

Identification of a MAGE-2-encoded Human Leukocyte Antigen-A24-binding Synthetic Peptide That Induces Specific Antitumor Cytotoxic T Lymphocytes

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ABSTRACT

Because *MAGE-2* gene is expressed in a wide variety of malignant tumors and HLA-A24 is the most common allele in the Japanese population and is also frequently present in Caucasians, the identification of MAGE-2-encoded peptide presented by HLA-A24 is, therefore, considered to be important in order to develop specific immunotherapy for malignant tumors using peptides as a vaccine. By using a MHC-binding assay, eight peptides derived from MAGE-2 were found to bind with sufficient affinity to the HLA-A24 molecule. When the induction of specific cytotoxic T lymphocytes (CTLs) was examined using a simplified method, the highest human lymphocyte antigen (HLA) binder (EYLQLVFGI) in these peptides was able to elicit CTLs from unseparated peripheral blood mononuclear cells in HLA-A24 healthy donors by stimulation with freshly isolated, peptide-pulsed peripheral blood mononuclear cells as antigen-presenting cells and also by using interleukin 7 and keyhole-limpet hemocyanin in a primary culture. The induced CTL could, thus, lyse HLA-A24 tumor cells expressing MAGE-2, as well as the peptide-pulsed target cells, with antigen specificity in a HLA class I-restricted manner. The identification of this peptide may, thus, be of therapeutic value in peptide-based vaccines for the treatment of several types of malignant tumors expressing MAGE-2.

INTRODUCTION

The *MAGE* gene family comprises a series of at least 12 closely related genes (1). These genes are expressed in a number of malignant tumors of various histological origins but not in

normal tissues with the exception of the testis (2). The antigenic peptides derived from MAGE-1 or -3 have been shown to elicit a CTL² response in the context of HLA class I molecules (3–7). Therefore, immunization using these peptides has been investigated for specific immunotherapy in melanoma patients, and some clinical effectiveness has been reported (8–11). Regarding the *MAGE-2* gene, two MAGE-2-derived synthetic peptides presented by HLA-A2 were shown to be capable of eliciting a CTL response in transgenic mice, although no recognition of HLA-A2 human tumor cell lines could be detected (12). However, one of these peptides has recently been demonstrated to be able to induce CTLs that kill human tumor cells expressing both HLA-A2 and MAGE-2 (13).

One way to identify potential peptides from tumor antigens is by means of an *in vitro* induction protocol of peptide-specific CTLs which consists of the stimulation of PBMCs using synthetic peptides with binding affinity to target HLA alleles (5). In line with this assumption, we developed a simplified method to efficiently induce MAGE-specific CTLs from PBMCs by *in vitro* stimulation with MAGE peptide, in which the CTLs were able to lyse human tumor cells expressing MAGE (14). Using this procedure, we have previously characterized MAGE-1 and -3-encoded peptides presented by HLA-A24 (15, 16).

The *MAGE-2* gene was shown to be expressed in a relatively high proportion of melanomas as well as in several other types of malignant tumors (17). On the other hand, HLA-A24 is the most common allele in the Japanese population (18) and is also frequently present in Caucasians (19). Therefore, it is considered important to identify potential antigenic peptide derived from MAGE-2 presented by HLA-A24 for specific immunotherapy using the peptide as vaccine. In this study, we identified eight MAGE-2-derived peptides that bind with sufficient affinity to the HLA-A24 molecule, after defining the MHC binding assay based on the screening of sequences for the presence of MHC-binding motifs. We then investigated the induction of MAGE-2-specific CTLs from the PBMCs of HLA-A24 healthy donors by *in vitro* stimulation with the synthetic peptides using our newly developed method.

MATERIALS AND METHODS

Cell Lines. The TISI cells, a human B-lymphoblastoid cell line showing HLA-A24, were provided by the Takara Shuzo Co., Ltd. (Otsu, Shiga, Japan). The esophageal carcinoma cell line KY150, the gastric carcinoma cell lines MKN-1 and

Received 3/18/99; accepted 5/21/99.

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² The abbreviations used are: CTL, cytotoxic T lymphocyte; HLA, human lymphocyte antigen; PBMC, peripheral blood mononuclear cell; IL, interleukin; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

KATOIII, the colon carcinoma cell line WiDr, the breast carcinoma cell line MRKnu1, Raji, and K562 were supplied by the Japanese Cancer Research Bank (Tokyo, Japan). The esophageal carcinoma cell line TE11 was provided by the Tohoku University Cell Bank (Sendai, Japan). These cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum and antibiotics.

MHC-binding Assay. The peptides derived from MAGE-2 sequence that were used for the MHC-binding assay were purchased from Chiron/Mimotopes (Clayton, Victoria, Australia). The peptides used as radioactive probes were synthesized in-house, purified by high-performance liquid chromatography, and radioiodinated. The peptide-binding assay specific for the class I molecule was described previously (20). In brief, the assay is based on the inhibition of the binding of radiolabeled standard peptides to detergent solubilized MHC molecules. The sequence of the standard peptide used for the MHC binding study for HLA-A24 was AYIDNYNKF. The standard peptide was radioiodinated using ^{125}I by the chloramine-T method. HLA-A concentrations, yielding approximately 15% of bound peptide, were used in the inhibition assay. Various doses of the test peptides (10 μM to 1 nM) were incubated together with 5 nM of radiolabeled standard peptide and HLA-A24 molecules for 2 days at room temperature in the presence of the cocktail of protease inhibitors and 1 μM β_2 -microglobulin. At the end of the incubation period, the percentage of MHC-bound radioactivity was determined by gel filtration.

CTL Induction Using Synthetic MAGE-2 Peptides. The CTL induction *in vitro* was performed according to the previously described procedures (14). Briefly, the PBMCs of HLA-A24 healthy donors were collected by centrifugation on a Ficoll-Paque density gradient. The PBMCs were prepulsed by purified peptides at a final concentration of 20 $\mu\text{g}/\text{ml}$ for 2 hr at 37°C. The cells were cultured in an RPMI 1640 supplemented with 5% heat-inactivated AB serum, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate, and 50 μM 2-mercaptoethanol (Wako Pure Chemical, Osaka, Japan), with the addition of keyhole limpet hemocyanin (5 $\mu\text{g}/\text{ml}$, Calkbiochem-Novobiochem, San Diego, CA) and IL-7 (25 ng/ml, Upstate Biotechnology, Lake Placid, NY). On day 4, recombinant IL-2 was then added to the culture at 30 IU/ml. The responder cells were stimulated every 7 days with freshly isolated autologous PBMCs that had been prepulsed with peptides and treated with mitomycin C (Kyowa Hakko, Osaka, Japan). The cultures were fed with a fresh medium containing IL-2 1 day after every stimulation. The CTL activity was assessed on day 42.

Phenotypic Analysis. Flow cytometry was performed using a FACScan. The cells were stained with murine anti-human monoclonal antibodies against CD3, CD4, and CD8 (Becton Dickinson, San Jose, CA). Isotype-matched murine antibodies (Becton Dickinson) served as negative control.

Cytotoxicity Assay. The target cells were labeled with 100 μCi of sodium ^{51}Cr chromate (^{51}Cr) for 1 hr at 37°C, and the labeled cells were then washed and resuspended. The peptide-pulsed targets, TISI cells, were prepared by incubating the cells with the peptides overnight at 37°C and then labeling them with ^{51}Cr . The effector cells were placed in each well of round-bottomed microtiter plates. The labeled target cells were then added to the well at a concentration of 3×10^3 cells/well to

produce a total volume of 0.2 ml. After a 4-h incubation period, the release of the ^{51}Cr label was measured by collecting the supernatant, followed by quantitation in an automated gamma counter. Antigen specificity was determined by cold-target inhibition analysis by determining the capacity of peptide-pulsed, unlabeled TISI cells to block the lysis of carcinoma cells. The percentage of specific cytotoxicity was calculated as the percentage of specific ^{51}Cr release: [(experimental release – spontaneous release)/(maximum release – spontaneous release)] \times 100. To eliminate any specific lysis due to natural killer-like effectors, the cytolytic activity was tested in the presence of a 30-fold excess of unlabeled K562 cells. Some target cells were preincubated for 48 h in the medium containing 300 units/ml IFN- γ (Pepro Tech, Rocky Hill, NJ) to enhance HLA expression.

Inhibition of the Cytotoxicity with Monoclonal Antibodies. Appropriate target cells were incubated with monoclonal antibodies at a final concentration of 1/20 for 1 hr at 4°C before the assay of cytotoxicity. The monoclonal antibodies used were anti-HLA class I antibody (Immunotech, Marseilles, France).

RT-PCR Analysis of MAGE-2 Expression. Total RNA was isolated from the tumor cell lines using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (21). cDNA was synthesized by RT from 2.5 μg of total RNA as described previously (22, 23). MAGE-2 cDNA was detected by PCR amplification using oligonucleotide primers specific for the different exons of the MAGE-2 gene. The primer sequences were 5'-AAGTAGGACCCGAGGCACTG-3' and 5'-GAA-GAGGAAGAAGCGGTCTG-3'. PCR was performed for 33 cycles (30 sec at 94°C, 20 sec at 68°C, and 30 sec at 72°C). The PCR product was size-fractionated on 2% agarose gel. To ensure that the RNA had not degraded, a PCR assay with primers specific for the gene GAPDH cDNA was, thus, carried out in each case. The primers used for the amplification of GAPDH were 5'-GTCAACGGATTTGGTCGTATT-3' and 5'-AGTCT-TCTGGGTGGCAGTGAT-3' (24).

RESULTS

Expression of MAGE-2 mRNA. The expression of MAGE-2 mRNA in all of the cell lines used in this study was analyzed using RT-PCR (Fig. 1). The MAGE-2 gene was expressed in KY150 (HLA-A24⁺), MKN-1 (HLA-A24⁺), WiDr (HLA-A24⁺), MRKnu1 (HLA-A24⁺), TE11 (HLA-A24⁺), and KATOIII (HLA-A24⁻). However, no expression of the MAGE-2 gene was detected in Raji (HLA-A24⁻) and TISI (HLA-A24⁺).

Identification of Peptides from MAGE-2 That Bind to HLA-A24. The known sequences of MAGE-2 were initially screened for peptides containing the anchor motif for HLA-A24. Eight peptides consisted of 9 or 10 residues were found to contain the binding motif for HLA-A24. These peptides were synthesized and tested for binding to purified HLA-A24 molecules. The results from the binding assay showed that three peptides bound to HLA-A24 with high affinity (<100 nM required to achieve 50% inhibition), and five peptides were intermediate HLA-A24 binders (100–1000 nM required for 50% inhibition; Table 1).

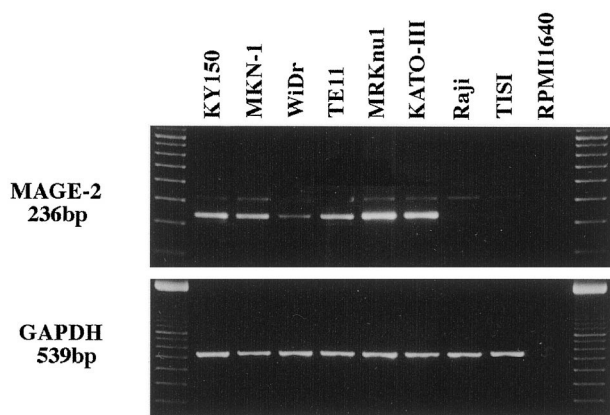


Fig. 1 Expression of the *MAGE-2* gene in cell lines detected by RT-PCR and agarose gel electrophoresis. PCR amplification was performed with specific oligonucleotides.

Table 1 HLA-A24 binding of motif containing peptides derived from the sequence of *MAGE-2*

Sequence	Position ^a	Binding ^b (IC ₅₀ , nM)
EYLQLVFGI	156	3.4
VMPKTGLLI	195	52.2
SYVKVLHHTL	282	75.0
VMPKTGLLII	195	206.9
IFSKASEYL	150	521.7
SFSTTINYTL	70	800.0
LYILVTCLGL	175	857.1
MFPDLESEF	97	857.1

^a Refer to the residue number of the first position of the peptide in relation to the sequence of the entire gene product.

^b Concentration of the peptide necessary to inhibit 50% binding of the radiolabeled test peptide to purified HLA-A24 molecules.

Induction of CTL Recognizing MAGE-2-encoded Synthetic Peptides. We stimulated the PBMC from at least three donors with the synthetic peptides using the simplified method for CTL induction. Of the eight peptides tested, the highest MHC binder (EYLQLVFGI) was able to elicit CTLs. After 42 days of culture, the induced effector cells lysed the TISI cells preincubated with different concentrations (0.04–20 μ g/ml) of the peptide at various E:T ratios in a dose-related fashion, and, as shown in Fig. 2, the cells demonstrated approximately a 70% cytotoxicity against the TISI cells pulsed with 20 μ g/ml of the peptide, compared with less than 10% cytolysis against the TISI cells alone at an E:T ratio of 60:1. No anti-peptide or anti-tumor reactivities were apparently detected in the case of the remaining seven peptides.

Antigen Specificity of the Induced CTL. Antigen specificity of CTLs elicited with peptide EYLQLVFGI was further studied by testing its capacity to recognize various carcinoma cell lines and by determining the ability of peptide-pulsed, nonradiolabeled targets to inhibit the lysis of a *MAGE-2*⁺ and HLA-A24⁺ carcinoma cell line, MKN-1. The results presented in Fig. 3 show that the induced cells were capable of killing KY150, MKN-1 and WiDr cells, which were *MAGE-2*⁺ and HLA-A24⁺. However, the cytolysis was minimal against

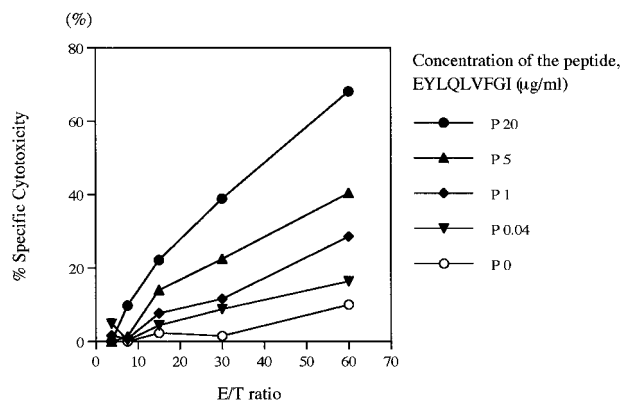


Fig. 2 The cytotoxic activity of the induced cells by stimulation with MAGE-2/HLA-A24 peptide, EYLQLVFGI, against TISI cells pulsed with the peptide. The induced cells were obtained by stimulating PBMCs for 42 days with mitomycin C-treated autologous PBMCs pulsed with the peptide. TISI cells were pulsed, either with different concentrations (0.04–20 μ g/ml) of or without peptide, overnight and then were labeled with ⁵¹Cr. The cytotoxic activity was assessed at various E:T ratios.

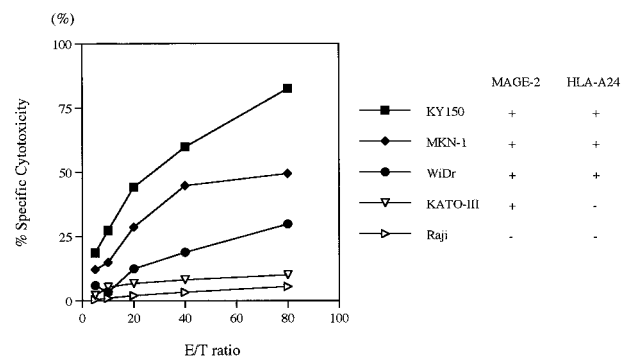


Fig. 3 Lysis of various cell lines by the induced cells by using the peptide EYLQLVFGI. The cytotoxic activity of the induced cells was assessed against KY150 (esophageal carcinoma cell line, HLA-A24⁺, *MAGE-2*⁺), MKN-1 (gastric carcinoma cell line, HLA-A24⁺, *MAGE-2*⁺), WiDr (colorectal carcinoma cell line, HLA-A24⁺, *MAGE-2*⁺), KATOIII (gastric carcinoma cell line, HLA-A24⁻, *MAGE-2*⁺), and Raji (B-lymphoblastoid cell line, HLA-A24⁻, *MAGE-2*⁻) at various E:T ratios.

KATOIII (*MAGE-2*⁺, HLA-A24⁻) and Raji (*MAGE-2*⁻, HLA-A24⁻) cells. When the HLA-A24 carcinoma cell lines expressing *MAGE-2* that showed even relatively low percentages of lysis by the CTLs were preincubated with IFN- γ , a significant level of lysis was observed (Fig. 4). Furthermore, the reactivity of the CTLs against carcinoma cells was apparently blocked by cold targets loaded with this peptide but not by cold targets pulsed with an irrelevant peptide (Fig. 5).

Characterization of the Induced Effectors. A flow cytometry analysis was performed before and after the culture in the case of the peptide EYLQLVFGI. The number of CD3⁺ cells gradually increased after the culture. The percentage of CD4⁺ gradually decreased, whereas the CD8⁺ cells increased after day 14. On day 42, 99% of the cell population was CD3⁺, and approximately 80% of these cells were positive for CD8.

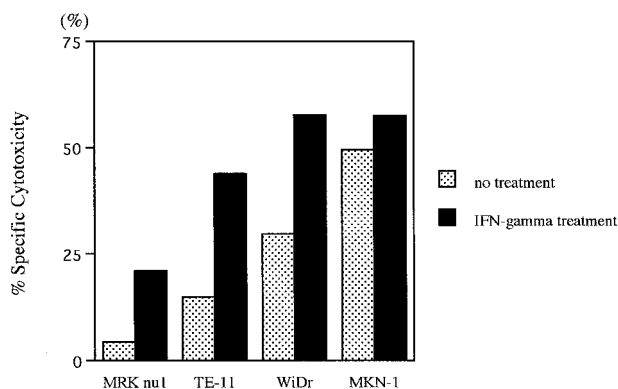


Fig. 4 Effect of the pretreatment of target cells with IFN- γ on cytotoxicity. Target cells (MRK nu1, HLA-A24⁺, MAGE-2⁺; TE-11, HLA-A24⁺, MAGE-2⁺; WiDr; and MKN-1) pretreated with or without IFN- γ (300 units/ml), and the cytotoxic activity was assessed against these cells at an E:T ratio of 80:1.

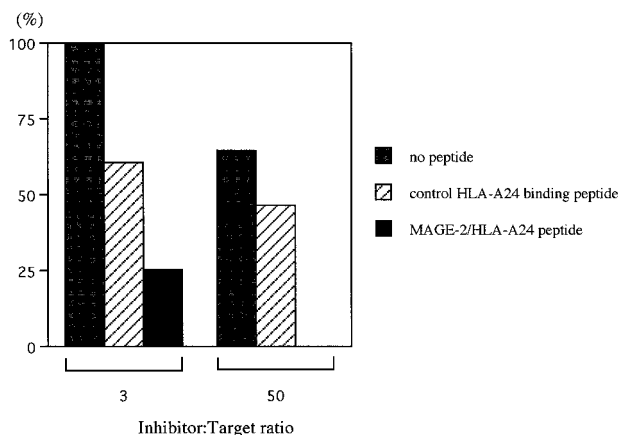


Fig. 5 The antigen specificity of the induced cells demonstrated by cold-target inhibition studies. Lysis of MKN-1 cells (HLA-A24⁺, MAGE-2⁺) at an E:T ratio of 20:1 by the induced cells was blocked by unlabeled TISI cells, unlabeled TISI cells pulsed with MAGE-2/HLA-A24 peptide, EYLQLVFGI, and unlabeled TISI cells pulsed with control HLA-A24-binding peptide.

Inhibition of the Recognition of Effectors by Monoclonal Antibody. To determine whether the induced effector cells recognized the MAGE-2-expressing targets in a HLA-restricted manner, the monoclonal antibody generated against the HLA class-I molecules was used to block the recognition by effectors. The cytotoxic activity of effectors against the peptide-pulsed TISI cells was, thus, significantly eliminated by the anti-HLA class I antibody (Fig. 6). These results suggested that the induced effector cells lysed the targets in a HLA class-I-restricted manner.

DISCUSSION

The antigenic peptides derived from *MAGE* genes have been previously identified using several methods for the development of specific immunotherapy in cancer patients. The characterization of peptides encoded by MAGE-1 pre-

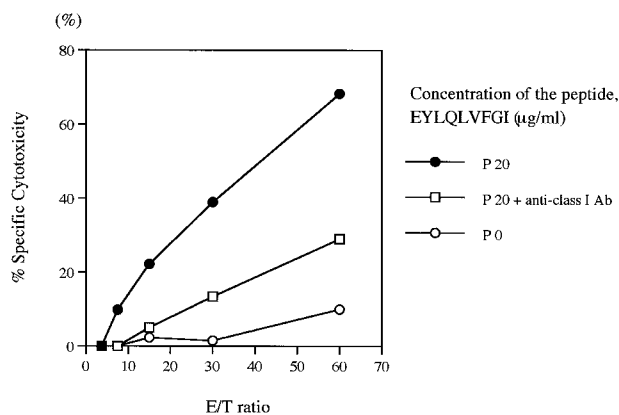


Fig. 6 The inhibited recognition of the induced cells by the anti-HLA class I antibody. TISI cells prepulsed with the peptide EYLQLVFGI were incubated with or without anti-HLA-class I antibody at a final concentration of 1:20 for 1 h at 4°C. The cytotoxic activity of the induced cells was assessed against these target cells at various E:T ratios.

sented by HLA-A1 (3), HLA-A24 (16), and HLA-Cw1601 (25) have thus already been reported, and HLA-A1- (4, 5), HLA-A2- (6), HLA-A24- (15), and HLA-B44- (7) restricted peptides encoded by MAGE-3 have also been identified. With respect to MAGE-2, two MAGE-2-derived HLA-A2-binding peptides (KMVELVHFL and YLQLVFGIEV) that were capable of eliciting a CTL response in transgenic mice were shown to be processed and presented by HLA-A2, although the CTLs did not recognize human tumor cell lines (12). However, one of these peptides (YLQLVFGIEV) has recently been reported to elicit CTLs that could lyse tumor cells expressing both HLA-A2 and MAGE-2 by stimulation of PBMCs with the peptide (13). In the present study, we identified a MAGE-2-encoded peptide presented by HLA-A24 by means of the *in vitro* primary induction of peptide-specific CTLs, which were able to kill HLA-A24 human carcinoma cells expressing MAGE-2, from the PBMCs of healthy donors using HLA-A24-binding synthetic peptides.

For the identification of MAGE-encoded antigenic peptides, specific CTLs have been induced *in vitro* by the stimulation of the PBMCs from healthy donors with synthetic peptides by using rather complicated procedures (5–7). However, we have developed a simplified method for the induction of specific CTLs, in which the CTL responses could be induced from unseparated PBMCs by stimulation with freshly isolated, peptide-pulsed PBMCs as antigen-presenting cells and by using IL-7 and keyhole limpet hemocyanine for a primary culture (14). Using this procedure, we identified a HLA-A24-restricted MAGE-3 peptide (15) and also recently characterized a MAGE-1-encoded peptide presented by HLA-A24 (16). In this study, we demonstrated the induction of peptide-specific CTLs that were able to kill HLA-A24 human tumor cells expressing MAGE-2, as well as peptide-pulsed cells, with antigen specificity in a HLA class-I-restricted manner, when stimulated with the highest-affinity binding synthetic peptide derived from MAGE-2, EYLQLVFGI, by using our simplified method.

The HLA-A24-restricted MAGE-2 peptide identified here and the HLA-A2-restricted MAGE-2 peptides previously identified—both by using transgenic mice (12) and by stimulation of PBMCs with the peptide (13)—are derived from almost the same protein region. In fact, the peptide reported here (EYLQLVFGI) covers amino acids 156–164 and the amino acid sequence of the peptide presented by HLA-A2 (YLQLVFGIEV) is located at position 157–166. These results are reminiscent of the observation that a MAGE-3-encoded peptide presented by HLA-A1 is entirely included in the sequence of an HLA-B44-restricted MAGE-3 peptide (7).

Because of the tumor-specific expression of the *MAGE* genes, the antigens encoded by these genes may be potential targets for tumor-specific immunotherapy. Peptide-specific CTLs have been shown to be generated against MAGE-1- and -3-encoded peptides, and the antigenic peptides were demonstrated to be useful for the treatment of melanoma patients (8–11). Because peptide-specific CTLs were shown to be induced by a MAGE-2-encoded peptide presented by HLA-A24 in this study, immunization with this peptide may thus prove to be effective in vaccine therapy for patients with malignant tumors expressing MAGE-2.

Although two MAGE-2-derived peptides have been reported to be presented in the context of HLA-A2, the proportion of HLA-A2 individuals is 44% in Japanese and 49% in Caucasians (19). However, HLA-A24 is present in 61% of Japanese (18) but is only expressed in 10% of Caucasians (19). Therefore, the identification of a HLA-A24-restricted MAGE-2 peptide described in this study would result in an increased number of patients with MAGE-2 positive malignant tumors, especially Japanese patients, who could thus benefit from immunization with the MAGE-2-encoded peptide.

The *MAGE-2* gene is expressed in 70% of metastatic melanomas (26), in 41% of head and neck squamous cell carcinomas (27), in 37% of lung carcinomas (28), and in 30% of transitional carcinomas of the urinary bladder (29). Regarding gastrointestinal carcinomas, which are one of the most common malignant tumors in Japan, the *MAGE* gene expression was shown to be relatively high. *MAGE-2* was thus expressed in 31% of gastric carcinomas (23) and in 43% of esophageal carcinomas (22). Therefore, immunization based on this MAGE-2-encoded peptide presented by HLA-A24 could be of therapeutic importance in patients with these carcinomas as well as in patients with melanoma.

Because we have recently identified the HLA-A24-restricted MAGE-3 and MAGE-1 peptides (15, 16), some HLA-A24 patients bearing malignant tumors expressing MAGE-1, -2, and -3 could be further immunized with a combination of these antigens, which may lead to a potentially more successful outcome. Moreover, immunotherapy with a synthetic peptide plus IL-2 in melanoma patients was reported to provide significantly higher tumor regression rates than those seen with either agent alone (30). Because the pretreatment of tumor cells with IFN- γ could enhance the cytolysis by CTLs as shown in this study, immunization with separate peptides derived from MAGE, including MAGE-2, in combination with cytokines, such as IL-2, may serve for the development of vaccine therapy in patients with various malignant tumors.

ACKNOWLEDGMENTS

We thank Dr. Masaki Mori for giving us appropriate advice. We also thank T. Shimoka, J. Miyake, K. Ogata, and K. Sato for their valuable technical assistance.

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