

A New Family of Genes Coding for an Antigen Recognized by Autologous Cytolytic T Lymphocytes on a Human Melanoma

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Summary

Human melanoma MZ2-MEL expresses several distinct antigens that are recognized by autologous cytolytic T lymphocytes (CTL). Some of these antigens are encoded by genes *MAGE-1*, *MAGE-3*, and *BAGE*, which are expressed in a large fraction of tumors of various histological types but are silent in normal adult tissues with the exception of testis. We report here the identification of the gene coding for MZ2-F, another antigen recognized by autologous CTL on MZ2-MEL cells. This gene, which was named *GAGE-1*, is not related to any presently known gene. It belongs to a family of genes that are expressed in a variety of tumors but not in normal tissues, except for the testis. Antigenic peptide YRPRPRRY, which is encoded by *GAGE-1*, is recognized by anti-MZ2-F CTL on class I molecule HLA-Cw6. The two genes of the *GAGE* family that code for this peptide, namely *GAGE-1* and *GAGE-2*, are expressed in a significant proportion of melanomas (24%), sarcomas (25%), non-small cell lung cancers (19%), head and neck tumors (19%), and bladder tumors (12%). About 50% of melanoma patients carry on their tumor at least one of the presently defined antigens encoded by the *MAGE*, *BAGE*, and *GAGE* genes.

Human melanoma cells bear antigens that are recognized by autologous CD8⁺ CTL, which can be derived either from blood lymphocytes or from tumor-infiltrating lymphocytes (1). Two distinct strategies have been used to characterize these antigens. The first is a genetic approach based on transfection of genomic or cDNA libraries to identify the gene encoding the antigen (2–7). The antigenic peptide can then be identified on the basis of the protein sequence encoded by the gene (6, 8–12). The second approach involves acid elution of peptides from immunoprecipitated HLA molecules followed by separation of the peptides, evaluation of their ability to sensitize target cells to CTL, and sequencing of the active peptide (13, 14).

A first class of antigens that are recognized on melanoma by autologous CTL is encoded by genes that are expressed in various tumors but that are completely silent in normal adult tissues, except the testis. These genes include genes *MAGE-1*, *MAGE-3*, and *BAGE* (2, 6, 9, 11, 15). The mouse gene *P1A*, which codes for a tumor rejection antigen of mouse tumor P815, follows the same pattern of expression (16). The tumor specificity of these antigens may make them targets of choice for cancer immunotherapy based on specific immunization.

A second class of antigens represents differentiation antigens encoded by genes that are expressed in melanoma and

in normal melanocytes. Antigens derived from tyrosinase were the first examples of this class, which also comprises antigens encoded by *Melan-A*^{MART-1}, *gp100*^{pmel17}, and *gp75*^{TRP1} (3, 4, 7, 10, 12, 13, 17, 18).

We report here the identification of a new member of the first class of tumor antigens.

Materials and Methods

Cell Lines. Melanoma cell line MZ2-MEL was derived from an abdominal metastasis of patient MZ2 and a number of subclones were obtained. Subclone MZ2-MEL.3.0 was obtained by limiting dilution. Subline MZ2-MEL.3.1 was obtained by extending the culture of subclone MZ2-MEL.3.0 for more than 150 generations. Subline MZ2-MEL.43 was derived by limiting dilution from MZ2-MEL.3.0 cells that had survived to a mutagen treatment (19, 20). Clonal subline MZ2-MEL.4, which does not express antigen MZ2-F, was selected in vitro from subline MZ2-MEL.3.1 with autologous anti-MZ2-F CTL clone 76/6 (20). MZ2-E-negative clonal subline MZ2-MEL.2.2 was selected from subline MZ2-MEL.3.1 with an autologous anti-MZ2-E CTL clone (20). MZ2-MEL.2.2 was further treated in vitro with anti-MZ2-F CTL clone 76/6 and clonal subline MZ2-MEL.2.2.5, which does not express antigen MZ2-F was obtained. Melanoma cell lines were grown as previously described (20, 21). Autologous CTL clone 76/6 was derived from PBL of patient MZ2 by in vitro stimulation with irradiated MZ2-

MEL.3.1 cells (20). Lymphoblastoid cell lines MZ2-EBV and LB33-EBV were derived from the PBL of patients MZ2 and LB33 by standard techniques. IGR3-MEL, a melanoma line derived from an HLA-Cw6 patient (22), was kindly provided by Dr. D. Rimoldi (Ludwig Institute for Cancer Research, Lausanne, Switzerland). HeLa-S3 cells were obtained from the American Type Culture Collection. They were cotransfected by the calcium phosphate precipitate method with the *GAGE-1* cDNA cloned in plasmid pcDNA3 (Invitrogen, San Diego, CA), which contains the neomycin resistance gene, and with the *HLA-Cw*0601* cDNA cloned in plasmid pcDNA1/Amp (Invitrogen). A clonal subline was isolated from a G418-resistant transfected population.

Lysis Test. Lytic activity of CTL was tested in chromium release assays as previously described (23). We used indifferently as positive control target MZ2-MEL.43 (see Fig. 1) or MZ2-MEL.3.1 (see Fig. 6). Negative control target was MZ2-MEL.4.

Construction of the cDNA Library. Poly-A⁺ RNA was extracted from MZ2-MEL.43 cells using mRNA extraction kit FastTrack (Invitrogen). mRNA was converted to cDNA with the Superscript Choice System (GIBCO BRL, Gaithersburg, MD) using an oligo-dT primer containing a NotI site at its 5' end. cDNA were then ligated to BstXI adaptors and digested with NotI. After size fractionation, the cDNA were unidirectionally cloned into the BstXI and NotI sites of plasmid pcDNA1/Amp. Recombinant plasmids were electroporated into *Escherichia coli* DH5- α and selected with ampicillin (50 μ g/ml). The library was divided into 1,500 pools of ~ 100 cDNA clones. Each pool of bacteria was amplified to saturation, and plasmid DNA was extracted by a simplified alkaline lysis method without phenol extraction (24).

Transfection of COS-7 Cells. Transfection experiments were performed by the DEAE-dextran-chloroquine method (3, 4, 25). Briefly, 1.5×10^4 COS-7 cells were transfected with ~ 100 ng of plasmid DNA of a pool of the cDNA library and either 30 ng of plasmid pcD-SR α (26) containing the *HLA-A1* gene (27), 50 ng of plasmid pcDNA1/Amp containing the autologous *HLA-B37* cDNA, or 75 ng of plasmid pcDNA1/Amp containing the autologous *HLA-Cw*0601* cDNA. The *HLA-A1* gene, derived from another patient, was kindly provided by Dr. Girdlestone (Medical Research Council Centre, Hills Road, Cambridge, UK). The *HLA-B37* and the *HLA-Cw*0601* cDNA derive from patient MZ2 and were isolated from the cDNA library described above by hybridization with specific oligonucleotides. Transfected COS-7 cells were tested in a CTL stimulation assay after 24–48 h. The data shown in Figs. 2 and 7 were obtained by transfecting 100 ng of the *HLA-Cw*0601* construct with 100 ng of the *GAGE* cDNA, and by testing the transfected COS-7 cells after 24 h.

CTL Stimulation Assay. Transfectants were tested for their ability to stimulate the production of TNF by the CTL as described (21). Briefly, 3,000 CTL were added to microwells containing transfected cells in 100 μ l of Iscove's modified Dulbecco's medium (GIBCO BRL) containing 10% human serum and 30 U/ml rIL-2. After 18 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on WEHI 164 clone 13 cells (28) in a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay (21, 29). Positive control stimulator cells were indifferently MZ2-MEL.3.1 (see Fig. 2) or MZ2-MEL.43 (see Fig. 7). Negative control stimulator cells were MZ2-MEL.2.2.5. The inhibition with mAb W6/32 (30) was performed by addition of a 1/20 dilution of W6/32 ascites to the CTL stimulation assay.

DNA Sequence Analysis. DNA sequencing was performed by the dideoxy-chain termination method (T7 Sequencing Kit; Pharmacia LKB, Uppsala Sweden and Δ TaqTMCycle-Sequencing Kit; U.S. Biochemical Corp., Cleveland, OH) using specific oligonu-

cleotides as primers. The computer search for sequence homology was done with program blast @ncbi.nlm.nih.gov. Sequence alignments were performed with Geneworks[®] software (Intelligenetics, Mountain View, CA).

Northern Blot Analysis. Total RNA was isolated by the guanidine-isothiocyanate procedure as described (31). Northern blots were prepared as described (16) and were hybridized with ³²P-labeled cDNA 2D6. The membranes were washed twice for 5 min at room temperature in 2 $\times$ SSC and twice for 15 min at 60°C in 2 $\times$ SSC supplemented with 1% SDS, and were autoradiographed overnight. Control hybridization was performed on the same membrane with a mouse β -actin probe.

PCR Assay for the Expression of *GAGE* Genes. Total RNA was extracted by the guanidine-isothiocyanate procedure as described (31). Reverse transcription was performed on 2 μ g of total RNA in a reaction volume of 20 μ l with 4 μ l of 5 $\times$ reverse transcriptase buffer (GIBCO BRL), 1 μ l each of 10 mM deoxynucleotides, 2 μ l of a 20- μ M solution of oligo(dT)₁₅ primer, 20 U of RNasin (Promega Biotech, Madison, WI), 2 μ l of 0.1 M dithiothreitol, and 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). The reaction was incubated at 42°C for 60 min. 1/20 of the cDNA product was then supplemented with 5 μ l of 10 $\times$ thermostable DNA polymerase buffer (Finnzymes OY, Espoo, Finland), 1 μ l each of 10 mM dNTP, 2 μ l each of 20- μ M primer solutions, 1 U of DynaZymeTM (Finnzymes OY), and water to a final volume of 50 μ l. The PCR primers for the amplification of all *GAGE* genes were either 5'-GCGGCCCCGAGCAGTTCA-3' (VDE43, sense, nucleotide 126–142) or 5'-AGACGCTACGTAGAG-CCT-3' (VDE18, sense, nucleotide 88–105), as indicated, and antisense primer 5'-CCATCAGGACCATCTTCA-3' (VDE24, antisense, nucleotide 309–326). For the specific amplification of *GAGE-1* and *GAGE-2*, sense primer 5'-GACCAAGACGCTACGTAG-3' (VDE44, sense, nucleotide 83–100) was used with antisense primer VDE24. A first denaturation step was done for 5 min at 94°C. 30 cycles were then performed as follows: 1 min at 94°C, 2 min at 56°C (55°C when primer VDE18 was used), and 3 min at 72°C. A final extension step of 15 min at 72°C was done. 10- μ l aliquots of the PCR products were size separated on agarose gels. RNA integrity was checked by reverse transcription and amplification of the β -actin mRNA.

Production of Truncated *GAGE-1* cDNA. Progressive 3' deletions were produced with the Erase-a-Base System (Promega) as described (11). A minigene containing the first 118 nucleotides of *GAGE-1* was constructed by PCR using specific primers and was cloned in plasmid pcDNA3.

Antigenic Peptides and CTL Assay. Peptides were synthesized on solid phase using F-moc for transient NH₂-terminal protection, as described by Atherton et al., and they were characterized by mass spectrometry (32). All peptides were >90% pure as indicated by analytical HPLC. Lyophilized peptides were dissolved at 20 mg/ml in DMSO, diluted at 2 mg/ml in 10 mM acetic acid, and stored at –80°C. Peptides were tested in CTL stimulation assays with COS-7 cells transfected with *HLA-Cw*0601* and incubated with the peptides. They were also tested in chromium release assays, where 1,000 ⁵¹Cr-labeled target cells were incubated for 30 min at 37°C in 96-well microplates with various concentrations of peptide before addition of CTL 76/6 at a lymphocyte/target ratio of 10:1. The assay was terminated after a 4-h incubation at 37°C.

Results

A panel of stable autologous CTL clones was obtained against the human melanoma cell line MZ2-MEL by stim-

ulating PBL from patient MZ2 with irradiated autologous tumor cells (19). Tumor cell variants selected in vitro for resistance to some of these CTL clones remained sensitive to others, indicating that several different antigens were present on the MZ2-MEL cells (20). One of these antigens, named MZ2-F, was recognized by CD8⁺ CTL clone 76/6, which lysed autologous MZ2-MEL cells, but neither autologous EBV-B cells nor NK target K562 cells (Fig. 1). Upon contact with MZ2 melanoma cells, this CTL clone released TNF, and this was inhibited by an mAb directed against HLA class I molecules (Fig. 2).

Identification of a cDNA Encoding Antigen MZ2-F. To clone the gene encoding antigen MZ2-F, we used RNA from MZ2-MEL to prepare a cDNA library with plasmid pcDNA1/Amp. This plasmid contains the origin of replication of SV40 resulting in considerable episomal multiplication in COS-7 cells and therefore high expression of cloned genes (25). To allow presentation of the antigen to CTL, COS cells must express the appropriate HLA molecule, and this can be achieved by cotransfection of the relevant gene (3, 4). A prerequisite for this method is therefore the knowledge of the restricting MHC molecule. For antigen MZ2-F, only three of the six HLA class I specificities of patient MZ2, namely A1, B37, and Cw*0601, were possible candidates. This came from the finding that subline MZ2-MEL.3.1, which had lost the *HLA-A29*, *B*4403*, and *Cw*1601* genes, was still lysed by CTL 76/6 (6, 11, 20).

The library was divided into pools of 100 recombinant clones, and DNA from each pool was cotransfected into microcultures of COS-7 cells with either the *HLA-A1* gene, the *HLA-B37* cDNA, or the *HLA-Cw*0601* cDNA cloned in pcDNA1/Amp. 48 h later, we screened the transfectants for the expression of antigen MZ2-F by adding CTL 76/6 to the microcultures and by measuring TNF production after 18 h. One out of the 500 cDNA pools that were tested proved positive when cotransfected with *HLA-Cw*0601*. By subcloning the bacteria of this pool and repeating the transfection and screening procedures outlined above with individual plasmid DNA, we obtained several clones that transferred the expression of the antigen. The result obtained with rep-

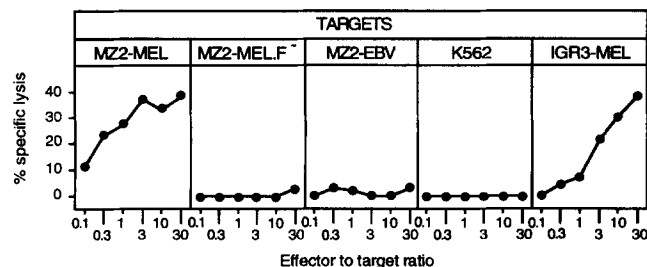


Figure 1. Specific lysis of autologous melanoma cell line MZ2-MEL by CTL 76/6. Variant MZ2-MEL.F⁻ was selected in vitro for resistance to CTL 76/6. Control targets include the autologous EBV-transformed lymphoblastoid line MZ2-EBV and the NK-sensitive line K562. IGR3-MEL is an allogeneic melanoma line expressing *GAGE-1/2* and *HLA-Cw6*. Chromium release was measured after 4 h. Results are pooled from two representative experiments.

resentative cDNA clone 2D6 is shown in Fig. 2. When this clone was cotransfected into HeLa cells with the *HLA-Cw*0601* cDNA, it produced stable transfectants that were also recognized by CTL 76/6 (Fig. 2).

This cDNA was 650 bp long and appeared to be full length, since it hybridized with a similarly sized band on a Northern blot prepared with RNA from MZ2-MEL (Fig. 3). Its sequence did not show significant homology with any gene reported in data banks, and this new gene was named *GAGE*. It contained an open reading frame coding for a protein of 138 amino acids.

The expression of gene *GAGE* was analyzed by Northern blot with RNA from various tissues. No *GAGE* mRNA was detected in the normal tissues that were tested, but it was found in a number of melanoma lines derived from different patients (Fig. 3). It appeared therefore that expression of antigen MZ2-F results from the activation in melanoma cells of a gene that is silent in normal tissues.

The *GAGE* Gene Family. By hybridizing 5,000 clones of the MZ2-MEL cDNA library with the *GAGE* cDNA, we obtained 20 other cDNA clones carrying five new homologous sequences that were 80–98% identical to the *GAGE* cDNA. Accordingly, we renamed *GAGE-1* the gene corresponding to our first cDNA and named the homologous sequences *GAGE-2–6*. An alignment of the six cDNA sequences is shown in Fig. 4. They differ mainly by single nucleotide substitutions scattered throughout the sequence. This fea-

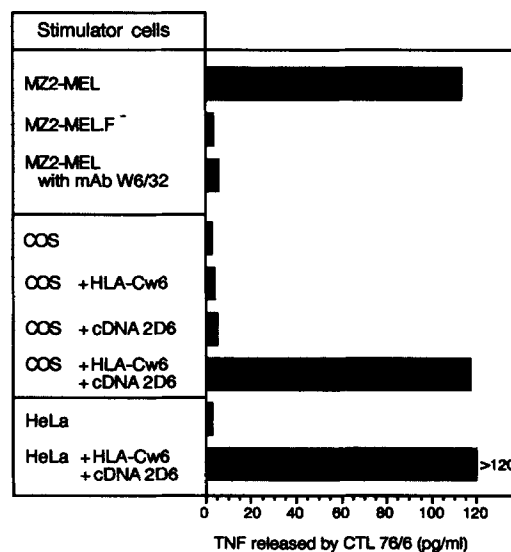


Figure 2. Stimulation of CTL 76/6 by cells transfected with the *HLA-Cw*0601* cDNA and with cDNA 2D6. COS-7 cells were transiently transfected with the indicated cDNA cloned in pcDNA1/Amp. HeLa cells were stably transfected with the indicated cDNA. The production of TNF by CTL 76/6 was measured after 18 h of coculture with the transfected cells by testing toxicity of the supernatants for TNF-sensitive cells WEHI 164.13. Control stimulator cells included autologous MZ2-MEL and variant MZ2-MEL.F⁻, which was selected in vitro for resistance to CTL 76/6. The production of TNF by CTL 76/6 was inhibited in the presence of mAb W6/32, which binds to all HLA class I molecules.

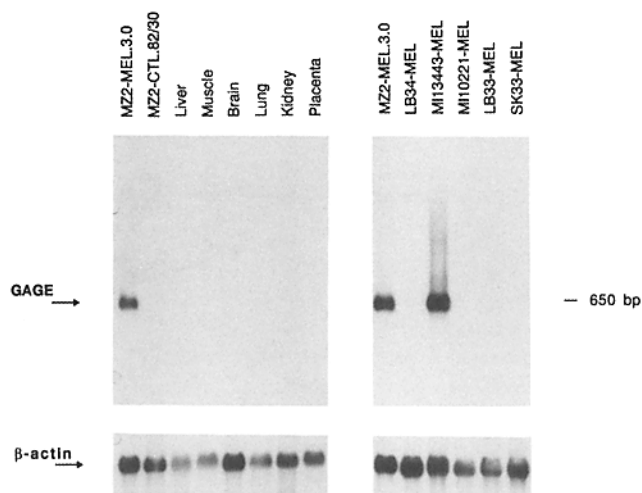


Figure 3. Northern blot analysis of the expression of gene *GAGE*. Each lane contains 10 μ g of total RNA of the cells indicated on top. MZ2-MEL.3.0 is a clone derived from melanoma MZ2-MEL. MZ2-CTL.82/30 is a CTL clone derived from patient MZ2. LB34-MEL, MI13443-MEL, MI10221-MEL, LB33-MEL, and SK33-MEL are melanoma lines derived from other patients. Hybridizations were performed successively with *GAGE* cDNA 2D6 and with a β -actin probe. A series of other melanoma lines were tested similarly and several of them expressed the *GAGE* message (not shown).

ture makes it unlikely that the different *GAGE* cDNA result from alternative splicing of distinct exons of a single gene, but some of them could correspond to different alleles of the same gene, so that the actual number of *GAGE* genes may not exceed 3.

As shown in Fig. 4, a sequence of 143 nucleotides that is located near the termination codon of the *GAGE-1* coding sequence is absent in the other *GAGE* cDNA. Because of this insertion, the *GAGE-1* putative protein is 20–22 amino acids longer than the five other predicted proteins (Fig. 5). The first 35 bases of this stretch (nucleotide 376–410) show significant homologies with Alu repeats, and could therefore result from the lack of splicing of an intron (33). However, the sequence of a genomic *GAGE* clone suggests that the *GAGE-1* stretch rather corresponds to an additional exon that is homologous to a small region of an intron of the other *GAGE* genes. A similar situation has been observed with the genes *MAGE-1* and *MAGE-2* (34).

Identification of the Antigenic Peptide Encoded by Gene *GAGE*. To identify the *GAGE-1*-encoded peptide presented by *HLA-Cw*0601* to anti-MZ2-F CTL, we generated progressive deletions from the 3' end of the *GAGE-1* cDNA by digestion with exonuclease III. Plasmids containing the truncated cDNA were then cotransfected into COS-7 cells with the *HLA-Cw*0601* cDNA, and the transfected cells were tested for recognition by the CTL in a TNF production assay. The smallest truncated cDNA that was positive contained the first 168 nucleotides of *GAGE-1*. Since the open reading frame started in position 49, this result localized the peptide in the first 40 amino acids of the protein. A minigene

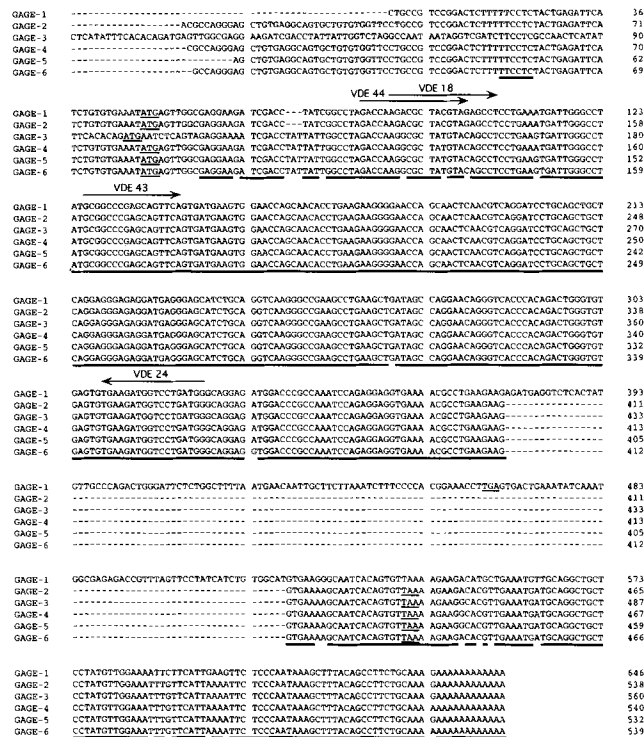


Figure 4. Alignment of the nucleotide sequences of the six *GAGE* cDNA. Bold lines indicate the regions that are conserved in the six sequences. The initiation and termination codons are underlined. Primers VDE44, VDE18, VDE43, and VDE24 used for the analysis of *GAGE* expression by PCR are indicated by arrows. The nucleotide sequences of the six *GAGE* cDNA are available from EMBL/Genbank/DBJ under accession numbers U19142, U19143, U19144, U19145, U19146, and U19147.

encoding only the first 23 amino acids of *GAGE-1* was synthesized by PCR, cloned into plasmid pcDNA3, and tested similarly. It conferred expression of the antigen. Two overlapping synthetic peptides containing residues 1–15 and 8–23 were synthesized and tested for their ability to render COS-7 cells, which had been transfected with the *HLA-Cw*0601* cDNA, capable of stimulating the release of TNF by CTL 76/6. The second peptide was effective. Several peptides of nine residues included in this peptide were tested, and

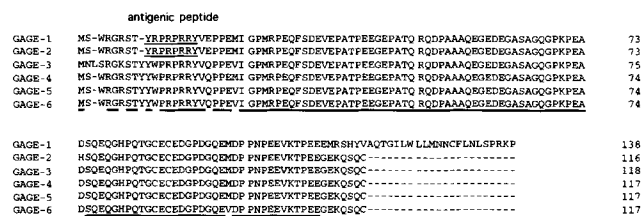


Figure 5. Alignment of the protein sequences encoded by the six *GAGE* cDNA. Bold lines indicate the regions that are conserved in the six sequences. The antigenic peptide derived from the *GAGE-1* and *GAGE-2* proteins is underlined.

two overlapping nonamers, namely TYRPRPRRY and YRPRPRRYV, were positive. We then synthesized the octameric peptide common to the two nonamers and the deca-meric peptide containing both. The four peptides were compared for their ability to sensitize HLA-Cw*0601⁺ EBV lymphoblastoid cells to lysis by CTL 76/6 (Fig. 6). Octamer YRPRPRRY was found to be the optimal peptide. Half maximal lysis was obtained at a peptide concentration of ~100 nM. The two nonamers were one order of magnitude less efficient and the deca-mer was even less efficient. Another anti-MZ2-F CTL clone derived from a different blood sample of patient MZ2 also lysed HLA-Cw*0601⁺ lymphoblastoid cells pulsed with peptide YRPRPRRY (data not shown).

The gene *GAGE-2* codes for the same antigenic peptide as *GAGE-1*, but the homologous peptides encoded by *GAGE-3-6* have tryptophan instead of arginine in position 2 (Fig. 5). When transfected into COS-7 cells with the HLA-Cw*0601 cDNA, only *GAGE-1* and *GAGE-2* cDNA were able to confer recognition by CTL 76/6, showing that the arginine in position 2 is an essential element of the MZ2-F antigenic peptide (Fig. 7). 9 of the 21 *GAGE* cDNA were either *GAGE-1* or *GAGE-2* sequences.

Expression of *GAGE* Genes. The expression of the *GAGE* genes was tested in a panel of normal tissues by reverse transcription PCR (RT-PCR)¹ using primers common to the six *GAGE* sequences. We found no expression in any normal adult tissue except testis (Table 1). With another set of primers that amplified only *GAGE-1* and *GAGE-2*, we found that a significant proportion of tumors of various histological types express at least one of these genes (Table 1). The highest proportions of positive tumors were found among sarcomas (25%), melanomas (24%), non-small cell lung carcinomas (19%), head and neck tumors (19%), and testicular seminomas (five out of six), but the genes are also expressed in bladder tumors and breast tumors. No expression was found in colorectal carcinomas and renal cell carcinomas. Melanoma line IGR3-MEL, which expresses *GAGE-1/2* and HLA-Cw6, triggered TNF release by CTL 76/6 (data not shown) and was lysed by it (Fig. 1).

Discussion

Two members of the *GAGE* gene family, *GAGE-1* and *GAGE-2*, code for a tumor-specific antigenic peptide presented to CTL by HLA-Cw*0601. Previous results obtained with mouse tumors revealed two mechanisms that can produce such antigens. One is the occurrence of a point mutation (35-37); the other is the activation in the tumor of a gene that is silent in normal cells (16). For *GAGE*, all our evidence supports the second mechanism. *GAGE* genes are expressed in a number of tumors, but not in normal tissues, except the testis. Furthermore, the fact that two distinct

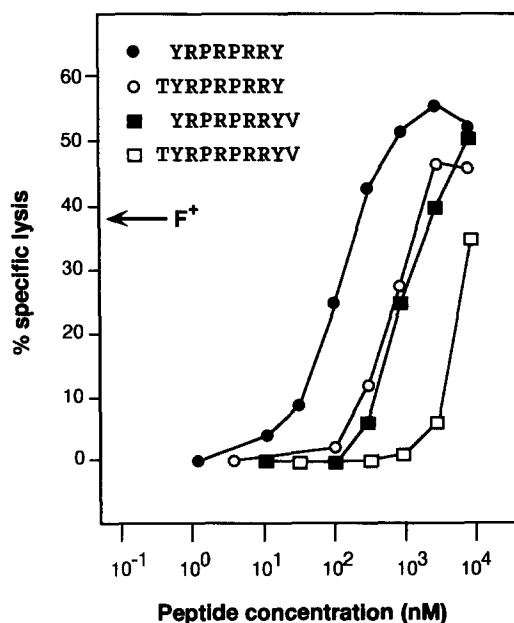


Figure 6. Lysis by CTL 76/6 of HLA-Cw*0601⁺ cells pulsed with *GAGE*-encoded peptides. Chromium-labeled HLA-Cw*0601⁺ EBV-transformed lymphoblastoid cells (LB33-EBV) were pulsed 30 min with the indicated peptides at various concentrations before addition of CTL 76/6 at an E/T ratio of 10. Chromium release was measured after 4 h. The arrow indicates the level of lysis of MZ2.MEL cells (F⁺) at the same E/T ratio. Similar results were obtained when autologous lymphoblastoid cells MZ2-EBV were used as peptide-pulsed target cells.

members of the *GAGE* gene family code for this antigen rules out the possibility that the antigen appeared by mutation.

Because of its specific expression in tumors, the *GAGE* antigen may constitute a useful target for specific cancer immunotherapy. The expression of *GAGE* genes in testis, however, raises the issue of undesirable auto-immune effects. The

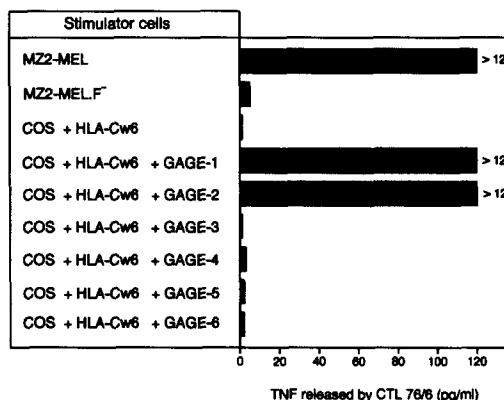


Figure 7. Transfection of the *GAGE-1-6* cDNA into COS cells. The *GAGE* cDNA were transiently cotransfected into COS-7 cells with the HLA-Cw*0601 cDNA. CTL 76/6 was added after 24 h and the production of TNF was measured 18 h later. Control stimulator cells included autologous MZ2-MEL and variant MZ2-MEL.F⁻, which was selected in vitro for resistance to CTL 76/6.

¹ Abbreviation used in this paper: RT-PCR, reverse transcription PCR.

Table 1. Expression of *GAGE* Genes by Normal and Tumoral Tissues

Normal tissues		Tumors		
Histological type	Expression of <i>GAGE-1-6</i> *	Histological type	Number of tumors expressing	
			<i>GAGE-1-6</i> †	<i>GAGE-1/2</i> §
Adult tissues		Tumor samples		
Adrenal gland	—	Melanomas primary lesions	5/39	5/39 (13%)
Benign naevus	—	metastases	47/130	36/129 (28%)
Bone marrow	—	Sarcomas	7/24	6/24 (25%)
Brain	—	Lung carcinomas (NSCLC	18/77	15/77 (19%)
Breast	—	Bladder tumors superficial	1/36	1/36
Cerebellum	—	infiltrating	10/39	8/39 (21%)
Colon	—	Head and neck tumors	14/59	11/58 (19%)
Heart	—	Mammary carcinomas	18/162	14/162 (9%)
Kidney	—	Testicular seminomas	6/6	5/6
Liver	—	Prostatic carcinomas	2/20	2/20
Lung	—	Colorectal carcinomas	0/43	
Melanocytes	—	Leukemias and Lymphomas	1/71	
Muscle	—	Renal carcinomas	0/45	
Ovary	—			
Prostate	—			
Skin	—			
Splenocytes	—			
Stomach	—	Tumor cell lines		
Testis	+			
Thymocytes	—	Melanomas	45/74	40/74 (54%)
Urinary bladder	—	Sarcomas	1/4	1/4
Uterus	—	Lung carcinomas NSCLC	1/2	1/2
		SCLC**	7/24	7/24 (29%)
Fetal tissues [¶]		Mesotheliomas	5/19	5/19 (26%)
		Head and neck tumors	0/2	
Fibroblasts	—	Mammary carcinomas	1/4	0/4
Brain	—	Bladder tumors	0/3	
Liver	—	Colorectal carcinomas	5/13	5/13
Spleen	—	Leukemias	3/6	1/6
Thymus	—	Lymphomas	0/6	
Testis	+	Renal carcinomas	0/6	

* Expression of genes *GAGE-1-6* was tested by RT-PCR on total RNA with sense primer VDE43 and antisense primer VDE24 (Fig. 4). These primers are common to the six *GAGE* sequences. They are located in different exons and amplify a 201-bp product that is not observed when genomic DNA is tested.

† Expression of genes *GAGE-1-6* was tested by RT-PCR on total RNA with sense primer VDE18 and antisense primer VDE24 (Fig. 4). These primers amplify the six *GAGE* sequences. They are located in different exons and give a 239-bp product that is not observed when genomic DNA is tested.

§ Expression of *GAGE-1/2* was tested by RT-PCR on total RNA with sense primer VDE44 and antisense primer VDE24 (Fig. 4). These primers amplify *GAGE-1* and *GAGE-2*, but not *GAGE-3-6*. They are located in different exons and give a 244-bp product that is not observed when genomic DNA is tested.

^{||} NSCLC, non-small cell lung carcinoma.

[¶] Fetal tissues derived from fetuses older than 20 w.

** SCLC, small cell lung carcinoma.

testis is an immunoprivileged site, however, and germ-line cells do not express MHC class I molecules and therefore should not express antigens recognized by T cells (38, 39). In male mice, it was possible to generate CTL responses against a tumor antigen encoded by gene P1A, which is also expressed in testis but not in other normal tissues. We observed neither inflammation of testicular tissues nor reduction of fertility (Uyttenhove, C., B. Lethé, T. Boon, and B. Van den Eynde, manuscript in preparation). In our view, it is therefore likely, but not certain, that immunization against the GAGE antigen will not produce autoimmune effects.

Tumors expressing *GAGE-1/2* and HLA-Cw6 can be identified by typing patients for HLA and by testing the expression of *GAGE-1/2* by RT-PCR amplification on RNA from a small tumor sample. Because HLA-Cw*0601 is present in 16% of Caucasian individuals and 24% of melanomas express *GAGE-1* or *GAGE-2*, ~4% of all melanomas should express this antigen. This brings to ~51% the fraction of Caucasian melanoma patients eligible for immunotherapy directed against defined tumor antigens encoded by genes *MAGE-1*, *MAGE-3*, *BAGE*, or *GAGE* (Table 2). The histological distribution of *GAGE*-positive tumors is rather similar to that of *MAGE-1*- or *MAGE-3*-positive tumors. In view of the very high incidence of non-small cell lung cancer, it is noteworthy that 49% of these cancers express at least one of the *MAGE-1*, *MAGE-3*, *BAGE*, *GAGE-1*, or *GAGE-2* genes (40). Accordingly, patients with lung cancer represent the largest cohort of patients that could benefit from specific immunotherapy directed against antigens encoded by these genes.

Melanoma lines studied in vitro were found to simultaneously express several antigens recognized by CTL (20, 41, 42). Many tumors expressing *GAGE-1/2* also express *MAGE-1*, *MAGE-3*, or *BAGE* (data not shown). Some of the patients bearing such tumors could therefore be immunized simultaneously against several antigens encoded by these genes. This might ensure a more effective tumor rejection response.

It should also reduce the emergence of antigen loss variants arising by loss of antigen expression, since it is unlikely that tumor cells could simultaneously delete or mutate several of these genes or lose their expression. Nevertheless, the simultaneous loss of several antigens could still occur after the loss of MHC class I molecules. Fortunately, MHC-negative variants appear to be highly sensitive to NK cells, which may eliminate these variants (43, 44). In support of this notion, Levitsky et al. observed the very frequent emergence of MHC-negative variants in immunized mice that had been depleted of NK1⁺ cells before challenge with tumor cells. These variants were less frequently observed in mice that had not been NK depleted (45).

To the best of our knowledge, the GAGE peptide is the first antigenic peptide presented by HLA-Cw6 that has been identified. A consensus motif for binding to HLA-Cw6 was proposed by Falk et al. on the basis of peptide elution studies (46). A dominant residue of this motif was L at position 9. Strong residues were P in position 4, I or L in position 5, and V, I, or L in position 6. Our peptide does not fit this motif. A first difference is the presence of tyrosine instead of leucine at the COOH terminus of the GAGE peptide. Although tyrosine was also detected at position 9 in the pool of eluted peptides, it was not considered a dominant or strong residue. Another difference is the fact that the GAGE peptide that is most effective in vitro is an octamer rather than a nonamer, but this does not prove that the octamer is the natural peptide. The cells used by Falk et al. (46) for the peptide elution expressed the HLA-Cw*0602 allele, which was first reported to be distinct from Cw*0601 (47). However, after correction of sequence uncertainties, the two Cw6 sequences proved identical (48). In any case, allelic diversity could not explain the divergence of the GAGE peptide from the proposed motif because the cells used by Falk et al. (46) can present the GAGE peptide to CTL 76/6 (data not shown).

Among the class I binding antigenic peptides that have so far been identified in humans, most are presented by HLA-A

Table 2. Proportion of Melanomas Expressing Antigens Encoded by the *MAGE*, *BAGE*, or *GAGE* genes

Genes	Expressing the gene	Fractions of melanomas				Reference
		Expressing an antigen presented by HLA				
		A1 (26%)*	A2 (49%)	Cw16 (7%)	Cw6 (16%)	
MAGE-1	36%	9%		3%		8, 11
MAGE-3	64%	17%	31%			9, 15
BAGE	22%			2%		6
GAGE-1/2	24%				4%	this report
Corrected [‡] total of melanomas expressing at least one antigen: 51%						

* The frequency of each HLA specificity in Caucasians is indicated in parentheses.

‡ After correction for melanomas expressing both HLA-A1 and HLA-A2, and for melanomas expressing both *MAGE-1* and *MAGE-3*.

or HLA-B molecules, and only very few by HLA-C (49–52). Since surface expression of HLA-C molecules was reported to be lower than that of HLA-A and HLA-B, this led to the suggestion that HLA-C molecules do not contribute significantly to antigen presentation (53). However, in MZ2-MEL melanoma cells, we have observed that among five antigens recognized by autologous CTL, three are presented by HLA-C molecules, and both HLA-C alleles are involved: HLA-Cw*1601 presents a peptide derived from MAGE-1 and another derived from BAGE, whereas HLA-Cw*0601 presents a GAGE-encoded antigen (6, 11).

Like MAGE and BAGE, the GAGE genes form a family of very closely related genes. The MAGE family is made up of 12 genes, none of which are expressed in normal adult tissues besides the testis (6, 34). In addition to these genes

located in the q27-qter region of the X chromosome, several additional related genes are located in the p21.3 region of the same chromosome (54, 55). Hydrophobic cluster analysis of the proteins encoded by the different MAGE genes showed a remarkable conservation of the main hydrophobic regions, suggesting conservation of function of these proteins. Higher variation was observed in the promoter region of the MAGE genes, and this led to the suggestion that duplication of a MAGE gene into a large gene family placed the same function under different transcriptional controls, possibly to allow it to occur at a number of very specific times and locations (34). The apparent absence of expression in adult somatic tissues and in fetuses older than 20 wk raises the possibility that MAGE, BAGE, and GAGE gene products play a role during early stages of embryonic development.

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