Brief Definitive Report

A Nonapeptide Encoded by Human Gene MAGE-1 Is Recognized on HLA-A1 by Cytolytic T Lymphocytes Directed against Tumor Antigen MZ2-E

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Summary

We have reported the identification of human gene MAGE-1, which directs the expression of an antigen recognized on a melanoma by autologous cytolytic T lymphocytes (CTL). We show here that CTL directed against this antigen, which was named MZ2-E, recognize a nonapeptide encoded by the third exon of gene MAGE-1. The CTL also recognize this peptide when it is presented by mouse cells transfected with an HLA-A1 gene, confirming the association of antigen MZ2-E with the HLA-A1 molecule. Other members of the MAGE gene family do not code for the same peptide, suggesting that only MAGE-1 produces the antigen recognized by the anti-MZ2-E CTL. Our results open the possibility of immunizing HLA-A1 patients whose tumor expresses MAGE-1 either with the antigenic peptide or with autologous antigen-presenting cells pulsed with the peptide.

Mixed lymphocyte–tumor cell cultures carried out with human melanoma cells and lymphocytes of the same patient often generate CTL that lyse the autologous tumor cells (1–4). Using blood lymphocytes of melanoma patient MZ2, we have obtained a panel of $CD8^+$ CTL clones that lyse the autologous tumor cell line MZ2-MEL (3). Antigen-loss variants of MZ2-MEL were obtained by selecting tumor cells that were resistant to some of these CTL clones. The pattern of resistance of these antigen-loss variants demonstrated that six different antigens are recognized on the tumor cells by the autologous CTL (5). To identify the gene of one of these antigens, which was named MZ2-E (E), a cosmid library prepared with the DNA of a MZ2-MEL subclone was transfected into E^- antigen-loss variant MZ2-MEL.2.2. This led to the isolation of gene MAGE-1, which directs the expression of antigen MZ2-E (6, 7). The gene was found to be deleted in the antigen-loss variant.

Gene MAGE-1 comprises three exons. A large open reading frame is entirely contained in the third exon. The sequence of the gene found in normal cells of the patient appears to be identical to that found in the melanoma cells. The gene does not appear to be expressed in normal tissues, but it is expressed in ~40% of the melanoma cell lines and tumor samples that have been analyzed (8).

Gene MAGE-1 belongs to a family of several closely related genes. Melanoma MZ2-MEL expresses, in addition to MAGE-1, two other members of this family, called MAGE-2 and MAGE-3. These two genes do not direct the expression of antigen MZ2-E. Like MAGE-1, they do not appear to be expressed in normal tissues.

There is now overwhelming evidence that $CD8^+$ CTL recognize their antigen in the form of a small peptide bound to a class I MHC molecule (9–11). This implies that each MAGE gene expressed in tumor cells could provide one or more peptides binding to some class I molecule, thus providing a tumor-specific antigen. As a first step in the analysis of these potential antigens, we report here the identification of a MAGE-1-encoded nonapeptide that combines with HLA-A1 to form antigen MZ2-E.

Materials and Methods

Cell Lines. Melanoma cell line MZ2-MEL was derived from patient MZ2, and a number of subclones were obtained (3). Clonal

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subline MZ2-MEL.2.2 was selected from subclone MZ2-MEL.3.1 with an autologous CTL clone (5). Anti-MZ2-E autologous CTL clone 82/30 and its culture conditions have been described elsewhere (6). Mouse cell line P1.HTR was derived from mastocytoma P815 (12). P1.HTR was cotransfected with an HLA-A1 gene (13), inserted in expression vector pcD-SRα (14), and with pSVtkneoβ (15). The neo^r transfectants were cloned and tested for HLA class I expression by flow cytometry using F(ab')$_2$ fragments of mAb B.9.12.1 (16). One of these clones expressing HLA class I was then cotransfected with a MAGE-1 cDNA, inserted in expression vector pcD-SRα, and with pHMR272 (17). The hygromycin-resistant transfectants were cloned and tested for the expression of antigen MZ2-E on the basis of their ability to stimulate the production of TNF by CTL 82/30 (6).

Cloning of Subgenic Fragments of Gene MAGE-1. The 2.4-kb BamHI fragment containing exons 2 and 3 of gene MAGE-1 and smaller parts of this fragment were cloned in plasmid vector pTZ18R (Pharmacia Fine Chemicals, Piscataway, NJ). Expression vector pSVK3 (Pharmacia Fine Chemicals) was used to clone a 300-bp fragment, obtained by PCR amplification of MAGE-1 with oligonucleotides VDB14 (5'-CAGGGAGCCAGTCACAAAG-3') and CHO9 (5'-ACTCAGCTCCTCCCAGATTT-3') (positions 422–722 in the third exon).

Transfection of MAGE-1 Fragments and Screening of the Transfectants. Transfections were performed by the calcium phosphate precipitation method (6, 18). Briefly, 4 × 10^6 cells were treated with 3 μg of pSVtkneoβ (15) as selective marker and 30 μg of pTZ18R or pSVK3 containing a MAGE-1 insert. The neo^r transfectants were selected in medium containing 2 mg/ml of neomycin analogue G418 (Gibco Laboratories, Grand Island, NY). 15 d after transfection, the geneticin-resistant transfectants were tested for their ability to stimulate the production of TNF by anti-E CTL 82/30 (6). Briefly, 100 μl containing 1,500 CTL 82/30 was added to 4 × 10^4 transfected cells in flat-bottomed microwells. After 24 h, 50 μl of the supernatant was harvested and added to 3 × 10^4 cells of WEHI 164 clone 13 (19) to evaluate the presence of TNF. The mortality of WEHI cells was estimated 24 h later in a MTT colorimetric assay (6, 20).

Antigenic Peptides and CTL Assay. Peptides were synthesized on solid phase using F-moc for transient NH_2-terminal protection as described by Atherton et al. (21), purified by C-18 reverse-phase HPLC, and characterized by amino acid analysis. Lysis of target cells by CTL was tested by chromium release as previously described (22). In the peptide sensitization assay, 1,000 ^{51}Cr-labeled E$^-$ target cells were incubated in 96-well microplates in the presence of various concentrations of peptide for 30 min at 37°C. An equal volume containing the CTL was then added. Chromium release was measured after 4 h at 37°C. We have indicated in Figs. 2 and 3 the final concentration of peptides during the incubation of the target cells with the CTL.

Results

Identification of the MAGE-1 Region Coding for the Antigenic Peptide. We reported previously, that a 2.4-kb BamHI fragment containing only exons 2 and 3 of gene MAGE-1 transfers at high efficiency the expression of antigen MZ2-E when it is transfected into E$^-$ antigen-loss variant MZ2-MEL.2.2 (7). This confirmed our previous experience with several genes coding for antigens recognized by CTL on mouse tumors: the transfection of small gene fragments that contain the sequence coding for an antigenic peptide results regularly in

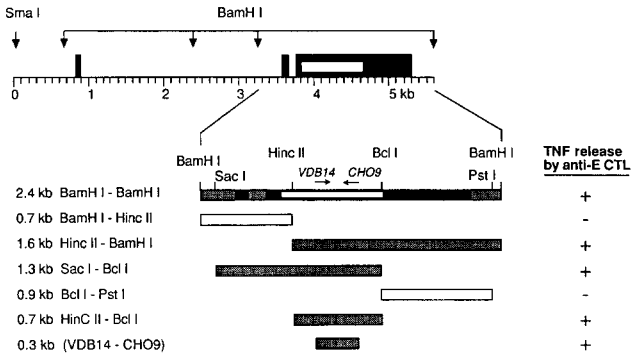

Figure 1. Fragments of gene MAGE-1 that transfer the expression of antigen MZ2-E. The gene is represented with exons as black boxes and the open reading frame of exon 3 as a white box. The restriction fragments were cloned in pTZ18R. The 300-bp fragment obtained by PCR amplification with oligonucleotides VDB14 and CHO9 was cloned in expression vector pSVK3. Each construct was cotransfected into MZ2-MEL.2.2 (E$^-$) cells with pSVtkneoβ as selective marker. The neo^r populations expressing antigen MZ2-E were identified by their ability to stimulate anti-E MZ2-CTL clone 82/30 to produce TNF. For the DNA fragments marked +, the CTL stimulated with the transfectants produced supernatants causing the lysis of 17–93% of the WEHI test cells in the conditions described in Materials and Methods (this corresponds to 12.5–100 pg/ml rhuTNF-β). With the fragments marked –, <4% lysis was observed (corresponding to <1.3 pg/ml rhuTNF-β). When control E$^+$ and E$^-$ MZ2-MEL cells were tested in the same conditions as the transfectants, they produced CTL supernatants lysing, respectively, 78 and 2% of the WEHI cells (corresponding to 46 and 1.1 pg/ml rhuTNF-β).

the expression of the antigen, even when these fragments are cloned in vectors that are not expression vectors (23, 24). Accordingly, we cloned smaller MAGE-1 fragments obtained with restriction enzymes or by PCR amplification, as indicated in Fig. 1. These fragments were cotransfected with

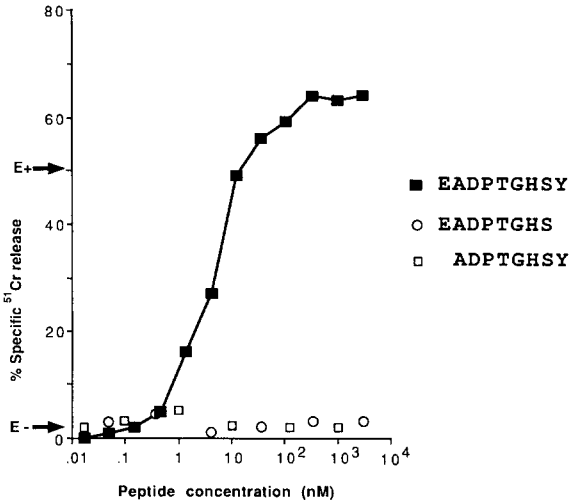

Figure 2. Lysis by anti-MZ2-E CTL of cells incubated with MAGE-1-derived peptides. ^{51}Cr-labeled MZ2-MEL.2.2 (E$^-$) cells were incubated with CTL 82/30 at an E/T ratio of 5:1, in the presence of the synthetic peptides shown on the right at the concentrations indicated. Chromium release was measured after 4 h. The arrows indicate the level of lysis of E$^+$ and E$^-$ MZ2-MEL cells incubated without peptides.

pSVtkneoβ into the E^- line, and the geneticin-resistant population was tested for its ability to stimulate TNF release by autologous anti-E CTL clone 82/30 (6). Thus, we identified a 300-bp fragment of exon 3 that was capable of transferring efficiently the expression of antigen MZ2-E. In agreement with the results obtained with several mouse genes (23, 25–27), this fragment was located in the large open reading frame of gene MAGE-1 (Fig. 1).

The Antigenic Peptide. In the search for potential peptide sequences, we focused on those parts of the 300-bp fragment where the MAGE-1 amino acid sequence differs from that of MAGE-2 and -3. This was based on the observation that genes MAGE-2 and -3 do not direct the expression of antigen MZ2-E. Several 15-amino acid peptides were synthesized. Sensitization of E^- cells to lysis by the anti-E CTL was observed with a peptide that corresponds to codons 158–172 of the large open reading frame of MAGE-1. Shorter peptides were prepared, and efficient lysis of E^- cells was observed with nonapeptide EADPTGHSY (codons 161–169) (Figs. 2 and 3). Half-maximal lysis was obtained at a peptide concentration of 5 nM (Fig. 2). In agreement with the evidence that most peptides recovered from MHC class I molecules are nonapeptides (11), we found that the removal of either the NH_2-terminal Glu or the COOH-terminal Tyr abolished the ability of the peptide to sensitize cells to lysis (Fig. 2).

Presentation of the Peptide by HLA-A1. Previous experiments involving the transfection of gene MAGE-1 into tumor cells of various haplotypes suggested that HLA-A1 was the presenting molecule of antigen MZ2-E (7). To definitively prove this point, we transfected an HLA-A1 gene (13) into P1.HTR, a highly transfectable variant derived from mouse tumor cell line P815. When these P1.HTR.A1 cells were incubated in the presence of the MZ2-E nonapeptide, they were lysed by the anti-MZ2-E CTL (Fig. 3), as were P1.HTR.A1 cells transfected with a MAGE-1 cDNA, indicating that the MZ2-E peptide can be produced and transported in these mouse cells (Fig. 3).

MAGE 1	E	A	D	P	T	G	H	S	Y
MAGE 2	E	V	V	P	I	S	H	L	Y
MAGE 21	E	V	V	R	I	G	H	L	Y
MAGE 3	E	V	D	P	I	G	H	L	Y
MAGE 4	E	V	D	P	A	S	N	T	Y
MAGE 41	E	V	D	P	T	S	N	T	Y
MAGE 5	E	A	D	P	T	S	N	T	Y
MAGE 51	E	A	D	P	T	S	N	T	Y
MAGE 6	E	V	D	P	I	G	H	V	Y

MAGE 1	GAA	GCA	GAC	CCC	ACC	GGC	CAC	TCC	TAT
MAGE 2	GAA	GTG	GTC	CCC	ATC	AGC	CAC	TTG	TAC
MAGE 21	GAA	GTG	GTC	CGC	ATC	GGC	CAC	TTG	TAC
MAGE 3	GAA	GTG	GAC	CCC	ATC	GGC	CAC	TTG	TAC
MAGE 4	GAA	GTG	GAC	CCC	GCC	AGC	AAC	ACC	TAC
MAGE 41	GAA	GTG	GAC	CCC	ACC	AGC	AAC	ACC	TAC
MAGE 5	GAA	GCG	GAC	CCC	ACC	AGC	AAC	ACC	TAC
MAGE 51	GAA	GCG	GAC	CCC	ACC	AGC	AAC	ACC	TAC
MAGE 6	GAA	GTG	GAC	CCC	ATC	GGC	CAC	GTG	TAC

Figure 4. Comparison of peptide MZ2-E encoded by gene MAGE-1 with the peptides encoded by the homologous regions of other genes of the MAGE family. These MAGE genes were isolated from cosmid libraries prepared with genomic DNA isolated from a MZ2-MEL melanoma cell line or from blood lymphocytes of patient MZ2.

Discussion

To our best knowledge, only one other HLA-A1 binding peptide has been identified so far. This is a 13-amino acid peptide corresponding to residues 89–101 of the influenza A nucleoprotein (28). The sequence of this peptide (PKKTGGPIYKRVD) shows no obvious similarity with the MZ2-E peptide, except possibly for the Gly and Tyr residues at positions 6 and 9 of the MZ2-E peptide. The COOH-terminal Tyr may serve as anchoring residue, as reported for K^d- and K^b-restricted antigenic peptides (29, 30).

Because MAGE-1 belongs to a family of several highly related genes, it was of interest to compare these genes in the region that is homologous to the region of MAGE-1 that codes for the MZ2-E peptide. As shown in Fig. 4, other MAGE genes code for peptides that are not identical to the MZ2-E

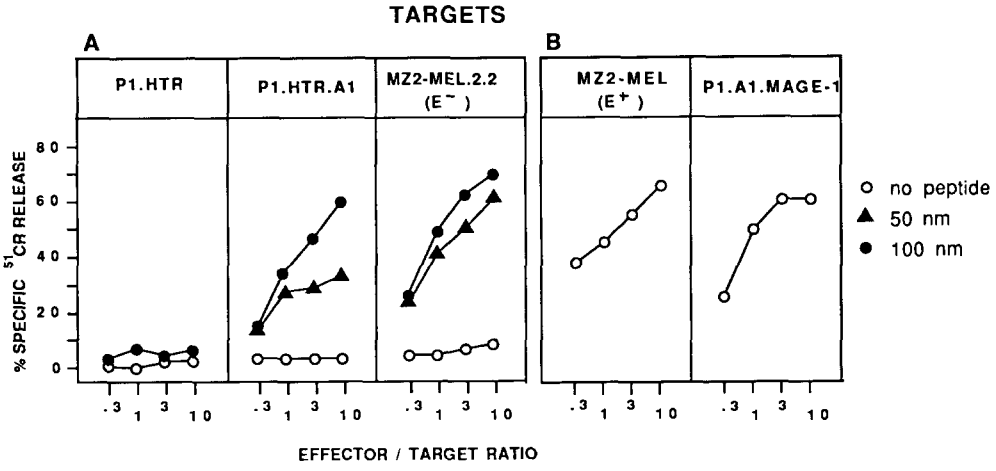

Figure 3. (*A*) Lysis by anti-MZ2-E CTL of mouse cells expressing HLA.A1 in the presence of the MZ2-E peptide. P1.HTR mouse cells expressing HLA-A1 were obtained by transfection. These P1.HTR.A1 cells, as well as control cells P1.HTR and MZ2-MEL.2.2 (E^-), were chromium labeled and incubated with anti-MZ2-E CTL 82/30 at various E/T ratios in the presence of the MZ2-E nonapeptide EADPTGHSY. Chromium release was measured after 4 h. (*B*). Lysis by anti-MZ2-E CTL of mouse cells transfected with HLA-A1 and MAGE-1.

peptide. It is therefore likely that none of these genes codes for the antigen recognized by the CTL directed against MZ2-E. In agreement with this, we have observed that HLA-A1 cells expressing one or several of the genes MAGE-2, MAGE-3, and MAGE-4 are not recognized by the anti-E CTL. The conservation of the terminal Glu and Tyr in all the peptides displayed in Fig. 4 is worth noting. Preliminary experiments carried out with some of these peptides containing terminal Glu and Tyr residues failed to compete with the MZ2-E peptide for sensitization to the anti-E CTL. This suggests that binding to HLA-A1 requires more than these two constant residues.

The availability of a gene encoding a tumor antigen recognized by CTL provides the possibility of selecting for immunization against this antigen for those patients whose tumor expresses the antigen. For antigen MZ2-E, these are HLA-A1 patients whose tumor expresses MAGE-1. These patients can be identified by HLA typing and PCR analysis of the messenger RNA of a small tumor fragment (7). We expect that ~10% of the melanoma patients bear a tumor that expresses the antigen, since MAGE-1 is expressed in 40% of the melanoma tumors, and since the HLA-A1 allele is present in 26% of the Caucasian population. Attempts will be made to immunize these patients with irradiated cells expressing antigen MZ2-E. The availability of the antigenic peptide opens new possibilities for immunization, since effective priming of CTL has been reported in mouse systems after immunization with peptides or with presenting cells pulsed with peptides (31–36).

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