

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
Plasmacytoma	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 pep-

ptide (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										Total
			1 CLL	2 CLL	3 MCL	4 NHL	5 B-ALL	6 MM	7 MDS	8 ATL	9 ATL	10 T-ALL	
SART-1 690	EYRGFTQDF	7	0	<u>486</u>	0	53	8	<u>141</u>	0	55	<u>155</u>	26	3/10
SART-2 93	DYSARWNEI	9	0	<u>440</u>	9	63	20	28	0	0	<u>354</u>	<u>192</u>	3/10
SART-2 161	AYDFLYNYL	9	0	<u>177</u>	49	13	0	28	0	0	<u>198</u>	<u>227</u>	3/10
SART-2 899	SYTRLFLIL	9	0	15	12	<u>220</u>	0	0	0	12	<u>346</u>	<u>526</u>	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	<u>137</u>	6	0	11	0	0	22	60	6	1/10
Lck 208	HYTNASDGL	11	0	2	22	30	0	0	0	0	<u>445</u>	<u>153</u>	2/10
Lck 486	TFDYLRSLV	11	0	0	0	0	0	0	0	<u>140</u>	<u>2099</u>	<u>140</u>	3/10
Lck 488	DYLRSVLEDF	11	0	0	0	0	6	<u>182</u>	<u>102</u>	<u>476</u>	<u>304</u>	0	4/10
ART-1 170	EYCLKFTKL	10	<u>501</u>	33	0	0	0	94	0	69	<u>1487</u>	3	2/10
HIV	RYLRDQQLGI	15	0	71	8	0	0	0	9	0	0	2	0/10
EBV	TYGPFVMCL	15	0	<u>100</u>	0	<u>100</u>	0	<u>418</u>	<u>115</u>	<u>264</u>	<u>264</u>	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 IFN γ production by the effector PBMCs in response to C1R-A24 cells prepulsed with the corresponding peptide. Background IFN γ 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.

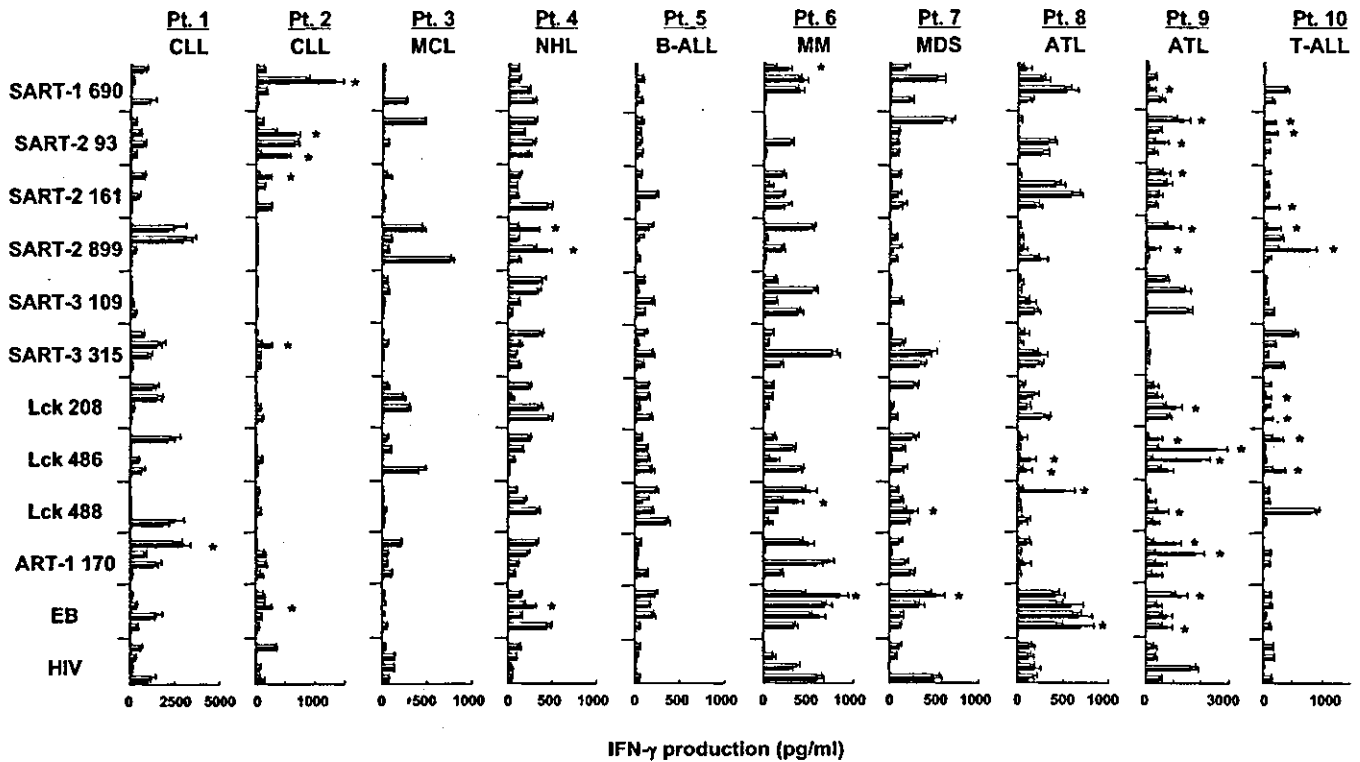


FIGURE 3. Induction of peptide-specific CTLs from the PBMCs of HLA-A24⁺ patients. PBMCs from 10 patients with hematologic malignancy were stimulated *in vitro* with the peptides indicated, as described in text. Values represent IFN γ production. The open and closed bars represent IFN γ production in 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).

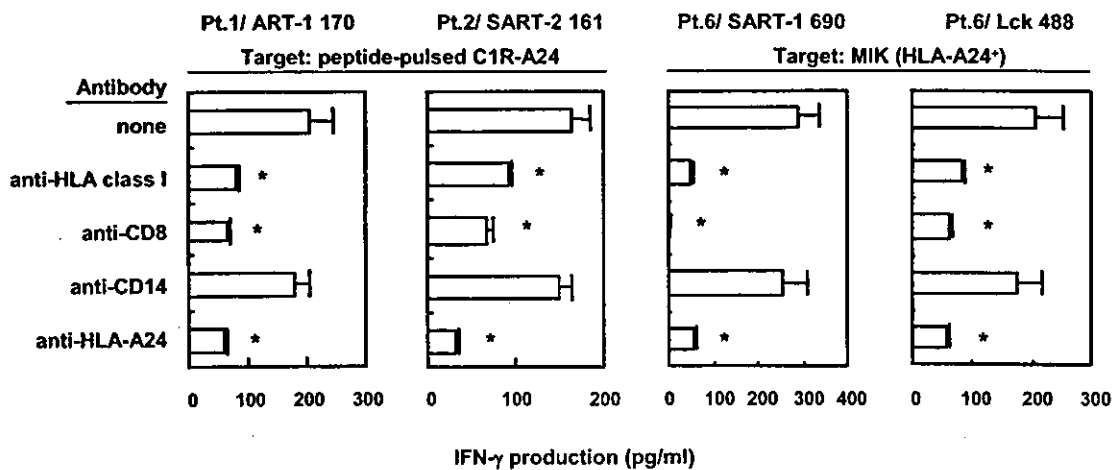


FIGURE 4. HLA class I-restricted response of peptide-stimulated CTLs. CD8⁺ T cells, which were purified from peptide-stimulated PBMCs, were examined for their IFN γ production in response to peptide-pulsed C1R-A24 cells or tumor cell lines. The assay was carried out at an effector:target ratio of 10:1, and the indicated monoclonal antibody was added at a dose of 20 μ g/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 IFN γ production by the effector cells alone was subtracted from the values. **P* < 0.05 by two-tailed Student *t* test.

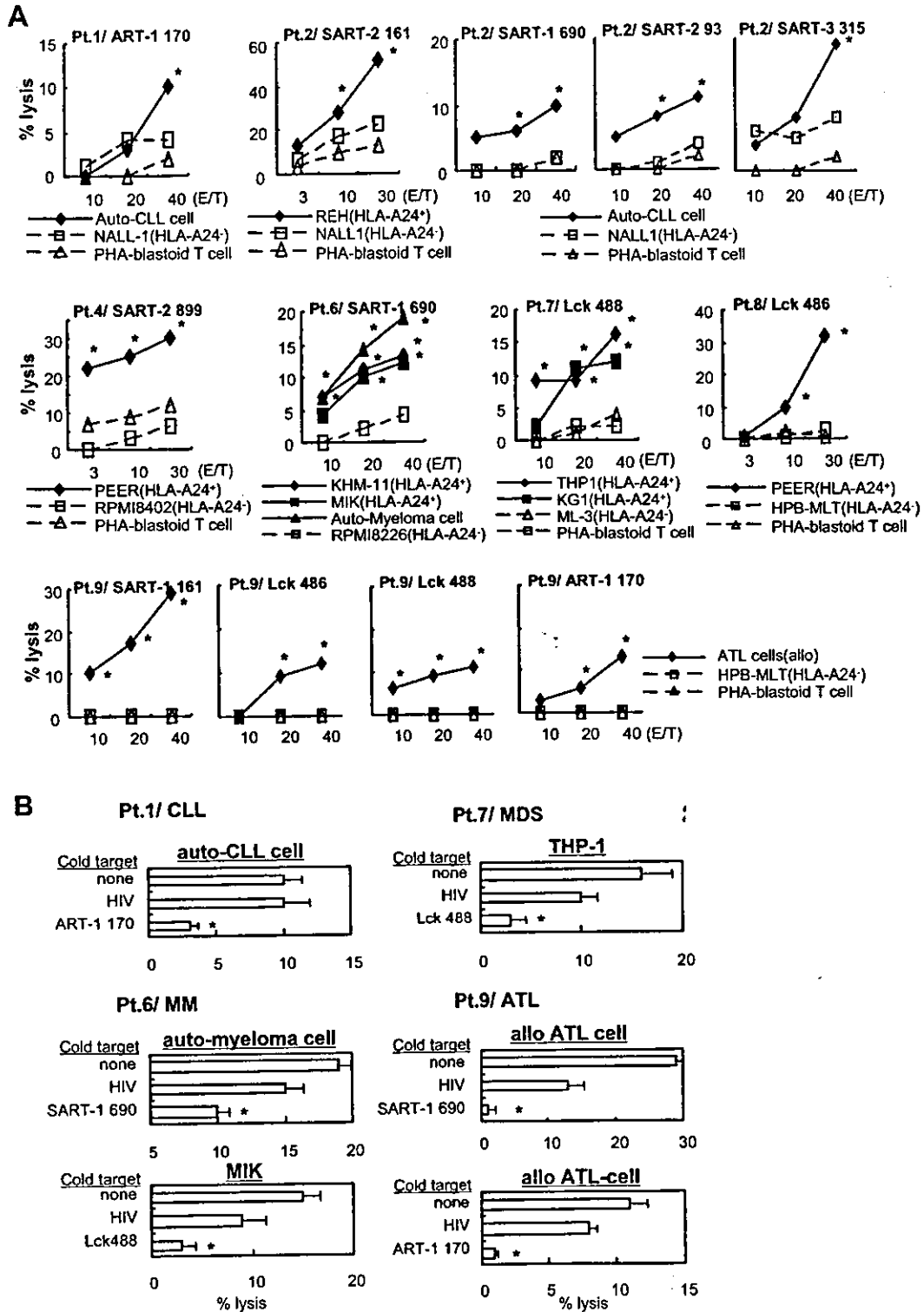


FIGURE 5. Cytotoxic activity of peptide-stimulated PBMCs. (A) PBMCs from seven patients (patients 1, 2, 4, 6, 7, 8, and 9) were stimulated in vitro with the indicated peptides and examined for their cytotoxic activity against hematologic malignant cell lines, fresh malignant cells, and PHA-activated 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/RAR α , 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 vac-

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

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

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

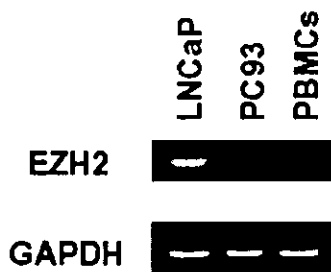


Fig. 2. The expression of the EZH2 gene in tumor cells. The EZH2 mRNA expression in the LNCaP cells, PC93 cells, and peripheral blood mononuclear cells (PBMCs) was examined by the RT-PCR method.

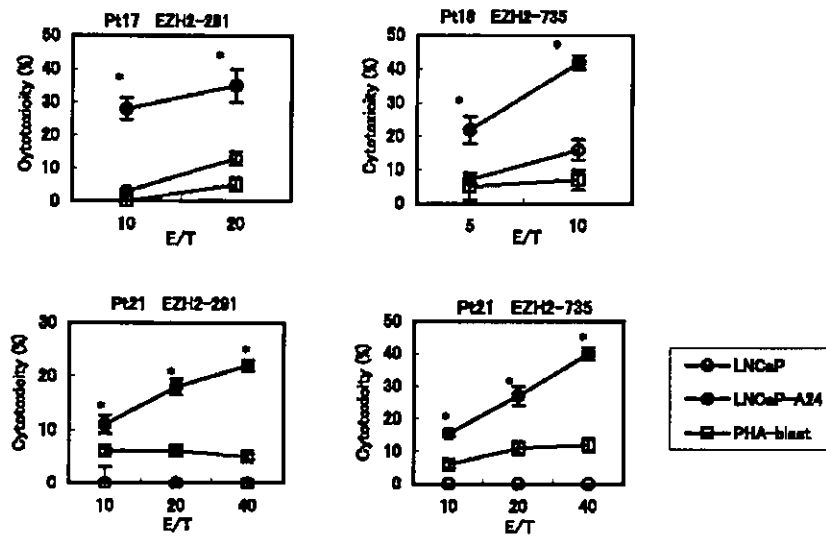


Fig. 3. Cytotoxicity of EZH2 peptide-stimulated PBMCs from cancer patients. EZH2 peptide-stimulated PBMCs from three HLA-A24⁺ prostate cancer patients were tested for their cytotoxicity toward three different targets by a 6-hr ⁵¹Cr-release assay. *Statistically significant at *P* < 0.05.

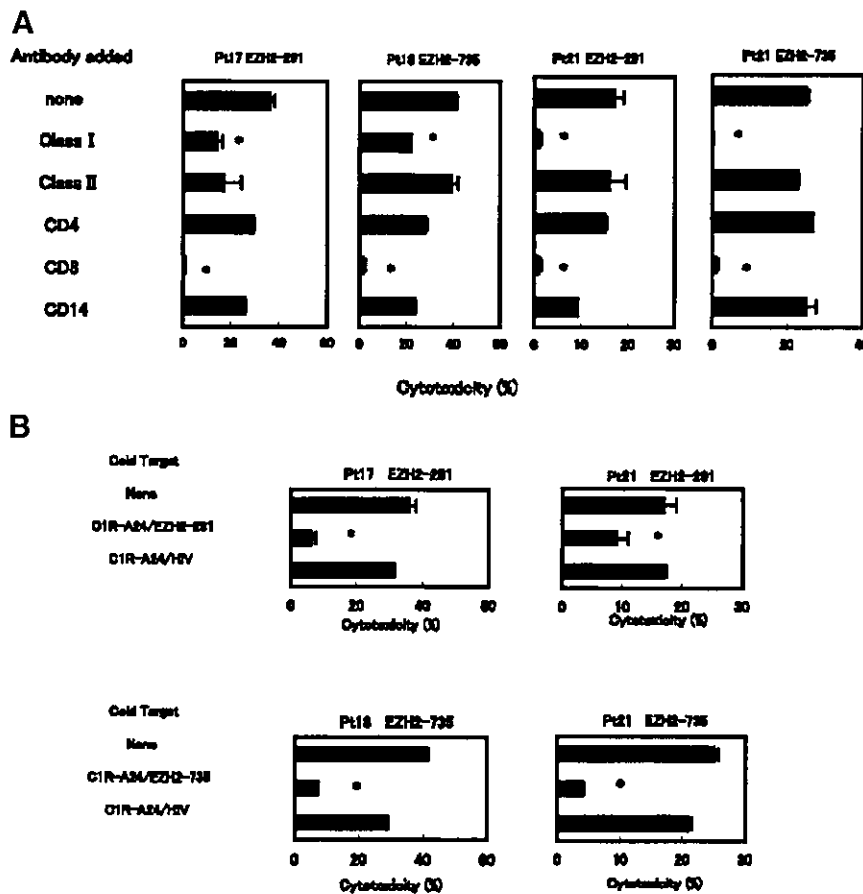


Fig. 4. Peptide-specific and CD8⁺ T cell-dependent cytotoxicity of EZH2 peptide-stimulated PBMCs from cancer patients. **A:** EZH2 peptide-stimulated PBMCs from Patients 17, 18, and 21 were tested for their cytotoxicity against the LNCaP-A24. The 6-hr cytotoxicity assay was performed in the presence 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 CIR-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.

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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-ribose nucleotide transferase/inosinase (AICRT/I); and phosphoglycerate kinase 1 (PGK1), which was secreted by tumor cells and involved in the angiogenic process. TYMS was preferentially expressed in tumor cells, whereas AICRT/I and PGK1 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 PGK1 and two from AICRT/I) 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; Multi-SerTM; 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-CTL_e, was also generated by incubation of OK-CTL with 100 units/mL IL-2 and 10 μ g/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-CTL_e subline. In brief, poly(A)⁺ RNA of the SW620 colon tumor cells was converted to cDNA, ligated to the *Sall* 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 μ L of Opti-MEM (Invitrogen) with 0.5% of LipofectAMINE (Invitrogen) for 30 minutes. A 50- μ L 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-CTL_e cells (5×10^3); OK-CTL_e is a subline of the parental OK-CTL line. After an 18-hour incubation, 100 μ L 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 μ g 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) \times (β -actin density of SW620/mRNA density of SW620) \times 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 μ g/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-CTL_e) 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-CTL ϵ 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-CTL ϵ line, 1,000 pools of 100–150 cDNA clones (a total of $1-1.5 \times 10^5$ 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-ribose nucleotide transferase/inosinase (AICRT/I; accession number D82348). 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transferase/inosin monophosphate cyclohydrolase (ATIC), the product of the *purH* gene, is a bifunctional enzyme that catalyzes the two activities in *de novo* purine biosynthesis, AICAR transferase and inosine 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-CTL ϵ subline did not react to COS-7 cells cotransfected with

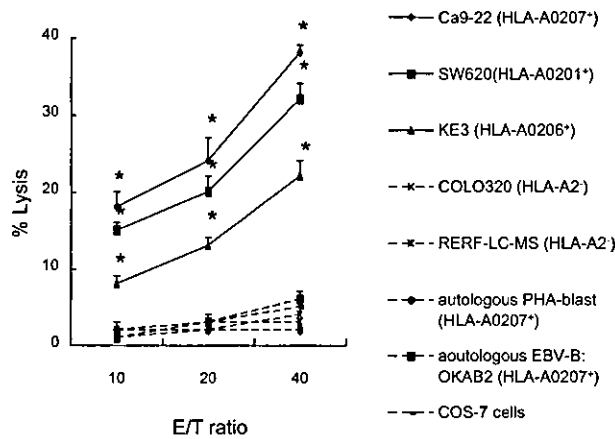


Fig. 1 Characteristics of HLA-A2-restricted OK-CTL. The cytotoxicity of OK-CTL ϵ against various target cells was tested by a 6-hour ^{51}Cr release assay at different effector to target cell ratios in triplicate assays. Target cells were HLA-A0207 $^+$ Ca9-22 head and neck SCC, HLA-A0201 $^+$ SW620 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).

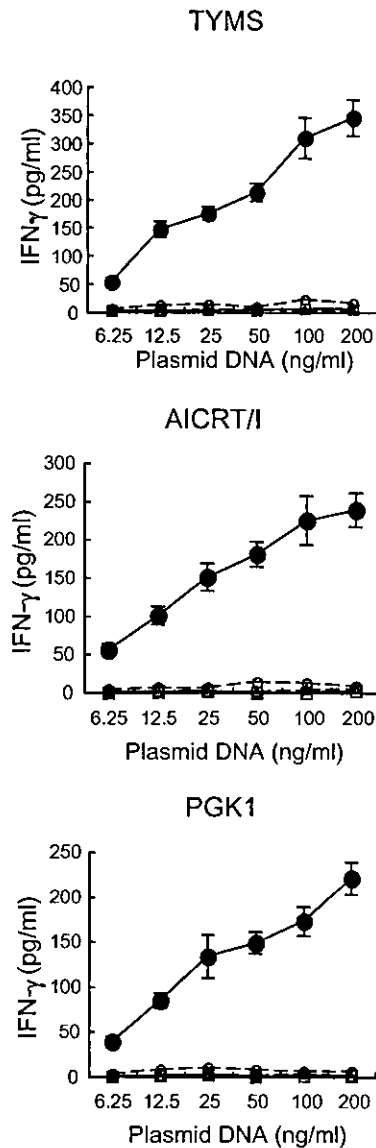


Fig. 2 Genes coding for tumor epitopes. Different amounts of each of three cDNA clones derived from SW620 tumor cells and 100 ng of HLA-A0207, HLA-A2402, or HLA-A2601 cDNA were cotransfected into COS-7 cells, incubated for 48 hours, and then tested for their ability to stimulate IFN- γ release by the OK-CTL ϵ 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 (\circ); cloned cDNA with HLA-A2601 (\square) or cloned cDNA alone (\triangle) was not recognized by the OK-CTLs. Values represent the means of triplicate assays of the data from cDNA cotransfected with HLA-A0207 (\bullet), 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, \circ). This subline also failed to react to COS-7 cells transfected with any of the three cDNA clones (Fig. 2, \triangle) or cotransfected with any of these three cDNA clones and HLA-A2601 cDNA as the other negative controls (Fig. 2, \square).

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-CTL. Eight peptides (three from *TYMS*, three from *AICRT/I*, and two from *PGK1*) among them were recognized by OK-CTL (Fig. 4A). The peptide sequence and position of the first amino acid of the peptides are shown on the left of the column. In *TYMS*, *AICRT/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 μ g/mL *PGK1*₂₃₅ (Fig. 4B). 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. 5A).

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. 5B). Furthermore, the activated PBMCs of the TT case also recognized three (one from *TYMS* and two from *AICRT/I*) of these eight peptides (Fig. 5B). IFN- γ production by these cells in response to peptides was inhibited by 20 μ g/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 μ g/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 ⁵¹Cr 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 ⁵¹Cr 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. 6A 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

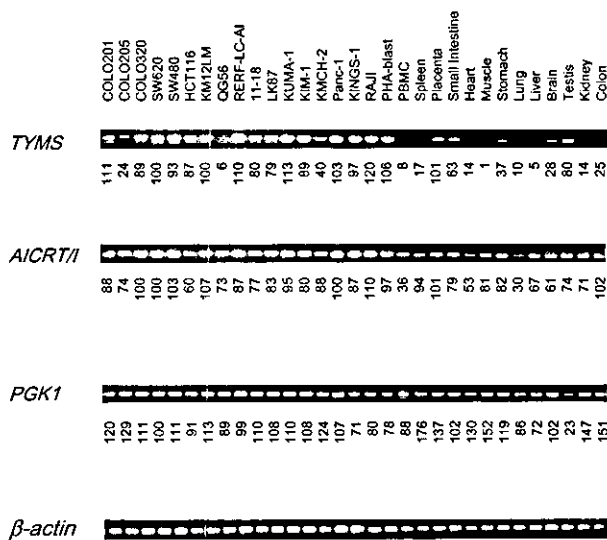


Fig. 3 Messenger RNA expression by semiquantitative RT-PCR. The relative expression of mRNA is shown as the ratio of the density of each sample to the β -actin density of RT-PCR products. The primer pairs were as follows: 029-186S, 5'-GCAGATCCAACACATCCTCC-3'; 029-795AS, 5'-CGCAATCATGTACGTGAGC-3'; 086-329S,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

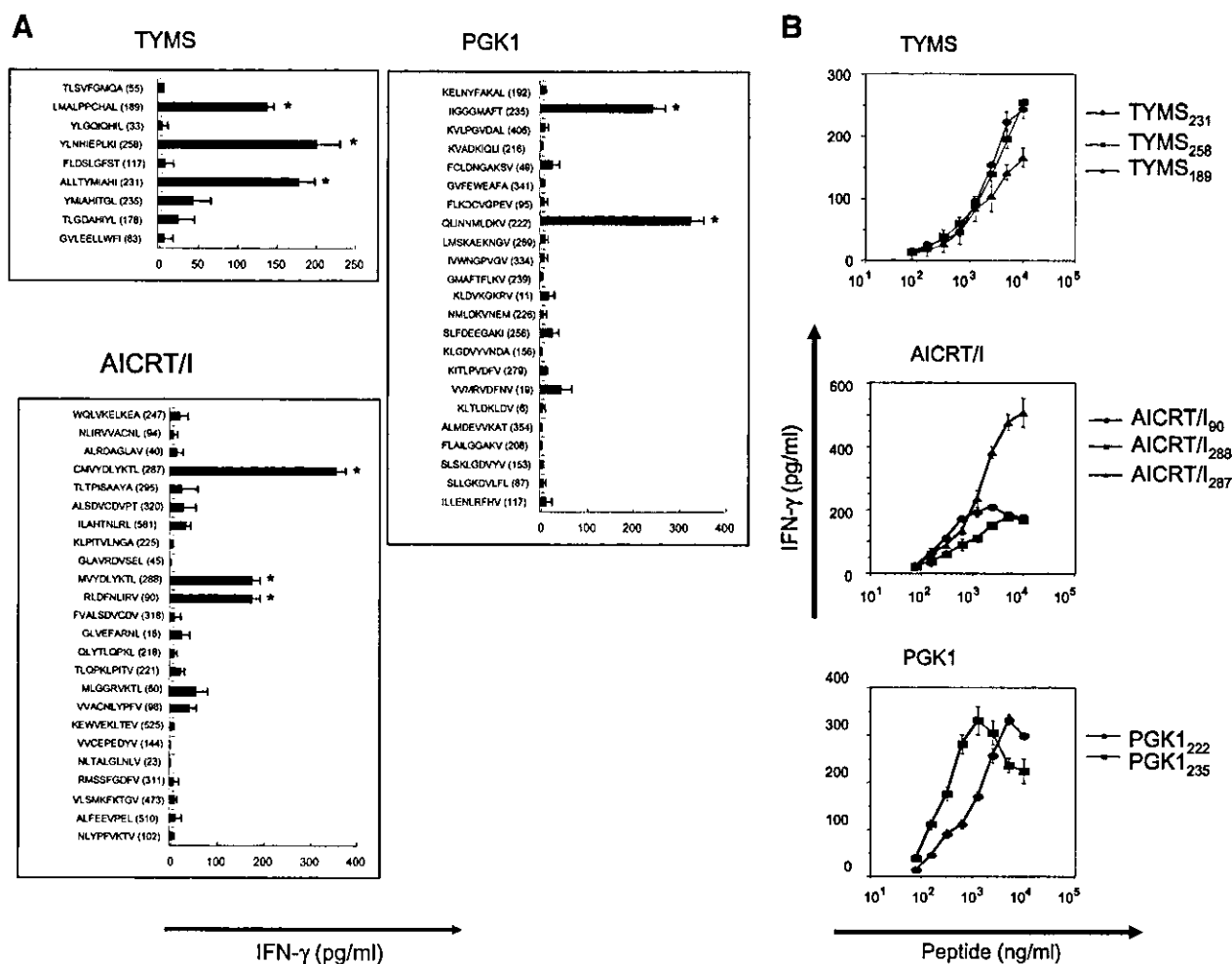


Fig. 4 Determination of epitopes. **A**. Each of the peptides (9–10-mer) or a HIV-derived peptide with an HLA-A2-binding motif as a negative control was loaded onto T2 cells at various concentrations of peptides for 2 hours. The OK-CTLs were then added and incubated for 18 hours, followed by collection 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).

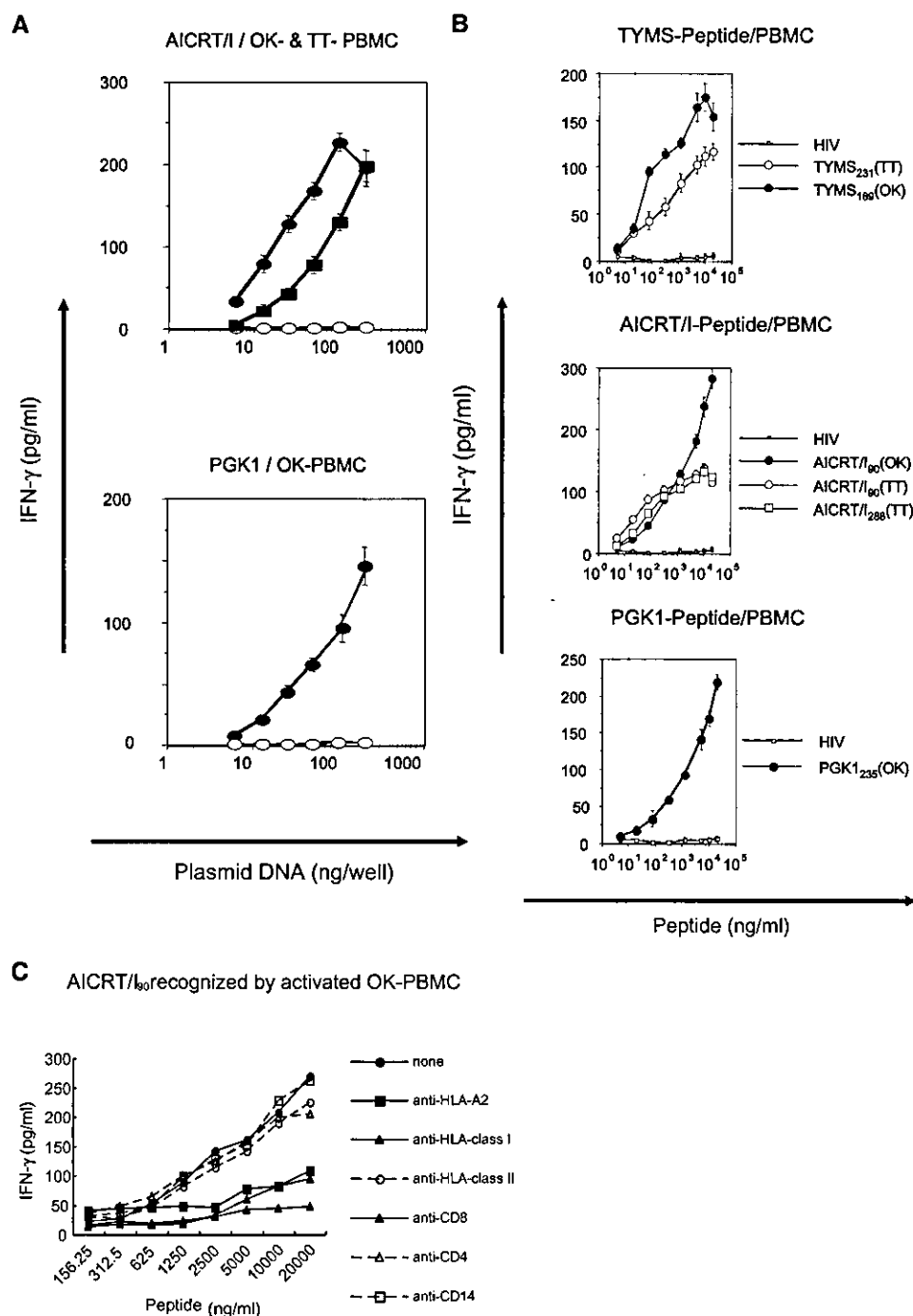
DISCUSSION

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/190 is shown. AICRT/190-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 PGK1 proteins are appropriate for use as CTL-directed tumor antigens because of their possible roles in tumor growth, whereas the involvement of AICRT/190 protein in proliferation of colon cancer cells remains uncertain.

It is well known that colonic epithelial stem cells vigor-

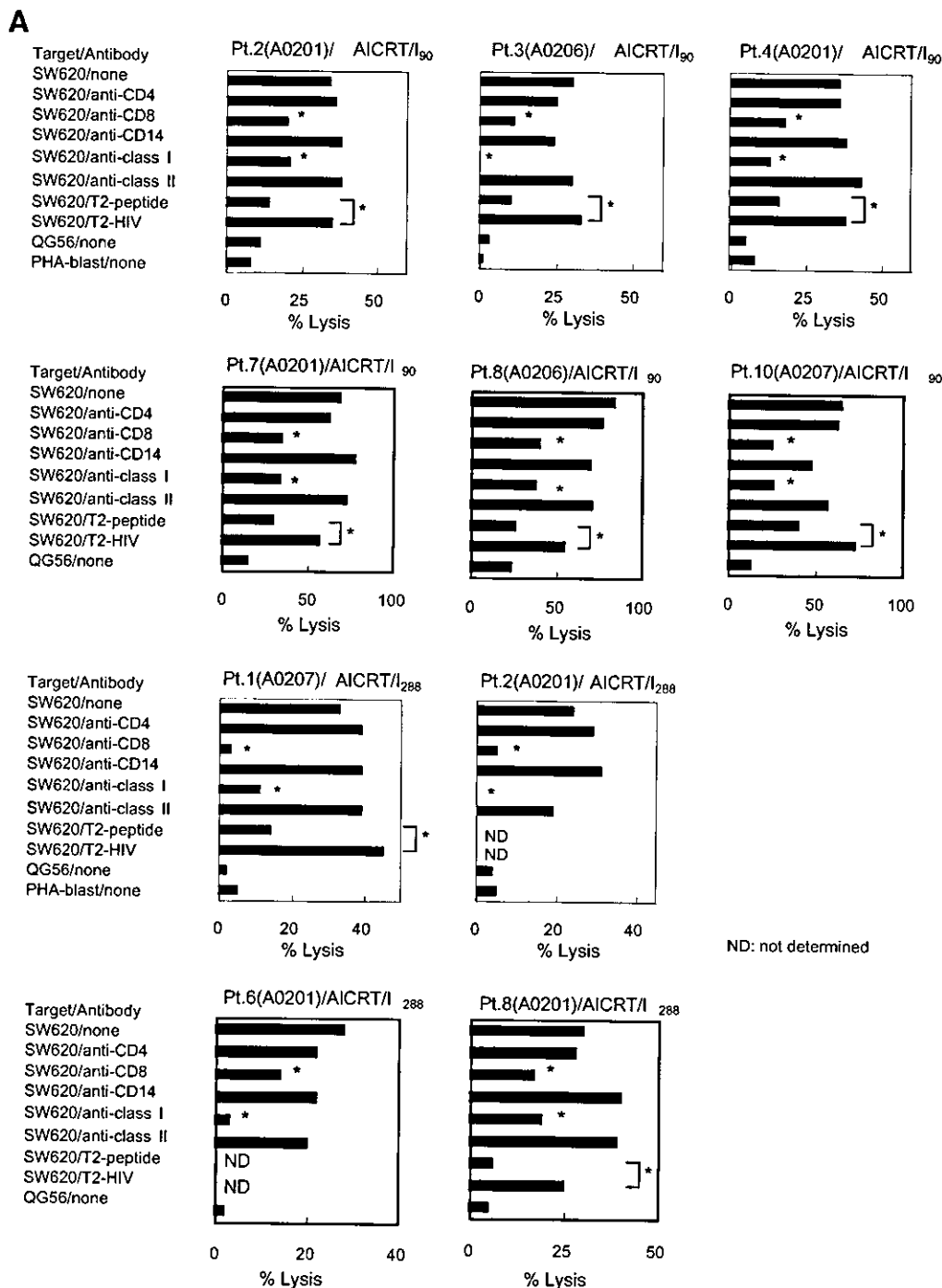


Fig. 6 Cytotoxicity by the peptide-stimulated PBMCs. Each of these eight peptides was tested for its ability to induce CTL activity in PBMCs of five HLA-A2⁺ colon cancer patients. Their HLA-A2 genotypes were determined by a previously reported method (10); patients 2 and 4 had the A0201 genotype; patients 3 and 5 had the A0206 genotype; and patient 1 had the A0207 genotype. PBMCs were stimulated with each of the eight peptides (10 μ g/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 against HLA-A2⁺ SW620 tumor cells in the presence or absence of various mAbs by a standard 6-hour ⁵¹Cr release assay at an effector to target cell ratio of 10:1 in triplicate assays. HLA-A2⁻ QG56 tumor cells and HLA-A24⁺ PHA-blast cells were used as negative controls for the ⁵¹Cr release assay. The peptide specificity of the cytotoxicity was also confirmed by competition assay by addition of T2 cells pulsed with a corresponding peptide or a HIV peptide as a negative control at a hot to cold cell ratio of 20:1 in triplicate assays. Results for the AICRT/I peptides (panel A) and for the TYMS or PKG1 (panel B) peptides that had the ability to induce CTLs from at least two patients are shown in Fig. 6. A two-tailed Student's *t* test was used for the statistical analysis in this study; *P* values of <0.05 were considered to indicate statistical significance. Values represent the means \pm SD of the percentage of specific lysis.

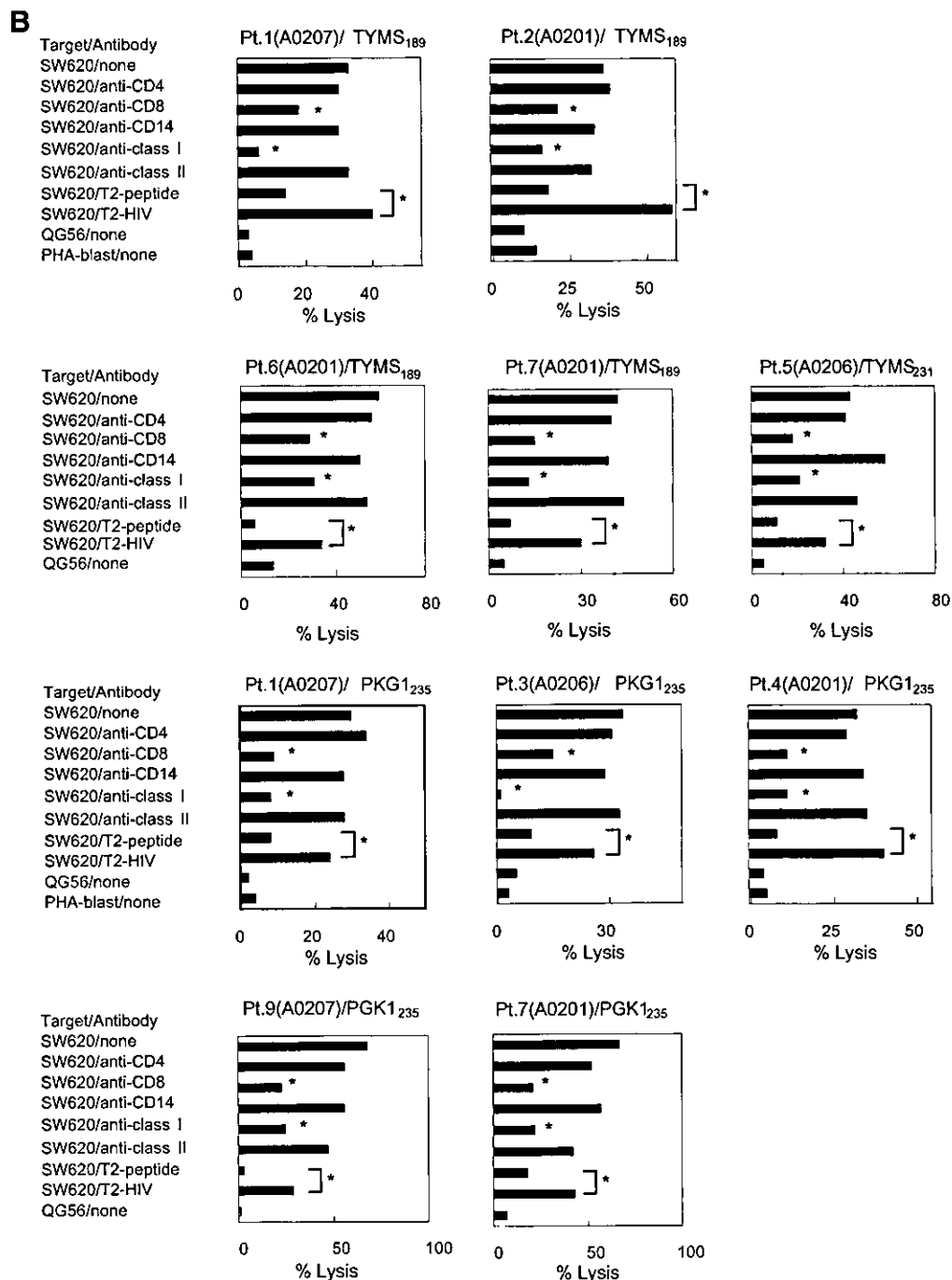


Fig. 6 Continued.

ously proliferate, differentiate, and mature and then quickly turnover via apoptosis. Therefore, adverse events such as diarrhea are expected to be associated with vaccination of peptides derived from proliferation-related proteins. However, no severe adverse effects were observed in our phase I clinical trials of individualized peptide vaccination for advanced cancer patients, which were performed using peptides from proliferation-related proteins (9, 21–25). However, whether or not proliferation-

related peptides are suitable as vaccines for the treatment of colon cancer needs to be carefully evaluated through additional basic and clinical studies. It also remains to be elucidated whether or not these CTL epitope peptides derived from proteins ubiquitously expressed in both tumor cells and normal cells at the mRNA level are suitable for use in peptide-based cancer immunotherapy. Among the previously reported CTL-directed antigens, most were ubiquitously expressed in both

tumor cells and normal tissues at the mRNA level, with preferential or exclusive expression in tumor cells at the protein level (2, 4–8), whereas *MRP3* showed preferential expression at the mRNA level (3). However, in our above-mentioned clinical trials of peptide vaccinations for several epithelial cancers, including colon cancer, there were no apparent differences in safety or tumor regression between the peptides from the former group and those from the latter group, and major tumor regression was observed in a few patients with no severe adverse events (21–25).

The HLA-A2 allele is found in 23% of Africans, 53% of Chinese, 40% of Japanese, and 50% of Caucasians (12). With regard to HLA-A2 subtypes, HLA-A0201 is found in the majority of HLA-A2⁺ Caucasians and in 45% of HLA-A2⁺ Japanese, whereas HLA-A0206 is found in 36% of HLA-A2⁺ Japanese (26). PGK1-derived peptide at positions 235 to 243 induced CTL activity in PBMCs from patients with all three HLA-A2 subtypes, whereas AICRT/I at positions 90 to 98 induced CTL activity in PBMCs of HLA-A0201 and HLA-A0206⁺ patients. Two peptides (TYMS at positions 189 to 198 and AICRT/I at positions 288 to 296) induced CTL activity in HLA-A0201 and HLA-A0207⁺ patients. These results suggest that each of the four peptides has the ability to induce HLA-A2-restricted CTLs reactive to tumor cells, in agreement with our previous observation (8). However, additional studies with more samples with different subtypes will be needed to confirm whether or not each of these four peptides has the ability to induce HLA-A2-restricted and tumor-reactive CTLs from HLA-A2 colon cancer patients irrespective of the HLA-A2 subtype.

The results of these studies showed that four peptides from three proliferation-related proteins had the ability to induce HLA-A2-restricted CTLs reactive to colon tumor cells in PBMCs of colon cancer patients. These results may provide a better understanding of the molecular features of colon cancer antigens and CTL-directed peptides.

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Ran, a Small GTPase Gene, Encodes Cytotoxic T Lymphocyte (CTL) Epitopes Capable of Inducing HLA-A33–restricted and Tumor-Reactive CTLs in Cancer Patients

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ABSTRACT

Purpose: The purpose is to identify a gene coding for tumor-associated antigen and peptide capable of inducing CTLs reactive to tumor cells with a HLA-A33–restricted fashion to provide scientific basis for specific immunotherapy to HLA-A33⁺ cancer patients.

Experimental Design: An expression gene-cloning method was used to identify the tumor-associated antigen gene. Northern blot analysis and immunohistochemistry were used to examine the mRNA and protein expression levels in various cells and tissues, respectively. Synthetic peptides were examined for their ability to induce HLA-A33⁺ tumor-reactive CTLs in peripheral blood mononuclear cells from cancer patients.

Result: A gene of small GTPase, *Ran*, which controls the cell cycle through the regulation of nucleocytoplasmic transport, mitotic spindle organization, and nuclear envelope formation, was found to encode epitopes recognized by the HLA-A33–restricted CTLs established from T cells infiltrating into gastric adenocarcinoma. The expression of the *Ran* gene was increased in most cancer cell lines and cancer

tissues at both the mRNA and protein levels. However, it was not enhanced in the surrounding normal cells or tissues. It was also undetectable in normal tissues as far as tested. Ran-derived peptides at positions 48–56 and 87–95 could induce CD8⁺ peptide-specific CTLs reactive to tumor cells from HLA-A33⁺ epithelial cancer patients in a HLA class I-restricted manner.

Conclusions: Because of its increased expression in cancer cells and involvement in malignant transformation and/or the enhanced proliferation of cancer cells, the two Ran-directed peptides could be potent candidates in use for specific immunotherapy against HLA-A33⁺ epithelial cancers.

INTRODUCTION

There is growing evidence that human tumors express antigenic peptides recognized by CTLs, and some of these peptides have been used as peptide vaccines for cancer patients with the *HLA-A2* or *HLA-A24* allele (1–7). In contrast to the many reports on epitope peptides recognized by *HLA-A2* or *HLA-A24* CTLs (8–10), information on the antigens and peptides recognized by *HLA-A33*–restricted CTL is very limited (11–13). This lack of information has been hampering the development of peptide-based specific immunotherapy for *HLA-A33*⁺ cancer patients, despite the relatively wide expression of the *HLA-A33* allele in people of various ethnicities (14% of Asians, 4% of Caucasians, and 16% of blacks; refs. 14, 15). To identify CTL epitope peptides in *HLA-A33*⁺ cancer patients, we established a *HLA-A33*–restricted CTL line from the tumor-infiltrating lymphocytes (TILs) of a patient with scirrhous-type gastric adenocarcinoma, and identified a gene, *Ran* (16, 17), as a gene that codes for tumor-associated antigen (13). We have demonstrated in this study that the two Ran-derived peptides are capable of inducing *HLA-A33*–restricted CTL activity reactive to tumor cells in epithelial cancer patients.

MATERIALS AND METHODS

CTL Line and Tumor Cell Lines. The parental *HLA-A33*–restricted and tumor-reactive 850B-CTL line was established from the TILs of patient with scirrhous-type gastric adenocarcinoma (*HLA-A*2402/A*3303*, B7/B44, and Cw7/Cw14) by incubating TILs with interleukin 2 alone for >50 days by methods reported previously (13). Briefly, the patient's TILs were incubated with a culture medium (45% RPMI 1640, 45% AIM-V medium; Invitrogen, San Diego, CA; 10% FCS with 100 units/mL interleukin 2). These interleukin 2-activated TILs were tested for CTL activity every 7 days to various tumor cells by the standard 6-hour ⁵¹Cr release assay. The cancer cell lines used for this experiment were as follows: gastric adenocarcinoma (MKN-28, MKN-45, SSTW-9, KATO-III, KWS, and

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