TABLE 1. Summary of the Expression of Epithelial Cancer-Related Antigens in Hematologic Malignant Cell Lines

Sample	SART-1	SART-2	SART-3	ART-1	Lck	Lck (Type I/Type II)			
						−/−	+/−	−/+	+/+
ATL	1/1	1/1	1/1	NT	1/1	0/1	0/1	0/1	1/1
T-ALL	1/1	2/2	7/7	2.0	2/2	0/2	0/2	0/2	2/2
B-ALL	2/2	4/4	6/6	NT	1/2	0/2	2/2	0/2	0/2
B-lymphoma	1/1	1/1	1/1	NT	0/1	0/1	1/1	0/1	0/1
Plasmocytoma	5/5	5/5	2/2	2.8	3/5	1/4	2/4	0/4	1/4
AML	2/2	5/5	7/7	1.4	1/5	3/4	0/4	0/4	1/4
CLL*	2/2	NT	2/2	NT	NT	NT	NT	NT	NT
CML	NT	NT	2/2	4.6	NT	NT	NT	NT	NT
Burkitt lymphoma	NT	2/2	1/1	2.8	2/2	0/2	2/2	0/2	0/2
PBMCs	0/2	0/2	2/2	1.0	2/2	0/2	0/2	2/2	0/2
PHA blastoid	2/2	0/2	2/2	NT	2/2	0/2	0/2	2/2	0/2

The expression of 5 epithelial cancer-related antigens was examined. The expression of SART-1, SART-2, and Lck proteins was examined by Western blot analysis. SART-3 expression was examined by flow cytometry. mRNA expression of the *ART-1* gene was examined by Northern blot analysis, and the data are shown as an index. The index was calculated based on a ratio of ART-1/β-actin density, and the index of PBMCs from a healthy donor was defined as 1.0. Two Lck transcripts from different promoters were examined by RT-PCR.

*PBMCs from CLL patients were used as a source of CLL.

NT, not tested.

A24-negative cells or HLA-A24-positive PHA-blastoid T cells in any of the seven cases tested (Fig. 5A). Such CTL activity, however, was not observed when the PBMCs were stimulated with either an EBV-derived or an HIV-derived peptide (data not shown). Because of the limited availability of samples, peptide-stimulated PBMCs from the other six patients were not examined for CTL activity against fresh tumor cells.

TABLE 2. Summary of CTL Induction by Peptides

Peptide	Sequence	Reference	Patient										
			1 CLL	2 CLL	3 MCL	4 NHL	5 B-ALL	6 MM	7 MDS	8 ATL	9 ATL	10 T-ALL	Total
SART-1 690	EYRGFTQDF	7	0	486	0	53	8	141	0	55	155	26	3/10
SART-2 93	DYSARWNEI	9	0	440	9	63	20	28	0	0	354	192	3/10
SART-2 161	AYDFLYNYL	9	0	177	49	13	0	28	0	0	198	227	3/10
SART-2 899	SYTRLFLIL	9	0	15	12	220	0	0	0	12	346	526	3/10
SART-3 109	VYDYNCHVDL	8	49	0	7	0	0	0	11	49	0	0	0/10
SART-3 315	AYIDFEMKI	8	39	137	6	0	11	0	0	22	60	6	1/10
Lck 208	HYTNASDGL	11	0	2	22	30	0	0	0	0	445	153	2/10
Lck 486	TFDYLRSVL	11	0	0	0	0	0	0	0	140	2099	140	3/10
Lck 488	DYLRSVLEDF	11	0	0	0	0	6	182	102	476	304	0	4/10
ART-1 170	EYCLKFTKL	10	501	33	0	0	0	94	0	69	1487	3	2/10
HIV	RYLRDQQLLGI	15	0	71	8	0	0	0	9	0	0	2	0/10
EBV	TYGPVFMCL	15	0	100	0	100	0	418	115	264	264	0	6/10

The PBMCs from patients were tested for their reactivity to a corresponding peptide after in vitro stimulation with each peptide for 12 days. Values represent the IFNγ production by the effector PBMCs in response to C1R-A24 cells prepulsed with the corresponding peptide. Background IFNγ response to HIV peptide was subtracted, and the results that showed the best response among 4 wells are shown. Significant values ($P < 0.05$ by the two-tailed Student t-test) are underlined.

MCL, mantle cell lymphoma; NHL, non-Hodgkin's lymphoma.

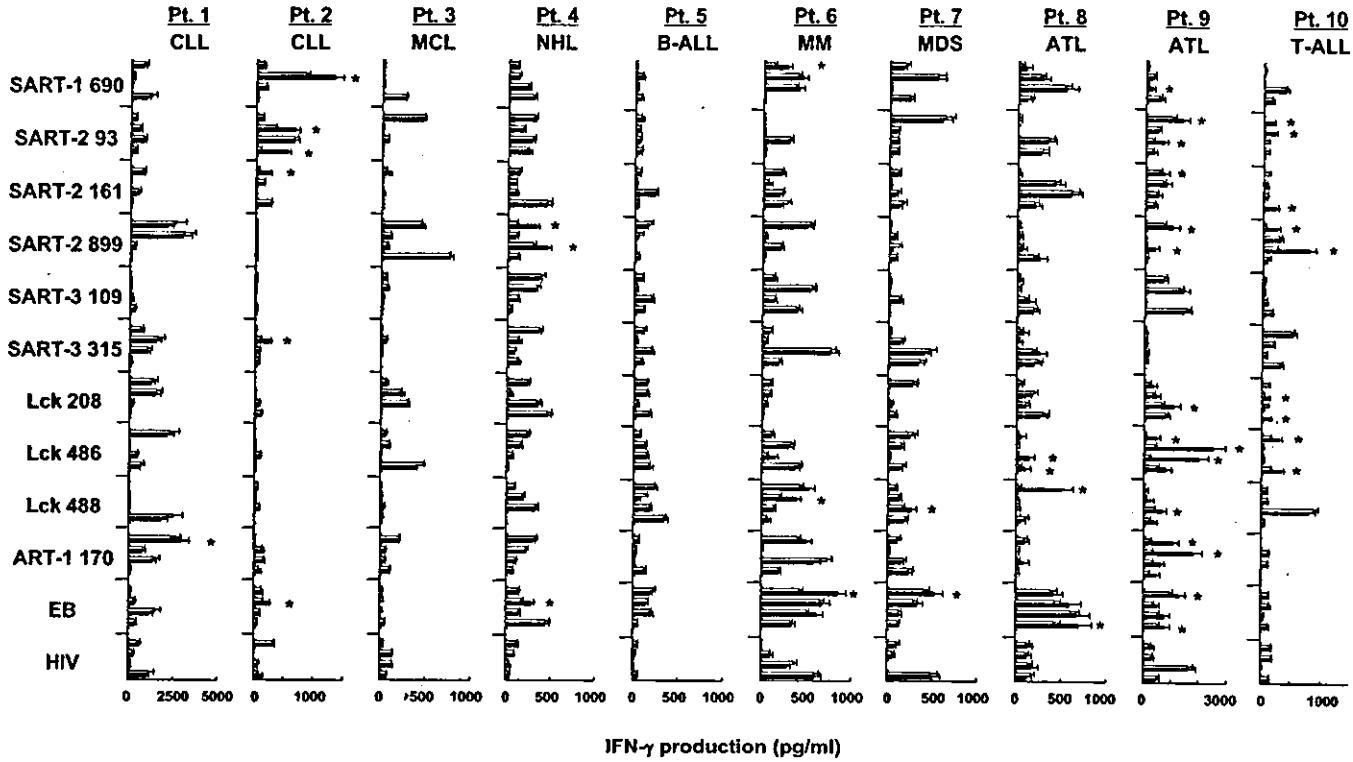

FIGURE 3. Induction of peptide-specific CTLs from the PBMCs of HLA-A24$^+$ patients. PBMCs from 10 patients with hematologic malignancy were stimulated in vitro with the peptides indicated, as described in text. Values represent IFNγ production. The open and closed bars represent IFNγ production in response to HIV peptide-pulsed C1R-A24 cells and corresponding peptide-pulsed C1R-A24 cells, respectively. *$P < 0.05$ by two-tailed Student t test.

A cold inhibition assay was further performed to confirm that cytotoxicity against tumor cells was dependent on peptide-specific CTLs. The cytotoxicity against various target cells, including fresh tumor cells, was significantly blocked by the addition of unlabeled C1R-A24 cells loaded with the corresponding peptide, but not by unlabeled C1R-A24 cells loaded with an HIV peptide, taken as a negative control (see Fig. 5B).

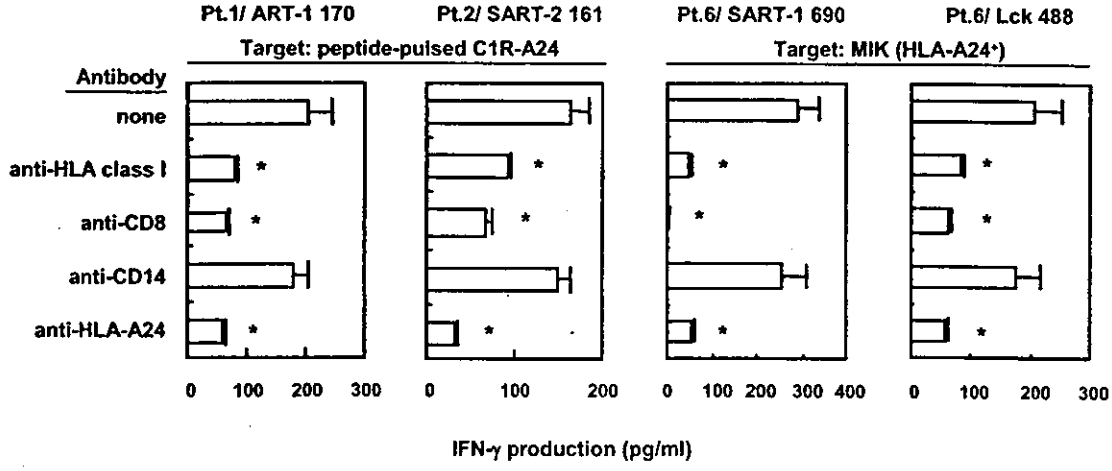

FIGURE 4. HLA class I-restricted response of peptide-stimulated CTLs. CD8$^+$ T cells, which were purified from peptide-stimulated PBMCs, were examined for their IFNγ production in response to peptide-pulsed C1R-A24 cells or tumor cell lines. The assay was carried out at an effector:target ratio of 10:1, and the indicated monoclonal antibody was added at a dose of 20 μg/mL. The results of two CLL patients (patients 1 and 2) and an MM patient (patient 6) are shown. These values represent the means of triplicate assays. The background IFNγ production by the effector cells alone was subtracted from the values. *$P < 0.05$ by two-tailed Student t test.

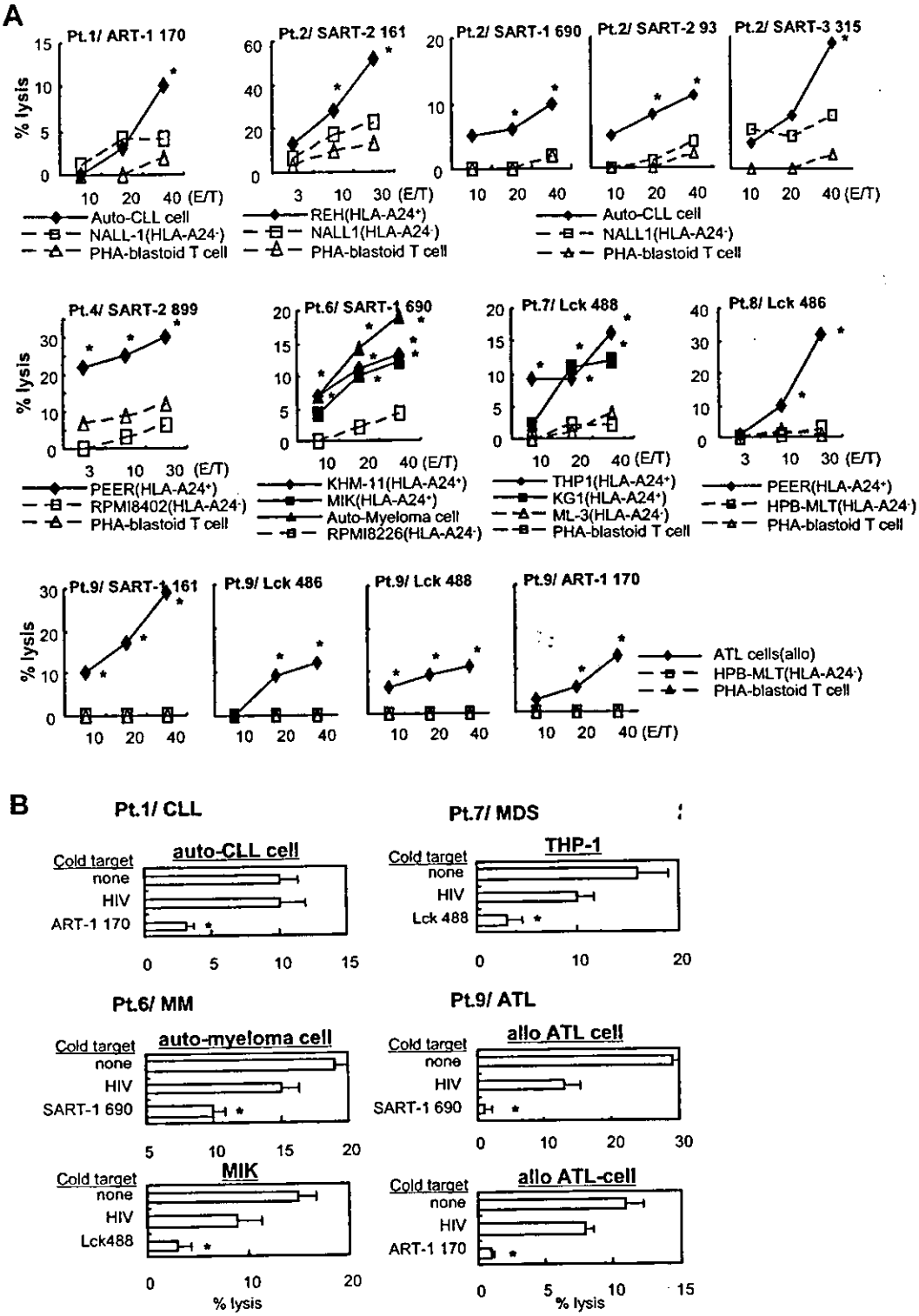

FIGURE 5. Cytotoxic activity of peptide-stimulated PBMCs. (A) PBMCs from seven patients (patients 1, 2, 4, 6, 7, 8, and 9) were stimulated in vitro with the indicated peptides and examined for their cytotoxic activity against hematologic malignant cell lines, fresh malignant cells, and PHA-activated blastoid T cells. $^*P < 0.05$ by two-tailed Student *t* test. (B) Cold inhibition assay was carried out to confirm the specificity of peptide-specific CTLs. Unlabeled C1R-A24 cells that were preloaded with either a corresponding peptide or the control HIV peptide were added to the culture, and the cytotoxicity of peptide-specific CTLs against cell lines or fresh tumor cells was determined. The ratio of cold and hot targets was 10:1. $^*P < 0.05$ by two-tailed Student *t* test.

DISCUSSION

Hematologic malignancies are thought to be ideal candidates for immunotherapy for several reasons: the immunocompetent cells have much easier access to leukemic cells in the circulation than to epithelial cancer cells at tumor sites, the hematologic malignant cells are usually monoclonally originated, and HLA molecules are expressed on most of them, whereas epithelial cancer cells are polyclonally originated and HLA molecules are frequently deleted.[16] Donor lymphocyte infusion following allogeneic stem cell transplantation and the use of nonmyeloablative conditioning regimens have been associated with a graft-versus-leukemia or graft-versus-myeloma effect, demonstrating that these malignant cells can be a target of T-cell-mediated immune responses.[17] In addition, Ig-derived peptides can be targets of T-cell response against B-cell malignant cells.[3,5] Mutational antigens, such as bcr/abl and PML/RARα, are considered targets for T-cell immunotherapy against CML and acute promyelocytic leukemia, respectively.[2,18] Finally, several other leukemia-associated antigens have been reported: proteinase 3 in CML, ALK in lymphoma, and Wilms' tumor-suppressor (WT1) in leukemia.[18,19] Nevertheless, the clinical responses are unsatisfactory at present, and the development of new treatment modalities is required.

Recent advances in tumor immunology have identified many melanoma-related or epithelial cancer-related antigens and their epitopes, each having the potential to generate tumor-reactive CTLs.[20,21] Some of them, including MAGE, NY-ESO-1, and MUC-1, have been reported to be expressed in hematologic malignancies.[22,23] To extend the potential for peptide-based immunotherapy against hematologic malignancies, we here investigated whether the epithelial cancer-related antigens identified by our group, all of which are nonmutated proliferation-related proteins,[7–11] could be appropriate target molecules for immunotherapy of patients with hematologic malignancies. The SART-1 and SART-2 antigens were expressed in all samples except for normal PBMCs. The SART-3 was expressed in the majority of hematologic malignant cell lines. Fresh CLL cells that were gated on $CD19^+$ cells in the PBMCs of a CLL patient were positive for the antigen. The Lck protein was also expressed in the majority of cell lines tested, with the exception of AML. Most of the hematologic malignant cell lines were suggested to use the type I promoter of the *lck* gene, which is preferentially used in epithelial and metastatic cancer cells.[11] In addition, MM cells, which were gated on $CD38^+$ cells in the PBMCs of a patient, expressed the Lck protein. Taken together, these results clearly show that these epithelial cancer-derived antigens are highly expressed in hematologic malignant cells. Further studies of the expression on fresh tumor samples are needed to confirm this observation.

We then determined whether these antigen-derived peptides, which are being tested in clinical trials as potential vaccines for epithelial cancer patients,[12,13] could generate peptide-specific and hematologic malignant cell-reactive CTLs. All peptides except for the SART-3 109 peptide induced peptide-specific CTLs from PBMCs of at least 1 of 10 $HLA\text{-}A24^+$ patients with various types of hematologic malignancies, resulting in a positive percentage of 20% to 40%. More importantly, peptide-stimulated PBMCs from the majority of patients exerted a cytotoxic activity against $HLA\text{-}A24^+$ leukemia or lymphoma cell lines. Moreover, peptide-stimulated PBMCs from four patients with CLL, MM, or ATL showed a cytotoxicity against HLA-matched fresh tumor cells. These results indicate that these peptide candidates can generate CTLs cytotoxic to hematologic malignant cells. These lines of evidence demonstrate that these five epithelial cancer-related antigens can be employed as target molecules against hematologic malignancies.

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REFFERENCES

1. Powles RL, Russell JA, Selby PJ, et al. Maintenance of remission in acute myelogenous leukaemia by a mixture of B.C.G. and irradiated leukaemia cells. *Lancet*. 1977;2:1107–1110.
2. Pinilla-Ibarz J, Cathcart K, Korontsvit T, et al. Vaccination of patients with chronic myelogenous leukemia with bcr-abl oncogene breakpoint fusion peptides generates specific immune responses. *Blood*. 2000;95:1781–1787.
3. Hsu FJ, Caspar CB, Czerwinski D, et al. Tumor-specific idiotype vaccines in the treatment of patients with B-cell lymphoma—long-term results of a clinical trial. *Blood*. 1997;89:3129–3135.
4. Reichardt VL, Okada CY, Liso A, et al. Idiotype vaccination using dendritic cells after autologous peripheral blood stem cell transplantation for multiple myeloma—a feasibility study. *Blood*. 1999;93:2411–2419.
5. Bendandi M, Gocke CD, Kobrin CB, et al. Complete molecular remissions induced by patient-specific vaccination plus granulocyte-monocyte colony-stimulating factor against lymphoma. *Nat Med*. 1999;5:1171–1177.
6. Trojan A, Schultze JL, Witzens M, et al. Immunoglobulin framework-derived peptides function as cytotoxic T-cell epitopes commonly expressed in B-cell malignancies. *Nat Med*. 2000;6:667–672.
7. Kikuchi M, Nakao M, Inoue Y, et al. Identification of a SART-1-derived peptide capable of inducing HLA-A24-restricted and tumor-specific cytotoxic T lymphocytes. *Int J Cancer*. 1999;81:459–466.
8. Yang D, Nakao M, Shichijo S, et al. Identification of a gene coding for a protein possessing shared tumor epitopes capable of inducing HLA-A24-restricted cytotoxic T lymphocytes in cancer patients. *Cancer Res*. 1999;59:4056–4063.
9. Nakao M, Shichijo S, Imaizumi T, et al. Identification of a gene coding for a new squamous cell carcinoma antigen recognized by the CTL. *J Immunol*. 2000;164:2565–2574.
10. Nishizaka S, Gomi S, Harada K, et al. A new tumor-rejection antigen recognized by cytotoxic T lymphocytes infiltrating into a lung adenocarcinoma. *Cancer Res*. 2000;60:4830–4837.
11. Harashima N, Tanaka K, Sasatomi T, et al. Recognition of the Lck tyrosine kinase as a tumor antigen by cytotoxic T lymphocytes of cancer patients with distant metastases. *Eur J Immunol*. 2001;31:323–332.
12. Mine T, Gouhara R, Hida N, et al. Immunological evaluation of CTL

precursor-oriented vaccines for advanced lung cancer patients. *Cancer Sci.* 2003;94:548–556.
13. Noguchi M, Kobayashi K, Suetsugu N, et al. Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination. *Prostate.* 2003;57:80–92.
14. Koga M, Komatsu N, Kawamoto N, et al. Analysis of cellular localization of SART3 tumor antigen by a newly established monoclonal antibody: heterotopic expression of SART3 on the surface of B-lineage leukemic cells. *Oncol Rep.* 2004;11:785–789.
15. Maeda Y, Hida N, Niiya F, et al. Detection of peptide-specific CTL-precursors in peripheral blood lymphocytes of cancer patients. *Br J Cancer.* 2002;87:796–804.
16. Janeway CA, Travers P, Walport M, et al. Using the immune response to attack tumors. In: Immunobiology: The Immune System in Health and Disease. London: Elsevier Science; 1999:550–560.
17. Khouri IF, Keating M, Korbling M, et al. Transplant-lite: induction of graft-versus-malignancy using fludarabine-based nonablative chemotherapy and allogeneic blood progenitor-cell transplantation as treatment for lymphoid malignancies. *J Clin Oncol.* 1998;16:2817–2824.
18. Appelbaum FR. Haematopoietic cell transplantation as immunotherapy. *Nature.* 2001;411:385–389.
19. Passoni L, Scardino A, Bertazzoli C, et al. ALK as a novel lymphoma-associated tumor antigen: identification of 2 HLA-A2.1-restricted $CD8^+$ T-cell epitopes. *Blood.* 2002;99:2100–2106.
20. Rosenberg SA, Yang JC, Schwartzentruber DJ, et al. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med.* 1998;4:321–327.
21. Wang RF, Rosenberg SA. Human tumor antigens for cancer vaccine development. *Immunol Rev.* 1999;170:85–100.
22. van Baren N, Brasseur F, Godelaine D, et al. Genes encoding tumor-specific antigens are expressed in human myeloma cells. *Blood.* 1999;94:1156–1164.
23. Brossart P, Schneider A, Dill P, et al. The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. *Cancer Res.* 2001;61:6846–6850.

Identification of Polycomb Group Protein Enhancer of Zeste Homolog 2 (EZH2)-Derived Peptides Immunogenic in HLA-A24$^+$ Prostate Cancer Patients

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BACKGROUND. Antigens overexpressed in metastatic prostate cancer are appropriate targets in anti-cancer immunotherapy, and one candidate is the polycomb group protein enhancer of zeste homolog 2 (EZH2).

METHODS. Eleven EZH2-derived peptides were prepared based on the HLA-A24 binding motif. These peptide candidates were screened first by their ability to be recognized by immunoglobulin G (IgG), and then by their ability to induce peptide-specific cytotoxic T lymphocytes (CTLs).

RESULTS. IgGs reactive to three EZH2 peptides (EZH2-243 to -252, EZH2-291 to -299, and EZH2-735 to -742) were detected in the plasma of almost half of prostate cancer patients. Among them, the EZH2-291 to -299 and EZH2-735 to -742 peptides effectively induced HLA-A24-restricted and prostate cancer-reactive CTLs from prostate cancer patients. The cytotoxicity was mainly dependent on EZH2 peptide-specific and CD8$^+$ T cells.

CONCLUSIONS. These EZH2-291 to -299 and EZH2-735 to -742 peptides could be promising candidates for peptide-based immunotherapy for HLA-A24$^+$ prostate cancer patients with metastases. *Prostate 60: 273–281, 2004.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; EZH2; cytotoxic T lymphocyte; peptide; antibody; HLA-A24**

INTRODUCTION

Prostate cancer is one of the most common cancers among elder men [1]. Prostate cancer frequently metastasizes to bone, and androgen withdrawal therapy has been applied for such patients. However, there is no efficient therapy against hormone-refractory and metastatic prostate cancer. Therefore, there is an urgent need for the development of new therapeutic modalities, and specific immunotherapy is one candidate. Indeed, prostate tissue-specific antigens expressed in the normal prostate can be target molecules for specific immunotherapy [2]. Specific immunotherapy targeting prostate tissue-specific antigens has been carried out, but the clinical responses remain unsatisfactory [3–7].

Enhancer of zeste homolog 2 (EZH2) is a polycomb group protein homologue to the *Drosophila* enhancer of zeste, and is involved in gene silencing [8]. Dysregulation of this gene-silencing machinery can lead to cancer [9–11]. In addition, it has been reported that EZH2 is overexpressed in metastatic prostate

Abbreviations: CTLs, cytotoxic T lymphocytes; ELISA, enzyme-linked immunosorbent assay; EZH2, enhancer of zeste homolog 2; Flu, influenza; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; HIV, human immunodeficiency virus; Ig, immunoglobulin; IL, interleukin; mAb, monoclonal antibody; OD, optical density; PBMCs, peripheral blood mononuclear cells.

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cancer and functions as a transcriptional repressor, and that inhibition of EZH2 blocks prostate cell growth [12]. These lines of evidence suggest that EZH2 could be a promising target molecule in specific immunotherapy of prostate cancer patients, and particularly in those with metastases.

In our clinical trials against several types of cancer, several class I-binding tumor antigen-derived peptides that had originally been identified by their ability to induce tumor-reactive cytotoxic T lymphocytes (CTLs) were also recognized by immunoglobulin g (IgG) [13,14]. Additional clinical trials revealed that the induction of IgG reactive to administered peptides was positively correlated with the overall survival of cancer patients [15–17]. These lines of evidence suggest that peptides that can be recognized by both the humoral and cellular immune systems might be more useful for immunotherapy than peptides that can be recognized by only one of the two systems. In addition, the assay for peptide-specific IgG is much simpler and easier than the in vitro sensitization experiment to induce peptide-specific CTLs [18]. For these reasons, we first screened EZH2-derived peptide candidates by their ability to be recognized by the humoral immune system, and then determined their potential to induce peptide-specific and prostate cancer-reactive CTLs. As a consequence, we identified two EZH2-derived peptides that are effectively recognized by both the humoral and cellular immune systems in HLA-A24$^+$ prostate cancer patients.

MATERIALS AND METHODS

Patients

All prostate cancer patients in this study provided their informed consent before enrollment. None of these participants was infected with human immunodeficiency virus (HIV). Twenty milliliters of peripheral blood was obtained, and the peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll–Conray density gradient centrifugation. The expression of HLA-A24 molecules on the PBMCs of cancer patients and healthy donors was determined by flow cytometry.

Cell Lines

C1R-A24 is an HLA-A*2402-expressing subline of C1R lymphoma. The LNCaP is an HLA-A24-negative prostate cancer cell line, and LNCaP-A24 is a subline which was stably transfected with the *HLA-A*2402* gene (Yao et al., submitted for publication). All cell lines were maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% FCS.

TABLE I. List of Enhancer of Zeste Homolog 2 (EZH2)-Derived Peptides

Position	Amino acid sequence	Score
291–299	KYDCFLHPF	200.0
735–743	KYVGIEREM	115.5
657–665	VYDKYMCSF	100.0
446–454	TYYDNFCAI	72.0
170–178	IFVELVNAL	51.8
90–98	DFPTQVIPL	30.0
660–669	KYMCSFLFNL	600.0
243–252	KYKELTEQQL	576.0
725–734	DYRSQADAL	200.0
657–666	VYDKYMCSFL	200.0
119–128	NFMVEDETVL	30.0

Scores represent the estimated half-time dissociation of the EZH2 peptide binding to HLA-A24 molecules.

Peptides

Eleven EZH2-derived peptides (listed in Table I) were prepared based on the HLA-A24 binding motif [19]. All peptides were of >90% purity and were purchased from Biologica Co. (Nagoya, Japan). Influenza (Flu) virus-derived (RFYIQMCYEL), EBV-derived (TYGPVFMCL), and HIV-derived peptides (RYLRQQLLGI) with the HLA-A24 binding motif were used as controls. All peptides were dissolved with DMSO at a dose of 10 mg/ml.

Detection of Peptide-Specific IgG

Peptide-specific IgG levels in the plasma was measured by enzyme-linked immunosorbent assay (ELISA) using a previously reported method [20]. In brief, peptide (20 μg/well)-immobilized plates were blocked with Block Ace (Yukijirushi, Tokyo, Japan) and washed with 0.05% Tween 20–PBS, after which 100 μl/well of plasma sample diluted with 0.05% Tween 20–Block Ace was added to the plates. After a 2-hr incubation at 37°C, the plates were washed and further incubated for 2 hr with a 1:1,000-diluted rabbit anti-human IgG (γ-chain-specific) (Dako, Glostrup, Denmark). The plates were washed, and then 100 μl of 1:100-diluted goat anti-rabbit IgG-conjugated horseradish peroxidase (EnVision; Dako) was added to each well, and the plates were incubated at room temperature for 40 min. After the plates were washed once, 100 μl/well of tetramethyl benzidine substrate solution (KPL, Guildford, UK) was added, and the reaction was stopped by the addition of 1 M phosphoric acid. The values are shown as optical density (OD) U/ml. To confirm the specificity of IgG to the indicated EZH2 peptide, sample plasma was cultured with plates coated with either the corresponding EZH2 peptide or an irrelevant EZH2 peptide. Thereafter, the levels of

EZH2 peptide-specific IgG in the resulting supernatant were determined by ELISA.

Assay for Peptide-Specific CTLs in PBMCs

The assay for the detection of peptide-specific CTLs in PBMCs was performed according to a previously reported method [21]. In brief, PBMCs (1×10^5 cells/well) were incubated with 10 μg/ml of each peptide in a U-bottom-type 96-well microculture plate (Nunc, Roskilde, Denmark) at a volume of 200 μl of culture medium. The culture medium consisted of 45% RPMI-1640, 45% AIM-V medium (Gibco BRL), 10% FCS, 100 U/ml of interleukin (IL)-2, and 0.1 mM MEM nonessential amino acid solution (Gibco BRL). Half of the culture medium was removed and replaced with new medium containing a corresponding peptide (20 μg/ml) every 3 days. On the 15th day of culture, the cultured cells were separated into four wells, and two of which were used for EZH2 peptide-pulsed C1R-A24 cells, and the other two of which were used for the HIV peptide-pulsed C1R-A24 cells. After an 18-hr incubation period, the supernatants were collected and the level of IFN-γ was determined by ELISA.

RT-PCR

Total RNA was isolated from cancer cell lines using RNAzol™ B (Tel-Test, Inc., Friendswood, TX). The cDNA was prepared using the SuperScript™ Preamplification System for first strand cDNA synthesis (Invitrogen), and it was amplified using the primers 5′-AATGTGGAATGGAGTGGTGC-3′(sense) and 5′-ACGAACTGTCACAAGGCTGC-3′(anti-sense) for EZH2, and 5′-ACAACAGCCTCAAGATCATCAG-3′(sense) 5′-GGTCCACCACTGACACGTTG-3′(anti-sense) for glyceraldehydes 3-phosphate dehydrogenase (GAPDH). Because the PCR primer pair for EZH2 spans three distinct introns, the predicted PCR products from cDNA and genomic DNA are 316 and 2,368 bp, respectively. PCR was performed using Taq DNA polymerase in a DNA thermal cycler (iCycler, Bio-Rad Laboratories, Hercules, CA) for 28 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min.

Cytotoxicity Assay

After in vitro stimulation with the EZH2 peptides, the peptide-stimulated PBMCs were additionally cultured with 100 U/ml IL-2 for approximately 10 days in 96 round-well plates in order to obtain a sufficient number of cells to carry out a cytotoxicity assay. Then, these cells were tested for cytotoxicity against both LNCaP and LNCaP-A24 by a 6-hr ^{51}Cr-release assay. Two thousand ^{51}Cr-labeled cells per well were cultured with effector cells in 96 round-well plates at the indicated effector/target ratios. In some experiments, either anti-HLA class I (W6/32: mouse IgG2a), anti-HLA-DR (L243: mouse IgG2a), anti-CD4 (NU-TH/I: mouse IgG1), anti-CD8 (NU-TS/C: mouse IgG2a), or anti-CD14 (H14: mouse IgG2a) mAb was added to the wells at a dose of 20 μg/ml at the start of the assay.

Cold Inhibition Assay

The specificity of EZH2 peptide-stimulated CTLs was confirmed by a cold inhibition assay. In brief, ^{51}Cr-labeled target cells (2×10^3 cells/well) were cultured with the CTLs (4×10^4 cells/well) in 96 round-well plates with 2×10^4 cold target cells. C1R-A24 cells that were pre-pulsed with either the HIV peptide or a corresponding EZH2 peptide were used as cold targets.

Statistics

The statistical significance of the data was determined using a two-tailed Student's *t*-test. A *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

IgG Reactive to EZH2-Derived Peptides

We prepared 11 kinds of EZH2-derived peptides based on the binding affinity to the HLA-A*2402 molecules (Table I). In the following experiments, only the starting amino acid of the EZH2 peptide is shown. In addition, as described in the "Introduction," we first screened EZH2-derived peptide candidates that can be recognized by the humoral immune system in cancer patients. We investigated whether IgG reactive to each of the 11 EZH2-derived peptides could be detected in the plasma of 10 prostate cancer patients (Table II). These patients were not limited to the HLA-A24$^+$ subjects, because peptide-specific IgG was not restricted to MHC class I molecules, as reported previously [22]. The mean + 2SD value (OD: 0.04), which was determined as the level of IgG reactive to the HIV peptide in plasma from HIV-negative healthy donors, was used as a cut-off value at a 1:100 diluted plasma in the ELISA. As a result, IgGs reactive to the EZH2-291, EZH2-735, and EZH2-243 peptides were detected in the plasma of 4, 5, and 6 of 10 patients, respectively. These three EZH2 peptides were recognized by IgG more efficiently than the other eight EZH2 peptides. Eight patients other than Patients 4 and 6 were diagnosed to suffer from bone metastases (data not shown). The representative results of Patients 2, 7, and 8 are shown in Figure 1A. The level of IgG reactive to each of these EZH2 peptides was decreased by culturing in corresponding peptide-coated plates, but not in irrelevant peptide-coated plates (Fig. 1B). These results indicate that the ELISA system detected peptide-specific IgG in an antigen-specific manner. Taken together, these results indicated that these three EZH2 peptides

TABLE II. Humoral Response to the EZH2 Peptides

	Prostate cancer patient										
Peptide	1	2	3	4	5	6	7	8	9	10	Total
EZH2-291	0.040	0.041	0.074	—	—	—	—	0.067	—	—	4/10
EZH2-735	—	0.050	—	0.120	—	0.045	0.060	0.144	—	—	5/10
EZH2-657	—	—	—	0.106	—	—	—	0.076	—	—	2/10
EZH2-446	—	—	—	—	—	—	—	—	—	—	0/10
EZH2-170	—	—	—	—	—	—	—	0.043	—	—	1/10
EZH2-90	—	—	—	—	—	—	—	—	—	—	0/10
EZH2-660	—	0.105	—	—	—	—	—	—	—	0.058	2/10
EZH2-243	0.055	0.080	—	—	—	—	0.041	0.150	0.089	0.067	6/10
EZH2-725	—	0.049	—	—	—	—	—	0.056	—	—	2/10
EZH2-657	—	—	—	—	—	—	—	—	—	0.0625	1/10
EZH2-119	—	—	—	—	—	—	—	0.047	—	—	1/10

Immunoglobulin G (IgG) reactive to a corresponding peptide was judged to be positive when a difference of OD at a 1:100-diluted plasma was more than 0.04, which was the mean + 2SD value as described in the "Materials and Methods."

(EZH2-291, EZH2-735, and EZH2-243) can be effectively recognized by IgG in the plasma of prostate cancer patients.

Induction of EZH2 Peptide-Specific CTLs From Cancer Patients

We next determined whether these three EZH2 peptides had the potential to generate peptide-specific CTLs from the PBMCs of HLA-A24$^+$ cancer patients. The EZH2-170 peptide was included in this experiment as an EZH2 peptide that was less frequently recognized by the humoral immune system. The PBMCs from 10 HLA-A24$^+$ prostate cancer patients were incubated in vitro with each of the EZH2 peptides, followed by examination of their IFN-γ production in response to the corresponding peptide-pulsed C1R-A24 cells (Table III). The assay was carried out in four wells, and the values that showed more than 100 pg/ml IFN-γ production and $P < 0.05$ are underlined in this table. The result was that the EZH2-291 and EZH2-735 peptides induced peptide-specific CTLs in 6 of 10 and 4 of 9 cancer patients, respectively. The EZH2-243 peptide induced peptide-specific CTLs in 2 of 10 cancer patients, and the EZH2-170 peptide induced peptide-specific CTLs in 1 of 10 cancer patients. Patients 11, 14, and 20 were diagnosed to suffer from bone metastases (data not shown). These results indicate that both the EZH2-291 and EZH2-735 peptides have the potential to effectively induce peptide-specific CTLs from HLA-A24$^+$ prostate cancer patients, even with bone metastases.

Induction of Prostate Cancer-Reactive CTLs From HLA-A24$^+$ Cancer Patients

We further determined whether these EZH2 peptide-stimulated PBMCs showed any cytotoxicity against EZH2-expressing tumor cells. Before assay of cytotoxicity, the expression of *EZH2* gene in tumor cell lines was examined using the RT-PCR method. As shown in Figure 2, the LNCaP cells were clearly positive for the EZH2 mRNA expression. However, expression of EZH2 mRNA in PC-93 cells, another prostate cancer cell line, was very faint, and was almost the same as that in normal PBMCs included as a negative control. Because the PCR primer pair for EZH2 spans three distinct introns, the possibility that the faint band was due to contamination of genomic DNA could be excluded. To investigate HLA-A24-restricted cytotoxicity, the level of cytotoxicity against the LNCaP was compared with the level of cytotoxicity against its HLA-A24-expressing transfectant, LNCaP-A24. The PBMCs from three HLA-A24$^+$ prostate cancer patients were then stimulated in vitro with the indicated EZH2 peptide, and examined for their cytotoxicity against HLA-A24$^-$ LNCaP cells, HLA-A24$^+$ LNCaP-A24, and HLA-A24$^+$ PHA-stimulated T cell blasts. The results are shown in Figure 3. The EZH2 peptide-stimulated PBMCs exhibited higher levels of cytotoxicity against the HLA-A24$^+$ LNCaP-A24 cells than against the HLA-A2$^-$ LNCaP and HLA-A2$^+$ PHA-stimulated T cell blasts. These results indicated that the EZH2-291 and EZH2-735 peptides have the potential to effectively induce HLA-A24-restricted and prostate cancer-reactive CTLs from HLA-A24$^+$ prostate cancer patients.

Peptide-Specific and CD8$^+$ T Cell-Dependent Cytotoxicity of EZH2 Peptide-Stimulated PBMCs

We further tried to confirm which effector cells were responsible for the cytotoxicity. The cytotoxicity of the PBMCs from three prostate cancer patients, which

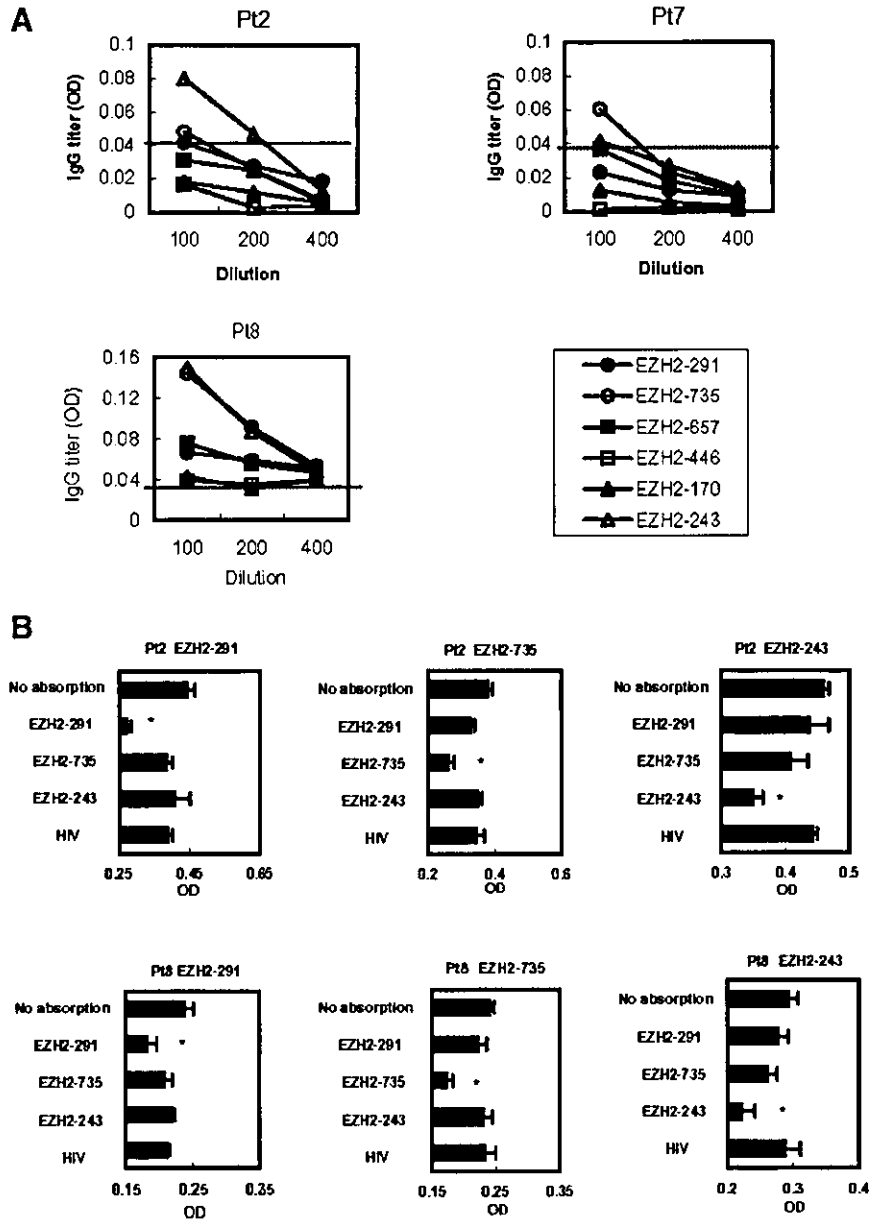

Fig. 1. Immunoglobulin G (IgG) reactive to enhancer of zeste homolog 2 (EZH2) peptides. **A**: The levels of IgG reactive to EZH2 peptides in the plasma from Patients 2, 7, and 8 were examined by enzyme-linked immunosorbent assay (ELISA). The values represent optical density (OD). The cut-off level (OD: 0.04) was determined based on the levels of anti-human immunodeficiency virus (HIV) peptide IgG in HIV-negative healthy donors, as described in the "Materials and Methods." **B**: The plasma samples from Patients 2 and 8 were cultured in plates that were pre-coated with the indicated peptides, and the levels of IgG reactive to the indicated EZH2 peptide in the resultant supernatants were determined by ELISA. *Statistically significant at $P < 0.05$.

were stimulated in vitro with each of the EZH2-291 and EZH2-735 peptides, against the LNCaP-A24 cells were significantly inhibited by the addition of anti-class I or anti-CD8 mAb, but not by the addition of anti-class II, anti-CD4, or anti-CD14 mAb (Fig. 4A). In addition, the cytotoxicity of these EZH2 peptide-stimulated PBMCs against the LNCaP-A24 cells was significantly suppressed by the addition of the relevant peptide-pulsed unlabeled C1R-A24 cells, but not by the addition of the HIV peptide-pulsed unlabeled C1R-A24 cells. Collectively, these results indicate that cytotoxicity of the EZH2 peptide-stimulated PBMCs was mainly ascribed to peptide-specific and $CD8^+$ T cells.

DISCUSSION

Prostate cancer appears to be a good target for the development of specific immunotherapies [2]. In recent

TABLE III. Reactivity of EZH2 Peptide-Stimulated Peripheral Blood Mononuclear Cells (PBMCs) From HLA A24$^+$ Prostate Cancer Patients

	Peptides					
Patient	EZH2-291	EZH2-735	EZH2-243	EZH2-170	Influenza (Flu)	EBV
	IFN-γ production (pg/ml)					
11	111	239	124	10	1,638	597
12	67	54	25	15	186	36
13	1,597	236	0	181	75	42
14	276	26	13	35	58	971
15	319	168	27	10	25	197
16	463	460	27	30	441	263
17	85	22	44	87	35	111
18	132	0	13	0	30	25
19	0	10	71	16	24	14
20	12	N.D.	171	82	26	10
Total	6/10	4/9	2/10	1/10	3/10	5/10

The PBMCs of HLA-A24$^+$ prostate cancer patients were stimulated in vitro with the indicated EZH2 peptide, as described in "Materials and Methods." On day 15, the cultured PBMCs were tested for their reactivity to CIR-A24 cells, which were pre-pulsed with a corresponding peptide or the HIV peptide. The values represent the mean of 2-wells, and the background IFN-γ production in response to the HIV peptide was subtracted. Significant values ($P < 0.05$ by two-tailed Student's t-test and <100 pg/ml of IFN-γ production) are underlined. N.D., not done.

years, our group has identified several epitope peptides derived from prostate-related antigens that are able to generate prostate cancer-reactive CTLs from prostate cancer patients [23–26]. However, one major obstacle encountered when treating prostate cancer patients is the treatment of bone metastases, as prostate cancer frequently metastasizes to the bone tissue [1]. Therefore, we undertook the present study to identify epitope peptides which could potentially be suitable for specific immunotherapeutic treatment of HLA-A24$^+$ prostate cancer patients with metastases.

EZH2 is a polycomb group protein that is overexpressed in metastatic prostate cancer and functions as a transcriptional repressor [12]. Therefore, EZH2 could be a good target for the development of specific immunotherapies against metastatic prostate cancer. In this study, we revealed that both the EZH2-291 and EZH2-735 peptides have the potential to induce prostate cancer-reactive CTLs in HLA-A24$^+$ prostate cancer patients. More importantly, PBMCs that were stimulated with these two EZH2 peptides showed cytotoxicity against prostate cancer cells in an HLA-A24-restricted manner. The cytotoxicity was revealed to depend on peptide-specific and CD8$^+$ T cells. These results indicate that these two EZH2 peptides are potentially useful in specific immunotherapy for HLA-A24$^+$ prostate cancer patients.

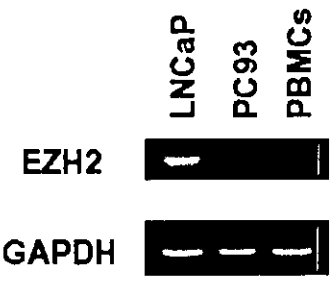

Fig. 2. The expression of the *EZH2* gene in tumor cells. The EZH2 mRNA expression in the LNCaP cells, PC93 cells, and peripheral blood mononuclear cells (PBMCs) was examined by the RT-PCR method.

We first investigated whether or not IgG against 11 EZH2 peptide candidates would be detectable in the plasma of prostate cancer patients, because antibodies against class I-binding tumor peptides had already been observed in certain cancer patients and healthy donors [18,20]. We previously reported that IgG reactive to peptides derived from prostate-related antigens was frequently detectable in healthy donors and prostate cancer patients [23–25]. In this study, IgG reactive to either the EZH2-291, the EZH2-735, or the EZH2-243 peptide was detected in almost half of prostate cancer patients. The potential of the EZH2-243 peptide to induce peptide-specific CTLs from HLA-A24$^+$ prostate cancer patients was lower than those of the former two EZH2 peptides. This means that both the EZH2-291 and EZH2-735 peptides were recognized efficiently by both the cellular and the humoral immune system. Interestingly, our clinical trials revealed that

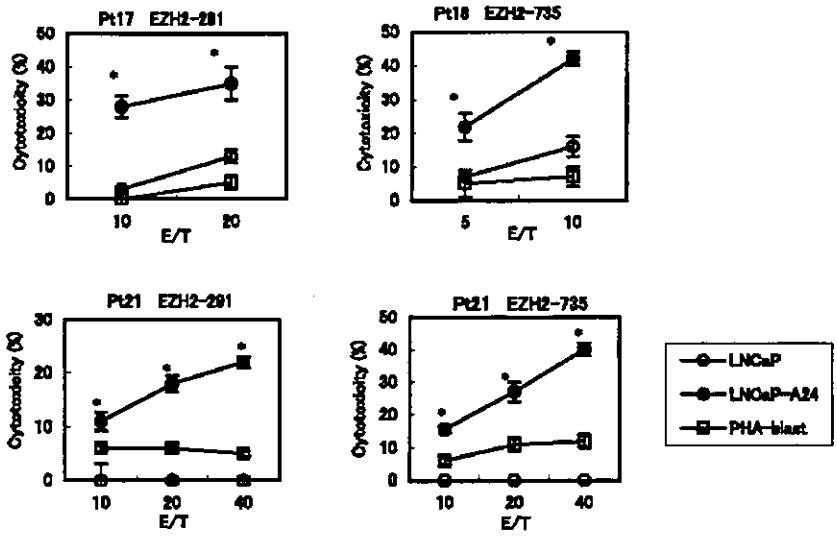

Fig. 3. Cytotoxicity of EZH2 peptide-stimulated PBMCs from cancer patients. EZH2 peptide-stimulated PBMCs from three HLA-A24$^+$ prostate cancer patients were tested for their cytotoxicity toward three different targets by a 6-hr ^{51}Cr-release assay. *Statistically significant at $P < 0.05$.

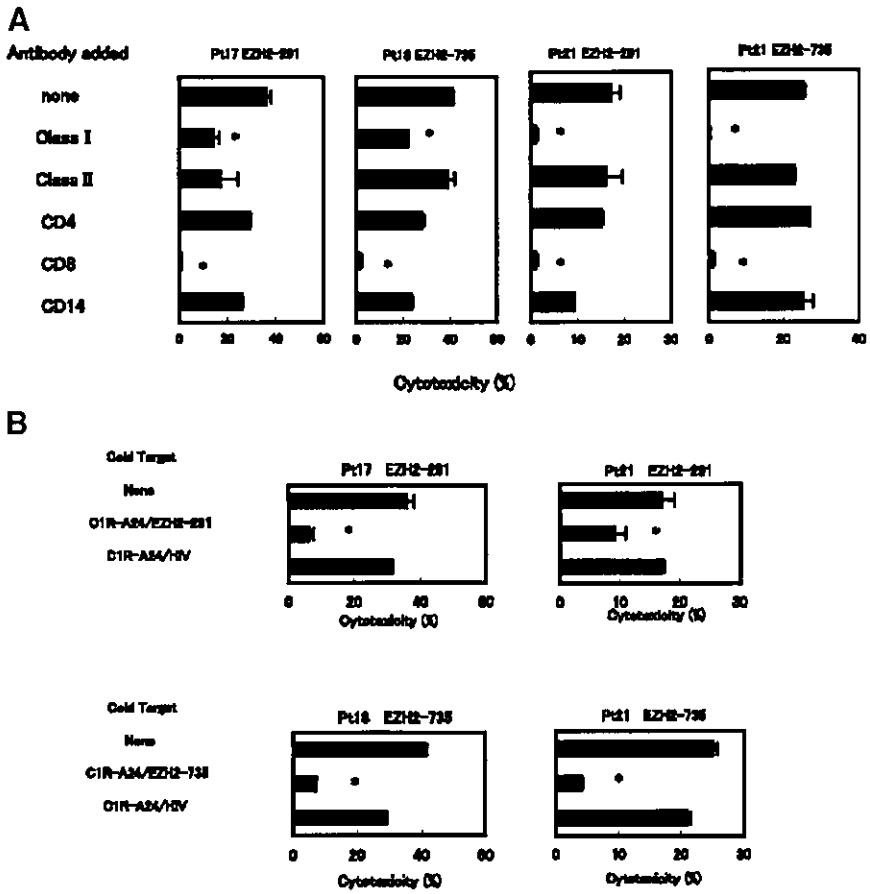

Fig. 4. Peptide-specific and CD8$^+$ T cell-dependent cytotoxicity of EZH2 peptide-stimulated PBMCs from cancer patients. **A**: EZH2 peptide-stimulated PBMCs from Patients 17, 18, and 21 were tested for their cytotoxicity against the LNCaP-A24. The 6-hr cytotoxicity assay was performed in the presence of the indicated mAb. The assay was done at an effector/target ratio of 10/1. *Statistically significant at $P < 0.05$. **B**: EZH2 peptide-stimulated PBMCs from Patients 17, 18, and 21 were tested for their cytotoxicity against the LNCaP-A24 in the presence of unlabeled C1R-A24 cells, which were pre-loaded with either the corresponding EZH2 peptide or the HIV peptide. The assay was done at an effector/target ratio of 10/1. *Statistically significant at $P < 0.05$.

peptide vaccination frequently resulted in the induction of IgG reactive to administered peptides [13,14]. The induction of IgG reactive to vaccinated peptides was positively correlated with longer survival of patients with advanced lung or gastric cancer [15,16]. In addition, the induction of IgG reactive to the administered peptides was also correlated with a clinical response among patients with recurrent gynecologic cancer [17]. Therefore, vaccination with the EZH2-291 and EZH2-735 peptides into HLA-A24$^+$ prostate cancer patients could efficiently elicit the induction of both prostate cancer-reactive CTLs and peptide-specific IgG, and subsequently lead to clinical responses.

We do not yet have a clear understanding of the roles played by peptide-specific IgG in anti-tumor immune responses. As described above, several CTL epitope peptides have elicited humoral and cellular immune responses in our clinical trials, and the levels of anti-peptide IgG in post-vaccination plasma correlated with clinical responses of patients with several types of cancer. Until now, we have not obtained evidence that peptide-specific IgG could react to the mother protein. It seems that IgG specific to class I-binding tumor peptides may not show any direct effect on tumor cells. On the other hand, we recently observed that peptide vaccination with a 9-mer peptide could induce peptide-specific and HLA-DR-restricted CD4$^+$ T cells in vivo (Harada et al. [27]). Such CD4$^+$ T cells may participate in the augmentation of peptide-specific humoral responses in vaccinated patients. Further study could shed light on the role of peptide-specific IgG in the anti-tumor immune response.

In conclusion, we identified two EZH2-derived peptides that are efficiently recognized by both the humoral and cellular immune systems in HLA-A24$^+$ prostate cancer patients. The frequencies of the HLA-A24 allele are relatively high throughout the world [28]. The information provided here might increase the possibility of treating HLA-A24$^+$ prostate cancer patients with metastases using peptide-based immunotherapy.

REFERENCES

1. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics 2000. CA Cancer J Clin 2000;50:7–33.
2. Harada M, Noguchi M, Itoh K. Target molecules in specific immunotherapy against prostate cancer. Int J Clin Oncl 2003;8: 193–199.
3. Gulley J, Chen AP, Dahut W, Arlen PM, Bastian A, Steinberg SM, Tsang K, Panicali D, Poole D, Schlom J, Hamilton MJ. Phase I study of a vaccine using recombinant vaccinia virus expressing PSA (rV-PSA) in patients with metastatic androgen-independent prostate cancer. Prostate 2002;53:109–117.
4. Murphy G, Tjoa B, Ragde H, Kenny G, Boynton A. Phase I clinical trial: T-cell therapy for prostate cancer using autologous dendritic cells pulsed with HLA-A0201-specific peptides from prostate-specific membrane antigen. Prostate 1996;29:371–380.
5. Tjoa BA, Simmons SJ, Bowes VA, Ragde H, Rogers M, Elgamal A, Kenny GM, Cobb OE, Ireton RC, Troychak MJ, Salgaller ML, Boynton AL, Murphy GP. Evaluation of phase I/II clinical trials in prostate cancer with dendritic cells and PSMA peptides. Prostate 1998;36:39–44.
6. Murphy GP, Tjoa BA, Simmons SJ, Jarisch J, Bowes VA, Rogers M, Elgamal A, Kenny GM, Cobb OE, Ireton RC, Troychak MJ, Salgaller ML, Boynton AL. Infusion of dendritic cells pulsed with HLA-A2-specific prostate-specific membrane antigen peptides: A phase II prostate cancer vaccine trial involving patients with hormone-refractory metastatic disease. Prostate 1999;38:73–78.
7. Small EJ, Fratesi P, Reese DM, Strang G, Laus R, Peshwa MV, Valon FH. Immunotherapy of hormone-refractory prostate cancer with antigen-loaded dendritic cells. J Clin Oncol 2000; 18:3894–3903.
8. Laible G, Wolf A, Dorn R, Reuter G, Nislow C, Lebersorger A, Popkin D, Pillus L, Jenuwein T. Mammalian homologue of the polycomb-group gene enhancer of zeste mediate gene silencing in *Drosophila* heterochromatin and at *S. cerevisiae* telomeres. EMBO J 1997;16:3219–3232.
9. Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, Ghosh D, Sewalt RGAB, Otte AP, Hayes DF, Sabel MS, Livant D, Weiss SJ, Rubin MA, Chinnaiyan AM. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci USA 2003; 100:11606–11611.
10. Jacobs JJ, Kieboom K, Mario S, DePinho RA, van Lohuizen M. The oncogene and polycomb-group gene *bmi-1* regulates cell proliferation and senescence through the ink4a locus. Nature 1999;397:164–168.
11. Jacob JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. Genes Dev 1999;13:2678–2690.
12. Varambally S, Dhanasekaran SM, Zhou M, Barrette TB, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RGAB, Oyye AP, Rubin MA, Chinnaiyan AM. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 2002; 419:624–629.
13. Noguchi M, Mine T, Suetsugu N, Tomiyasu K, Suekane S, Yamada A, Itoh K, Noda S. Induction of cellular and humoral immune responses to tumor cells and peptides in HLA-A24 positive hormone-refractory prostate cancer patients by peptide vaccination. Prostate 2003;57:80–92.
14. Tanaka S, Harada M, Mine T, Noguchi M, Gohara R, Azuma K, Tamura M, Yamada A, Morinaga A, Nishikori M, Katagiri K, Itoh K, Yamana H, Hashimoto T. Peptide vaccination for patients with melanoma and other types of cancer based on pre-existing peptide-specific cytotoxic T lymphocyte precursors in the periphery. J Immunother 2003;26:357–366.
15. Mine T, Gouhara R, Hida N, Imai N, Azuma K, Rikimaru T, Katagiri K, Nishikori M, Sukehiro A, Nakagawa M, Yamada A, Aizawa H, Shirouzu K, Itoh K, Yamana H. Immunological evaluation of CTL precursor-oriented vaccines for advanced lung cancer patients. Cancer Sci 2003;94:548–556.
16. Sato Y, Shomura H, Maeda Y, Mine T, Ueno Y, Akasaka Y, Kondo M, Takahashi S, Shinohara T, Katagiri K, Sato M, Okada S, Matsui K, Yamada A, Yamana H, Itoh K, Todo S. Immunogical evaluation of peptide vaccination for patients with gastric cancer

based on pre-existing cellular response to peptide. Cancer Sci 2003;94:802–808.

17. Tsuda N, Mochizuki K, Harada M, Sukehiro A, Kawano K, Yamada A, Ushijima K, Sugiyama T, Nishida T, Yamana H, Itoh K, Kamura T. Vaccination with pre-designated or evidence-based peptides for patients with recurrent gynecologic cancers. J Immunother 2004;27:60–72.
18. Nakatsura T, Senju S, Ito M, Nishimura Y, Itoh K. Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method. Eur J Immunol 2002;32:826–836.
19. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol 1994;152:163–175.
20. Ohkouchi S, Yamada A, Imai N, Mine T, Harada K, Shichijo S, Maeda Y, Saijyo Y, Nukiwa T, Itoh K. Non-mutated tumor rejection antigen peptides elicit type-I allergy in the majority of healthy individuals. Tissue Antigens 2002;59:259–272.
21. Hida N, Maeda Y, Katagiri K, Takasu H, Harada M, Itoh K. A new culture protocol to detect peptide-specific cytotixic T lymphocyte precursors in the circulation. Cancer Immunol Immunother 2002;51:219–228.
22. Kawamoto N, Yamada A, Ohkouchi S, Maeda T, Tanaka S, Hashimoto T, Saijo Y, Saijo S, Nukiwa T, Shichijo S, Aizawa H, Itoh K. IgG reactive to CTL-directed epitopes of self-antigens is either lacking or unbalanced in atopic dermatitis patients. Tissue Antigens 2003;61:352–361.
23. Harada M, Kobayashi K, Matsueda S, Nakagawa M, Noguchi M, Itoh K. Prostate-specific antigen-derived epitopes capable of inducing cellular and humoral responses in HLA-A24$^+$ prostate cancer patients. Prostate 2003;57:152–159.
24. Kobayashi K, Noguchi M, Itoh K, Harada M. Identification of a prostate-specific membrane antigen-derived peptide capable of eliciting both cellular and humoral immune responses in HLA-A24$^+$ prostate cancer patients. Cancer Sci 2003;94:622–627.
25. Matsueda S, Kobayashi K, Nonaka Y, Noguchi M, Itoh K, Harada M. Identification of new prostate stem cell antigen-derived peptides immunogenic in HLA-A2$^+$ patients with hormone-refractory prostate cancer. Cancer Immunol Immunother 2004 (In press).
26. Inoue Y, Takaue Y, Takei M, Kato K, Kanai S, Harada Y, Tobisu K, Noguchi M, Kakizoe T, Itoh K, Wakasugi H. Induction of tumor specific cytotoxic T lymphocytes in prostate cancer using prostatic acid phosphatase derived HLA-A2402 binding peptide. J Urol 2001;166:1508–1513.
27. Harada M, Matsueda S, Muto A, Gohara R, Oda T, Iwamoto Y, Itoh K. In vivo evidence that peptide vaccination can induce HLA-DR-restricated CD4$^+$ T cells reactive to a class I tumor peptide. J Immunol 2004;172:2659–2667.
28. Imanishi T, Akazawa T, Kimura A. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: Tsuji K, Aizawa M, Sasazuki T, editors. HLA 1991, Vol. 1. Oxford: Oxford Scientific Publications; 1992. pp 1065–1220.

Two Proliferation-Related Proteins, TYMS and PGK1, Could Be New Cytotoxic T Lymphocyte-Directed Tumor-Associated Antigens of HLA-A2$^+$ Colon Cancer

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ABSTRACT

Purpose: **The purpose of this work was to provide a scientific basis for specific immunotherapy of colon cancer.**

Experimental Design: **This study focused on identification of colon tumor-associated antigens and HLA-A2–restricted and tumor-reactive cytotoxic T lymphocytes (CTLs) generated from tumor-infiltrating lymphocytes of a colon cancer patient. A gene expression cloning method was used to identify genes coding for tumor antigens. Fifty-six peptides with HLA-A2–binding motifs encoded by these proteins were examined for their ability to induce HLA-A2–restricted and tumor-reactive CTLs.**

Results: **We identified the following three genes coding for proliferation-related proteins: thymidylate synthase (*TYMS*), which is involved in chemoresistance (5-fluorouracil); 5′-aminoimidazole-4-carboxamide-1-β-D-ribonucleotide transfolmylase/inosinicase (*AICRT/I*); and phosphoglycerate kinase 1 (*PKG1*), which was secreted by tumor cells and involved in the angiogenic process. *TYMS* was preferentially expressed in tumor cells, whereas *AICRT/I* and *PKG1* were equally expressed in both cancer cells and normal tissues at the mRNA level. Among 56 peptides with HLA-A2–binding motifs encoded by these proteins, 8 peptides were recognized by the CTLs, and 5 of 8 peptides were also recognized by the CTL precursors without *ex vivo* activation in the peripheral blood of colon cancer patients. Furthermore, four of them (one each from TYMS and PKG1 and two from AICRT/1) possessed the ability to induce HLA-A2–restricted and peptide-specific CTLs cytotoxic to colon tumor cells in peripheral blood mononuclear cells of colon cancer patients.**

Conclusions: **TYMS and PGK1, as well as their epitope peptides, might be appropriate target molecules for specific immunotherapy of HLA-A2$^+$ colon cancer patients because of the positive role of TYMS and PGK1 in chemoresistance (5-fluorouracil) and angiogenesis of tumor cells, respectively.**

INTRODUCTION

Colorectal cancer is one of the most commonly occurring malignancies in the world, and the prognosis for patients with advanced colorectal cancer with distant metastasis is extremely poor, despite recent clinical trials with chemotherapeutic agents (1). There is thus a need for the development of new treatment modalities, possibly involving specific immunotherapy. Determination of genes encoding tumor-associated antigens is pivotal for development of a specific immunotherapy. To our knowledge, however, there have been only a few studies that attempted to identify genes encoding tumor-associated antigens in colon cancer. We reported 16 genes encoding tumor-associated antigens by cloning from cDNAs of epithelial cancer cells other than colon cancer cells (2–8), and some of the peptides from these antigens were used as peptide vaccines for colon cancer patients, but no major tumor regression was obtained in this initial clinical trial (9). The other proliferation-related antigens, including breast (HER2/neu) and colon cancer (carcinoembryonic antigen) antigens, were reported to have cytotoxic T lymphocyte (CTL)-directed epitopes (10, 11). However, as far as we know, tumor regression was also not obtained in the clinical trials with those peptides. In the present study, we attempted to determine the molecular features of colon cancer antigens and CTL-directed peptides, and we report herein three proliferation-related proteins and four peptides applicable for use in specific immunotherapy of HLA-A2$^+$ colon cancer.

MATERIALS AND METHODS

Cytotoxic T Lymphocyte Line and Tumor Cell Lines. The parental HLA-A2–restricted and tumor-reactive OK-CTL line was established from tumor-infiltrating lymphocytes (TILs) of a patient with colon cancer (HLA-A0207/3101, HLA-B46/51, HLA-Cw1), as reported previously (8). In brief, TILs were cultured with 45% AIM-V (Invitrogen, Carlsbad, CA) and 45% RPMI 1640 (Invitrogen), 10% fetal calf serum (FCS; MultiSerTM; Trace Scientific Ltd., Melbourne, Australia), 0.1

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mmol/L minimal essential medium nonessential amino acids solution (Invitrogen), and interleukin (IL)-2 (100 units/mL) for more than 50 days. The OK-CTL line with an 80% $CD3^+CD4^-CD8^+$ phenotype showed both HLA-A2–restricted and tumor-specific CTL activity as measured by both chromium-51 release and interferon (IFN)-γ production assays (8). The tumor cell lines used in this study were HLA-A0201$^+$ SW620 colon adenocarcinoma cells, HLA-A0206$^+$ KE3 esophageal squamous cell carcinoma (SCC) cells, HLA-A0207$^+$ Ca9-22 oral SCC cells, HLA-A2$^-$ tumor cells (COLO320 and RERF-LC-MS), autologous Epstein-Barr virus (EBV)-transformed B cells, and phytohemagglutinin P (PHA; Becton Dickinson and Company, Sparks, MD)-activated T cells. One subline of this OK-CTL line, OK-CTLe, was also generated by incubation of OK-CTL with 100 units/mL IL-2 and 10 μg/mL PHA in the presence of irradiated (45 Gy) allogenic peripheral blood mononuclear cells (PBMCs; 2×10^5 cells/well) donated from three HLA-A2$^+$ healthy volunteers as feeder cells. The CTL line was then used as effector cells in the following experiments. SW620 tumor cells were used for preparation of the cDNA library. COS-7 cells or T2 cells were used as the target of a gene expression cloning method or for pulsing peptides, as reported previously (8).

Identification of cDNA Clones and Analyses. A previously reported gene expression cloning method (2, 8) was used to identify genes coding for tumor antigens recognized by the OK-CTLe subline. In brief, poly(A)$^+$ RNA of the SW620 colon tumor cells was converted to cDNA, ligated to the *Sal*I adapter, and inserted into the expression vector pCMV-SPORT-2 (Invitrogen). The cDNA clone of *HLA-A0207, HLA-A2601*, or *HLA-A2402* was obtained by reverse transcription-polymerase chain reaction (RT-PCR) and inserted into the eukaryotic expression vector pCR3 (Invitrogen). Both 200 ng of plasmid DNA pools or clones of the SW620 library and 200 ng of the *HLA-A0207* cDNA were mixed in 100 μL of Opti-MEM (Invitrogen) with 0.5% of LipofectAMINE (Invitrogen) for 30 minutes. A 50-μL aliquot of the mixture was then added to the COS-7 cells (1×10^3), which were then incubated for 6 hours. RPMI 1640 containing 10% FCS was then added and cultured for 2 days, followed by the addition of the OK-CTLe cells (5×10^3); OK-CTLe is a subline of the parental OK-CTL line. After an 18-hour incubation, 100 μL of supernatant were collected to measure IFN-γ by enzyme-linked immunosorbent assay (ELISA) in a duplicate assay. DNA sequencing was performed with a dideoxynucleotide sequencing method by using a DNA Sequencing kit (Perkin-Elmer, Foster, CA) and analyzed by an ABI PRISM 377 DNA Sequencer (Perkin-Elmer). The homology of the sequences of cloned genes derived from the SW620 cDNA library was analyzed using databases on the European Molecular Biology Laboratory GenBank/DDBJ homepage.

For the study of mRNA expression by RT-PCR, total cellular RNA was isolated from colon cancer (COLO201, COLO205, COLO320, SW620, SW480, HCT116, and KM12LM), lung cancer (QG56, RERF-LC-AI, 11-18, and LK87), head and neck SCC (Kuma-1), liver cancer (KIM-1 and KMCH-2), pancreatic cancer (Panc-1), brain tumor (KINGS-1), and leukemia (RAJI) cell lines, and PHA-blast cells and PBMCs were obtained from healthy donors (HDs) by the RNA-Bee RNA isolation reagent (Tel-Test, Inc., Friendswood, TX) method according to the manufacturer's instructions. Total RNAs from normal tissues (spleen, placenta, small intestine, heart, muscle, stomach, lung, liver, brain, testis, kidney, and colon) were purchased from Sawaday Technology (Tokyo, Japan). Complementary DNA of mRNA was prepared from 2 μg of total RNA using a SuperScript Preamplification System (Invitrogen) according to the manufacturer's instructions. Amplification was performed for 25 cycles (1 minute at 94°C, 2 minutes at the annealing temperature for each pair of primer sets, and 3 minutes at 72°C; the annealing temperatures for *SW#029*, *SW#086*, *SW#110*, and β-*actin* were 58°C, 62°C, 60°C, and 58°C, respectively). The separated band corresponding to each of the amplified mRNAs was analyzed by National Institutes of Health Image 1.55f software and integrated to calculate the area. The expression of each mRNA in SW620 tumor cells from which either of the two cDNAs was cloned was defined as 100, respectively. The formula is as follows: percentage of expression of mRNA = (mRNA density of samples/β-*actin* density of sample) × (β-*actin* density of SW620/mRNA density of SW620) × 100.

Peptides and Cytotoxic T Lymphocyte Assay. The only difference in the peptide sequence between HLA-A0201 and HLA-A0207 was the 123rd amino acid, which was Tyr or Cys, respectively. This amino acid was localized in the coil region in the second structure but not in the α-helix or β-sheet, which were involved in peptide binding, and thus this difference might not influence the binding of the peptide. Consequently, in an effort to identify peptides capable of binding to the HLA-A0207 molecules, an internet search was performed for peptides deriving from the thymidylate synthase (TYMS), PGK1 antigens with HLA-A0201–binding motifs (12), and 56 different peptides (>70% purity) were synthesized for screening. To identify CTL-directed epitopes, the CTLs were incubated for 18 hours with T2 cells prepulsed with each peptide at different doses for 2 hours followed by harvesting of supernatant to measure IFN-γ by ELISA. Eight peptides with >90% purity were thereby obtained for the CTL induction experiments. To inhibit IFN-γ production, 20 μg/mL anti-HLA class I (W6/32), anti-HLA class II (H-DR-1), anti-CD4 (Nu-Th/I), anti-CD8 (Nu-Ts/c), and anti-HLA-A2 (BB7.2) was used. Anti-CD14 (JML-H14, IgG2a) served as an isotype-matched control monoclonal antibody (mAb). A two-tailed Student's *t* test was used for the statistical analysis. PBMCs were obtained from the HLA-A2$^+$ cancer patients and HDs and used for CTL induction by the methods reported previously (8). In brief, PBMCs were incubated for 15 to 25 days with 45% AIM-V and 45% RPMI 1640 with 10% FCS and 100 units/mL IL-2 at concentrations of 2 to 4×10^5 cells/well. These activated PBMCs contained 20% to 35% $CD4^-CD8^+$ T cells, and the other cells were mostly $CD4^+CD8^-$ T cells. The IFN-γ production of these activated PBMCs in response to peptide was measured by ELISA.

RESULTS

Identification of cDNA Clones. An HLA-A2–restricted and tumor-specific CTL line was established from the TILs of a patient with colon adenocarcinoma, as reported previously (8). One of the sublines (OK-CTLe) with an 80% $CD3^+CD4^-CD8^+$ phenotype showed HLA-A2–restricted and tumor-specific CTL

activity as measured by IFN-γ production assay (data not shown) and ^{51}Cr release assay (Fig. 1). Namely, OK-CTLe showed cytotoxicity against tumor cells with different HLA-A2 subtypes (HLA-A0201, HLA-A0206, and HLA-A0207), but not to any of the HLA-A2$^-$ tumor cells, autologous PHA-blasts, autologous EBV-transformed B cells, or COS-7 fibroblast cells. Using this OK-CTLe line, 1,000 pools of 100–150 cDNA clones (a total of 1–1.5 × 10^5 cDNA clones) from the cDNA library of SW620 tumor cells were screened by the gene expression cloning method followed by selection to identify specific cDNAs within positive pools. After repeated experiments, we identified the following three known genes encoding for proliferation-related proteins. Complementary DNA *SW#029* (accession number AB062290) encoded *Homo sapiens* TYMS (accession number: XM008753), which is considered to be a measure of 5-fluorouracil (5-FU) chemosensitivity for colorectal carcinoma (13). Complementary DNA *SW#086* (accession number AB062403) encoded *H. sapiens* 5'-aminoimidazole-4-carboxamide-1-β-D-ribonucleotide transfolmylase/inosinicase (AICRT/I; accession number D82348). 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transfolmylase-inoshin monophosphate cyclohydrolase (ATIC), the product of the *purH* gene, is a bifunctional enzyme that catalyzes the two activities in *de novo* purine biosynthesis, AICAR transformylase and inosone 5'-monophosphate (IMP) cyclohydrolase (14). Complementary DNA *SW#110* (accession number AB062432) encoded the *H. sapiens* phosphoglycerate kinase 1 (PGK1; accession number XM010102). PGK influences DNA replication and repair in mammalian cell nuclei (15–17). It is also secreted by tumor cells and participates in tumor angiogenesis (18). Representative results of the CTL assays are shown in Fig. 2. This OK-CTLe subline did not react to COS-7 cells cotransfected with

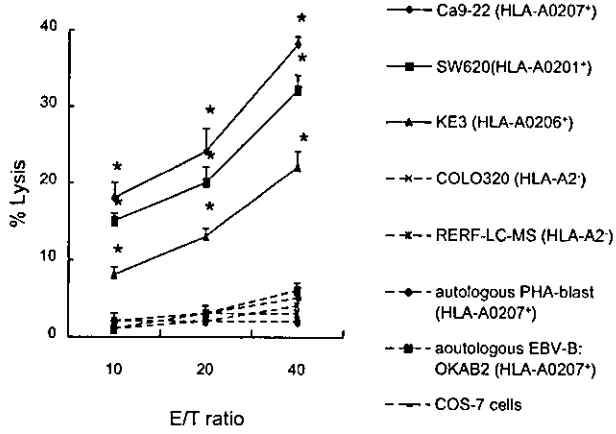

Fig. 1 Characteristics of HLA-A2-restricted OK-CTLe. The cytotoxicity of OK-CTLe against various target cells was tested by a 6-hour ^{51}Cr release assay at different effector to target cell ratios in triplicate assays. Target cells were HLA-A0207$^+$ Ca9-22 head and neck SCC, HLA-A0201$^+$ SW620 colon adenocarcinoma, HLA-A0206$^+$ KE3 esophageal SCC, HLA-A2402$^+$ COLO320 colon adenocarcinoma, and HLA-A1101$^+$ RERF-LC-MS lung adenocarcinoma cell lines; autologous PHA-blastoid cells; autologous EBV-transformed B cells (OKAB2); and COS-7 cells. Values represent the mean of percentage of specific lysis of triplicate determinations. A two-tailed Student's *t* test was used for the statistical analysis (*, $P < 0.05$) against a negative control (COLO320, HLA-A2$^-$ cell line).

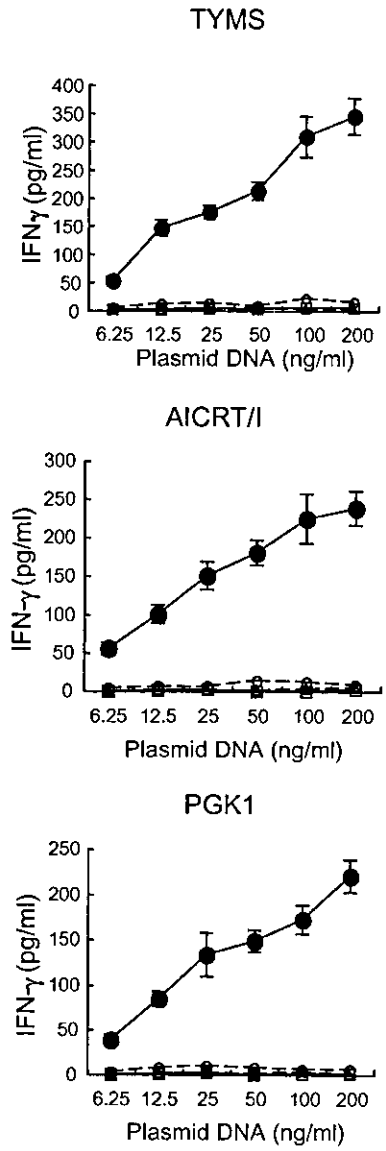

Fig. 2 Genes coding for tumor epitopes. Different amounts of each of three cDNA clones derived from SW620 tumor cells and 100 ng of *HLA-A0207*, *HLA-A2402*, or *HLA-A2601* cDNA were cotransfected into COS-7 cells, incubated for 48 hours, and then tested for their ability to stimulate IFN-γ release by the OK-CTLe lines. The background of IFN-γ release by the CTLs in response to COS-7 cells (under 50 pg/mL) was subtracted from the values in the figure. Complementary DNA *clone #001* represents the irrelevant clones cotransfected with HLA-A0207 (○); cloned cDNA with HLA-A2601 (□) or cloned cDNA alone (△) was not recognized by the OK-CTLs. Values represent the means of triplicate assays of the data from cDNA cotransfected with *HLA-A0207* (●), which values were significantly higher than those for the negative control cDNA *clone #001* at two or more different concentrations of plasmid DNAs. A two-tailed Student's *t* test was used.

HLA-A0207 cDNA and cDNA *clone #001* taken as a negative control (Fig. 2, ○). This subline also failed to react to COS-7 cells transfected with any of the three cDNA clones (Fig. 2, △) or cotransfected with any of these three cDNA clones and *HLA-A2601* cDNA as the other negative controls (Fig. 2, □).

Gene Expression Levels. The mRNA expression of these genes was investigated by the semiquantitative RT-PCR method using 17 tumor cell lines and 14 normal cells or tissues (Fig. 3). *TYMS* was highly expressed in the majority of tumor cells, with a few exceptions, whereas *TYMS* expression was very low in the majority of normal tissues with a few exceptions (testes, placenta, and small intestine). In contrast, *AICRT/I* and *PGK1* were equally expressed in both tumor cells and normal tissues.

Determination of Cytotoxic T Lymphocyte Epitopes. To determine CTL-directed epitopes, each of the 56 different synthesized peptides with HLA-A2 molecule-binding motifs derived from these proteins or a human immunodeficiency virus (HIV)-derived peptide with an HLA-A2-binding motif as a negative control was loaded onto T2 cells followed by testing for their ability to stimulate IFN-γ release by OK-CTLe. Eight peptides (three from TYMS, three from AICRT/I, and two from PGK1) among them were recognized by OK-CTLe (Fig. 4*A*). The peptide sequence and position of the first amino acid of the peptides are shown on the *left* of the column. In TYMS, AICR/I, and $PGK1_{222}$ cases, the amounts of IFN-γ increased in a peptide concentration-dependent manner. On the other hand, the ability to stimulate IFN-γ production was observed at the maximal level with 1 μg/mL $PGK1_{235}$ (Fig. 4*B*). The high avidity CTL capable to recognize at low-dose peptide-pulsed target cells, and these cells sometime suppressed at higher-dose of peptides.

Cytotoxic T Lymphocyte Precursors in the Circulation. We also investigated whether CTL precursors reactive to these antigens would be detectable in PBMCs of HLA-$A2^+$ colon cancer patients. PBMCs from three patients [a colon cancer patient (OK) from whose tumor the parental CTL line was obtained, the other colon cancer patient (TT, HLA-A0201), and a metastatic melanoma patient (KM, HLA-A0201) as a negative control] and an HLA-$A2^+$ HD (HLA-A0201) as the other negative control were expanded *in vitro* with IL-2 alone for 15 to 25 days and then tested for their reactivity to each of these three cDNA clones. None of the clones was recognized by the activated PBMCs of a melanoma patient or a HD (data not shown). However, the activated OK PBMCs recognized *AICRT/I* and *PGK1*, and furthermore, those of the colon cancer patient (TT) recognized *AICRT/I* (Fig. 5*A*).

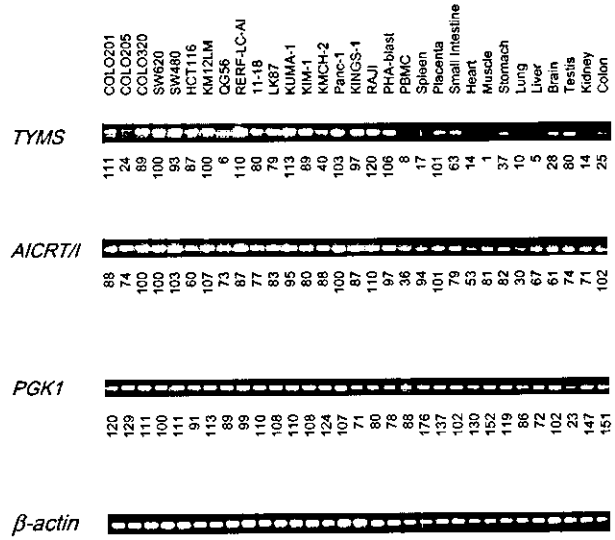

Fig. 3 Messenger RNA expression by semiquantitative RT-PCR. The relative expression of mRNA is shown as the ratio of the density of each sample to the β-*actin* density of RT-PCR products. The primer pairs were as follows: 029-186S, 5′-GCAGATCCAACACATCCTCC-3′; 029-795AS, 5′-CGCAATCATGTACGTGAGC-3′; 086-329S, 5′-GTTGCCTGCAATCTCTATCCC-3′; 086-798AS, 5′-CCTTGAGTTCCTTCACCAGC-3′; 110-194S, 5′-AGAGGATTAAGGCTGCTGTCC-3′; 110-838AS, 5′-TCTCCATGTTGTTGAGCACC-3′; β-*actin*-60S, 5′-CTTCGCGGGCGACGATGC-3′; and β-*actin*-381AS, 5′-CGTACATGGCTGGGGTGTTG-3′.

Each of the 56 peptides described above was then tested for its ability to stimulate IFN-γ production by PBMCs incubated with IL-2 (100 units/mL) alone. These IL-2–activated PBMCs from either a melanoma patient or a HD did not react to any peptides directed at the three gene products tested (data not shown). In contrast, the IL-2–activated OK PBMCs reacted to three (one from each of the three proteins) of the eight peptides that were also recognized by the parental CTL line (Fig. 5*B*). Furthermore, the activated PBMCs of the TT case also recognized three (one from TYMS and two from AICRT/I) of these eight peptides (Fig. 5*B*). IFN-γ production by these cells in response to peptides was inhibited by 20 μg/mL anti-HLA class I, anti-CD8, or anti-HLA-A2 mAb, but not by anti-HLA class II, anti-CD4, or anti-CD14 serving as an isotype-matched control mAb (data not shown). These results suggest that CTL precursors reactive to at least one peptide from each of these three proliferation-related proteins are detectable in the circulation of colon cancer patients without *ex vivo* stimulation by the corresponding peptides.

Cytotoxic T Lymphocyte Induction by Peptides. Based on these results, these eight peptides were tested for their ability to induce CTL activity in PBMCs of 10 HLA-$A2^+$ colon cancer patients (subtypes: 4 HLA-A0201, 3 HLA-A0206, and 3 HLA-A0207). PBMCs were stimulated *in vitro* with each of the eight peptides (10 μg/mL) and IL-2 (100 units/mL) every 3 days for 15 days, followed by incubation with IL-2 alone for an additional 15 days, and tested for their cytotoxicity in triplicate experiments by a 6-hour ^{51}Cr release assay against HLA-$A2^+$ SW620 tumor cells in the presence or absence of various mAbs. HLA-$A2^-$ QG56 tumor cells and HLA-$A2^+$ PHA-blast cells were used as negative controls for the ^{51}Cr release assay. The peptide specificity of the cytotoxicity was also confirmed by competition assay by the addition of excess numbers of T2 cells pulsed with a corresponding peptide or a HIV peptide as a negative control. Among the eight peptides tested, AICRT/I at positions 90–98, PGK1-derived peptide at positions 235–243, TYMS at positions 189–198, AICRT/I at positions 288–296, and TYMS at positions 231–240 induced HLA-A2–restricted and peptide-specific cytotoxicity against SW620 tumor cells in the PBMCs from 6, 5, 4, 4, and 1 of 10 patients irrespective of the different HLA-A2 subtypes, respectively (Fig. 6*A* and *B*). The levels of cytotoxicity of the peptide-stimulated PBMCs were significantly inhibited by anti-class I (W6/32) or anti-CD8 mAb, but not by the other mAbs tested in the assay. The cytotoxicity was also inhibited by the addition of the corresponding peptide-pulsed T2 cells, but not by that of the HIV peptide-pulsed cells in all of the cases tested. These results

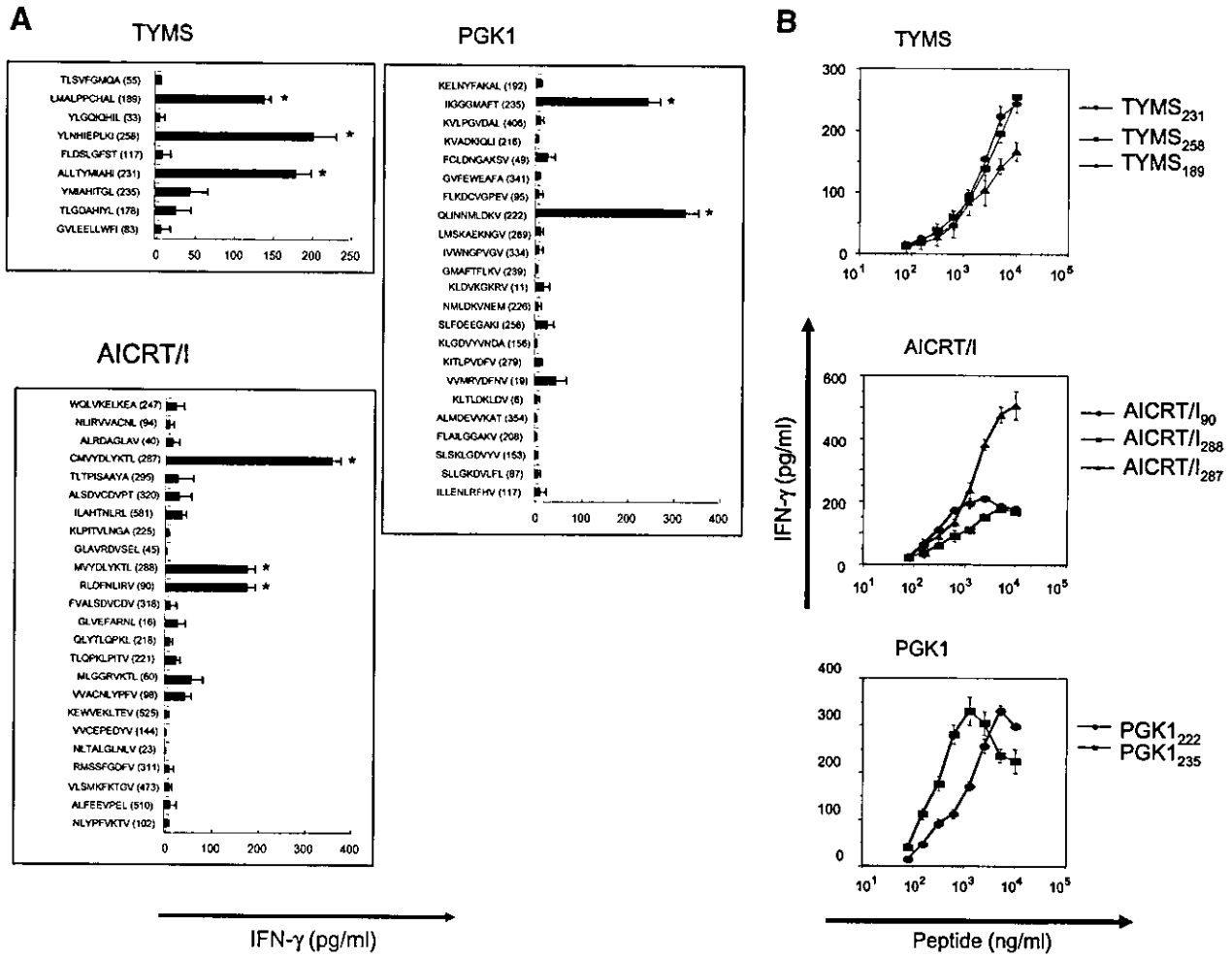

Fig. 4 Determination of epitopes. *A*. Each of the peptides (9–10-mer) or a HIV-derived peptide with an HLA-A2–binding motif as a negative control was loaded onto T2 cells at various concentrations of peptides for 2 hours. The OK-CTLe cells were then added and incubated for 18 hours, followed by collection of cell-free supernatant for measurement of IFN-γ. Values are the means of triplicate assays. The background of IFN-γ release by the CTLs (<50 pg/mL) in response to the T2 cells alone was subtracted from the values in the figure. Peptides on T2 cells that could stimulate a significant amount of IFN-γ production are indicated (*, $P < 0.05$ by two-tailed Student's *t* test and 100 pg/mL > IFN-γ). *B*. In all cases, the amount of IFN-γ increased in a peptide concentration-dependent manner. Representative results are shown in the figure. Values are the mean of triplicate assays.

suggest that the CTL activity induced by each of these four peptides was largely mediated by the peptide-specific and tumor-reactive $CD8^+$ T cells in an HLA class I-restricted manner. In contrast, the remaining three peptides induced such CTL activity in the PBMCs from any patient tested (data not shown).

DISCUSSION

In this study we reported three proliferation-related proteins as new CTL-directed tumor-associated antigens that were recognized by the CTL line from T cells infiltrating into colon tumor. These cell proliferation-related proteins would be vigorously synthesized, used, and then processed in tumor cells. Consequently, the processed peptides might be loaded onto major histocompatibility complex class I molecules at higher levels than those of immunologic ignorance but still at levels lower than those to induce immunologic tolerance. In this case, a few proteins involved in proliferation might act as colon tumor-associated antigens recognized by CTLs. Thus, there might be relatively low but distinct adaptive immunity against autologous colon tumor cells.

Colon tumor cells often become resistant to 5-FU–based chemotherapy. The up-regulated expression of TYMS, one of the antigens identified in this study, might be considered to be a parameter of 5-FU chemosensitivity for colorectal carcinoma (13). Indeed, the chemosensitivity of 5-FU for colorectal carcinomas with a low expression of TYMS has been reported to be better than that for tumors showing high expression of TYMS. Overall survival is reported to be better in the TYMS−(−) group than in TYMS−(+) group. Recent systemic review and meta-analysis provided evidence that colorectal tumors expressing high levels of TYMS appeared to have a poor overall survival compared with tumors expressing low levels of TYMS (15). Uchida *et al.* (16) also provide further support to the growing body of evidence that TYMS expression may be used

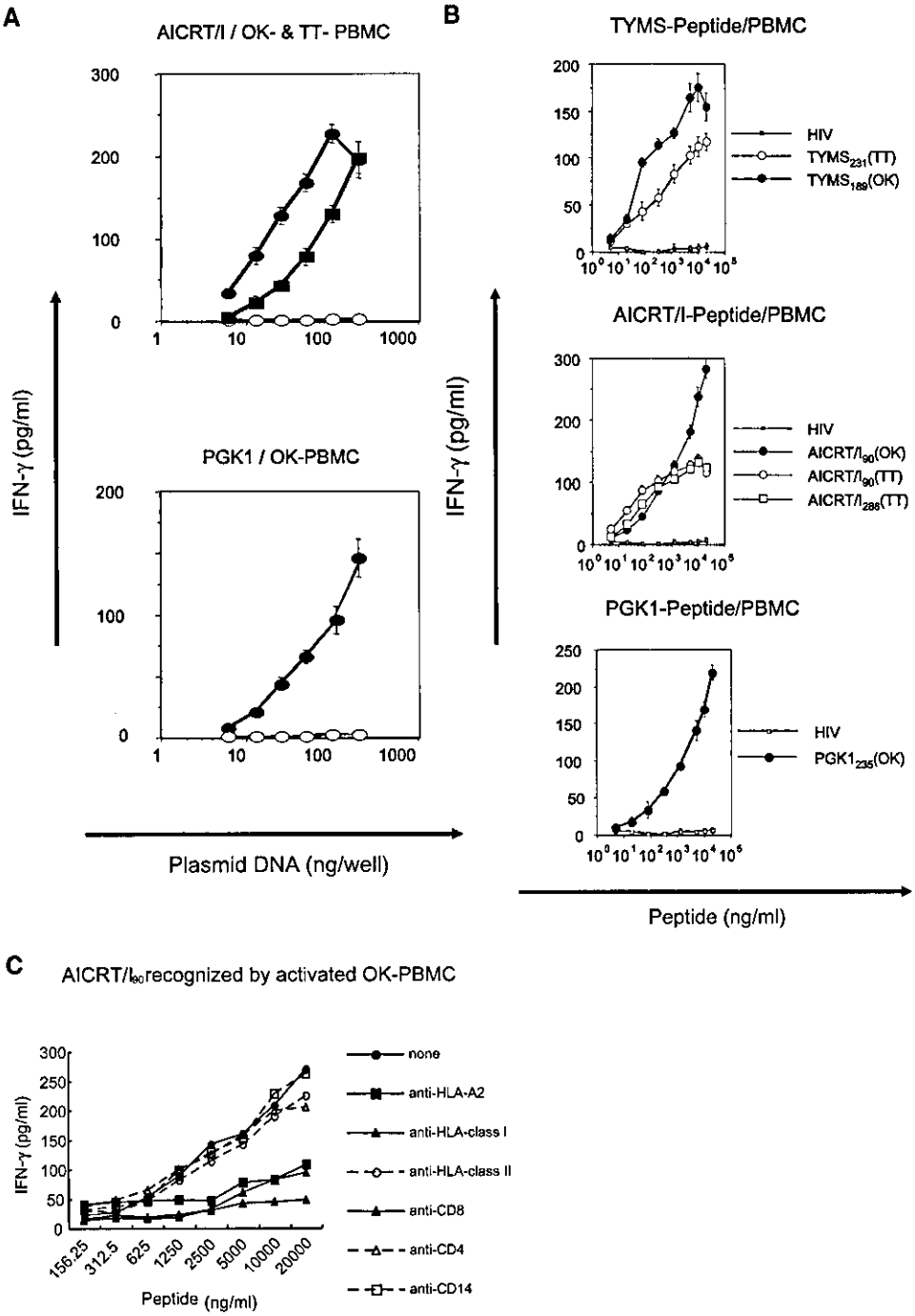

Fig. 5 Recognition of gene products and peptides by the PBMCs. *A*. Different amounts of each of the three cDNA clones and 100 ng of *HLA-A0207* or *HLA-A02402* cDNA were cotransfected into COS-7 cells, followed by a test of their ability to stimulate IFN-γ release by the activated OK-PBMCs (●) or TT PBMCs (■). Results for cDNA clones and HLA-A0207 are shown. Complementary DNA *clone #001* represents the irrelevant clones that were not recognized by the OK-CTLs (○). Values represent the means of triplicate assays of the data from cDNA cotransfected with *HLA-A0207* (●), which values were significantly higher than those for the negative control cDNA *clone #001* at two or more different concentrations of plasmid DNAs. A two-tailed Student's *t* test was used. *B*. Recognition of peptides by the activated OK PBMCs was tested. Each of the peptides or a HIV-derived peptide with an HLA-A2–binding motif as negative control was loaded onto T2 cells at various concentrations of peptides for 2 hours. The activated PBMCs were then added and incubated for 18 hours, followed by collection of cell-free supernatant for measurement of IFN-γ. Values represent the means of triplicate assays. The ability to stimulate IFN-γ production by the activated OK PBMCs (*closed symbols*) or TT PBMCs (*open symbols*) was assayed by ELISA. *C*, inhibition of IFN-γ production by mAbs. A representative result for AICRT/I_{90} is shown. AICRT/I_{90}-pulsed T2 cells were used as target cells for activated OK PBMCs (2×10^5 cells). The methods for preparation of peptide-pulsed T2 cells and inhibition assay are described in Materials and Methods.

as a molecular biomarker of response to TYMS-directed chemotherapy. PGK is known as the sixth enzyme of the glycolytic pathway, in which it equilibrates transfer between position 1 of 1,3-bisphosphoglycerate and the γ-phosphate of $MgATP^{2-}$. PGK also influences DNA replication and repair in mammalian cell nuclei (17, 18). The hypoxic nature of solid tumors triggers expression of vascular endothelial cell growth factor, which in turn stimulates both angiogenesis and glycolytic enzyme activity, including that of PGK, with the ability to facilitate anaerobic production of ATP (19). PGK not only functions in glycolysis but also is secreted by tumor cells and participates in the angiogenic process as a disulfide reductase (20). All these results suggest that both TYMS and PKG1 proteins are appropriate for use as CTL-directed tumor antigens because of their possible roles in tumor growth, whereas the involvement of AICRT/I protein in proliferation of colon cancer cells remains uncertain.

It is well known that colonic epithelial stem cells vigor-