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Multidrug Resistance-associated Protein 3 Is a Tumor Rejection Antigen Recognized by HLA-A2402-restricted Cytotoxic T Lymphocytes[1]

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ABSTRACT

The identification of tumor rejection antigens recognized by CTLs and its application in peptide-based specific immunotherapy against melanomas have been extensively investigated in the past decade. However, only a small number of studies regarding these issues in other epithelial cancers have been reported. In this study, we show that a multidrug resistance-associated protein 3 (MRP3) is a tumor rejection antigen recognized by HLA-A2402-restricted CTLs established from T cells infiltrating into lung adenocarcinoma. MRP3 is expressed in differing quantities in tumor cells of various tissue types and origins. Four dominant MRP3-derived antigenic peptides that are recognized by the CTLs have been identified, each possessing *in vitro* immunogenicity. Namely, these four peptides (MRP3–503, MRP3–692, MRP3–765, and MRP3–1293) can induce peptide-specific CTLs after *in vitro* stimulation with these peptides in peripheral blood mononuclear cell cultures of HLA-A24$^+$ cancer patients, with the CTLs expressing cytotoxicity against HLA-A2402$^+$ MRP3$^+$ tumor cells but not against either HLA-A2402$^-$ or MRP3$^-$ target cells. The peptide specificity of the cytotoxicity of the CTLs was further confirmed by using peptide-loaded HLA-A24$^+$ EBV-transformed B cells. Widespread MRP3 expression in various tumor cell lines and tumor tissues at the mRNA level was confirmed. Furthermore, reactivities of the MRP3-peptide-induced CTLs against tumor cells correlated with MRP3 expression in the tumor cells. These results suggest that MRP3 and its derived peptides described in the present paper are potential candidates for cancer vaccines in regard to HLA-A24$^+$ patients with various tumors, particularly for those tumors that show anticancer drug resistance.

INTRODUCTION

In the past decade, the identification of genes encoding tumor rejection antigens that can be recognized by CTLs has been extensively investigated (1, 2). Most of these genes are from melanomas and belong to a family of genes that encode cancer/testis antigens or melanoma differentiation antigens (1, 2). The antigenic peptides of these gene products that can be recognized by CTLs in a class-I HLA-restricted manner have also been identified, and some of them have been used as a peptide-based cancer vaccine in clinical trials for patients with melanomas (1, 2). Although many studies in the field of melanomas have been performed, only a small number of studies (3–10) regarding these issues in epithelial cancers other than melanomas have been reported. SART-1, SART-2, and SART-3 have been identified from the cDNA libraries of squamous cell carcinoma cells using a CTL line established from a patient with esophageal cancer (6, 7, 9). SART-1 and SART-2 are preferentially expressed in squamous cell carcinomas rather than in adenocarcinomas, whereas the expression of SART-3 is more widespread in both squamous cell carcinomas and adenocarcinomas. Cyclophilin B and ART-4 are identified using a CTL line established from a patient with lung adenocarcinoma (8, 10). Both the molecules are ubiquitously expressed in normal and cancer tissues at mRNA levels, although the expression of ART4 at protein levels has been rarely observed in normal cells. These molecule-derived antigenic peptides that can be recognized by tumor-specific CTLs in a HLA-A24-restricted manner have been reported (6–10). Furthermore, Phase I clinical trials using these peptides as cancer vaccines in patients with various cancers, such as lung, esophagus, and colon cancers, have begun in Kurume University Hospital.

At the present time, chemotherapy is one of the major treatment modalities for patients with cancer. However, MDR[3] is a serious problem associated with this therapy. MDR is a phenomenon in which cancer cells acquire cross-resistance to a variety of structurally unrelated cancer chemotherapeutic agents after selection for resistance to a single anticancer agent (11). This phenomenon is observed not only *in vitro* but also *in vivo* and often results in cancer treatment failure. In the present study, we report that MRP3 is a tumor rejection antigen recognized by CTLs in an HLA-A2402-restricted manner. We discuss the potential application of MRP3-derived peptides as cancer vaccines in specific immunotherapy.

MATERIALS AND METHODS

Cell Lines. A lung adenocarcinoma cell line, 11-18, was used for preparation of the cDNA library. COS7, VA13 (fibroblast), 293T, and C1R-A2402 (an HLA-A2402 transfectant) cells were used for the transfection and peptide-pulse experiments, respectively. C1R-A2402 cells were kindly provided by Dr. Masafumi Takiguchi (Kumamoto University, Kumamoto, Japan). The other cell lines used in this study were as follows: lung adenocarcinomas (PC-9, A549, LC-1, RERF-LCMS, and 1-87), lung squamous cell carcinomas (Sq-1, RERF-LCA1, QG56, and LC1-Sq), lung small cell carcinoma (LK79), renal cell carcinomas (Kur-11, Caki-1, PC93, RC30-14, PC3, VMRC-RCW, TUHR-4TKB, TUHR-10TKB, RCC-10RGB, and LNCap), ovarian cancers (KOC-3S, KOC-5C, KOC-7C, TYK-nu, RMUG-S, RMG-I, TOC-2, MCAS, RTSG, and RKN), bladder carcinoma (HT1376), esophageal squamous cell carcinoma (KE4), and EBV-transformed B-cell line (SS-EBB). The origins and HLA genotypes of these cell lines have been described previously (9, 10).

Identification of the *MRP3* Gene. The expression-gene cloning method was used to identify a gene that encodes tumor rejection antigens recognized by the CTL line, GK-CTL, as reported elsewhere (6, 10). Briefly, mRNA of the 11-18 lung adenocarcinoma cells was converted to cDNA, ligated to *Sal*I adapter, and inserted into the expression vector *pCMV-SPORT2.0* (Life Technologies, Inc., Gaithersburg, MD). A total of 1×10^5 clones from the cDNA library of the 11-18 cells was divided into 1000 wells (the expected number of clones/well was 100) and subjected to the first screening. Purified DNA from the divided pools and 100 ng of *HLA-A2402* cDNA were cotransfected into VA13 cells and analyzed for their activity to stimulate IFN-γ production by the GK-CTLs. Nineteen positive pools were obtained from the first screening and subdivided into smaller pools for further screening. Finally, one cDNA clone, *clone 5*, was obtained. DNA sequencing was performed using the dideoxynucleotide sequencing method employing an AutoRead Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) and analyzed using an ALF express DNA Sequencer (Pharmacia Biotech). The nt sequence of *clone 5* was almost

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[3] The abbreviations used are: MDR, multidrug resistance; MRP, MDR-associated protein; aa, amino acid; nt, nucleotide; PBMC, peripheral blood mononuclear cell; ABC, ATP-binding cassette.

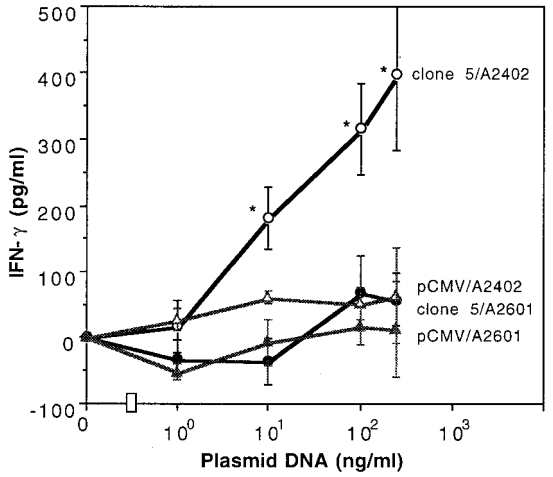

Fig. 1. Bioactivity of *clone 5*. VA13 cells were transfected with various doses of *clone 5* or vacant vector *pCMVSPORT2.0* DNA and 100 ng of *HLA-A2402* or control *HLA-A2601* cDNA, and their stimulatory effects on IFN-γ production by the GK-CTLs were tested. Values represent the means of triplicate assays. Two-tailed Student's *t* test was used for statistical analysis between IFN-γ production by the GK-CTLs in response to VA13 cells transfected with the *clone 5* and *HLA-A2402* cDNA and that transfected with *clone 5* and *HLA-A2402* cDNA. *, $P < 0.05$.

identical to that of MRP3 (accession nos. Y17151, AF104943, AF085692, AF085690, AF009670, and NM003786).

Northern Blot Analysis. Total RNA (5 μg/lane) extracted from various cells or tissue specimens using RNA zol B (TEL-TEST, Friendswood, TX) was separated on formaldehyde-agarose gel and transferred to nylon membranes (Hybond-N^+; Amersham, Buckinghamshire, United Kingdom). The membranes were further hybridized overnight at 65°C in a hybridization buffer [7% SDS, 1 mM EDTA, and 0.5 M NaH_2PO_4 (pH 7.2)] containing a ^{32}P-labeled 800-bp fragment of *Xho*I and *Eco*RI cut *clone 5* cDNA as a probe. The membranes were washed three times at room temperature and once at 65°C with a washing buffer [1% SDS and 40 mM NaH_2PO_4 (pH 7.2)] and then autoradiographed. Human *β-actin* cDNA (Clontech, Tokyo, Japan) was also used as a control probe. The relative expression of the *MRP3* mRNA was calculated using the following formula: index = (*MRP3* density of a sample/*β-actin* density of a sample) × (*β-actin* density of the 11-18 cells/*MRP3* density of the 11-18 cells).

Peptides and Assays. Thirty-one different synthetic peptides (purity, >70%) derived from the deduced aa sequence of MRP3 with binding motifs for HLA-A2402 molecules as described in the literature (12), including motifs of tyrosine or phenylalanine at position 2 and of isoleucine, leucine, phenylalanine, or tryptophan at position 9, searched using BIMAS (BioInformatics and Molecular Analysis Section, NIH) software (Center for Information Technology, NIH, Bethesda, MD) were obtained from Sawady (Tokyo, Japan). An HIV-derived peptide (RYPLTFGWCF) capable of binding to HLA-A2402 molecules was used as a negative control (8). Peptides of >95% in purity were used for experiments regarding dose dependency and CTL induction. The estimated scores of half-time of dissociation of each MRP3 peptide for HLA-A24-molecules were calculated using BIMAS software (13). For detection of antigenic peptides recognized by the GK-CTLs, the peptides were loaded onto C1R-A2402 cells by incubation at a concentration of 10 μM, unless stated otherwise. Two h later, the supernatant was removed, and the GK-CTLs (1×10^5) were added to the culture, incubated for an additional 18 h, and the concentration of IFN-γ in the culture supernatants was measured by ELISA (the limit of sensitivity, 10 pg/ml) in triplicate assays. Two-tailed Student's *t* test was used for statistical analysis.

CTL Induction by Peptides. PBMCs (1×10^5/well) obtained from HLA-$A2402^+$ healthy donors or cancer patients were incubated with 10 μM of the peptide in a 96-well plate in the presence of 100 units/ml interleukin-2 at day 0 as reported previously (10). At culture days 3, 6, and 9, the cells were restimulated by 10 μM of the peptide. The culture supernatant of each well was removed, resuspended in fresh medium, and separated into two wells. Each of the two wells was stimulated with the corresponding peptide or control HIV-peptide. After 18 h of culture, the concentration of IFN-γ in the culture supernatants was measured by ELISA in triplicate assays. The concentration of IFN-γ in the supernatant of the assay culture stimulated by control HIV peptide-loaded C1R-A2402 cells or HLA-$A24^+$ tumor cells (11-18, Sq-1) was subtracted from that of specific peptide-loaded C1R-A2402 cells or that of HLA-$A24^-$ QG56 cells, respectively. For cytotoxicity tests, the cells were further cultured in a 96-well U-bottomed microculture plate in the presence of

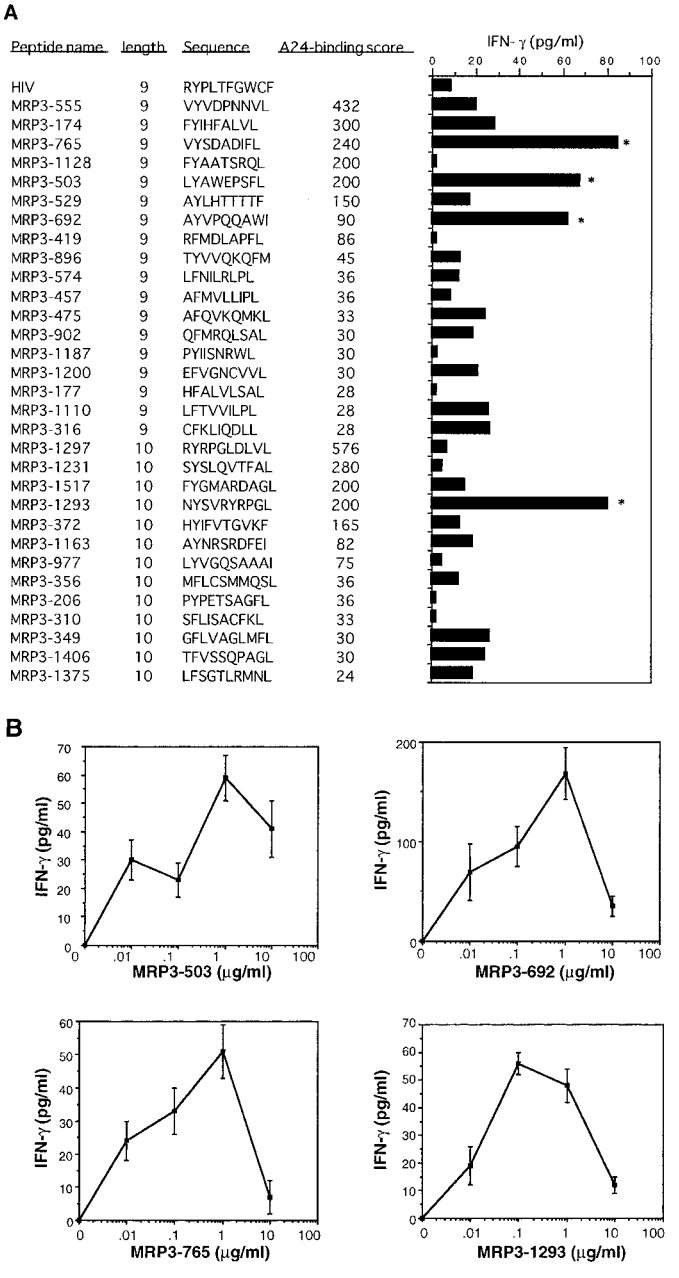

Fig. 2. Identification of MRP3-derived antigenic peptides recognized by the GK-CTLs. *A*, determination of the antigenic peptides. Each of the 31 different MRP3-derived peptides was loaded onto C1R-A2402 cells at a concentration of 10 μM. The GK-CTLs were cultured with the peptide-loaded C1R-A2402 for 18 h, and the culture supernatant was harvested to measure IFN-γ production using ELISA. Values represent the means of triplicate assays. The background of IFN-γ production by the GK-CTLs in response to peptide-unloaded C1R-A2402 cells was subtracted from the values. The two-tailed Student's *t* test was used for the statistical analysis between the IFN-γ production by the GK-CTLs in response to peptide-loaded C1R-A2402 cells and that in response to unloaded C1R-A2402 cells. *, $P < 0.05$. The A24-binding score shows estimated score of half-time of dissociation of each peptide for HLA-A24 molecules. *B*, dose response of the MRP3 peptides. Indicated doses of the peptides were loaded onto C1R-A2402 cells, and the ability of the peptides to stimulate IFN-γ production by the GK-CTLs was tested. Values represent the means ± SD of triplicate assays. The background of IFN-γ production by the GK-CTLs in response to peptide-unloaded C1R-A2402 cells was subtracted from the values.

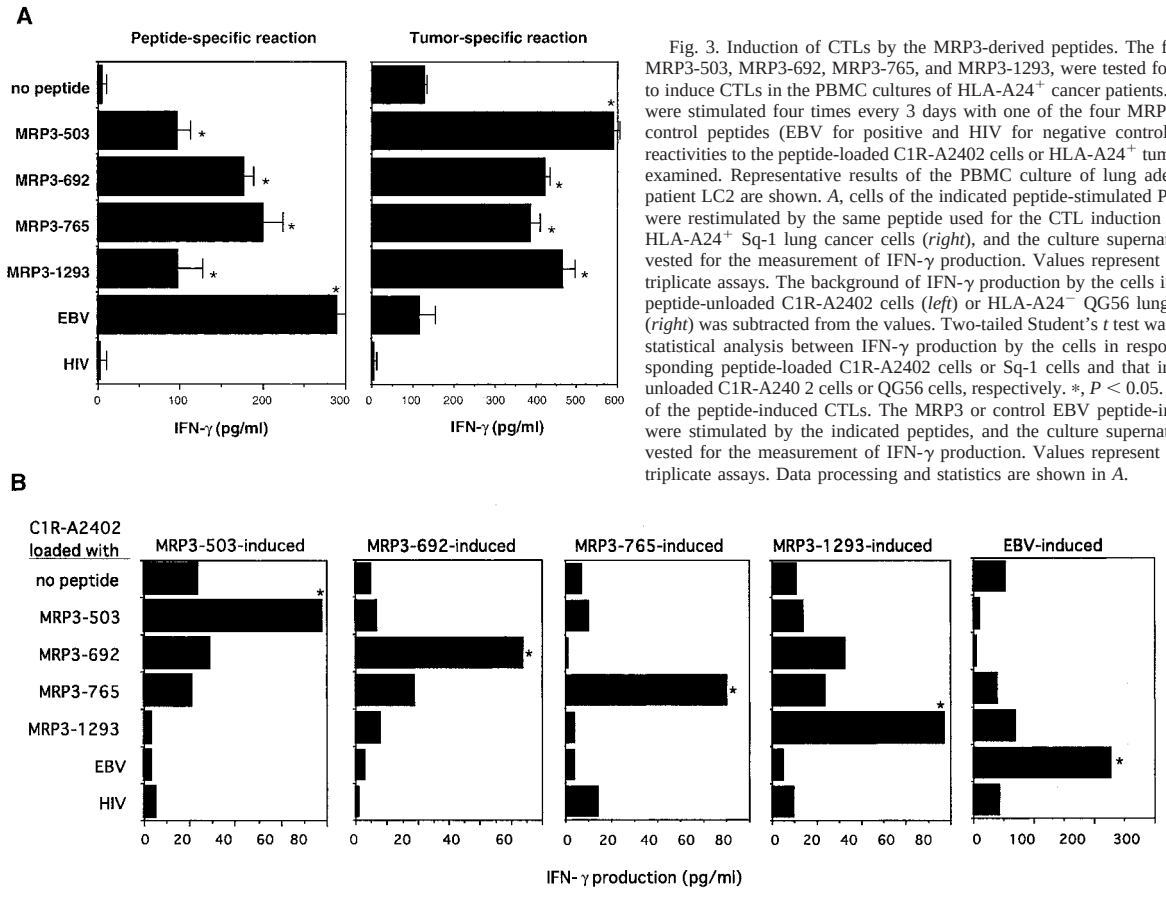

Fig. 3. Induction of CTLs by the MRP3-derived peptides. The four peptides, MRP3-503, MRP3-692, MRP3-765, and MRP3-1293, were tested for their ability to induce CTLs in the PBMC cultures of HLA-A24$^+$ cancer patients. The PBMCs were stimulated four times every 3 days with one of the four MRP3-peptides or control peptides (EBV for positive and HIV for negative controls), and their reactivities to the peptide-loaded C1R-A2402 cells or HLA-A24$^+$ tumor cells were examined. Representative results of the PBMC culture of lung adenocarcinoma patient LC2 are shown. *A*, cells of the indicated peptide-stimulated PBMC culture were restimulated by the same peptide used for the CTL induction (*left*) or with HLA-A24$^+$ Sq-1 lung cancer cells (*right*), and the culture supernatant was harvested for the measurement of IFN-γ production. Values represent the means of triplicate assays. The background of IFN-γ production by the cells in response to peptide-unloaded C1R-A2402 cells (*left*) or HLA-A24$^-$ QG56 lung cancer cells (*right*) was subtracted from the values. Two-tailed Student's *t* test was used for the statistical analysis between IFN-γ production by the cells in response to corresponding peptide-loaded C1R-A2402 cells or Sq-1 cells and that in response to unloaded C1R-A240 2 cells or QG56 cells, respectively. *, $P < 0.05$. *B*, specificity of the peptide-induced CTLs. The MRP3 or control EBV peptide-induced CTLs were stimulated by the indicated peptides, and the culture supernatant was harvested for the measurement of IFN-γ production. Values represent the means of triplicate assays. Data processing and statistics are shown in *A*.

irradiated autologous PBMCs (2×10^6 cells/well) as antigen-presenting cells that had been pulsed by a corresponding peptide. The cells were stimulated with the peptide without antigen-presenting cells at days 3 and 7 of the second culture, and the cells were further cultured with interleukin-2 alone. The cells were harvested at culture days 28–42, and the cytotoxic activity was measured using a standard 6-h ^{51}Cr-labeled release assay at different E:T ratios as reported previously (10).

RESULTS

Identification and Characterization of the *MRP3* Gene. The CTL line used for identification of the *MRP3* gene was the HLA-A24-restricted and tumor-specific CTL (GK-CTL) line with CD3$^+$CD4$^-$CD8$^+$ phenotype that was established from T cells infiltrating into the lung adenocarcinoma, and its characteristics have been reported elsewhere (8). Reactivities of the GK-CTLs to various tumor cells are described previously (8). Briefly, the GK-CTLs recognized HT1376 (HLA-A2402/-A2402) bladder cancer cells and 11-18 (HLA-A0201/-A2402), PC9 (HLA-A0206/-A2402), and Sq-1 (HLA-A1101/-A2402) lung cancer cells when assessed by an IFN-γ production, whereas none of HLA-A2402$^-$ cells, including COS-7 and VA13 cells, stimulated GK-CTLs. The 11-18 lung adenocarcinoma cells were used as a source of cDNA library. A total of 10^5 cDNA clones from the cDNA library of the 11-18 cells were tested for their ability to stimulate IFN-γ production by the GK-CTLs after cotransfection with *HLA-A2402* cDNA into the VA13 cells. After repeated cycles of the screening, one clone, *clone 5*, was confirmed to encode a tumor antigen recognized by the GK-CTLs when cotransfected with *HLA-A2402*, but not with control *HLA-A2601* (Fig. 1). The vacant vector, *pCMVSPORT2*, served as a negative control in this experiment.

Clone 5 contained a 4948-bp-long cDNA insert with 4365 bp of open reading frame encoding 1455 aas. The obtained nt sequence was identical to those of MRP3 (European Molecular Biology Laboratory/GenBank/DNA Data Bank of Japan accession no. AF085690) but lacked the 5′ end; *i.e.*, the nt sequence of *clone 5* corresponds to position 175-5142 of the MRP3 cDNA, whereas the predicted aa sequence of *clone 5* lacked position 1-73 of the MRP3 protein. The reading frame of *clone 5* was identical to that of *MRP3*, thus indicating that *clone 5* encodes a large part of the MRP3 protein. Two alternative splicing variants of MRP3, MRP3A, and MRP3B have been reported (14). However, the nt sequence of *clone 5* differed from those of the two variants.

Identification of MRP3-derived Antigenic Peptides Recognized by the CTLs. Each of the 31 different MRP3-derived synthetic peptides with binding motifs to HLA-A2402 molecules was loaded onto an HLA-A2402 stable transformant, C1R-A2402 cells, at a concentration of 10 μM, and its ability to induce IFN-γ production by GK-CTL was tested. Four of these peptides, MRP3-503, MRP3-692, MRP3-765, and MRP3-1293, induced significant levels of IFN-γ production (Fig. 2*A*) in a dose-dependent manner (Fig. 2*B*). The optimal concentration of the four peptides for loading C1R-A2402 cells varied in each peptide ranging from 0.1–1 μg/ml (compatible to 0.1–1 μM). The binding affinity of the four peptides to the HLA-A2402 molecules is relatively intermediate. The estimated score of half-time of dissociation of each peptide ranged between 90 to 240 (Fig. 2*A*).

Induction of CTLs by the MRP3-derived Peptides. The four peptides, MRP3-503, MRP3-692, MRP3-765, and MRP3-1293, were tested for their ability to induce CTLs in the PBMC cultures of nine HLA-A24$^+$ patients with cancer (three with lung adenocarcinomas,

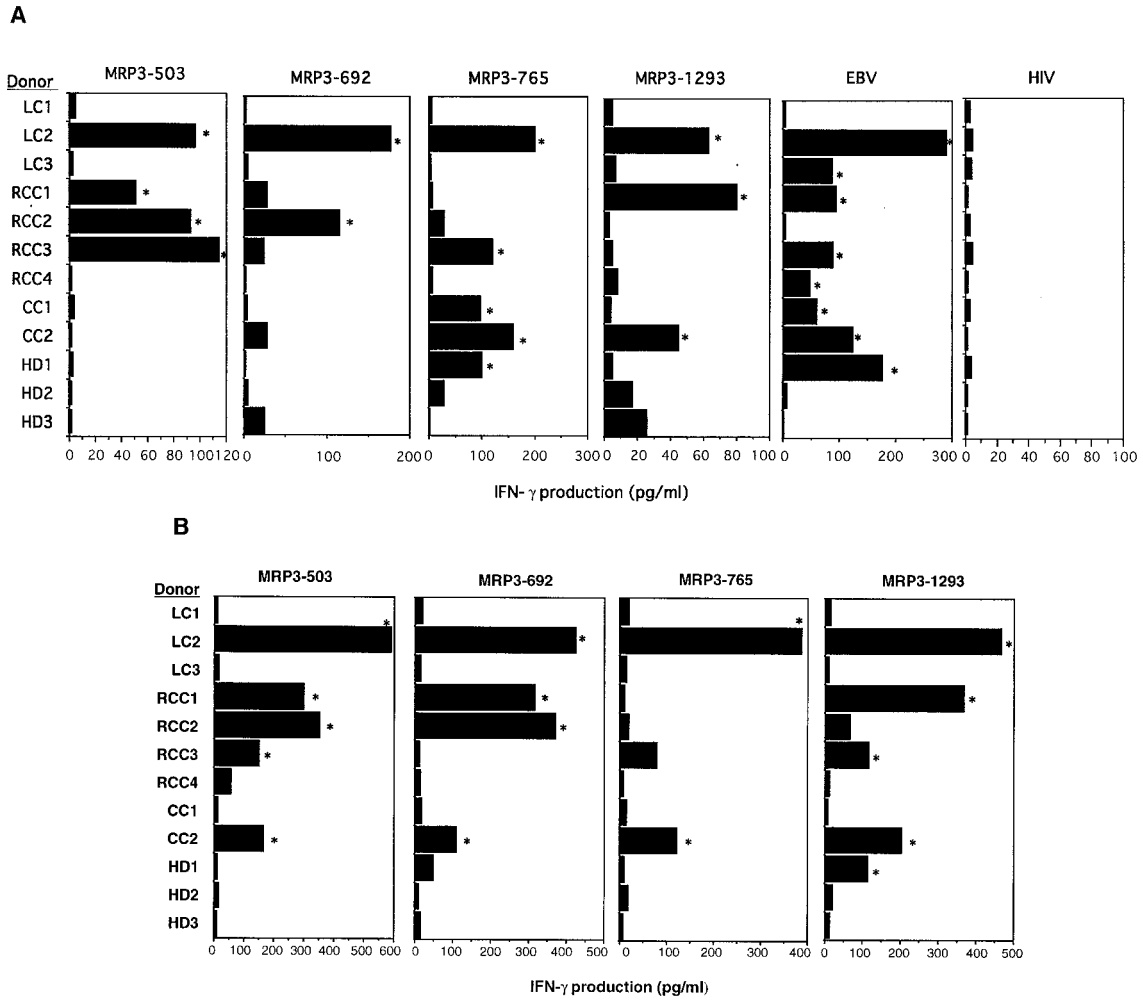

Fig. 4. Summary of the results of CTL induction by the MRP3 peptides. PBMCs of nine HLA-A24$^+$ patients with cancer (three with lung adenocarcinomas, four with renal cell carcinomas, and two with colon adenocarcinomas) and three HLA-A24$^+$ healthy donors were subjected to CTL induction by the MRP3 peptides or control EBV and HIV peptides. The reactivities of the cells to specific peptide-loaded C1R-A2402 cells (*A*) or HLA-A24$^+$ Sq-1 lung cancer cells (*B*) were shown. Experimental details are the same as those described in Fig. 3.

four with renal cell carcinomas, and two with colon adenocarcinomas) and three HLA-A24$^+$ healthy donors. The PBMCs were stimulated four times every 3 days by one of the four peptides, and their reactivities to the peptide-loaded C1R-A2402 cells or HLA-A24$^+$ tumor cells were examined. Representative results are shown in Fig. 3*A*. The PBMCs of a patient with lung adenocarcinoma (patient LC2) cultured with any of the four peptides produced significant levels of IFN-γ in response to the corresponding peptide-loaded C1R-A2402 cells in this case (Fig. 3*A*, *left*). EBV-derived peptide, which can bind to HLA-A2402 molecules, also induced EBV-peptide reactive T cells in the culture, whereas HIV-derived HLA-A2402 binding peptide did not induce the specific T cells after being stimulated four times. The PBMCs cultured with any of the MRP-peptides were also reactive to HLA-A24$^+$ lung cancer cells Sq-1 (Fig. 3*A*, *right*). Reactivity of the EBV-stimulated PBMCs was similar to that of unstimulated PBMCs (no peptide). These results suggest that the four MRP3-derived peptides can induce peptide-specific T cells in the PBMC culture of a lung cancer patient.

The specificity of the peptide-induced T cells was further analyzed. Reactivities of the peptide-induced T cells to C1R-A2402 cells loaded with each of the four MRP3-peptides or control EBV- and HIV-peptides were assessed in terms of IFN-γ production (Fig. 3*B*). The MRP3-503 peptide-induced T cells in the PBMC culture of patient LC2 produced significant levels of IFN-γ in response to stimulation by MRP3-503-loaded C1R-A2402 cells. Stimulation by the other MRP3-peptide-loaded or control EBV- and HIV-peptide-loaded C1R-A2402 cells did not elicit significant levels of IFN-γ production by the MRP3-503-induced T cells. Thus, the T cells were specific for the MRP3-503 peptide used for the induction. Similar results were also observed in the other three MRP3-peptide-induced T cells and control EBV-peptide-induced T cells. HIV-peptide-induced T cells in the PBMCs did not show reactivity against any of the peptides used in this study including HIV-peptide.

A summary of the results of CTL induction by the MRP3-peptides in the PBMC cultures of nine patients with cancer and three healthy donors is shown in Fig. 4. The EBV-peptide used as a positive control efficiently induced peptide-specific T cells in the PBMC cultures of seven of the nine patients with cancer and one of the three healthy donors (Fig. 4*A*). In contrast, the HIV-peptide used as a negative control did not induce HIV-peptide-specific T cells in any of the PBMC cultures used in this experiment. The MRP3-503 induced peptide-specific T cells in the PBMC cultures of one lung cancer patient (LC2) and three patients with renal cell carcinomas (RCC1-3). The apparent induction of peptide-specific T cells was also observed in the PBMC cultures stimulated by the other three MRP3 peptides. The peptide-reactive T cells specific for MRP3-692, MRP3-765, and MRP3-1293 were induced in the PBMC cultures of two (LC2 and RCC2), four (LC2, RCC3, and colon cancer patients, CC1 and CC2),

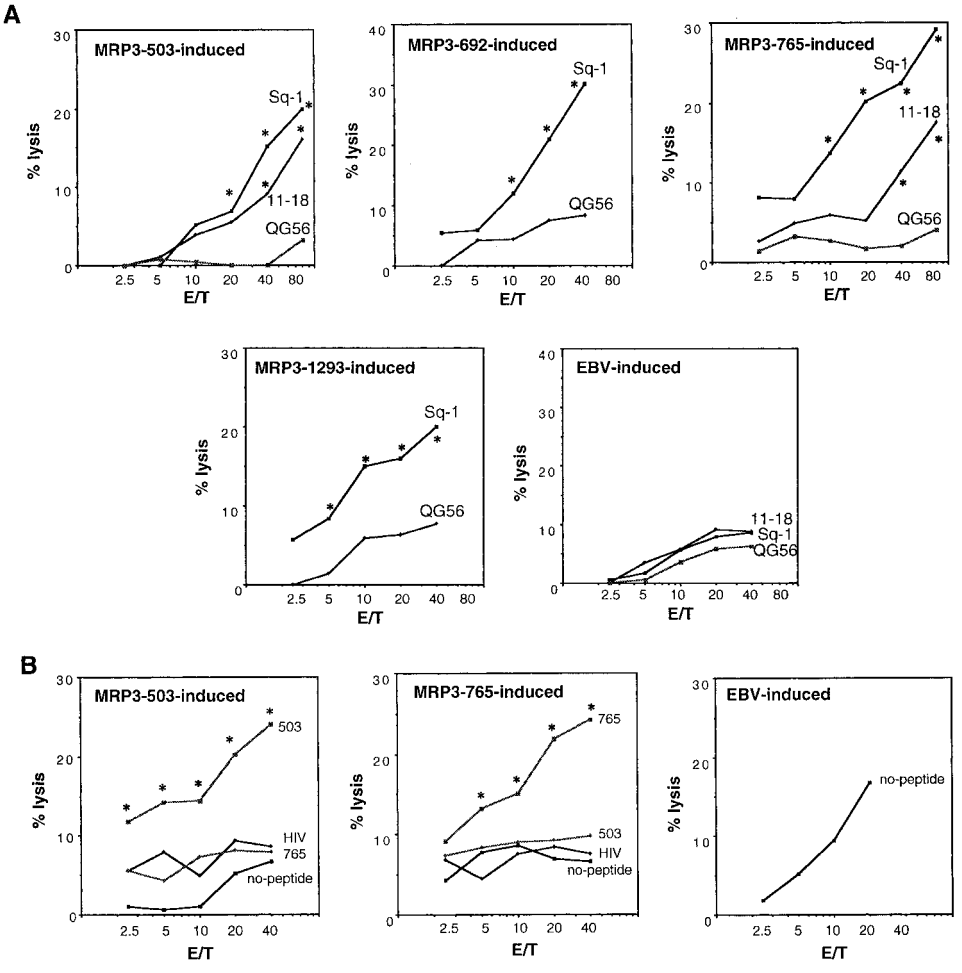

Fig. 5. Cytotoxic activities of the MRP3 peptide-induced CTLs. The peptide-induced CTLs shown in Fig. 5 were restimulated by the specific peptide and HLA-A24$^+$ irradiated PBMCs and grown to obtain a sufficient number of cells for the assay. Representative results of PBMC culture of lung cancer patient LC2 (MRP3-503 and MRP3-765-induced CTLs) and renal cancer patient RCC1 (MRP-692, MRP-1293, and EBV-induced CTLs) are shown. *A*, cytotoxicity of the cells against HLA-A24$^+$ lung cancer (Sq-1 and 11-18) and HLA-A24$^-$ lung cancer (QG56) cells was measured by a 6-h ^{51}Cr-labeled release assay at different E:T ratios. Values represent the means of triplicate assays. Two-tailed Student's *t* test was used for the statistical analysis between the percentage lysis of Sq-1 or 11-18 cells and that of QG56 cells. *, $P < 0.05$. *B*, effect of peptide loading on the cytotoxicity of the cells against HLA-A24$^+$ EBV-transformed B-cells (SS-EBB). The MRP3 peptides or control HIV peptide (10 μM) were loaded on SS-EBB cells and used as target cells for the cytotoxicity assay. A two-tailed Student's *t* test was used for the statistical analysis between the percentage lysis of peptide-loaded SS-EBB cells and that of unloaded SS-EBB cells. *, $P < 0.05$. Other details of the experiment are same as *A*.

and three (LC2, RCC1, and CC2) cancer patients, respectively. The MRP3-765 induced peptide-specific T cells in the PBMC culture of healthy donor HD1. Reactivities of the peptide-induced T cells to the HLA-A24$^+$ Sq-1 lung cancer cells are shown in Fig. 4*B*. Similar to the peptide-specific T cells, MRP3-peptide-induced T cells also reacted to the Sq-1 cells. In contrast, the EBV- or HIV-peptide-induced T cells did not react to the Sq-1 lung cancer cells (data not shown).

The cytotoxic activities of the MRP3-peptide-induced T cells were further examined. The MRP3-peptide-induced T cells specific to the peptide used for their induction in the PBMC cultures of cancer patients were restimulated by the specific peptide and HLA-A24$^+$-irradiated PBMCs and grown to obtain a sufficient number of cells for the cytotoxicity assay. Representative results against tumor cells are shown in Fig. 5*A*. The MRP3-peptide-induced T cells lysed HLA-A24$^+$ lung cancer cells, Sq-1, and/or 11-18 cells in an E:T ratio-dependent manner, whereas none of the T cells induced by the MRP3 peptides killed the HLA-A-24$^-$ QG56 cells. Control EBV-peptide-induced T cells did not show cytotoxicity against any target used in this experiment. These results suggest that the MRP3-peptide-induced T cells not only specifically react to the original peptide used for their induction, but also recognize HLA-A24$^+$ tumor cells, and result in lysis of the tumor cells in an HLA-A24-restricted manner. The cytotoxicity of the MRP3-peptide-induced CTLs against HLA-A24$^+$ EBV-transformed B-cell line was further examined (Fig. 5*B*). An HLA-A24$^+$ EBV-transformed B-cell line, SS-EBB, was used as target cells. None of the MRP3-peptide-induced CTLs lysed the SS-EBB cells. In contrast, EBV-peptide-induced T cells lysed SS-EBB. The effect of peptide loading to the SS-EBB on the cytotoxicity of the peptide-induced CTLs was analyzed. The MRP3-503-induced CTLs lysed the MRP3-503 peptide-loaded SS-EBB cells only in an E:T ratio-dependent manner, and MRP3-765 or control HIV-peptide-loaded SS-EBB cells were not lysed by the CTLs. In an opposite manner, the MRP3-765-induced CTLs lysed only MRP3-765-loaded SS-EBB cells (Fig. 5*B*). Similar peptide-specific lysis was observed in the other two MRP3-peptide-induced CTLs (data not shown).

Expression of MRP3 mRNA in Various Tumor Cell Lines. The expression of MRP3 in various tumor cell lines and tumor tissues at the mRNA levels was analyzed by Northern blotting (Fig. 6). The relative index of this expression is shown in Table 1. MRP3 was expressed in all of the tested lung cancer cell lines ($n = 10$), ovarian cancer cell lines ($n = 10$), and 8 of the 10 renal cancer cell lines. In contrast, its expression levels were very low in noncancerous cell lines, COS-7, VA13, and 293T cells, or the EBV-transformed B-cell line, SS-EBB. Widespread MRP3 expression in tumor cells of various tissue types and origins was also confirmed in tumor tissue specimens. Namely, MRP3 was expressed in all of the tested specimens of various tissue types and origins from patients with different clinical stages; lung cancers ($n = 6$), renal cancer ($n = 1$), colon cancers ($n = 2$), gastric cancers ($n = 4$), ovarian cancers ($n = 2$), esophageal cancer ($n = 1$), and oral cancer ($n = 1$).

Whether the expression of MRP3 in tumor cells correlates with IFN-γ production by MRP3-derived peptide-specific CTLs, the reactivity of the MRP3-peptide-induced CTLs against MRP3$^+$ and MRP3$^-$ tumor cells was further examined. Representative results of the reactivities of the MRP3-peptide-induced CTLs against HLA-A24$^+$ MRP3$^+$ (Sq-1, TUHR-10TKB), HLA-A24$^+$ MRP3$^-$ (Caki-1),

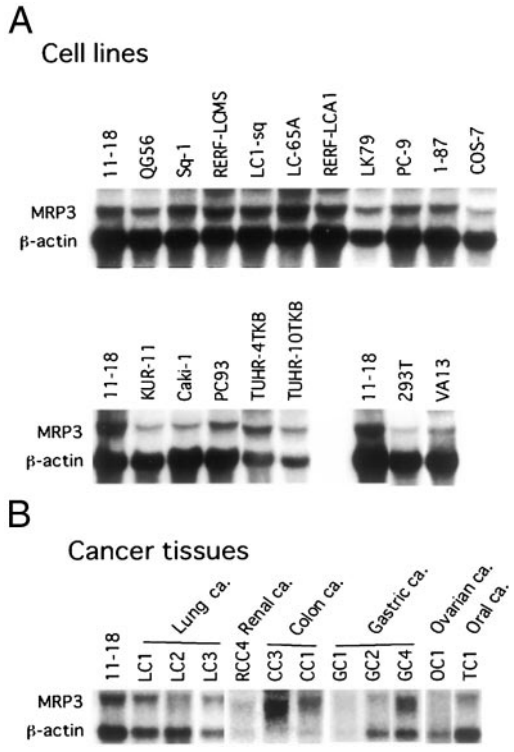

Fig. 6. Northern blot analysis of MRP3 expression in various tumor cell lines (*A*) and tumor tissues (*B*). Total RNA was separated on formaldehyde-agarose gel and transferred to nylon membranes. The membranes were further hybridized with ^{32}P-labeled fragment of *clone 5* and control β-actin cDNA. Representative results are shown in this figure.

HLA-A24$^-$ MRP3$^+$ (QG56), and HLA-A24$^-$ MRP3$^-$ (KUR-11) tumor cells are shown in Fig. 7*A*. The MRP3-peptide induced CTLs reacted to HLA-A24$^+$ MRP3$^+$ (Sq-1, TUHR-10TKB) tumor cells, whereas the CTLs induced by the MRP3-peptides recognized neither HLA-A-24$^-$ nor MRP3$^-$ tumor cells. Peptide loading of the MRP3-peptides, but not of the control HIV-peptide, onto HLA-A24$^+$ MRP3$^-$ Caki-1 cells led the CTLs to produce IFN-γ in a dose-dependent manner (Fig. 7*B*). These results suggest that MRP3 expression in target tumor cells correlates to IFN-γ production by MRP3-derived peptide-specific CTLs.

DISCUSSION

This study demonstrated that MRP3-derived peptides are tumor antigenic epitopes recognized by HLA-A24-restricted and tumor-specific CTLs established from T cells infiltrating into lung adenocarcinoma. Furthermore, we demonstrated that these MRP3-derived peptides could induce tumor-specific CTLs in the PBMC cultures of cancer patients in an HLA-A24-restricted manner.

The MRP family consists of at least seven ABC transporters, several of which have been demonstrated to transport amphipathic anions and to confer *in vitro* resistance to chemotherapeutic agents (15–18). Two prominent members of the ABC superfamily of transmembrane proteins, MDR1 P-glycoprotein (ABCB1) and MRP1 (ABCC1), can mediate the cellular extrusion of xenobiotics and anticancer agents from normal and tumor cells (11, 15–17, 19). MRP1 is a glutathione conjugate pump or multispecific organic anion transporter (20). MRP1 can mediate the transport of negatively charged hydrophilic compounds with large hydrophobic moieties such as glutathione *S*-, glucuronide, and sulfate conjugates of drugs (20–23). MRP1 is not only involved in reducing the passage of drugs across the membrane and some specialized epithelia (24, 25) but is also the major transporter for endogenous leukotriene C4, an important mediator of inflammatory response (26, 27). The roles of other members (MRP2-6, ABCC2-6) of the MRP family in MDR have been reported (28–30). MRP2 has been shown to confer low-level resistance to the anticancer drug cisplatin, etoposide, vincristine, and methotrexate (31–33), MRP3 to etoposide, vincristine, and methotrexate (28, 34, 35), MRP4 to acyclic nt phosphonates, such as 9-(2-phosphonylmethoxyethyl) guanine, and anti-HIV drug 9-(2-phosphonylmethoxyethyl) adenine (36), and MRP5 to thiopurine drugs, 6-mercaptopurine and thioguanine, and 9-(2-phosphonylmethoxyethyl) adenine (37). No resistance against anticancer or antiviral drugs has been reported for MRP6 and MRP7 at the present time.

The expression of several *MRP* genes at mRNA levels can be up-regulated after selection by anticancer drugs (15–17). Up-regulation of MRP3 expression has been observed in several cell lines after selection with doxorubicin, regardless of the apparent lack of correlation of the mRNA levels with resistance to either doxorubicin or cisplatin (29). In the present study, we demonstrated that MRP3 was expressed in most cell lines derived from lung cancers, ovarian cancers, and renal cancers at the mRNA levels. In contrast, the MRP3 message was very low in nontumorous cell lines (COS-7, VA13, and 293T) or EBV-transformed B cells (Table 1). MRP1 and MRP5 are ubiquitously expressed in normal tissues, whereas MRP3 expression in normal tissues is restricted to the liver, duodenum, colon, and

Table 1 *Relative expression levels of MRP-3 mRNA in various cell lines and cancer tissues*[a]

Cell lines	Relative index[b]	Cancer tissues (Patient ID)	Stage	Relative index
Lung cancer		Lung cancer		
11-18	1.0	LC1 (adeno)[c]	IIa	1.0
QG56	0.9	LC2 (adeno)	Ib	0.8
SQ-1	1.3	LC3 (adeno)	Ia	1.3
RERF-LCMS	1.5	LC4 (SCC)	Ib	2.1
LC1-Sq	1.3	LC5 (adeno)	IV	1.2
LC65A	1.7	LC6 (adeno)	IV	1.7
RERF-LCA1	1.2	Renal cell cancer		
LK79	0.9	RCC4	IV	1.4
PC-9	1.0	Colon cancer		
1-87	0.9	CC1 (adeno)	IV	2.5
Renal cancer		CC3 (adeno)	IIIb	2.6
KUR-11	<0.2	Gastric cancer		
Caki-1	<0.2	GC1 (adeno)	IV	1.5
PC93	0.5	GC2 (adeno)	II	0.8
RC30-14	0.3	GC3 (adeno)	II	1.9
PC3	0.5	GC4 (adeno)	IIIa	1.1
VMRC-RCW	0.6	Ovarian cancer		
TUHR-4TKB	1.0	OC1 (serus adeno)	IV	0.7
TUHR-10TKB	0.7	OC2 (serus adeno)	IIIc	2.1
RCC-10RGB	1.1	Esophagial cancer		
LNCap	0.9	EC1 (SCC)	III	0.4
Ovarian cancer		Oral (tongue) cancer		
KOC-3S	0.7	TC1 (SCC)	IV	0.4
KOC-5C	0.6			
KOC-7C	1.0			
TYK-nu	1.4			
RMUG-S	0.8			
RMG-I	0.9			
TOC-2	0.9			
MCAS	0.7			
RTSG	0.8			
RKN	0.6			
EBV-transformed B cells				
SS-EBB	<0.2			
Nontumorous cells				
COS-7	0.3			
VA13	<0.2			
293T	<0.2			

[a] Expression of the MRP3 mRNA in the various cell lines and cancer tissues was analyzed by Northern blotting. Expression levels of MRP3 mRNA in each sample were compensated with β-actin mRNA.

[b] Calculation method of relative index is described in "Materials and Methods."

[c] Histological types of cancer tissues. Adeno, adenocarcinoma; SCC, squamous cell carcinoma.

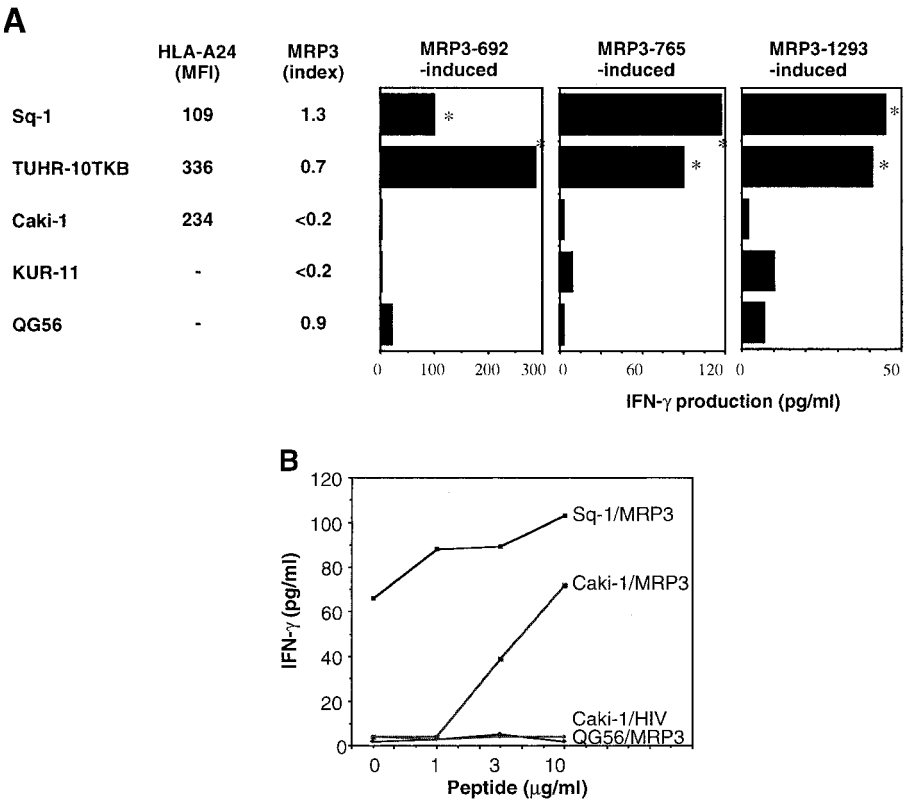

Fig. 7. Reactivity of the MRP3-peptide-induced CTLs against MRP3$^+$ and MRP3$^-$ tumor cells. *A*, representative reactivities of PBMC culture of lung cancer patient LC2 (MRP3-692-, MRP-765-, and MRP-1293-induced CTLs) against HLA-A24$^+$ MRP3$^+$ (Sq-1, TUHR-10TKB), HLA-A24$^+$ MRP3$^-$ (Caki-1), HLA-A24$^-$ MRP3$^+$ (QG56), and HLA-A24$^-$ MRP3$^-$ (KUR-11) tumor cells are shown. Values represent the means of triplicate assays. The background of IFN-γ production by the CTLs alone was subtracted from the values. Two-tailed Student's *t* test was used for the statistical analysis between IFN-γ production by the CTLs in response to indicated cells and that in response to QG56 cells. *, $P < 0.05$. *MFI*, mean fluorescence intensity. *B*, effect of peptide-loading onto the target tumor cells on IFN-γ production by the CTLs. Representative reactivities of MRP3-692-induced CTLs against MRP3-692 or control HIV-peptide loaded tumor cells are shown.

adrenal gland at relatively high levels and to the lung, kidney, bladder, spleen, stomach, pancreas, and tonsil at low levels (29).

Many genes encoding tumor antigens recognized by CTLs have been identified from melanoma cDNA (1, 2). CTL-directed tumor antigens have also been identified from tumors other than melanomas, including HER2/neu (3, 4), prostate-specific antigen (5), SART-1–3 (6, 7, 9), ART4 (10), and cyclophilin B (8), with some of these antigen-derived peptides being currently under Phase I clinical trials for development as cancer vaccines. The results shown in this study suggest that MRP3-derived peptides are possible candidates for cancer vaccines. MRP3 is a unique target molecule for cancer vaccines because the expression of MRP3 is associated with MDR, the most important problem in chemotherapy. As described above, MRP3 was expressed in most cancer cell lines tested, and overexpression of the MRP3 was observed in several tumor cell lines after acquisition of MDR (29). These results suggest that immunotherapy with MRP3-derived peptide vaccine is advantageous for MDR-acquired tumors. Patients with renal cancer may be particularly suitable subjects for the MRP3-peptide vaccine, because renal cancer is generally resistant to chemotherapy and radiation therapy. Our results regarding CTL induction by MRP3-peptides in the PBMC cultures of patients with renal cancer supported this suggestion. Namely, the MRP3-peptides induced tumor-specific CTLs in the PBMC cultures of three of the four patients with renal cancer tested. Furthermore, immunotherapy using MRP3-peptides in combination with chemotherapy might be possible if the immunosuppression induced by the chemotherapeutic agents is not severe in the patient. The effectiveness of the combination of humanized monoclonal antibody-mediated immunotherapy in accompaniment with chemotherapy for treatment of breast cancer and B-cell lymphoma has already been reported (38–40).

Because of the relatively high level expression of MRP3 in normal tissues, particularly the liver, duodenum, colon, and adrenal gland, these organs are possible targets of adverse effects of specific immunotherapy. However, it should be noted that no severe adverse effects in the normal tissues or organs have been reported in the clinical trials of cancer vaccines specific to the MAGE-1, MAGE-3, Melan-A, gp100, tyrosinase, and NY-ESO-1 in melanoma patients, although these molecules are expressed in the normal testis, retina, or melanocytes at both mRNA and protein levels (41–46). Similarly, no severe adverse effects on the function of normal organs have been observed in our clinical trials of peptide cancer vaccines, although some of the target molecules are ubiquitously expressed in normal organs.[4] Processing of the antigenic peptides in proteasomes of normal cells may differ from that of tumor cells in these cases. Alternatively, some molecules in normal cells, including a family of serpins (a group of serine-protease inhibitors), might be involved in normal cell resistance to CTL-mediated lysis (47).

The *HLA-A24* allele is found in 60% of Japanese (with 95% of these cases being genotypically A2402), in 20% of Caucasians, and in 12% of Africans (48). The four MRP3-derived peptides were able to induce HLA-A24-restricted and tumor-specific CTLs in the PBMCs of cancer patients. These MRP3 peptides might be appropriate molecules for use in specific immunotherapy for HLA-A24$^+$ patients with various cancers.

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