

## Human Gene MAGE-3 Codes for an Antigen Recognized on a Melanoma by Autologous Cytolytic T Lymphocytes

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### Summary

Human melanoma cell line MZ2-MEL expresses several antigens recognized by autologous cytolytic T lymphocyte (CTL) clones. We reported previously the identification of a gene, named MAGE-1, that codes for one of these antigens named MZ2-E. We show here that antigen MZ2-D, which is present on the same tumor, is encoded by another member of the MAGE gene family named MAGE-3. Like MAGE-1, MAGE-3 is composed of three exons and the large open reading frame is entirely located in the third exon. Its sequence shows 73% identity with MAGE-1. Like MZ2-E, antigen MZ2-D is presented by HLA-A1. The antigenic peptide of MZ2-D is a nonapeptide that is encoded by the sequence of MAGE-3 that is homologous to the MAGE-1 sequence coding for the MZ2-E peptide. Competition experiments using single Ala-substituted peptides indicated that amino acid residues Asp in position 3 and Tyr in position 9 were essential for binding of the MAGE-1 peptide to HLA-A1. Gene MAGE-3 is expressed in many tumors of several types, such as melanoma, head and neck squamous cell carcinoma, lung carcinoma and breast carcinoma, but not in normal tissues except for testes. It is expressed in a larger proportion of melanoma samples than MAGE-1. MAGE-3 encoded antigens may therefore have a wide applicability for specific immunotherapy of melanoma patients.

Mixed lymphocyte-tumor cell cultures carried out with human melanoma cells and lymphocytes from the same patient often generate cytolytic T lymphocytes (CTL)<sup>1</sup> that lyse the autologous tumor cells (1-4). Using blood lymphocytes of melanoma patient MZ2, a panel of CTL clones has been obtained that lyse autologous tumor cell line MZ2-MEL (3). These CTL clones were used to select antigen-loss variants and this led to the definition of several different antigens on the MZ2-MEL melanoma cells (5). Gene MAGE-1 that directs the expression of antigen MZ2-E was identified by transfecting a cosmid library prepared with the DNA of the MZ2-MEL cells into an E<sup>-</sup> antigen-loss variant and by testing the ability of the transfected cells to stimulate anti-MZ2-E CTL (6). Gene MAGE-1 is composed of three exons. The third exon contains an open reading frame coding for a protein of 309 amino acids (7). A MAGE-1 encoded nonapeptide composed of amino acids 161-169 binds to MHC class

I molecule HLA-A1 to form the complex recognized by the anti-MZ2-E CTL (8). Gene MAGE-1 is expressed in many melanoma tumors as well as in other types of tumors. No expression was detected on normal tissues with the exception of testes (7).

When the expression of gene MAGE-1 was analyzed in the MZ2-MEL cell line, two other cDNA species were found that crosshybridized with a MAGE-1 probe. The sequences of these cDNA proved to be closely similar to that of MAGE-1 and the corresponding genes were named MAGE-2 and MAGE-3 (6). We report here that gene MAGE-3 is responsible for the expression of another antigen present on the MZ2-MEL cell line, namely MZ2-D.

### Materials and Methods

**Cell Lines and Culture Conditions.** Melanoma cell line MZ2-MEL was derived from patient MZ2, and various clonal sublines were obtained (3). MZ2-MEL.61, which does not express antigen MZ2-D, was obtained by in vitro immunoselection of MZ2-MEL with anti-MZ2-D autologous CTL clone 20/38 (5). The derivation of

<sup>1</sup> Abbreviations used in this paper: CHO, Chinese hamster ovary; CTL, cytolytic T lymphocyte; RT-PCR, reverse transcriptase-polymerase chain reaction.

anti-MZ2-D CTL clone 20/38 and its culture conditions have been previously described (5). EBV-transformed B cell line BM21 was derived from a HLA-A1 individual (9).

**Screening of the Genomic Library and Sequencing.** The genomic library was constructed in cosmid c2RB with DNA from PBL of patient MZ2 as previously described (10). Cosmid 4.12, which contained the MAGE-3 sequence, was identified by colony hybridization with <sup>32</sup>P-labeled oligonucleotide Chinese hamster ovary (CHO)-3 (6) as described by Lurquin et al. (11). Cosmid 4.12 was digested with EcoRI, size-fractionated in agarose gels, blotted on nitrocellulose filters, and hybridized with <sup>32</sup>P-labeled oligonucleotides CHO-3 or VDB19 (5'-CGGATGGTTGAATGAGC-3': MAGE-3 positions 1323-1339 sense). A 5- and 1.9-kb fragment hybridizing with CHO-3 and VDB19, respectively, were then subcloned in plasmid pTZ18R (Pharmacia Fine Chemicals, Piscataway, NJ) and single-stranded DNA was produced. Nucleotide sequence was determined using the T7 sequencing kit (Pharmacia Fine Chemicals) and synthetic primers. Sequence alignments were performed with the GeneWorks computer software (IntelliGenetics, Inc./Betagen, Mountain View, CA).

**Transfection of COS-7 Cells.** Transfection experiments were performed by the DEAE-dextran-chloroquine method (12). Briefly, 1.5 × 10<sup>4</sup> COS-7 cells were treated with 100 ng of plasmid pcDNA1/Amp (Invitrogen Corporation, San Diego, CA) containing the HLA-A1 gene and 100 ng of plasmid containing the cDNA of MAGE-1, MAGE-3 (cloned in pcDNA1/Amp), or MAGE-2 (cloned in pcDSR $\alpha$ ). Plasmid pcDSR $\alpha$  was a gift of Kevin Moore (DNAX, Palo Alto). The COS-7 cells were incubated for 24 h at 37°C. The medium was then discarded and 1,500 CTL were added in 100  $\mu$ l of Iscove medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% human serum and 25 U/ml rIL-2 (Cetus Corp., Berkeley, CA). After 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxicity for WEHI-164-13 (13) as previously described (14).

**Antigenic Peptides and CTL Assay.** Peptides were synthesized on solid phase using F-moc for transient NH<sub>2</sub>-terminal protection as described by Atherton et al. (15), and characterized by mass spectrometry. All peptides were >90% pure as indicated by analytical HPLC. Lyophilized peptides were dissolved in 0.02 M PBS and stored at -20°C. Lysis of target cells by CTLs was tested by chromium release as previously described (16). In the peptide sensitization assay, target cells were <sup>51</sup>Cr-labeled for 1 h at 37°C and washed extensively. 1,000 target cells were then incubated in 96-well microplates in the presence of various concentrations of peptide for 30 min at 37°C. CTLs were then added in an equal volume. Chromium release was measured after 4 h at 37°C. In the peptide competition assay, 10<sup>6</sup> cells of the BM21 lymphoblastoid cell line (HLA-A1<sup>+</sup>) were labeled with <sup>51</sup>Cr during 1 h at 37°C in the presence of a 1:40 dilution of anti-class I mAb W6/32 ascites in Tris Dulbecco buffer. Labeled targets (1,000 cells/well) were incubated for 15 min at room temperature with various concentrations of competitor peptides, before addition of the antigenic MAGE-3 peptide. Then, after 15 min, cells from CTL clone 20/38 were added at a lymphocyte to target cell ratio of 10:1. The assay was terminated after a 4-h incubation at 37°C.

**mRNA Expression Analysis.** Total RNA was extracted by the guanidine-isothiocyanate procedure as described (17). For cDNA synthesis, RNA (2  $\mu$ g) was diluted with water, 4  $\mu$ l of 5 $\times$  reverse transcriptase buffer (GIBCO BRL), 1  $\mu$ l each of 10 mM dNTP, 2  $\mu$ l of a 20  $\mu$ M solution of oligo(dT), 20 U of RNasin (Promega Biotec, Madison, WI), 2  $\mu$ l of 0.1 M dithiothreitol, and 200 U of MoMLV reverse transcriptase (GIBCO BRL) in a 20- $\mu$ l reaction volume, and incubated at 42°C for 60 min. One twentieth of the

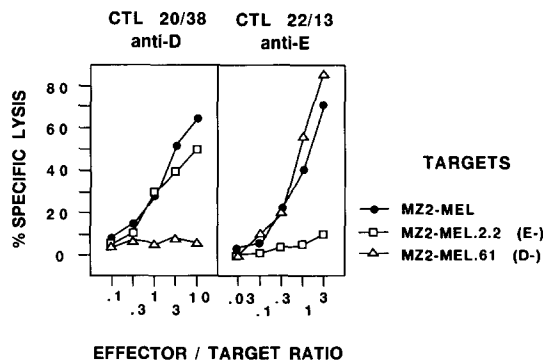
cDNA reaction was supplemented with 5  $\mu$ l of PCR buffer (Perkin-Elmer Cetus Instruments, Norwalk, CT), 0.5  $\mu$ l each of 10 mM dNTP, 1  $\mu$ l each of 20  $\mu$ M solutions of primers, 1.25 U of Taq polymerase (Perkin-Elmer Cetus Instruments) and water to a final volume of 50  $\mu$ l. Primers were 5'-TGGAGGACCAGAGGCCCCC-3' (sense, exon 2) and 5'-GGACGATTATCAGGAGGCCTGC-3' (anti-sense, exon 3) for MAGE-3, and 5'-GGGACCAGGAGACAC-GGAATA-3' (sense, exon 2) and 5'-AGCCCCGTCCACGCACCG-3' (anti-sense, exon 3) for HLA-A1. PCR was performed for 30 cycles (1 min at 94°C and 4 min at 72°C for MAGE-3; 1 min at 94°C, 2 min at 68°C and 2 min at 72°C for HLA-A1). The PCR product was size-fractionated on a 1% agarose gel. The conditions for PCR amplification of MAGE-1 and MAGE-2 were previously described (7, 18). The quality of RNA preparations was checked by PCR amplification of  $\beta$ -actin cDNA.

## Results

CTL clone 20/38, which was derived by stimulating PBL of patient MZ2 with the autologous melanoma cell line MZ2-MEL, lyses MZ2-MEL cells but not autologous fibroblasts, autologous EBV-transformed B cells, or K562 (5). This CTL clone recognizes an antigen named MZ2-D, which is different from MZ2-E, the antigen encoded by gene MAGE-1. This follows from the observation that an MZ2-MEL.E<sup>-</sup> variant selected for resistance to an anti-MZ2-E CTL was still lysed by CTL 20/38 (Fig. 1). Conversely, a MZ2-MEL.D<sup>-</sup> antigen-loss variant, which was selected in vitro for resistance to CTL 20/38, was still lysed by the anti-MZ2-E CTL.

**Anti-MZ2-D CTL Recognize HLA-A1 Melanomas That Express MAGE-3.** CTL clone 20/38 secreted TNF when put in the presence of the MZ2-MEL cells that express antigen MZ2-D, but not in the presence of the D<sup>-</sup> antigen-loss variant (Table 1). To identify the HLA class I molecule presenting antigen MZ2-D to CTL 20/38, we performed the same test with a number of melanoma lines of patients sharing one HLA specificity with patient MZ2. This patient carries HLA-A1, A29, B37, B44, Cw6, and C.c1.10. But we focused on melanomas expressing either A1, B37, or Cw6, because variant MZ2-MEL.2.2.5, known to have lost HLA-A29, B44, and C.c1.10, still expressed antigen MZ2-D. 8 of 10 melanoma cell lines derived from HLA-A1 patients stimulated CTL 20/38 (Table 1) and several of these tumor cell lines were also lysed by this CTL clone (Fig. 2), suggesting that HLA-A1 was the class I molecule presenting antigen MZ2-D. The gene coding for MZ2-D did not seem to be MAGE-1 because several allogeneic melanomas that stimulated the CTL did not express this gene (Table 1). By looking at the pattern of expression of genes MAGE-1, -2, and -3, we noticed that gene MAGE-3 was expressed by the eight HLA-A1 melanomas that were recognized by CTL 20/38, whereas the two lines that were not recognized did not express this gene. This suggested that antigen MZ2-D might be a MAGE-3-encoded peptide presented by HLA-A1.

**Expression of MZ2-D by COS Cells Transfected with HLA-A1 and MAGE-3.** To find out whether gene MAGE-3 directed the expression of antigen MZ2-D, we transfected COS-7 cells with the HLA-A1 gene and either MAGE-1, MAGE-2, or



**Figure 1.** Cytolytic activity of CTL clones 20/38 and 22/13 of patient MZ2 on MZ2-MEL cells. Antigen-loss variants MZ2-MEL.2.2 E<sup>-</sup> and MZ2-MEL.61 D<sup>-</sup> were obtained by in vitro immunoselection with CTL 22/13 and 20/38, respectively. Lysis of chromium-labeled cells was measured after 4 h.

MAGE-3 cDNA cloned in an expression vector. After 1 d we added CTL 20/38 and 24 h later, the amount of TNF present in the supernatant was assayed by testing its cytotoxicity for WEHI-164-13 cells (Fig. 3). CTL 20/38 was strongly stimulated by COS-7 cells transfected with HLA-A1 and MAGE-3, but not by cells transfected with HLA-A1 alone or with HLA-A1 and MAGE-1 or MAGE-2. We concluded

that antigen MZ2-D is a MAGE-3 product presented by HLA-A1.

We tested the expression of MAGE-3 in antigen-loss variant MZ2-MEL.61, which had lost the expression of antigen MZ2-D. RNA was reverse transcribed with an oligo(dT) primer, and the cDNA was amplified with a pair of primers specific for MAGE-3. The level of expression of MAGE-3 was considerably lower than that found in the D<sup>+</sup> MZ2-MEL cells. Quantitative PCR analysis revealed that D<sup>-</sup> cells express about 6% of the level of MAGE-3 mRNA found in D<sup>+</sup> cells (data not shown). This low level is presumably insufficient to provide enough antigenic peptide to ensure recognition by CTL 20/38.

**Sequence and Structure of Gene MAGE-3.** The gene coding for the MAGE-3 mRNA was isolated by screening a cosmid library prepared with the DNA of PBL from patient MZ2. A cosmid containing the gene was identified by colony hybridization with an oligonucleotide probe corresponding to a region where the MAGE-3 sequence differs significantly from that of MAGE-1 and MAGE-2. The sequence of the whole gene was determined and aligned to that of the MAGE-3 cDNA. This allowed us to deduce the structure of gene MAGE-3, which proved very similar to that of gene MAGE-1 with two small exons followed by a large one. The nucleotide sequence of MAGE-3 is 73% identical to that of MAGE-1,

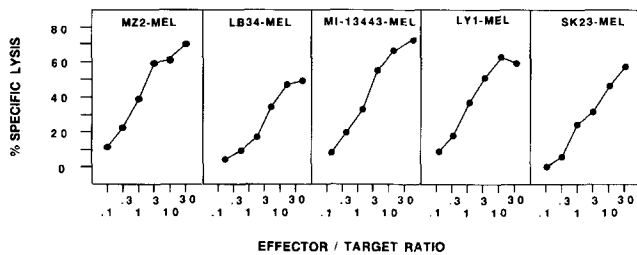
**Table 1.** TNF Release by CTL 20/38 Incubated with Allogeneic Melanomas

Melanoma	Expression of HLA-A1*	†Expression of			TNF release by CTL 20/38 <sup>§</sup>	
		MAGE-1	MAGE-2	MAGE-3	Exp. 1	Exp. 2
MZ2-MEL	+	+++	+++	+++	>120	>120
MZ2-MEL.61D <sup>-</sup>	+	++++	++++	±	1	4
LY1-MEL	+	+	-	+++	>120	>120
MI-10221-MEL	+	+	+++	+++	>120	
LY2-MEL	+	+++	+++	+++	57	
LY4-MEL	+	-	++	+++	>120	
SK23-MEL	+	-	++++	++++	112	
MI665/2-MEL	+	-	-	-	3	4
LB34-MEL	+	+	++++	++++	>120	
LB45-MEL	+	-	-	-	11	30
NA6-MEL	+	-	++	+++	77	98
MI-13443-MEL	+	++++	++++	++++	>120	
LB5-MEL	-	-	±	+	8	9
SK64-MEL	-	-	-	-	4	5
LB33-MEL	-	±	+++	+++		3.5
LB73-MEL	-	+	+++	+++	16	

\* Expression of HLA-A1 was tested by RT-PCR.

† Expression of MAGE genes was measured by RT-PCR analysis and scored according to band intensity of PCR products.

§ 1,500 cells of CTL clone 20/38 and 25 U/ml IL-2 were mixed with 30,000 cells of the different allogeneic melanomas, except for the two MZ2-MEL lines, where 50,000 stimulator cells were used. After 24 h, the amount of TNF present in the supernatant was assayed by testing its cytotoxicity for WEHI-164-13. Results are expressed in pg/ml equivalent TNFβ. TNF release in the absence of CTL added was <5 pg/ml in the two experiments.

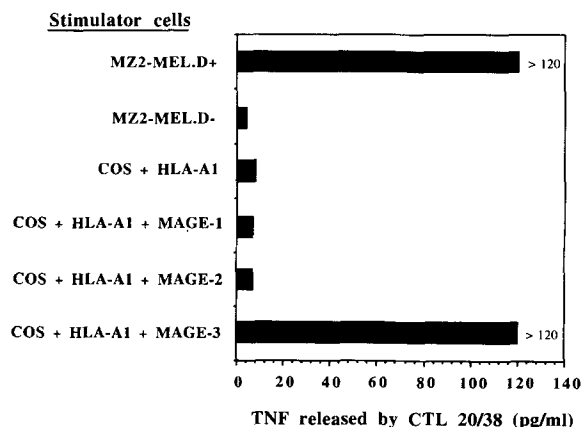


**Figure 2.** Lysis of melanoma target cells by CTL clone 20/38 of patient MZ2. Melanoma cell lines MI-13443-MEL, LB34-MEL, LY1-MEL, and SK23-MEL were derived from HLA-A1 patients. They all express MAGE-3. Lysis of chromium-labeled cells was measured after 4 h.

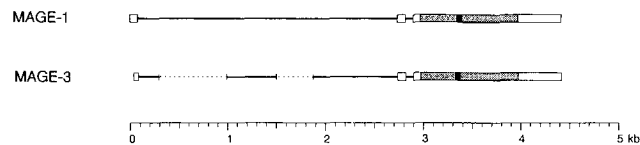
and in exon 3 the identity is 82%. The main structural difference between the two genes is the presence of two deletions in the first intron of MAGE-3, one of 700 bp and one of 400 bp (Fig. 4).

The cDNA sequence of MAGE-3 is shown in Fig. 5. Like MAGE-1, MAGE-3 contains a large open reading frame in the third exon. The MAGE-3 protein is 314 amino acids long and presents 66% identity with the MAGE-1 protein which is 309 amino acids long. The MAGE-3 protein is devoid of a signal sequence but contains a putative short transmembrane domain that is conserved in the MAGE-1 protein (De Plaen, E., K. Arden, C. Traversari, J. J. Gaforio, J.-P. Szikora, C. De Smet, F. Brasseur, P. van der Bruggen, B. Lethé, C. Lurquin, P. Chomez, R. Brasseur, W. Cavenee, and T. Boon, manuscript in preparation).

**Identification of the Antigenic Peptide of Antigen MZ2-D.** A 881-bp fragment of MAGE-3 cDNA (nucleotides 1-881 in Fig. 5) which did not contain the 3' end of exon 3 proved capable of transferring the expression of antigen MZ2-D when transfected into COS-7 cells (data not shown). In our attempt to find out which part of this fragment coded for the MZ2-D antigenic peptide, we were helped by the existence of another



**Figure 3.** Stimulation of CTL 20/38 by COS-7 cells transfected with expression vectors containing the cDNA of MAGE-3 and the HLA-A1 gene as described in Materials and Methods. As controls, COS-7 cells were transfected with HLA-A1 gene alone or mixed with the cDNA of MAGE-1 or of MAGE-2. CTL 20/38 was added and the TNF content of the supernatant was tested for its cytotoxicity on WEHI-164-13 cells.



**Figure 4.** Gene structures of genes MAGE-1 and MAGE-3. Exons are indicated as boxes, with the sequences encoding the proteins and the antigenic peptides in grey and black, respectively. MAGE-1 and MAGE-3 sequences were aligned and the stippled lines indicate regions that are deleted in MAGE-3 relative to MAGE-1.

member of the MAGE gene family named MAGE-6, which presented 99% identity with MAGE-3 (De Plaen, E. et al., manuscript in preparation). Despite this nearly complete identity, MAGE-6 did not seem to code for antigen MZ2-D because it was expressed at a high level in the D<sup>-</sup> antigen-loss variant (data not shown). This was confirmed by transfecting into COS-7 cells a fragment of MAGE-6 corresponding to the 881-bp fragment of MAGE-3 cDNA; no expression of antigen MZ2-D was observed (data not shown). We examined the few amino acid differences between MAGE-3 and MAGE-6 that were localized in the 881-bp fragment. Six of seven substitutions could not be included in a peptide sequence carrying a tyrosine. This amino acid residue has been suggested to serve as anchoring residue for several class I molecules (19-21) and it is present at position 9 in the MAGE-1 peptide that binds to HLA-A1 (8). It is interesting to note that the last substitution was located in the region homologous to the region of MAGE-1 that codes for the MZ2-E peptide (8). On this basis, nonapeptide EVDPIGHLY was synthesized and corresponds to codons 168-176 of the long open reading frame of MAGE-3. This peptide sensitized D<sup>-</sup> cells to lysis by the anti-D CTL clone (Fig. 6).

**Two Residues Involved in the Binding of the MAGE-1 Peptide to HLA-A1.** The availability of the MAGE-1 and MAGE-3 peptides, which both bind to HLA-A1 but are recognized by different CTL, enabled us to perform competition experiments in order to identify the residues involved in the binding to the HLA-A1 molecule. HLA-A1 positive cells were incubated with competitor MAGE-1 peptide at various concentrations. Then, the MAGE-3 peptide was added and the cells were tested for recognition by anti-D CTL 20/38. As shown in Fig. 7, the MAGE-1 peptide was able to inhibit the lysis of cells pulsed with the MAGE-3 peptide. We then tested a panel of MAGE-1 peptides substituted with alanine in single positions. Substitutions of P, T, G, H, and S (residues 4, 5, 6, 7, and 8) by A did not prevent the peptide from competing. In contrast, competition was reduced by the substitutions of D by A in position 3 and it was abolished by the substitution of Y by A in position 9, indicating that these two residues are involved in the binding to HLA-A1 (Fig. 7A).

We also tested peptides encoded by the homologous sequence of gene MAGE-2 and of additional genes that belong to the MAGE family (Fig. 7B). The MAGE-2 peptide, which does not carry D in position 3, did not compete with the MAGE-3 peptide. Replacement of V by D in position 3 of the MAGE-2 nonapeptide significantly improved its ability

to bind to HLA-A1 (data not shown), confirming the essential role of this residue for binding to HLA-A1. In contrast, the peptides encoded by MAGE-4a, 4b, 5, and 6, which do carry D in position 3 and Y in position 9, were able to compete. These results suggest that these MAGE genes are potential sources of antigens presented by HLA-A1.

**MAGE-3 Expression in Tumors and Normal Tissues.** The expression of gene MAGE-3 was evaluated in various tumors and normal tissues by reverse transcription and PCR amplification (RT-PCR) with primers that are specific for this gene. Primers located in different exons were chosen so as to prevent occasional false positives due to DNA contamination of the RNA. A panel of tumor cell lines and fresh-frozen samples from tumors of various histological types were tested. Gene MAGE-3 is expressed in a higher proportion of melanomas (69%) than gene MAGE-1 (40%). Like MAGE-1, gene MAGE-3 is expressed in several tumor types other than melanoma, such as small cell and nonsmall cell lung cancer and head and neck squamous cell carcinoma (Table 2). Lower percentages of positive tumors were found in mammary carcinomas and colorectal carcinomas. No renal carcinoma expressed MAGE-3. The expression of MAGE-3 was tested in a variety of normal adult tissues and in some tissues from fetuses of more than 20 wk. All were negative except testes.

## Discussion

The MAGE gene family is composed of at least 12 different closely related genes whose sequences show 64 to 85% identity with MAGE-1 (De Plaen, E., et al., manuscript in preparation). All these genes appear to be silent in normal adult tissues other than testis. Six of them, namely MAGE-1, -2, -3, -4, -6, and -12, are expressed in a significant proportion of human tumors of various histological types. Because these six genes code for proteins of more than 300 amino acids, it was surmised that they should produce many peptides combining with various HLA molecules to form tumor antigens recognized by T lymphocytes. This would be in line with the observations made with influenza proteins such as nucleoprotein which is known to produce several peptides recognized by CTL on different HLA alleles (22–26). On the other hand, it was impossible to exclude a priori that, because MAGE proteins are self proteins, the diversity of T lymphocytes recognizing MAGE-encoded antigens could be severely reduced by tolerance.

Our observation that MAGE-3 codes for an antigen recognized on a melanoma by autologous CTL fully confirms the notion that multiple antigens are coded by the MAGE gene family. Moreover, another antigen encoded by MAGE-1 and presented by the HLA-C<sub>1.10</sub> molecule has recently been identified (van der Bruggen, P., P. Boël, and T. Boon, manuscript in preparation). Accordingly, we will continue to test known antitumor CTL clones for their ability to crossreact with tumor cells sharing some HLA alleles with the syngeneic tumor target. By examining the pattern of expression of MAGE genes in the crossreacting tumors, we will try to identify new antigens encoded by MAGE genes.

An alternative approach should also prove useful. It is based

on the observation that a given class I molecule combines only with a restricted set of peptides that carry certain anchor residues at a few crucial positions. These consensus motives have been identified for a number of HLA alleles (20, 27–30). It is possible to identify in the MAGE protein sequences several peptides that should be capable of binding to certain HLA molecules. These peptides could then be synthesized and tested for their binding to the relevant HLA molecule, which results in stabilization of these molecules (31–33). The peptides that show good binding could then be used to stimulate T cells in order to generate CTL reactive with the peptide/HLA complex. This has been achieved for the identification of malaria epitopes recognized by CTL (21). However, the lymphocytes restimulated *in vitro* with these peptides came from individuals already immunized against malaria. With MAGE peptides, one would have to generate primary T cell responses *in vitro*. This should be more difficult, but it has been achieved using peptide-pulsed dendritic cells or mutants that are defective for antigen processing like RMA-S or T2 (34–36).

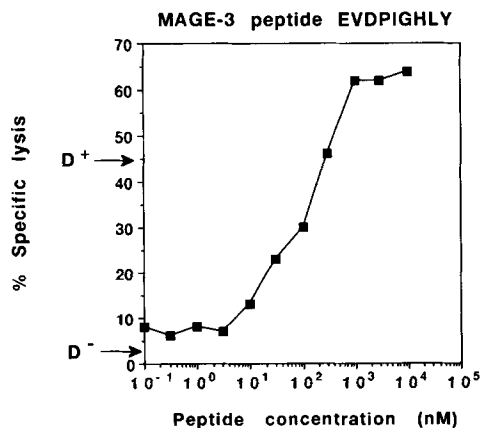
The antigenic peptide that is encoded by MAGE-3 and that binds to HLA-A1 is located at a position homologous to that of the MZ2-E peptide in the MAGE-1 protein. Two residues, which are shared by both peptides, appear to be essential for binding to HLA-A1, namely aspartic acid in position 3, and tyrosine in position 9. These residues may constitute a general motive for peptide binding to HLA-A1. This would be in line with mounting evidence that the COOH-terminal residue is always an anchor residue for peptide binding to class I molecules (20, 27–29). Homologous peptides derived from genes MAGE-4 and 6 carry the same motive and were shown to bind to HLA-A1. It may therefore be possible to obtain CTL that recognize antigens formed by these peptides on HLA-A1. The MAGE-6 derived peptide was not recognized by the anti-MZ2-D CTL clone, even though it differs from the MAGE-3 peptide only at position 8 (L→V). This leucine residue in the MAGE-3 peptide is therefore involved in the interaction of the peptide with the T cell receptor.

Antigen MZ2-D encoded by MAGE-3 may prove to be a useful target for specific antitumor immunization of melanoma patients. Patients expressing this antigen on their tumor cells will be identified by HLA typing and by performing reverse transcription and PCR amplification with MAGE-3 specific primers on small tumor samples frozen immediately after surgical removal. The proportion of melanoma patients expressing this antigen should be ~18% in Caucasian populations, since 26% of individuals are HLA-A1 positive and 69% of melanomas express MAGE-3. This compares with ~10% of melanoma patients whose tumor expresses MAGE-1 encoded antigen MZ2-E. It is noteworthy that all melanomas that express MAGE-1 also express MAGE-3. Thus, the availability of MAGE-3 in addition to MAGE-1, should only increase from 10 to 18% the proportion of patients eligible for vaccination. On the other hand, it will be possible to attempt to immunize 10% of melanoma patients against both the MAGE-1 and the MAGE-3 encoded antigens. This should reduce the risk of tumor escape due to the emergence of antigen-loss variants.

	exon1			exon2	
MAGE-1	CCATTCTGAGGGACGGCGTA	GAGTTCGGCCGAAGGAACCT	GACCCAGGCTCTGTGAGGAG	GCAAGGTTTTCAGGGGACAG	80
MAGE-3	-----	-----GAGGGAAGCC	GGCCAGGCTCGGTGAGGAG	GCAAGGTTCTGAGGGGACAG	50
	exon1			exon2	
MAGE-1	GCCAACCCAGAGGACAGGAT	TCCCTGGAGGCCACAGAGGA	GCACCAAGGAGAAGATCTGC	CTGTGGGTCTTTCATTGCCCA	160
MAGE-3	GCTGACCTGGAGGACCAGAG	GCCCCGGAGGAGCACTG--	----AAGGAGAAGATCTGC	CAGTGGGTCTCCATTGCCCA	123
				exon3	
MAGE-1	GCTCCTGCCCCACACTCCTGC	CTGCTGCCCTGACGAGAGTC	ATCATGTCTCTTGAGCAGAG	GAGTCTGCACTGCAAGCCTG	12
MAGE-3	GCTCCTGCCCCACACTCCCGC	CTGTTGCCCTGACCAGAGTC	ATCATGCCTCTTGAGCAGAG	GAGTCAGCACTGCAAGCCTG	240
			M S L E Q R	S L H C K P	12
			M P L E Q R	S Q H C K P	240
					193
					12
MAGE-1	E E A L E A Q	Q E A L G L V	C V Q A	A T	32
MAGE-3	AGGAAGCCCTTGAGGCCCAA	CAAGAGGCCCTGGGCCTGGT	GTGTGTGCAAGCT-----	-----GCCACC	299
	AAGAAGCCCTTGAGGCCCA	GGAGAGGCCCTGGGCCTGGT	GGGTGCGCAGGCTCCTGCTA	CTGAGGAGCAGGAGGCTGCC	273
	E E G L E A R	G E A L G L V	G A Q A P A	T E E Q E A A	39
MAGE-1	S S S S P L V	L G T L E E	V P T A G S T	D P P Q S P Q	59
MAGE-3	TCCTCCTCCTCCTCCTGGT	CCTGGGCACCCTGGAGGAGG	TGCCCACTGCTGGGTCAACA	GATCCTCCCCAGAGTCTCA	379
	TCCTCCTCCTCCTCCTAGT	TGAAGTCACCCTGGGGAGG	TGCTGCTGCCGAGTCACCA	GATCCTCCCCAGAGTCTCA	353
	S S S S T L V	E V T L G E	V P A A E S P	D P P Q S P Q	66
MAGE-1	G A S A F P	T T I N F T R	Q R Q P S E G	S S S R E E	85
MAGE-3	GGGAGCTCCGCCTTTCCCA	CTACCATCAACTTCACTCGA	CAGAGGCAACCCAGTGAGGG	TTCCAGCAGCCGTGAAGAGG	459
	GGGAGCTCCAGCCTCCCA	CTACCATGAATACCTCCTC	TGAGGCCAATCCTATGAGGA	CTCCAGCAACCAAGAAGAGG	433
	G A S S L P	T T M N Y P L	W S Q S Y E D	S S N Q E E	92
MAGE-1	E G P S T S C	I L E S L F R	A V I T K K	V A D L V G F	112
MAGE-3	AGGGCCAAGCACCTCTGT	ATCCTGGAGTCTCTGTCCG	AGCAGTAATCACTAAGAAGG	TGGCTGATTTGGTTGGTTTT	539
	AGGGCCAAGCACCTTCCCT	GACCTGGAGTCCGAGTTCCA	AGCAGCACTCAGTAGGAAGG	TGGCCGAGTTGGTTCATTTT	513
	E G P S T F P	D L E S E F Q	A A L S R K	V A E L V H F	119
MAGE-1	L L L K Y R A	R E P V T K	A E M L E S V	I K N Y K H C	139
MAGE-3	CTGCTCCTCAAATATCGAGC	CAGGGAGCCAGTCACAAAGG	CAGAAATGCTGGAGAGTGTC	ATCAAAAATTACAAGCACTG	619
	CTGCTCCTCAAGTATCGAGC	CAGGGAGCCGGTCACAAAGG	CAGAAATGCTGGGAGTGTC	GTCGGAATTTGGCAGTATTT	593
	L L L K Y R A	R E P V T K	A E M L G S V	V G N W Q Y F	146
MAGE-1	F P E I F G	K A S E S L Q	L V F G I D V	<u>K E A D P T</u>	165
MAGE-3	TTTTCCTGAGATCTTCGGCA	AAGCCTCTGAGTCTTGCAG	CTGGTCTTTGGCATTGACGT	GAAGGAAGCAGACCCACCG	699
	CTTTCCTGTGATCTTCAGCA	AAGCTTCCAGTTCCTTGCAG	CTGGTCTTTGGCATCGAGCT	GATGGAAGTGGACCCCATCG	673
	F P V I F S	K A S S S L Q	L V F G I E L	<u>M E V D P I</u>	172
MAGE-1	<u>G H S Y</u> V L V	T C L G L S Y	D G L L G D	N Q I M P K T	192
MAGE-3	GCCACTCCTATGTCCTTGTC	ACCTGCCCTAGGTCTCTCCTA	TGATGGCCTGCTGGGTGATA	ATCAGATCATGCCCAAGACA	779
	GCCACTTGTACATCTTTGCC	ACCTGCCCTGGGCCTCTCCTA	CGATGGCCTGCTGGGTGACA	ATCAGATCATGCCCAAGGCA	753
	<u>G H L Y</u> I F A	T C L G L S Y	D G L L G D	N Q I M P K A	199
MAGE-1	G F L I I V L	V M I A M E	G G H A P E E	E I W E E L S	219
MAGE-3	GGCTTCCTGATAAATGTCCCT	GGTCATGATTGCAATGGAGG	GCGGCCATGCTCCTGAGGAG	GAAATCTGGGAGGAGCTGAG	859
	GGCTTCCTGATAATCGTCCCT	GGCATAATCGCAAGAGAGG	GCGACTGTGCCCTGAGGAG	AAAATCTGGGAGGAGCTGAG	833
	G L L I I V L	A I I A R E	G D C A P E E	K I W E E L S	226
MAGE-1	V M E V Y D	G R E H S A Y	G E P R K L L	T Q D L V Q	245
MAGE-3	TGTGATGGAGGTGTATGATG	GGAGGGAGCACAGTGCCTAT	GGGGAGCCCAAGGAGCTGCT	CACCCAAGATTTGGTGCAGG	939
	TGTGTTAGAGGTGTTTGAGG	GGAGGGAAGACAGTATCTTG	GGGGATCCCAAGAAGCTGCT	CACCCAACATTTCTGTCAGG	913
	V L E V F E	G R E D S I L	G D P K K L L	T Q H F V Q	252

MAGE-1	<u>E K Y L E Y R</u>	<u>Q V P D S D P</u>	<u>A R Y E F L</u>	<u>W G P R A L A</u>	272
MAGE-3	AAAAGTACCTGGAGTACCGG	CAGGTGCCGGACAGTGTATCC	CGCACGCTATGAGTTCCTGT	GGGGTCCAAGGGCCCTCGCT	1019
MAGE-1	<u>E N Y L E Y R</u>	<u>Q V P G S D P</u>	<u>A C Y E F L</u>	<u>W G P R A L V</u>	279
MAGE-3	AAAACTACCTGGAGTACCGG	CAGGTCCCCGGCAGTGTATCC	TGCATGTTATGAATTCCTGT	GGGGTCCAAGGGCCCTCGTT	993
MAGE-1	<u>E T S Y V K V</u>	<u>L E Y V I K</u>	<u>V S A R V R F</u>	<u>F F P S L R E</u>	299
MAGE-3	GAAACCAGCTATGTGAAAGT	CCTTGAGTATGTGATCAAGG	TCAGTGC AAGAGTTCGCTTT	TTCTTCCCATCCCTGCGTGA	1099
MAGE-1	<u>E T S Y V K V</u>	<u>L H H M V K</u>	<u>I S G G P H I</u>	<u>S Y P P L H E</u>	306
MAGE-3	GAAACCAGCTATGTGAAAGT	CCTGCACCATATGGTAAAGA	TCAGTGGAGGACCTCACATT	TCCTACCCACCCCTGCATGA	1073
MAGE-1	<u>A A L R E E</u>	<u>E E G V</u>			309
MAGE-3	AGCAGCTTTGAGAGAGGAGG	AAGAGGGAGTCTGAGCATGA	GTTGCAGCCAAGGCCAGTGG	GAGGGGGACTGGGCCAGTGC	1179
MAGE-1	<u>W V L R E G</u>	<u>E E</u>			314
MAGE-3	GTGGGTTTGTGAGAGAGGGGG	AAGAGTGAGTCTGAGCACGA	GTTGCAGCCAGGGCCAGTGG	GAGGGGGTCTGGGCCAGTGC	1153
MAGE-1	ACCTTCCAGGGCCCGTCCA	GCAGCTTCCCCTGCCTCGTG	TGACATGAGGCCCATTCCTTC	ACTC--TGAAGAGAGCGGTC	1257
MAGE-3	ACCTTCCGGGGCCGCATCCC	TTAGTTPCCAC TGCCCTCCTG	TGACGTGAGGCCCATTCCTTC	ACTCTTTGAAGCGAGCAGTC	1233
MAGE-1	AGTGTTC TCAGTAGTAGGTT	TCTGTCTATTTGGGTGACTT	GGAGATTTATCTTTGTTCTC	TTTTGGAATTGTTCAAATGT	1337
MAGE-3	AGCATTCTTAGTAGTGGGTT	TCTGTCTGTGGATGACTT	TGAGATTATCTTTGTTTCC	TGTGGAGTTGTTCAAATGT	1313
MAGE-1	TTTTTTTTAAGGGATGGTTG	AATGAACTTCAGCATCCAAG	TTTATGAATGACAGCAGTCA	CACA--GTTCTGTGTATATA	1415
MAGE-3	TCC-TTTTAACGGATGGTTG	AATGAGCGTCAGCATCCAGG	TTTATGAATGACAGTAGTCA	CACATAGTGCTGTTTATATA	1392
MAGE-1	GTTTAAGGGTAAGAGTCTTG	TGTTTTATTTCAGATTGGGAA	ATCCATTCTATTTTGTGAAT	TGGG--ATAATAACAGCAGT	1493
MAGE-3	GTTTAGGAGTAAGAGTCTTG	TTTTTTACTCAAATTTGGGAA	ATCCATTCCATTTTGTGAAT	TGTGACATAATAATAGCAGT	1472
MAGE-1	GGATAAGTACTT-----AG	AAATGTGAAAAATGAGCAGT	AAAATAGATGAGATAAAGAA	CTAAAGAAATTAAGAGATAG	1568
MAGE-3	GGTAAAAGTATTTGCTTAAA	ATTGTGAGCGAATTAGCAAT	AACATACATGAGAT----AA	CTCAAGAAATCAAAGATAG	1548
MAGE-1	TCAATTCTTGCCCTTATACCT	CAGTCTATTCTGTAAAATTT	TTAAGATATATGCATACCT	GGATTTCCCTTGGCTTCTTTG	1648
MAGE-3	TTGATTCTTGCCCTTGTACCT	CAATCTATTCTGTAAAATTT	--AAACAAATATGCAAACCA	GGATTTCCCTTGACTTCTTTG	1625
MAGE-1	AGAATGTAAGAGAAATTAAA	TCTGAATAAAGAATTCTTCC	TGT		1691
MAGE-3	AGAATGCAAGCGAAATTAAA	TCTGAATAAATAATTCTTCC	TCTTC		1670

**Figure 5.** Comparison of the nucleotide sequence of the MAGE-3 cDNA with that of MAGE-1. Gaps indicated by dashes (-) were introduced for optimal alignment. The amino acid sequences of the proteins encoded by the large open reading frames are represented. The sequences corresponding to the MZ2-E and MZ2-D peptides are underlined. Exons boundaries are indicated by arrows. The MAGE-3 sequence is available from EMBL/GenBank/DBJ under accession number V03735.

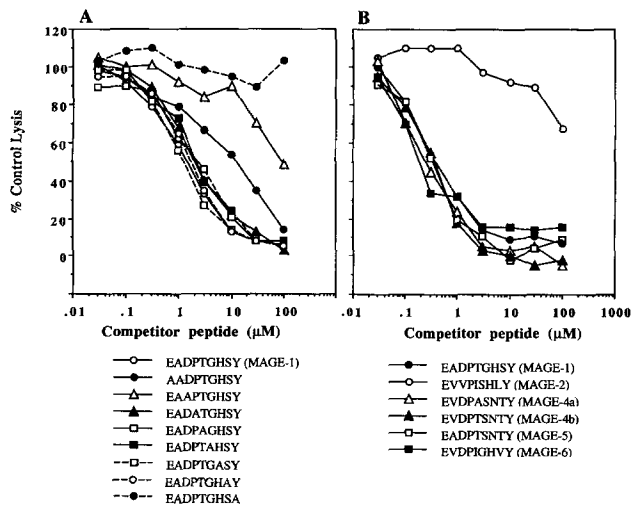


**Figure 6.** Lysis by anti-MZ2-D CTL of cells expressing HLA-A1 incubated with the MAGE-3-encoded peptide. MZ2-MEL.61 cells (D<sup>-</sup>) were <sup>51</sup>Cr-labeled and incubated with CTL 20/38 at an E/T ratio of 10:1 in the presence of the synthetic MAGE-3 peptide at the concentrations

Other types of tumor could also be targets for immunization with antigen MZ2-D: MAGE-3 is expressed in 56% of head and neck squamous cell carcinomas, 30% of nonsmall cell lung carcinomas, 16% of colorectal carcinomas, and 12% of breast tumors. The proportions of these tumors that express MAGE-1 are 25, 35, 0, and 17%, respectively (18, 37). By contrast with melanomas, some of these tumors express only MAGE-1 or only MAGE-3, so that considering the antigens encoded by both genes increases the proportion of patients eligible for immunization. Like MAGE-1, MAGE-3 is never expressed in kidney tumors, leukemias, and lymphomas.

The optimal method of immunization to generate efficient

indicated. Chromium release was measured after 4 h. The arrows indicate the level of lysis of D<sup>+</sup> and D<sup>-</sup> MZ2-MEL cells incubated without peptide.



**Figure 7.** Competition of the MAGE-3 peptide with single Ala-substituted MAGE-1 peptides (A) or with homologous nonapeptides from other MAGE genes (B). Chromium-labeled cells of HLA-A1 positive B lymphoblastoid cell line BM21 were incubated with the indicated concentrations of competitor peptides (0.03–100 μM) for 15 min before addition

CTL responses remains to be determined. Patients could be injected with irradiated tumor cells expressing antigen MZ2-D, or with the recombinant MAGE-3 protein combined with appropriate adjuvants. Effective priming of CTL has been reported in mouse systems after immunization with peptides either alone, associated with a lipid moiety, or mixed with β<sub>2</sub>-microglobulin (38–41). A precise knowledge of the residues involved in the binding to HLA-A1 may help in designing peptides with improved capabilities to bind to HLA-A1 resulting in better immunogenicity.

of the antigenic MAGE-3 peptide at a concentration of 0.25 μM. 15 min later, cells from anti-MZ2-D CTL clone 20/38 were added at a lymphocyte to target ratio of 10:1. Lysis was measured 4 h later. Results are presented as percentages of the control lysis obtained with the MAGE-3 peptide alone, which was 67% (A) and 42% (B). In the absence of the MAGE-3 peptide, lysis of BM21 cells was 2% (A) and 1% (B).

**Table 2.** Expression of Gene MAGE-3 by Tumoral, Normal, and Fetal Tissues

Histological type	Tumors		Normal tissues	
	Cell lines	Tumor samples	Histological type	MAGE-3 expression*
	Number of MAGE-3 positive tumors*			
			<u>Adult tissues</u>	
Melanomas	50/62 (81%)	72/105 (69%)	Brain	–
Head and neck squamous cell carcinomas	–	20/36 (56%)	Colon	–
Lung carcinomas			Stomach	–
NSCLC <sup>†</sup>	1/2	14/46 (30%)	Liver	–
SCLC	18/22 (82%)	2/3	Ovary	–
Colorectal carcinomas	5/16	5/31 (16%)	Skin	–
Mammary carcinomas	2/6	16/132 (12%)	Lung	–
Bladder tumors	–	2/6	Kidney	–
Sarcomas	1/4	3/10	Breast	–
Prostatic carcinomas	–	3/20	Testis	++
Renal carcinomas	0/5	0/38	<u>Fetal tissues</u>	
Leukemias	2/6	0/20	Brain	–
Lymphomas	0/6	0/5	Liver	–
			Spleen	–

\* Expression of gene MAGE-3 was tested by RT-PCR amplification on total RNA, with the primers described in Materials and Methods. These primers distinguish MAGE-3 from the 11 other MAGE genes that have been identified.

<sup>†</sup> NSCLC, nonsmall cell lung carcinomas; SCLC, small cell lung carcinomas.



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