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Cytolytic T Lymphocytes Recognize an Antigen Encoded by MAGE-A10 on a Human Melanoma

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From melanoma patient LB1751, cytolytic T lymphocytes (CTL) were generated that lysed specifically autologous tumor cells. To establish whether these CTL recognized one of the Ags that had previously been defined, a CTL clone was stimulated with cells expressing various MAGE genes. It produced TNF upon stimulation with target cells expressing MAGE-A10. The Ag was found to be nonapeptide GLYDGMEHL (codons 254–262), which is presented by HLA-A2.1. This is the first report on the generation of anti-MAGE CTL by autologous mixed lymphocyte-tumor cell culture (MLTC) from a melanoma patient other than patient MZ2, from whom the first MAGE gene was identified. MAGE genes are expressed in many tumors but not by normal tissues except male germline cells and placenta, which do not express HLA molecules. Therefore, the identification of an antigenic peptide derived from MAGE-A10 adds to the repertoire of tumor-specific shared Ags available for anti-tumoral vaccination trials. The Journal of Immunology, 1999, 162: 6849–6854.

The identification of tumor Ags is essential for active immunotherapy of cancers. In recent years intensive efforts have been made in this area, which has led to the identification of a wealth of Ags recognized on human tumors by autologous T cells, i.e., T cells obtained from the same patient (1). Among them, an Ag encoded by gene MAGE-1 was initially defined by cultivating blood lymphocytes of patient MZ2 in the presence of a melanoma cell line derived from this patient. A panel of cytolytic T lymphocyte (CTL) clones were generated, one of which recognizes a nonapeptide presented by HLA-A1 (2, 3). It was found later that MAGE-1 belongs to a family of at least seventeen related genes, namely MAGE-1 to -12 (now named MAGE-A1 to -A12) (4), MAGE-B1 to -B4 (5–7), and MAGE-C1 to -C12 (8). Genes of this family are expressed in various tumors of different histological types but are completely silent in normal tissues, with the exception of male germline cells and placenta (4, 6–8). Since male germline cells and placental trophoblast cells do not express MHC class I molecules (9), gene expression in these tissues should not lead to Ag expression. This view is supported by the observation that male mice immunized against an Ag encoded by mouse gene PIA, which has the same expression pattern as human MAGE genes, produced strong CTL responses that did not cause testicular inflammation or alteration of fertility (10). Ags encoded by MAGE genes are, therefore, highly tumor specific and are consequently suitable candidates for vaccine-based immunotherapy of cancers.

So far, however, only peptides encoded by MAGE-A1, -A3, and -A6 have been shown to be recognized by autologous CTL derived from mixed lymphocyte-tumor cell culture (MLTC), and all these CTL were generated from only a single patient, namely MZ2 (Refs. 3, 11, and 12; and P. van der Bruggen, unpublished data). We report here that CTL from another melanoma patient, LB1751, recognize an Ag that is encoded by MAGE-A10 and presented by HLA-A2.1.

Materials and Methods

Cell lines

Melanoma cell line LB1751-MEL was derived from a metastatic melanoma in axillary lymph nodes of 67-yr-old male patient LB1751 and grown by a method previously described (13). At passage 4 after the initiation of LB1751-MEL culture that grew as monolayer, aggregates of typical EBV-transformed lymphoblastoid cells appeared in the supernatant. They were collected and cultured separately to obtain B cell line LB1751-EBV. Melanoma culture LB1751-MEL was cleared of EBV-transformed B cells by limiting dilution cloning. DNA fingerprint confirmed that LB1751-MEL and LB1751-EBV originated from the same patient (data not shown).

Melanoma cell lines LB373-MEL and AVL3-MEL were derived from patients LB373 and AVL3, respectively, and were cultured in IMDM (Life Technologies, Gaithersburg, MD) containing 10% FCS. Medullary thyroid carcinoma cell line TT (ATCC No. CRL-1803) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM supplemented with 10% FCS.

CTL clone 447A/5 was generated by MLTC as described previously with minor modifications (14). Briefly, MLTC was conducted by culturing PBMC of patient LB1751 with irradiated LB1751-MEL cells in an 8% CO2 incubator in IMDM supplemented with 10 mM HEPES buffer, t-arginine (116 μM), t-asparagine (36 μM), t-glutamine (216 μM), 10% human serum, and 5 ng/ml of recombinant human (rh) IL-2 (Genzyme, Cambridge, MA). On day 3, rhIL-2 (Eurocetus, Amsterdam, The Netherlands) was added at a final concentration of 25 U/ml. Lymphocytes were restimulated weekly with irradiated LB1751-MEL cells in fresh medium containing 25 U/ml of rhIL-2 and 5 ng/ml of rhIL-7. On day 21, CD8+ T lymphocytes were sorted by using anti-CD8-conjugated MACS magnetic MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cloned by limiting dilution.

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4 Abbreviations used in this paper: CTL, cytolytic T lymphocyte; MLTC, mixed lymphocyte-tumor cell culture; ORF, open reading frame; rh, recombinant human.
Cloning of subgenic fragments of gene MAGE-A10

The 1.1-kb open reading frame (ORF) of MAGE-A10 was cloned in plasmid vector pcDNAI/Amp (Invitrogen, Oxon, U.K.). Three fragments containing the first 270, 546, and 825 nucleotides of MAGE-A10 ORF were amplified by PCR using sense primer 5′-GGAATTCATCATGCTCTCAGAGTCCAAAGC-3′ and three anti-sense primers 5′-GCTCTAACGAGTGGCTATCTGAGCACTCTG-3′, 5′-GCTTCTAGGCTTTAACCCTTTG-3′, and 5′-GCTTCTAGGCTTTAACCCTTTG-3′, respectively. For PCR amplification Pfu DNA polymerase (Stratagene, La Jolla, CA) was used. A first denaturation step was performed by incubation of hybridoma culture supernatant of mAb MA2.1 (24, 25).

RT-PCR was performed to detect the expression of MAGE-A10 in tumor tissues. Total RNA purification and cDNA synthesis were conducted as previously described (26). One fourth of the cDNA produced from 2 μg of total RNA was amplified using sense primer 5′-CACAGACGACCACTGGAAGGAG-3′ and anti-sense primer 5′-CTGGGAATTAAAGACTCAGTGTCTCAG-3′, which yielded a 485-bp specific fragment of MAGE-A10. For PCR, a first denaturation step was done for 4 min at 94°C, and then 30 cycles of amplification were performed as follows: 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C. Cycling was concluded with a final extension step of 15 min at 72°C.

Results

Autologous CTL clones directed against melanoma cell line LB1751-MEL

By stimulating PBL from patient LB1751 with irradiated autologous melanoma cell line LB1751-MEL, we obtained a panel of 23 CTL clones that specifically lysed LB1751-MEL cells, but not an autologous EBV-transformed B cell line LB1751-EBV or the NK-sensitive cell line K562. Among them, 5 clones were successfully maintained in long-term culture. Their lytic activities are shown by representative CTL clone 447A/5 in Fig. 1.

CTL clone 447A/5 produced TNF when stimulated with LB1751-MEL cells, and this production of TNF was inhibited by mAb W6/32, which recognizes all HLA class I molecules, and BB7.2, which is specific for HLA-A2, but not by mAb B1.23.2, which recognizes a common determinant of HLA-B and HLA-C molecules (data not shown), indicating that the target Ag is presented by HLA-A2.

Antigenic peptides and CTL assay

Peptides were synthesized on solid phase using F-moc for transient NH2-terminal protection and were characterized by mass spectrometry. All peptides were >90% pure as indicated by analytical HPLC. Lyophilized peptides were dissolved at 20 μM in DMSO, diluted to 2 μM with 0.01 M PBS, and stored at −20°C. Lysis of target cells by CTL was tested by chromium release as previously described (23). In the peptide sensitization assay, target cells were 51Cr-labeled for 1 h at 37°C and then washed extensively. Target cells (1000) were incubated in 96-well microplates in the presence of various concentrations of peptides for 30 min at 37°C. CTL were added at an E:T ratio of 20. Chromium release was measured after 4 h at 37°C. Enhancement of peptide binding to the HLA-A2 molecule was achieved by incubation of target cells during 51Cr-labeling with a 1/5 dilution of hybridoma culture supernatant of mAb MA2.1 (24, 25).

Table 1. Stimulation of CTL 447A/5 by COS-7 cells transfected with HLA-A2.1 and MAGE-A genes

<table>
<thead>
<tr>
<th>Stimulator Cells</th>
<th>TNF Released by CTL 447A/5 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB1751-MEL</td>
<td>28</td>
</tr>
<tr>
<td>COS</td>
<td></td>
</tr>
<tr>
<td>COS + HLA-A2.1</td>
<td>4</td>
</tr>
<tr>
<td>COS + HLA-A2.1 + MAGE-A1</td>
<td>3</td>
</tr>
<tr>
<td>MAGE-A2</td>
<td>4</td>
</tr>
<tr>
<td>MAGE-A3</td>
<td>4</td>
</tr>
<tr>
<td>MAGE-A4</td>
<td>4</td>
</tr>
<tr>
<td>MAGE-A6</td>
<td>4</td>
</tr>
<tr>
<td>MAGE-A8</td>
<td>30</td>
</tr>
<tr>
<td>MAGE-A9</td>
<td>3</td>
</tr>
<tr>
<td>MAGE-A10</td>
<td>&gt;120</td>
</tr>
<tr>
<td>MAGE-A11</td>
<td>4</td>
</tr>
<tr>
<td>MAGE-A12</td>
<td>2</td>
</tr>
</tbody>
</table>

a MAGE-A5, which is very weakly transcribed in various tumor cell lines as well as in tests, and MAGE-A7, which is not transcribed at all, were not tested.

COS-7 cells were transfected by the DEAE-dextran method with HLA-A2.1 and MAGE-A cDNAs cloned in pcDNAI/Amp. Control stimulator cells included autologous LB1751-MEL, untransfected COS-7 cells, and COS-7 cells transfected only with HLA-A2.1 gene.

After 24 h of coculture with stimulator cells, the production of TNF by CTL 447A/5 was measured by testing the toxicity of the supernatants for TNF-sensitive WEHI-164.13 cells.
CTL 447A/5 recognizes an Ag encoded by MAGE-A10

Because of the high expression level of almost all the MAGE-A genes in melanoma cell line LB1751-MEL (data not shown), we first tested the possibility that CTL 447A/5 recognizes an Ag encoded by one of the MAGE-A genes. We cotransfected into COS-7 cells the cDNA of each MAGE-A gene cloned in expression vector pcDNAI/Amp together with pcDNAI/Amp-A2, a construct encoding HLA-A2.1. The transfectants were tested for their ability to stimulate TNF production by CTL 447A/5. A very significant amount of TNF was produced by the CTL when stimulated with COS-7 cells transfected with MAGE-A10 (Table I). Transfectants obtained with MAGE-A8 also stimulated TNF release, but to a lesser extent. No stimulation was observed with COS-7 cells transfected with HLA-A2.1 alone or with the combination of HLA-A2.1 and any of the other MAGE-A genes.

We further examined the recognition by CTL 447A/5 of allogeneic HLA-A2+ tumor cell lines that express MAGE-A10 or MAGE-A8. The two MAGE-A10+ melanoma cell lines LB373-MEL and AVL3-MEL could stimulate the CTL to produce TNF, but MAGE-A8+ cell line TT could not (Fig. 2A). In addition, LB373-MEL cells were lysed by CTL 447A/5, though less efficiently than the autologous tumor line LB1751-MEL (Fig. 2B). AVL3-MEL cells showed a low level of sensitivity to lysis that could be increased by IFN-γ treatment. TT cells were not lysed by CTL 447A/5 even when pretreated with IFN-γ.

Identification of the MAGE-A10 antigenic peptide

In an attempt to identify the MAGE-A10 sequence that codes for the antigenic peptide, we generated by PCR amplification fragments of different lengths starting from the initiation codon. These subgenic fragments were cloned in pcDNAI/Amp and transfected into COS-7 cells together with the construct carrying the HLA-A2 gene. CTL stimulation assay was conducted with the transfectants. As shown in Fig. 3, a fragment of 825 bp rendered the transfectants capable of stimulating TNF production by CTL 447A/5, whereas a 546-bp fragment did not, indicating that the sequence coding for the antigenic peptide is located between nucleotide 522 and 825 of the MAGE-A10 ORF. In the amino acid sequence corresponding to nucleotides 522–825, we observed two nonapeptides, MLLVF-GIDV (codons 183–191) and GLYDGMEHL (254–262), which conformed to the HLA-A2.1 peptide binding motif, i.e., Leu or Met at position 2 and Leu, Val, or Ile at the C terminus (27).

**FIGURE 2.** Recognition of allogeneic tumor cell lines by CTL 447A/5. A, LB373-MEL (MAGE-A10+), AVL3-MEL (MAGE-A10+), and TT (MAGE-A8+) cell lines derived from HLA-A2 patients were used to stimulate CTL 447A/5. Autologous tumor cell line LB1751-MEL was included as a control. After 24 h of coculture, production of TNF by the CTL was measured by testing toxicity of the supernatants to TNF-sensitive WEHI-164.13 cells. B, Lysis of LB373-MEL, AVL3-MEL and TT cell lines was assessed by the chromium release assay. Autologous tumor cell line LB1751-MEL was included as a control target. Chromium release was measured after 4 h of incubation of chromium-labeled target cells with the CTL at different E:T ratios.

**FIGURE 3.** Identification of the region coding for the antigenic peptide recognized by CTL 447A/5. PCR fragments of different lengths were cloned into pcDNAI/Amp and cotransfected into COS-7 cells with gene HLA-A2.1. Transfected cells were incubated for 24 h with CTL 447A/5, and the TNF in the supernatants was measured by its toxicity to WEHI-164.13 cells.
MAGE-A10 is expressed in a variety of tumors

The expression of MAGE-A10 has been studied previously only in a small number of tumors. To obtain a better evaluation of the frequency of tumors expressing this gene, we tested a series of 314 samples of tumors of various histological types by RT-PCR with primers ensuring specificity for gene MAGE-A10. As shown in Table II, it was expressed in a high proportion of lung carcinomas, bladder carcinomas, head and neck and esophageal carcinomas, and melanomas. Of the 71 tumor samples expressing MAGE-A10, all but two expressed simultaneously at least one of genes MAGE-A1, -A2, -A3, -A4 or -A6 (data not shown).

Discussion

The MAGE, BAGE, and GAGE gene families have been identified on the basis of anti-tumoral CTL clones obtained following autologous MLTC conducted with a single melanoma patient, namely patient MZ2 (2, 3, 11, 12, 28, 29). Six different Ags encoded by these genes and presented by HLA-A1, Cw16, and Cw6 were identified. Curiously, when we later analyzed the CTL responses of several other patients bearing melanoma tumors expressing various MAGE-type genes, we obtained no CTL directed against MAGE, BAGE or GAGE Ags. The CTL were directed either against differentiation Ags such as tyrosinase or Melan-A/Mart-1 or against Ags encoded by mutated or overexpressed genes (30–35). However, autologous CTL were also obtained recently that were directed against Ags encoded by MAGE-type gene NY-ESO-1 (36, 37). CTL clone 447A/5 reported here is the first anti-MAGE CTL obtained by autologous MLTC with a patient other than MZ2. This CTL recognizes the MAGE-A10-encoded nonapeptide GLYDGMEHL, which is presented by HLA-A2. The CTL recognized not only the autologous melanoma cells but also other HLA-A2 tumor cells expressing MAGE-A10. We have confirmed that MAGE-A10 is expressed in a variety of tumor types including melanomas, lung cancers, head and neck and esophageal carcinomas, and bladder carcinomas, where the gene is expressed in more than 30% of the samples.

In a previous report, we suggested, on the basis of semiquantitative RT-PCR assays, that all the tumors expressing MAGE-A10 expressed the gene at a very low level (4). The level we observed was deemed unlikely to be sufficient for the production of enough antigenic peptides to allow recognition by CTL (38). However, it was later observed with a mAb that a tumor with a high level of MAGE-A1 expression contained similar amount of MAGE-A1 and MAGE-A10 protein (39). This led us to reconsider our MAGE-A10 assay, and we have now reached by two different methods the
conclusion that the tumors expressing MAGE-A10 express this gene at levels that are similar to those observed for MAGE-A1. In melanoma MZ2-MEL, for instance, the frequency of the MAGE-A10 message is 1/5500 and that of the MAGE-A10 message is 1/6600 (our unpublished data). We conclude that there is no discrepancy between the level of expression of MAGE-A10 and the fact that an Ag encoded by MAGE-A10 can be recognized by a CTL.

An immunotherapy trial of metastatic melanoma patients with detectable disease, which involves immunization with MAGE-A3 peptide presented by HLA-A2, has recently been completed (40, 41). Of the 25 patients who received the complete treatment, i.e., three monthly injections of peptide in skin location distant from the tumor, seven patients showed partial or complete tumor regression. Surprisingly, no CTL response against the MAGE-A3 Ag could be documented with lymphocytes present in the blood. In trials involving peptide-pulsed dendritic cells, it was reported that some patients developed delayed-type hypersensitivity or CTL responses (42–44). The identification of a MAGE-A10 Ag presented by HLA-A2 will make it possible to extend such immunotherapy trials to a wider group of patients.

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References


