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.

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 precipitation method with the GAGE-I 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 pCDNAI/Amp (Invitrogen). A clonal subline was isolated from MEL.3.1 cells (20). Lymphoblastoid cell lines MZ2-EBV and LB33-EBV were ligated to BstXI adaptors and digested with NotI. After size fractionation, the cDNA were unidirectionally cloned into the BstXI NotI sites of plasmid pCDNAI/Amp. Recombinant plasmids were electroporated into Escherichia coli DH5-α and selected with ampicillin (50 μg/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).

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 pCDNAI/Amp. Recombinant plasmids were electroporated into Escherichia coli DH5-α and selected with ampicillin (50 μg/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 × 10⁵ COS-7 cells were transfected with ~100 ng of plasmid DNA of a pool of the cDNA library and either 50 ng of plasmid pCD-SRα (26) containing the HLA-A1 gene (27), 50 ng of plasmid pCDNAI/Amp containing the autologous HLA-B37 cDNA, or 75 ng of plasmid pCDNAI/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 μl of Iscove's modified Dulbecco's medium (Gibco BRL) containing 10% human serum and 30 U/ml rhIL-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-diphenyltetrazolium bromide (MTT) colorimetric assay (29, 21). 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 in 24-h incubation at 37°C.

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 × SSC and twice for 15 min at 60°C in 2 × 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 from the guanidine-isothiocyanate procedure as described (31). Reverse transcription was performed on 2 μg of total RNA in a reaction volume of 20 μl with 4 μl of 5× reverse transcriptase buffer (GIBCO BRL), 1 μl each of 10 mM deoxynucleotides, 2 μl of a 20-μM solution of oligo(dT)17 primer, 20 U of RNAse (Promega Biotech, Madison, WI), 2 μl 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 μl of 10× thermostable DNA polymerase buffer (Finnzymes OY, Espoo, Finland), 1 μl each of 10 mM dNTP, 2 μl each of 20-μM primer solutions, 1 U of DynaZyme™ (Finnzymes OY), and water to a final volume of 50 μl. The PCR primers for the amplification of all GAGE genes were either 5′-GGCGGCGCGACGTCGTA-3′ (VDE43, sense, nucleotide 126–142) or 5′-AGACGCTACGTAGAGGAGGT-3′ (VDE43, sense, nucleotide 85–105), 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′-GAGACAGGATACTGAG-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. An additional extension step of 15 min at 72°C was done. 10-μl 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. Lysylated 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 pcDNAI/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 pcDNAI/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 representative 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 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 exons 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 166 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 encoding only the first 23 amino acids of GAGE-1 was synthesized by PCK, 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 3. Northern blot analysis of the expression of gene GAGE. Each lane contains 10 μg 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 1-MEL, LB33-MEL, and SK33-MEL are melanoma lines derived from other patients.](image)

![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/DDBJ under accession numbers U19142, U19143, U19144, U19145, U19146, and U19147.](image)

![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.](image)
two overlapping nonamers, namely TYRPRPRRY and YRPRPRRYV, were positive. We then synthesized the octameric peptide common to the two nonamers and the decameric 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 decamer 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 M2Z-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) 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 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
Table 1. Expression of GAGE Genes by Normal and Tumoral Tissues

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

I 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 PIA, 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

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

| Genes   | Expressing the gene | Expressing an antigen presented by HLA | Reference |
|---------|---------------------|---------------------------------------|-----------|
|         |                     | 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*0601 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.

The excellent technical assistance of Miss Anne Authom is gratefully acknowledged. We thank Dr. F. Brasseur and Mrs. M. Swinarska for preparation of RNA. We also appreciated helpful discussion with Drs. P. van der Bruggen, P. Coulie, and E. de Plaen. We also thank Saïda Khaoulali for her invaluable help in the preparation of the manuscript.

This work was partially supported by the Fonds J. Maisin (Belgium), by the Caisse Générale d’Epargne et de Retraite-Assurances (Belgium), and by the Association Contre le Cancer (Belgium). B. Gaugler was supported by an European Economic Community Grant, and O. Peeters was supported by a fellowship from the Institut pour l’Encouragement de la Recherche Scientifique dans l’Industrie et l’Agriculture (Belgium). S. Lucas is supported by the Fonds National de la Recherche Scientifique (Belgium).

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Received for publication 8 March 1995 and in revised form 13 April 1995.

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