection of antigen loss and presentation loss variants have been observed in both treated (24) and untreated patients (33, 38). However, escape from classical tumor-specific CTLs may be counteracted *in vivo* by the intervention of different immune effectors. Indeed, tumor cells that have lost expression of some but not all HLA class I molecules can be recognized by a new category of antitumor lymphocytes expressing killer-cell inhibitory receptors (39), whereas HLA-negative tumor cells can be targeted by NK cells.

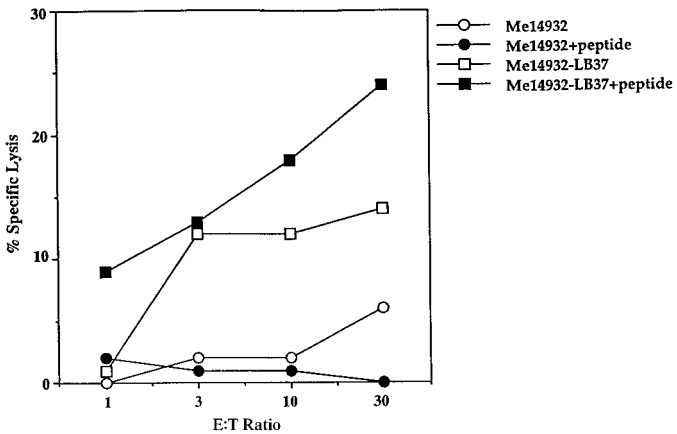

Fig. 7. Recognition of a *MAGE-6*-positive melanoma cell line by CTL 337. The HLA-B*3701-negative line Me14932 and the HLA-B*3701-positive line Me14932-LB37 were pulsed or not with 16 μM peptide $MAGE_{127–136}$ and used as target cells in a standard cytotoxicity assay at the indicated E:T ratios.

Table 1 *Expression of MAGE genes by fresh tumor samples*[a]

Histological type	% RT-PCR-positive tumors				
	MAGE-1	*MAGE-3*	*MAGE-2*	*MAGE-6*	*MAGE-2* or *MAGE-6* only
Lung carcinoma (28)[b]	35	39	32	29	0
Breast carcinoma (20)	30	10	10	15	5
Ovary carcinoma (25)	24	20	32	20	12
Bladder carcinoma (25)	28	28	20	24	0
Colon carcinoma (17)	0	5	5	5	5

[a] As determined by reverse transcriptase-PCR (RT-PCR) analysis.
[b] Numbers in parentheses represent numbers of fresh tumor samples analyzed.

The melanoma cell line used in this study belongs to the presentation loss variant class of HLA-negative tumor cells. The molecular defect responsible for the HLA class I phenotypes of MSR3-mel has not yet been identified; however, our melanoma line exhibits barely detectable levels of HLA class I expression by immunofluorescence analysis, which are not sufficient for stimulation of a tumor-specific T-cell response (data not shown). This altered phenotype does not seem to be due to β-2m or TAP alterations or to deletions of MHC genes but rather to a defect in the transcriptional machinery. Indeed, HLA class I expression in MSR3-mel can be restored by transfection of cDNAs encoding autologous HLA class I alleles.

The HLA-B*3701-transfected cell line (*i.e.*, MSR3-B37) allowed the isolation of HLA-B*3701-restricted and tumor-specific CTLs that recognized a nonapeptide encoded by the same region (*i.e.*, residues 127–136) of MAGE-1, -2, -3, and -6 proteins.

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To our knowledge, this is the first B*3701-restricted tumor-specific epitope that has been identified thus far. Note that the HLA-B*3701 molecule is present on both lymphocytes and tumor cells of patient MZ2 (7), from which a large variety of *MAGE*-specific CTL clones restricted by different HLA class I molecules were isolated (8–9, 40–42). Those results suggest a subdominant role of HLA-B*3701 in tumor antigen presentation in the MZ2 model that should be overcome in the MSR3 system by the absence on the stimulating cells of a HLA class I molecule other than HLA-B*3701. Indeed, dominance of a given HLA molecule in the tumor-specific stimulation of autologous CTL by melanoma cells has been described in several model systems (39, 43, 44).

Several members of the MAGE gene family are specifically expressed by tumors of various histological types and T-cell defined epitopes encoded by *MAGE-1* and *-3* have been identified. However, although *MAGE-2* and *-6* are expressed in a large percentage of tumor samples, thus far no *MAGE-2*- and *MAGE-6*-specific CTLs have been isolated. The only suggestion that *MAGE-2* behaves like a tumor-antigen comes from the study of Visseren *et al*. (45), who demonstrated the immunogenicity of *MAGE-2* in a HLA-A*0201Kb transgenic mouse model. Therefore, our study reports the first evidence for an immunogenic potential of *MAGE-2* and *-6* in humans. Indeed, CTL 337 cells were able to recognize Cos-7 cells transfected with HLA-B*3701 and *MAGE-2* or *-6* genes. Moreover, a stable HLA-B*3701-positive melanoma line expressing *MAGE-6* was recognized, whereas analogous experiments on *MAGE-2* and HLA-B*3701-positive melanoma lines could not be performed. It has been suggested that the proteasome specifically digests proteins into polypeptides with defined hydrophobic, basic, or acidic COOH termini, whereas the NH_2-terminal cleavage into smaller fragments occurs nonspecifically 8–10 amino acids further upstream. In view of the presence of hydrophobic residues (M and L) at the COOH-terminus of peptide REPVTKAEML, as well as the high degree of the amino acid sequence homology between *MAGE-2* and *MAGE-6* in the region around peptide REPVTKAEML, it is tempting to speculate that this peptide might indeed be processed in melanoma cells also from *MAGE-2* products. (46, 47).

The molecular analysis performed on tumor samples of various histotypes revealed a strong correlation between the expression of different *MAGE* genes. However, inclusion of *MAGE-2* and *MAGE-6* in the list of target antigens for cancer immunotherapy has practical implications for the enrollment of patients with ovarian carcinomas. Indeed, 12% of the ovarian carcinoma samples analyzed express *MAGE-2* and/or *-6*, without expressing *MAGE-1* and/or *-3* genes. On the other side, coexpression of more than one *MAGE* gene by a given tumor might prevent the development of antigen loss variants during vaccination treatment. Indeed, immune escape from a peptide-induced antitumor response might then be rare, because it would require the occurrence of several independent molecular alterations.

In conclusion, the identification of this new HLA-B*3701-restricted epitope not only increases the number of patients eligible for immunization but also may prove highly efficient for immunotherapy because of reduced risk of tumor escape due to the emergence of antigen loss variants.

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Identification of a Promiscuous T-Cell Epitope Encoded by Multiple Members of the *MAGE* Family

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