Induction of Antitumor Cytotoxic T Lymphocytes from the Peripheral Blood Mononuclear Cells of Cancer Patients Using HLA-A2-restricted MAGE-3 Peptide in Vitro

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ABSTRACT

As the basis for the application of MAGE antigens to therapeutic use, the induction of peptide-specific CTLs has been investigated by the stimulation of peripheral blood mononuclear cells (PBMCs) with antigenic peptides derived from MAGE genes. However, the cross-reactivity of the peptide-induced CTLs to the target cells endogenously presenting the MAGE epitope, especially in cancer patients, remains controversial, despite the use of complicated manipulations. Because we recently developed a new simplified method to induce peptide-specific CTLs that killed MAGE expressing tumor cells from the PBMCs of a healthy donor, we examined the induction of specific CTLs by stimulation of PBMCs with HLA-A2-restricted MAGE-3 peptide in HLA-A2+ cancer patients whose tumors expressed MAGE-3 by using the simple method. The CTL responses could thus be induced from unseparated PBMCs by stimulation with freshly isolated, peptide-pulsed PBMCs as antigen-presenting cells and by using interleukin 7 and keyhole limpet hemocyanin for the primary culture. All CTLs induced from the PBMCs of four cancer patients tested could thus lyse the HLA-A2 target cells pulsed with the peptide, and moreover, two of the CTLs were also able to kill HLA-A2 tumor cells expressing MAGE-3 in a HLA class I and A2-restricted manner. Therefore, these findings seem to indicate that HLA-A2-restricted MAGE-3 peptide may be potentially useful for specific immunotherapy in cancer patients.

INTRODUCTION

The MAGE genes that code for tumor antigens recognized by autologous CTLs have previously been isolated from a melanoma cell line (1, 2). The peptide epitopes derived from these tumor antigens encoded by MAGE-1 and MAGE-3 genes have also been further identified to serve as targets for CTLs in the context of HLA class I molecules (3–7). These genes have been shown to be expressed not only in melanomas but in other tumors as well, but not in normal tissue, except for the testis (8–10). For this reason, these tumor antigens seem to be potential targets for specific immunotherapy using antigenic peptides (11).

To use peptide-based immunotherapeutics and adoptive cellular therapy, the precursor CTLs capable of recognizing antigenic peptides expressed by tumors must first be determined. Therefore, the induction of specific CTLs by stimulation with these peptides has been investigated (12–16). Because the HLA-A2 allele is expressed in a relatively high proportion of both Japanese and Caucasians (17), the HLA-A2-restricted MAGE-3 peptide has recently been examined to induce specific CTLs from PBMCs in both healthy donors and cancer patients (6, 15). The CTLs that could lyse target cells pulsed with the peptide have thus been detected. However, the cross-reactivity of peptide-induced CTLs to target cells endogenously presenting the MAGE-3 epitope, especially in cancer patients, remains controversial, despite the complicated manipulations required for the induction.

We recently found that MAGE-3-specific CTLs, which were able to kill HLA-A2 tumor cells expressing MAGE-3 as well as the peptide-pulsed target cells, could be induced from the PBMCs of a healthy donor by stimulation with HLA-A2-restricted MAGE-3 peptide using our newly developed method (16). On the basis of these results, the present study was undertaken to investigate the induction of MAGE-3-specific CTLs, particularly those that lyse target cells endogenously presenting the MAGE-3 epitope, in HLA-A2+ cancer patients whose tumors expressed MAGE-3 using the MAGE-3/HLA-A2 peptide, as assessed by our simplified method.

MATERIALS AND METHODS

Cell Lines. The 221(A2.1) cells produced by transferring the HLA-A2.1 gene into HLA-A, HLA-B, and HLA-C null mutant human B-lymphoblastoid cell line 221 were supplied by Takara Shuzo Co., Ltd. (Shiga, Japan). The gastric carcinoma cell lines KATOII and AZ-521 and K562 and Raji were provided by the Japanese Cancer Research Bank (Tokyo, Japan). These cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics.

Received 4/29/97; revised 7/30/97; accepted 8/11/97.

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1 To whom requests for reprints should be addressed. Phone: 81-977-27-1600; Fax: 81-977-27-1607.

2 The abbreviations used are: PBMC, peripheral blood mononuclear cell; APC, antigen-presenting cell; IL, interleukin; TNF, tumor necrosis factor; KLH, keyhole limpet hemocyanin; MMC, mitomycin C.
Primary Induction of CTLs Using a Synthetic MAGE-3 Peptide. The CTL induction in vitro was performed according to the procedure described by Plebanski et al. (18) with minor modifications. Briefly, the PBMCs of HLA-A2" patients bearing MAGE-3-expressing carcinomas were collected by centrifugation on a Ficoll-Paque density gradient. The PBMCs were preincubated with HLA-A2-restricted MAGE-3 peptide at 40 μg/ml in the presence of β2-microglobulin at 3 μg/ml for 2 h at 37°C. The purified HLA-A2 immunodominant MAGE-3 peptide with a FLWGPRALV sequence (6) was provided by Takara Shuzo Co., Ltd. Free peptide was then removed by washing, and the cells were mixed at a ratio of 1:1 with autologous PBMCs. The cultures were maintained in α-MEM supplemented with 10% heat-inactivated AB serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, with the addition of KLH (5 μg/ml; Calbiochem-Novabiochem, La Jolla, CA) and IL-7 (25 ng/ml; Upstate Biotechnology, Inc., Lake Placid, NY). Recombinant IL-2 (Takeda Co., Ltd., Osaka, Japan) was added to the cultures at 20 units/ml on day 3. The responder cells were restimulated every 7 days with freshly isolated autologous PBMCs that had been prepulsed with the peptide and treated with MMC (Kyowa Hakko Co., Ltd., Osaka, Japan). The cultures were fed with fresh medium containing IL-2 1 day after every restimulation. The CTL activity was assayed on day 42.

Phenotypic Analysis. Flow cytometry was performed using a FACScan (Becton Dickinson, Franklin Lakes, NJ). The cells were stained with antihuman monoclonal antibodies against CD3, CD4, and CD8 (Becton Dickinson). Isotype-matched murine antibodies served as a negative control.

Cytotoxicity Assay. The target cells were labeled with 100 μCi of sodium 51chromate (51Cr) for 1 h at 37°C, and the labeled cells were then washed and resuspended. The peptide-pulsed targets, 221(A2.1) cells, were prepared by incubating the cells with MAGE-3/HLA-A2 peptide (20 μg/ml) overnight at 37°C and then labeling them with 51Cr. The effector cells were placed in each well of the round-bottomed microtiter plates. The labeled target cells were then added to the well at a concentration of 5 × 105 cells/well to produce a total volume of 0.2 ml. After a 4-h incubation period, the release of 51Cr was measured by collecting the supernatant, followed by quantitation in an automated gamma counter. The percentage of specific cytotoxicity was calculated as a percentage of specific 51Cr release: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. To eliminate nonspecific lysis due to natural killer-like effectors, the cytolytic activity was tested in the presence of a 30-fold excess of unlabeled K562 cells.

Cytokine Release. The CTLs prepared for a cytokine analysis were cultured for at least 2 days without IL-2 and washed twice thereafter. The effector cells (5 × 105 cells/ml) were cocultured with an equal number of MMC-treated target cells in RPMI 1640 supplemented with 5% human AB serum for 24 h at 37°C. The supernatants were collected and stored at −70°C until use. To determine the TNF-α activity in the culture supernatants, commercially available immunoenzymetric assay kits (Medgenix Diagnostic, Fleurus, Belgium) were used. The minimum detectable concentration was estimated to be 3 pg/ml.

Inhibition of Cytotoxicity with Monoclonal Antibodies. Appropriate target cells were incubated with monoclonal antibodies at a final concentration of 1:20 for 1 h at 4°C before the assay of cytotoxicity. The monoclonal antibodies used were anti-HLA class I antibody, anti-HLA class II antibody (Immunotech, Marseille, France), and anti-HLA-A2 antibody (One Lambda, Inc., Canoga Park, CA).

Reverse Transcription-PCR Analysis of MAGE-3 Expression. The tumor tissue samples obtained from surgical specimens were immediately frozen at −90°C until the RNA was extracted. Total RNA was isolated from the tumor tissues or the tumor cell lines using the acid guanidinium thiocyanate-phenol chloroform extraction procedure (19). cDNA was synthesized by reverse transcription from 2.5 μg of total RNA as described previously (9). MAGE-3 cDNA was detected by PCR amplification using oligonucleotide primers specific for the different exons of the MAGE-3 gene. The primer sequences were 5'-TGAGGACCAAGGCCCCC-3' (AB-5) and 5'-GGAGATTATCAGGGCCTGC-3' (BLE-5; Ref. 5). PCR was performed for 33 cycles (1 min at 94°C, 2 min at 71°C, and 2 min at 72°C). The PCR product was size-fractionated on a 2% agarose gel and visualized with ethidium bromide staining. To ensure that the RNA was not degraded, a PCR assay that was specific for glyceraldehyde-3-phosphate dehydrogenase cdNA was performed in each case. The primers used for the amplification of glyceraldehyde-3-phosphate dehydrogenase were 5'-GTCAACCGATTGTGCTGAT-3' and 5'-AGTCTTCTGGGTCGCCAGT-3' (20).

RESULTS

Expression of MAGE-3 mRNA. We analyzed the expression of MAGE-3 mRNA in all cell lines and tumor tissues used in this study. In the cell lines, the MAGE-3 gene was expressed in KATOIII (gastric carcinoma cells; HLA-A0201/0209, A0207); it was not detected in AZ-521 (gastric carcinoma cells; HLA-A0201/0209, A30), Raji (B-lymphoblastoid cells; HLA-A0301, A31/33), or 221(A2.1) (B-lymphoblastoid cells; HLA-A0201). Tumor tissue samples were obtained from the resected specimens of two gastric cancer patients designated GAS1 (HLA-A0201/0209, A1101) and GAS2 (HLA-A0201/0209, A2402), one patient with rectal carcinoma (CRE1: HLA-A0210, A2602), and one patient with breast carcinoma (BRE1: HLA-A0201/0209, A2602). The MAGE-3 gene was detected in all of the tumor tissue specimens tested.

Growth Kinetics and Characterization of the Induced Cells. After the first stimulation, the cells were continuously expanded by approximately 2-fold. As a result, a 20–30-fold increase compared to the starting cell number was thus achieved on day 42. A flow cytometric analysis of the cells was performed before and after the cultures. The number of CD3+ cells gradually increased after the culture. The percentage of CD4+ cells was elevated until day 14 and gradually decreased thereafter, whereas the percentage of CD8+ cells increased after day 14. On day 42, more than 95% of these cells were CD3+, and approximately 60% of the cell population was CD8+. The kinetics of these cell populations in the BRE1 patients are shown in Fig. 1.
Cytotoxic Activity and Cytokine Release of the Induced Cells. The cytotoxic activity of the induced cells stimulated with MAGE-3/HLA-A2 peptide was tested against the target cells, including 221(A2.1) cells pulsed with or without the peptide and KATOIII, AZ-521, and Raji cells, after 42 days of culture. In the case of the BRE1 patients, the induced cells demonstrated more than 90% cytotoxicity against peptide-pulsed 221(A2.1) cells, compared to the less than 10% cytolysis observed against the 221(A2.1) cells alone, at an E:T ratio of 80:1 (Fig. 2). Furthermore, the cells were also able to lyse KATOIII cells (HLA-A2+, MAGE-3+), although the cytotoxicity activity level was lower than that observed in the peptide-pulsed 221(A2.1) cells. However, cytotoxicity was minimal against AZ-521 cells (HLA-A2+, MAGE-3+) and Raji cells (HLA-A2+, MAGE-3+; Fig. 3). In the four MAGE-3+ carcinoma patients examined in this study, the induced cells from all four patients exhibited more than 15% cytolysis against peptide-pulsed 221(A2.1) cells, whereas two of the cells were also able to lyse the HLA-A2+, MAGE-3+ cell line, KATOIII (Table 1).

Because CTLs elicit the antigen-specific secretion of cytokines including TNF-α after coincubation with target cells, we examined the TNF-α release by induced cells from the PBMCs of BRE1 patients after stimulation with several target cells. A significant release of the cytokine was observed when the effector cells were coincubated with 221(A2.1) cells pulsed with the MAGE-3/HLA-A2 peptide and KATOIII, with virtually no reactivity against the 221(A2.1) cells alone or against AZ-521 or Raji cells (Fig. 4, a and b).

Inhibition of the Recognition of the Induced Effectors by Monoclonal Antibodies. To determine whether the induced effector cells recognized the MAGE-3-expressing target in a HLA-restricted manner, monoclonal antibodies generated against HLA molecules were used to block the cytotoxicity by effectors. The cytotoxic activity of the effector cells against the 221(A2.1) cells pulsed with the peptide was significantly eliminated by anti-HLA class I antibody or anti-HLA-A2 antibody, but not by anti-HLA class II antibody (Fig. 5). When the HLA-A2+, MAGE-3+ cell line, KATOIII, was used as a target, similar results were obtained (data not shown). These data suggested that the induced effectors lysed the targets expressing MAGE-3 in HLA class I- and HLA-A2-restricted manners.

DISCUSSION

The results described in this study demonstrated that the peptide-specific CTLs could be induced from the PBMCs of HLA-A2 patients with MAGE-3+ carcinoma by stimulation with HLA-A2-restricted MAGE-3 peptide using our simple method (16). With this method, we were able to detect CTLs that could lyse HLA-A2 carcinoma cells expressing MAGE-3 as well as the peptide-pulsed target cells in an HLA class I- and HLA-A2-restricted manner in some of the patients.

By using synthetic MAGE peptides, the in vitro induction of specific CTLs has been investigated in PBMCs from healthy donors. Celis et al. (12) demonstrated that specific CTLs could be induced in vitro from CD4+ cell-depleted PBMCs of a healthy donor by stimulation with Staphylococcus aureus Cowan-I and antihuman IgM antibody-treated autologous B cells pulsed with HLA-A1-restricted MAGE-3 peptide, and the CTLs lysed HLA-A1 tumor cells expressing MAGE-3 as well as
The 2TLs were obtained by stimulating PBMCs for 42 days with induction from cancer patients using MAGE peptide.

Fig. 3 Recognition by CTLs of MAGE-3⁺ and HLA-A2⁺ tumor cells. The CTLs were obtained by stimulating PBMCs for 42 days with MMC-treated autologous PBMCs pulsed with MAGE-3 peptide. The cytotoxic activity against KATOIII (□; gastric cancer cells; HLA-A2⁺ and MAGE-3⁺), AZ-521 (●; gastric cancer cells; HLA-A2⁺ and MAGE-3⁺), and Raji (○; lymphoma cells; HLA-A2⁺ and MAGE-3⁺) is shown.

HLA-A1 target cells pulsed with the peptide. Using a similar procedure, MAGE-3-specific CTLs were generated from the PBMCs of a healthy, HLA-A2⁺ donor by stimulation with a MAGE-3-derived peptide presented by HLA-A2 (6). The induced CTLs could lyse the peptide-pulsed target cells, whereas a significant level of lysis by the induced CTLs was only observed in the HLA-A2 tumor cells expressing MAGE-3 that had been pretreated with IFN-γ and TNF-α to induce a higher expression of HLA class I molecules. We recently found that the induced CTLs could kill the HLA-A2 tumor cells expressing MAGE-3 without pretreatment with these cytokines when unseparated PBMCs from a healthy donor were stimulated in vitro with HLA-A2-restricted MAGE-3 peptide by using the simplified method as described in this study (16).

The generation of peptide-specific CTLs has been further examined in PBMCs from cancer patients using the MAGE peptide. However, the recognition of target cells endogenously presenting the MAGE epitope by peptide-induced CTLs from cancer patients remains controversial. Recently, Valmori et al. (15) reported that MAGE-3 peptide-specific CTLs were obtained from the PBMCs of HLA-A2⁺ patients with melanoma when highly enriched CD8⁺ responder populations were stimulated with MAGE-3/HLA-A2 peptide-pulsed autologous PBMCs. In their study, however, the CTL lines derived from these populations from 4 of 10 patients lysed HLA-A2⁺ target cells that were pulsed with the peptide, whereas these were unable to recognize MAGE-3-expressing autologous or allogeneic HLA-A2⁺ melanoma cell lines as assessed by both cytolyis and cytokine release. However, our results showed that all CTL populations derived from the PBMCs of four cancer patients lysed MAGE-3/HLA-A2 peptide-pulsed target cells, and moreover, two of these cell populations could also lyse the HLA-A2 carcinoma cell line expressing MAGE-3, demonstrating the induction of specific CTLs from the PBMCs of cancer patients that killed MAGE-expressing tumor cells.

In contrast to previous reports on the generation of MAGE-3/HLA-A2 peptide-specific CTLs in which CD8⁺-enriched T cells were used as responders (6, 15), the induction of CTLs in this study was performed using unseparated PBMCs and a combination of IL-7 and KLH for primary culture according to the method described by Plebanski et al. (18). They showed that the IL-7 and CD4⁺ T cells primed in vitro by KLH acted together in the generation of primary and secondary responses with malarial peptides. The induced CTL lines that were CD8⁺ demonstrated a high level of the HLA class I-restricted killing of target cells either pulsed with peptides or infected with a recombinant vaccinia virus containing the full-length antigen. In the present study, the cytolytic activity of the effector cells was abrogated by anti-HLA class I and HLA-A2 antibodies, thus indicating the HLA class I- and HLA-A2-restricted lysis of the target cells, although approximately 60% of the induced cells were CD8⁺ after being cultured for 42 days. Although concomitant CD4⁺ T-cell activation has been shown to be able to promote CTL stimulation in vitro, it is still unclear as to whether or not the activation of the CD4⁺ T cells in this study could be related to the ability of CTLs to lyse target cells endogenously presenting the MAGE-3 epitope.

Various APCs have been used in the induction of peptide-specific CTLs in vitro, because effective antigen presentation may be crucial for the induction of T-cell-mediated tumor immunity. Recent studies have shown that dendritic cells pulsed with MHC class I peptides are potent inducers of tumor-specific CTL responses both in vitro and in vivo (21–23). However, no information exists on the in vitro induction of specific CTLs using MAGE-3/HLA-A2 peptide-pulsed dendritic cells, although autologous dendritic cells pulsed with HLA-B44-restricted MAGE-3 peptide were shown to stimulate the generation of MAGE-specific CTLs from the PBMCs of HLA-B44 healthy donors, which killed HLA-B44 tumor cells expressing MAGE-3 (7). For the induction of MAGE-3-specific CTLs from the PBMCs of melanoma patients using HLA-A2-restricted MAGE-3 peptide, positive CTL responses were shown to be obtained more frequently in cultures stimulated with acid-treated and peptide-loaded PBMCs than in cultures stimulated with peptide-pulsed PBMCs as APCs (15). However, the induced CTLs from both cultures were not able to lyse HLA-A2 melanoma cells expressing MAGE-3. In this study, we used freshly isolated autologous PBMCs pulsed with HLA-A2-restricted MAGE-3 peptide as APCs, and the CTLs that were able to kill the target cells endogenously presenting the MAGE-3 epitope were thus obtained. Additional comparable studies may be needed, however, to clarify the potential APCs for induction of the MAGE peptide-specific CTLs in vitro.

The expression of melanocyte differentiation antigens in melanomas has been reported to be associated with a lack of spontaneous antigen-specific CTL responses in vivo when assessed by a mixed lymphocyte-peptide culture (14). As shown in this study, we detected a specific CTL response against HLA-
A2-restricted MAGE-3 peptide by using our simple method in patients with carcinoma whose tumors expressed MAGE-3 antigen. However, the level of the CTL reactivity seemed to be relatively low compared to that previously observed in healthy donors (16). These results may account for the inadequate immunogenic environment that mediates the down-regulation of antigen-specific T-cell responses in cancer patients in vivo, including the selection of antigen-loss variants, a low frequency of precursor CTLs, and T-cell anergy or tolerance (14, 24, 25). However, such tumor-related down-regulation may be overcome with an adequate presentation of the peptide vaccine, because peptide immunization was reported to generate a pep-

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**Fig. 4** The CTLs exhibit HLA-A2- and MAGE-3-specific TNF-α secretion. The anti-MAGE-3 CTL culture was cocultured in combination with 221(A2.1) cells either with or without pulsed with MAGE-3 peptide (a) or different tumor cells (b). After 24 h of incubation, a portion of the supernatant was harvested, and its TNF-α content was measured by an ELISA kit.

**Fig. 5** The CTLs were obtained by stimulating the PBMCs for 42 days with MMC-treated autologous PBMCs pulsed with MAGE-3 peptide. The 221(A2.1) cells were pulsed with 20 µg/ml MAGE-3 peptide overnight and then labeled with 51Cr. CTLs were cultured thereafter with target cells alone (●) or in the presence of anti-HLA class I (○), anti-HLA class II (◇), or anti-HLA-A2 (▼) antibodies for 4 h. The cytotoxic activity was then assessed at E:T ratios ranging from 80:1 to 0.6:1.
tide-specific reaction (23, 26) and to also be effective in a significant proportion of melanoma patients (11).

The proportion of HLA-A2 individuals is reported to be 44% in Japanese and 49% in Caucasians (7). On the other hand, the MAGE-3 gene was expressed in 40% of gastric carcinomas (9), in 20% of colorectal carcinomas (10), and in 12% of breast carcinomas (5). Therefore, 5–20% of the patients with these carcinomas could potentially be candidates for specific immunotherapy directed against the MAGE-3 antigen. As shown in this study, specific CTLs that lysed MAGE-3-expressing carcinoma cells as well as peptide-pulsed target cells could thus be detected in vitro from PBMCs of two of four HLA-A2" patients bearing MAGE-3" carcinoma, using the HLA-A2-restricted MAGE-3 peptide and our simplified method. Therefore, these findings seem to indicate that immunization with the peptide in vivo may potentially become a possible modality for the treatment of carcinomas in such patients.

REFERENCES


Induction of antitumor cytotoxic T lymphocytes from the peripheral blood mononuclear cells of cancer patients using HLA-A2-restricted MAGE-3 peptide in vitro.

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