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Advances in Brief

HLA A2601-restricted CTLs Recognize a Peptide Antigen Expressed on Squamous Cell Carcinoma[1]

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Abstract

The $CD4^-CD8^+$ CTL (KE-4 CTL) cell line against autologous tumor cells was established in a patient with esophageal cancer. This KE-4 CTL recognized a peptide antigen on esophageal and lung squamous cell carcinomas in an HLA A2601-restricted manner, as evaluated by cytotoxicity against a panel of tumor cells, transfection experiments with *HLA A2601* cDNA, and reconstitution with eluted peptides. None of the normal cells tested was lysed by this CTL. These results suggest the existence of HLA A2601-restricted CTL precursors recognizing a peptide antigen on SCC in a patient with esophageal cancer.

Introduction

SCC[3] is one of the most common cancers in human. SCC, particularly, esophageal and lung SCC, are relatively resistant to currently available regimens of chemotherapy or radiation therapy. Therefore, the development of a specific immunotherapy using tumor specific CTLs would be important so that other treatment modalities may be offered. Tumor-specific CTL have been generated *in vitro* from PBMC or tumor-infiltrating lymphocytes of patients with melanomas and ovarian cancers (1–7). Genes encoding tumor-rejection antigens were also identified using these CTL (2–7). However, the generation of HLA class I-restricted CTL recognizing SCC has been reported rarely (1). We established the HLA A2601-restricted CTL cell line recognizing a peptide antigen expressed on SCC.

Materials and Methods

Patients and Cell Lines. KE-3 and KE-4 tumor cell lines were established from patients with esophageal cancer in our hospital. PBMC were isolated for the generation of CTL and the establishment of EBV-BCL. The tumor cell lines kindly provided for this study are as follows: TE-8, TE-9, TE-10, and TE-11 esophageal cancers by Dr. Nishihira (Tohoku University School of Medicine, Sendai, Japan); a Kuma-1 head and neck cancer by Dr. Eura (Kumamoto University School of Medicine, Kumamoto, Japan); a 1-87 lung cancer by Dr. Kobayashi (Tohoku University, The Research Institute for Tuberculosis and Cancer, Sendai, Japan); LK79 and LK87 lung cancers by Chugai Pharmaceutical Co. (Tokyo, Japan); a PC-9 lung cancer by Dainihon Pharmaceutical Co. (Tokyo, Japan); a QG-56 lung cancer by Dr. Yasumoto (University of Occupational and Environmental Health, Kitakyushu, Japan); OMC-4 and SKG-I cervical cancers by Dr. Ueda (Osaka Medical College, Osaka, Japan) and Dr. Nozawa (Keio University School of Medicine, Tokyo, Japan), respectively; a KWS gastric cancer by Dr. Sekiguchi (Tokyo University, Tokyo, Japan); a KM12LM colon cancer by Dr. Nakajima (Tokyo University); HAK-1B, KIM-1, and KYN-2 hepatocellular carcinomas from the First Department of Pathology; a KMG-A gall bladder cancer by Dr. Maruiwa; a R-27 breast cancer by Dr. Nomura (Kyushu Cancer Center, Fukuoka, Japan); TOC-2 and HOT ovarian cancers by Dr. Sato (Sapporo Medical College, Sapporo, Japan); and a VA-13 fibroblast cell line by Dr. Saya (Kumamoto University School of Medicine, Kumamoto, Japan). The other cell lines listed in Table 1 were either established in our laboratories or purchased from JCRB (Tokyo), ATCC (Rockville, MD), and Morinaga Co. (Tokyo).

HLA Class I Typing of Cell Lines. HLA A alleles of tumor cell lines were determined genetically by the PCR sequence-specific oligonucleotide probe method with tumor cell lines as reported (8). Serological HLA class I typing of PBMC was performed by HLA Monoclonal Reagent (One Lambda, Canoga Park, CA). The results of the genetic analyses of HLA A alleles were shown in this study when they were available.

Generation and Culture of CTL. One million PBMC from the KE-4 patient were incubated with 10^5 autologous tumor cells in a well of a 24-well tissue culture plate (Falcon, Lincoln park, NJ) containing 2 ml of RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with 10% FCS (Whittaker, Walkersville, MA), 5% human AB serum (GIBCO-BRL), 0.1 mM MEM nonessential amino acids solution (GIBCO-BRL), and 100 units/ml recombinant interleukin 2 (a generous gift from Shionogi Pharmaceutical Co., Osaka, Japan). Responder lymphocytes were collected, washed, resuspended in the medium, and restimulated every 7 to 10 days with autologous tumor cells at an effector:stimulator cell ratio of 10.

The surface phenotype of the KE-4 CTL was studied by a direct immunofluorescence assay with FITC-conjugated anti-CD3, -CD4, and -CD8 (Nichirei, Tokyo) mAbs and FACScan (Becton Dickinson, Mountain View, CA). A 6-h ^{51}Cr-release assay was used to measure CTL activity as reported (9). Anti-CD3 (OKT3; ATCC), -CD4 (Nichirei), -CD8 (Nichirei), anti-class I (W6/32) (ATCC), and anti-DR mAb (H-DR1; established in our laboratory) were used for the blocking experiments as reported (9).

Peptides of Tumor Cells. Peptides were extracted from the KE-4 and KE-3 tumor cell lines using the pH 3.3 acid elution technique (10). Briefly, extracted peptides were pretreated with C18 Sep Pak (Waters, Milford, MA), prefractionated with Centricon-3 (Amicon, Beverly, MA; consisting of peptides $\leq M_r$ 3000, ≈30 amino acids in length), and fractionated with reverse-phase HPLC. HPLC solvents were A = 99.92% water/0.08% trifluoroacetic acid and B = 99.94% acetonitrile/0.06% trifluoroacetic acid. Gradients consisted of the following linear step intervals: isocratic A solvent for 0 to 5 min; 0 to 10% B (in A) from 5 to 10 min; and 10 to 35% B (in A) from 10 to 60 min. The flow rate was 0.8 ml/min. Individual HPLC fractions were lyophilized and reconstituted in 200 μl HBSS. In a cytotoxic assay, 20 μl of individual HPLC fractions were added to a well containing ^{51}Cr-labeled autologous or allogeneic EBV-BCL cells, followed by incubation for 1 h at 37°C to allow the binding of peptides to target cells. Then the KE-4 CTL were added to the well.

Transfection of Cell Lines with *HLA A2601* cDNA. *HLA A2601* cDNA was isolated from the KE-4 tumor cell line. Briefly, 1×10^7 KE-4 tumor cells were used to prepare mRNA using a Quick Prep mRNA Purification Kit (Pharmacia, Uppsala, Sweden), and first-strand cDNA was synthesized using a Super Script Preamplification Kit (BRL, Gaithersburg, MD). *HLA A*-specific oligonucleotides (5′-primer, CCGAGATGGCCGTCATG; 3′-primer, TGTCT-

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[3] The abbreviations used are: SCC, squamous cell carcinoma; PBMC, peripheral blood mononuclear cells; EBV-BCL, EBV transformed B cell line; ATCC, American Type Culture Collection.

Table 1 *Cytotoxicity of the KE-4 CTL cell line*

Histology	Cell line	Origin	% Cx.[a]	HLA class I		
				A	B	C
Squamous cell carcinoma	KE-4	Esophagus	34	A2402/A2601	B54/B60	Cw1/Cw3
	KE-3	Esophagus	0	A2/A24	B7/B61	Cw7/
	TE-8	Esophagus	32	A2402/A2601	B7/B61	Cw7/
	TE-9	Esophagus	10	A33/-		
	TE-10	Esophagus	0	A2/A2402		
	TE-11	Esophagus	35	A2402/A2601		
	Kuma-1	Head and Neck	4	A2603/A3303	B39/B52	Cw7/
	Ca9-22	Oral	4	A24/		
	HSC-2	Oral	8	A24/		
	HSC-3	Oral	0	A2/A24		
	HSC-4	Oral	0	A31/A24		
	SAS	Oral	0	A24/		
	SCC-131	Oral	5	n.e.[b]		
	SCC-158	Oral	0	n.e.[b]		
	Sq-1	Lung	1	A11/A24		
	RERF-LC-AI	Lung	0	A2402/		
	QG-56	Lung	4	A2601/		
	OMC-4	Uterus	8	A0207/A2402		
	SKG-1	Uterus	7	A2402/		
Adenocarcinoma	A549	Lung	4	A2603/A3001		
	1-87	Lung	5	A0207/A1101		
	LK87	Lung	7	A0207/A1101		
	PC-9	Lung	8	A0206/A2402		
	MKN-45	Stomach	2	A2402/		
	MKN-28	Stomach	0	A3101/		
	KWS	Stomach	6	A0206/		
	COLO 201	Colon	6	A0101/A0201		
	COLO 205	Colon	0	A0101/A0201		
	COLO 320	Colon	1	A2402/		
	SW620	Colon	7	A0201/A2402		
	HCT116	Colon	5	A0101/A2402		
	KM12LM	Colon	6	A0201/A2402		
	Panc-1	Pancreas	3	A0201/A1102		
	KMG-A	Gall Bladder	18	A2601/		
	R-27	Breast	6	A0201/		
Large cell carcinoma	LC-99A	Lung	0	A2402/		
	86-2	Lung	3	A1101/		
Small cell carcinoma	LC-65A	Lung	0	A1101/A2402		
	LK79	Lung	0	A2402/		
Hepatocarcinoma	HAK-1B	Liver	0	A0201/A3301	B44/B54	Cw1/Cw4
	KIM-1	Liver	4	A11/A26		
	KYN-2	Liver	2	A24/	B52/B54	Cw1/
	PRC/PRF/5	Liver	2	A3303/		
	Hep-G2	Liver	0	A2402/A2601		
Renal cell carcinoma	KUR-11	Kidney	7	A2402/A3303		
	KUR-20	Kidney	5	A0201/A0211		
	KUR-21	Kidney	5	A0201/		
Ovarian Carcinoma	TOC-2	Ovarium	2	A26/A33	B7/B44	Cw7/
	HOT	Ovarium	8	A26/	B35/B60	Cw7/
Melanoma	M73	Melanocyte	0	A1/A25	B8/B18	Cw7/
Normal Cells	KE-4 EBV-BCL	B cell	0	A2402/A2601		
	TIG1-20	Fibroblast	5	A0206/A2402		
	VA-13	Fibroblast	1	n.e.[b]		
	011794 MIY	Keratinocyte	5	A0207/A2402		
	011794 KOJ	Keratinocyte	0	A0207/A2402		
	100394 SOM	Keratinocyte	0	A0207/A2402		
	110893 YOS	Keratinocyte	1	A0207/A2402		
	110893 SHI	Keratinocyte	1	A2402/A3303		
	HFK-CK-012	Keratinocyte	1	A0203/		
	HFK-CK-013	Keratinocyte	0	A0101/1101		

[a] Cytotoxicity of the KE-4 CTL against different types of cell lines was measured in a 6-h ^{51}Cr-release assay at an effector: target cell ratio of 20. Representative results of the repeated experiments (at least three times) were shown.

[b] n.e., no expression of HLA A alleles.

CACACTTTACAAGCTGTGAGAG) and PCR were used to amplify *HLA A* cDNA. *HLA A* cDNA was cloned using an Eukaryotic TA Cloning Kit (Invitrogen, San Diego, CA) according to the manufacturer's instruction and was sequenced using an A.L.F. DNA Sequencer (Pharmacia) as reported (11). Plasmid pCR 3 containing *HLA A2601* cDNA was transfected into the KE-3 esophageal SCC, RERF-LC-AI lung SCC, VA-13 fibroblast cell line, or HFK-CK-012 keratinocyte cell line in the presence of Lipofectin (GIBCO-BRL). Briefly, 5×10^5 cells in 2 ml of medium were transfected with 6 μg of the plasmid in the presence of 20 μg/ml of Lipofectin as reported (12). After 48 h, the VA-13 cells or HFK-CK-012 cells were harvested and used for the experiments. The KE-3 and RERF-LC-AI tumor cells were incubated for 14 days in the presence of 400 to 1000 mg/ml of Genetecin (GIBCO-BRL) and then were used as target cells.

Results and Discussion

The KE-4 CTL cell line was established by the repeated stimulation of PBMC from the KE-4 patient with cells of the autologous tumor

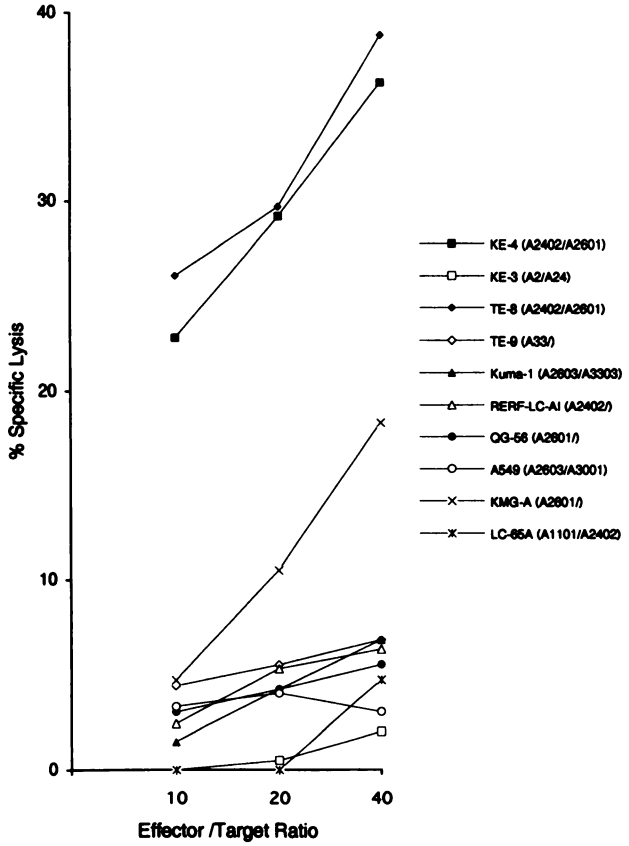

Fig. 1. Cytotoxicity of the KE-4 CTL cell line against HLA A2601$^+$ and HLA A2603$^+$ tumor cells. KE-4 CTL was tested for cytotoxicity in a 6-h ^{51}Cr-release assay at three different effector:target cell ratios (10, 20, and 40).

cell line in the presence of 100 units/ml of interleukin 2. This CTL proliferated continuously under these culture conditions for up to about 90 days. The KE-4 CTL was tested for phenotype and cytotoxicity 60 to 65 days after onset of culture. Phenotypes were >98% CD3$^+$, CD4$^-$, CD8$^+$, and T-cell receptor α/β^+ (data not shown). The CTL lysed esophageal SCC in a HLA A2601-restricted fashion (Table 1). Apparent HLA A2601-restricted lysis mediated by the KE-4 CTL was detected around day 35 after onset of culture in repeated experiments. This time frame was similar to that described for the generation of CTL in PBMC against melanomas (well-known as highly immunogenic tumors; Refs. 2 and 13). These results suggest that the frequency of CTL precursors in the PBMC of this patient is nearly equal to that of melanoma patients. However, there is little information relative to the CTL precursors in PBMC of SCC patients (1). Further studies are required to determine the frequency of CTL precursors in PBMC of SCC patients.

The autologous tumor lysis by the KE-4 CTL was inhibited by anti-CD3, anti-HLA class I mAb, or anti-CD8 but not by anti-CD4 or anti-HLA class II (DR) mAb (9, 9, 15, 32, or 40% lysis in the presence of each mAb, respectively, *versus* 37% lysis with an irrelevant mouse mAb at an effector:target cell ratio of 20). All cells of the KE-4 CTL were cryopreserved in liquid nitrogen at −130°C 70 to 80 days after onset of the culture. These cryopreserved cells were thawed in the morning of the experiments and used as effector cells in all of the following experiments.

A panel of human tumor cell lines with different HLA class I and different histology were tested for their susceptibility to the KE-4 CTL (Table 1). The KE-4 CTL showed significant levels of cytotoxicity against HLA A2601$^+$ KE-4, TE-8 and TE-11 esophageal SCC, and a low level of lysis against a HLA A2601$^+$ gall bladder adenocarcinoma (KMG-A). In contrast, this KE-4 CTL did not show significant levels of cytotoxicity (<10%) against any of the other 46 tumors tested. Resistant tumors included a HLA A2601$^+$ lung SCC (QG-56), a HLA A2601$^+$ hepatoblastoma (Hep-G2), a HLA A2603$^+$ head and neck SCC (Kuma-1), and a HLA A2603$^+$ lung adenocarcinoma (A549). The cytotoxicity was confirmed at three different effector:target cell ratios (Fig. 1). The results suggest that the KE-4 CTL preferentially lysed the HLA A2601$^+$ esophageal SCC.

Three HLA A26$^+$ tumors (KIM-1 hepatocellular carcinoma and TOC-2 and HOT ovarian serous cyst adenocarcinomas), in which the subtype of HLA A26 had not been genetically analyzed, were not lysed by the KE-4 CTL. Gene subtyping of these cell lines is in progress.

KE-4 CTL failed to lyse a HLA A2601$^+$ lung SCC (QG-56), even after treatment with 100 units/ml of IFN-γ for 2 days (5% lysis for

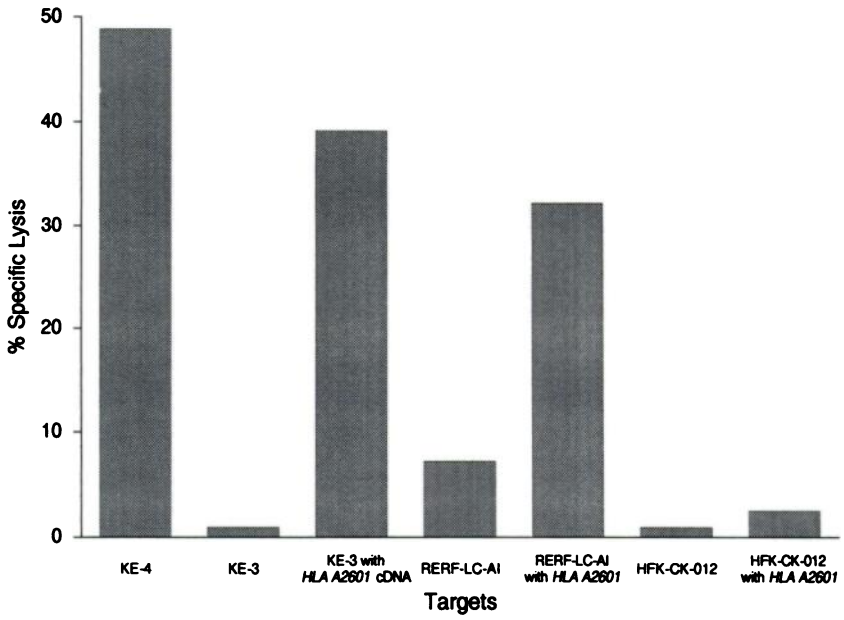

Fig. 2. Cytotoxicity of the KE-4 CTL cell line against *HLA A2601* cDNA transfected tumors. The KE-4 CTL was tested for cytotoxicity against the KE-4 autologous tumor alone, KE-3 esophageal SCC alone, or those transfected with *HLA A2601*, RERF-LC-AI lung SCC alone or those transfected with *HLA A2601*, HFK-CK-012 keratinocyte cells alone or those transfected with *HLA A2601* by a 6-h ^{51}Cr-release assay at an effector:target cell ratio of 20.

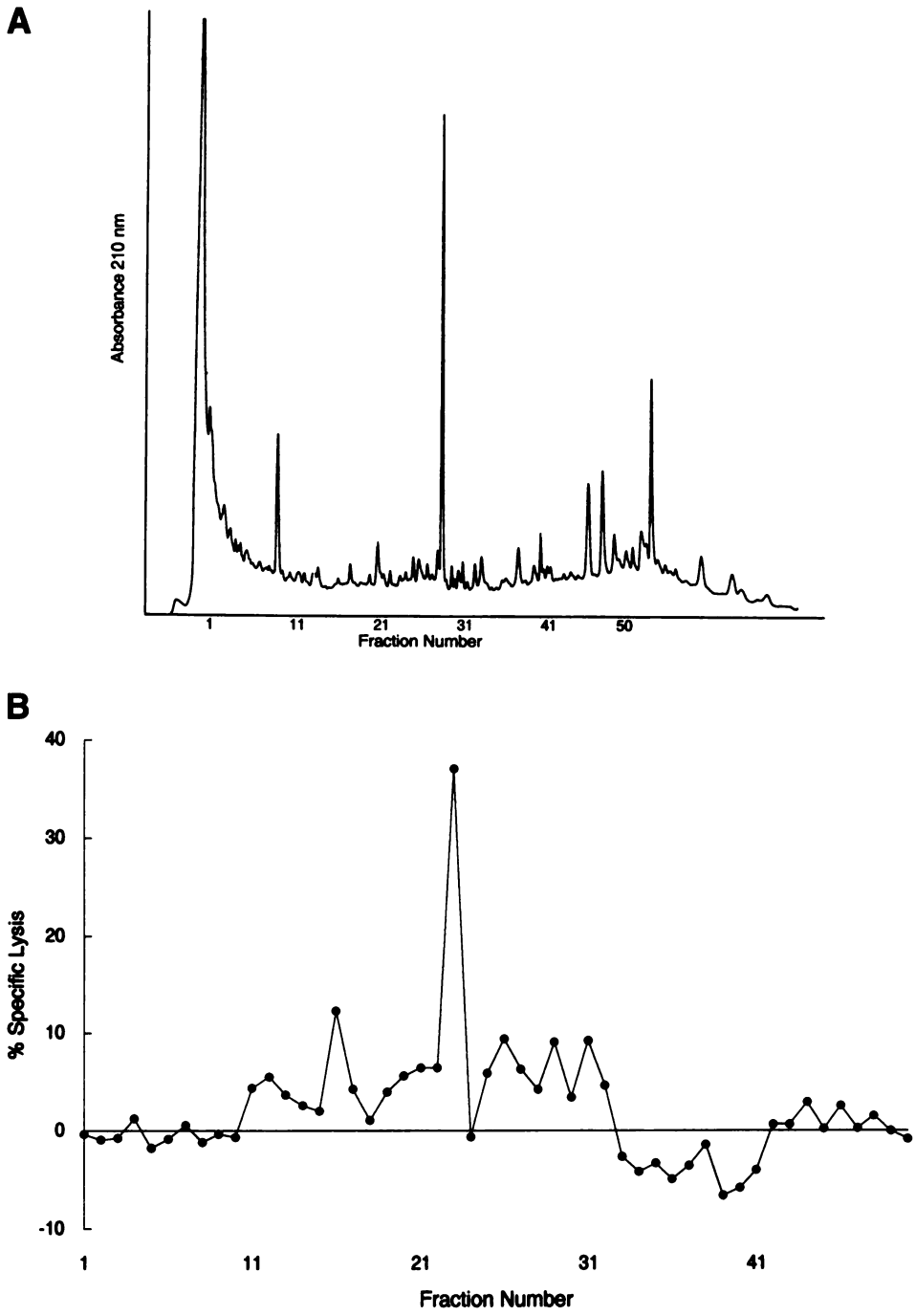

Fig. 3. HPLC fraction of the KE-4 peptides (*A*) and reconstitution of epitopes for the KE-4 CTL (*B*). *A*, peptides were eluted from the KE-4 tumor cell line by the treatment with pH 3.3 citrate-phosphate buffer, followed by fractionation on reverse-phase HPLC. *B*, KE-4 EBV-BCL was pulsed with peptides eluted from the KE-4 tumor cells, followed by testing their susceptibility to the KE-4 CTL at an effector:target cell ratio of 20 in a 6-h ^{51}Cr-release assay. Lysis of a negative control [peptide (−)] was less than 1%.

untreated *versus* 4% lysis for IFN-γ treated QG-56 at an effector: target cell ratio of 20), although the treatment significantly augmented their expression of MHC class I antigens (data not shown). These results suggest that a QG-56 lung SCC does not possess the antigen recognized by the KE-4 CTL.

To investigate if the HLA A2601$^-$ SCC possess the antigen recognized by the KE-4 CTL, *HLA A2601* cDNA was transfected into the KE-3 esophageal SCC (HLA A2/A24) and RERF-LC-AI lung SCC (HLA A2402), followed by testing their susceptibility to the KE-4 CTL (Fig. 2). The KE-4 CTL lysed both KE-3 and RERF-LC-AI tumor cells when *HLA A2601* cDNA was transfected. The results suggest that the antigen recognized by the KE-4 CTL was also distributed in some of the lung SCC in addition to the esophageal SCC. The failure of this CTL to lyse a HLA A2603$^+$ SCC (Kuma-1) may be due to the binding motif of a nonapeptide because the groove of the HLA A2603 molecule is different from that of HLA A2601.[4]

The biological significance of the modest susceptibility of a HLA A2601$^+$ gall bladder adenocarcinoma (KMG-A) to the KE-4 CTL is unclear at the present time. Some of adenocarcinomas might possess the antigen recognized by the KE-4 CTL in a HLA A2601-restricted manner.

Cytotoxicity of the KE-4 CTL against a panel of normal cells (autologous EBV-BCL, fibroblast cell lines, and keratinocyte cell lines) was investigated (Table 1). The KE-4 CTL was not cytotoxic to any of these normal cells. The human immune system appears to

[4] A. Kimura *et al.*, unpublished results.

recognize tissue differentiation antigens expressed on cancer cells (2–5, 14). Because none of the seven keratinocytes tested was HLA A2601$^+$, *HLA A2601* cDNA was transfected into HFK-CK-012 keratinocyte cell line (HLA A0203) to investigate if keratinocytes possess the antigen recognized by the KE-4 CTL. However, the KE-4 CTL did not lyse *HLA A2601* cDNA-transfected HFK-CK-012 keratinocytes (Fig. 2). The results suggest that keratinocytes do not possess the antigen recognized by the KE-4 CTL, although the other approaches, such as testing HLA A2601$^+$ keratinocytes or identification of a gene encoding the antigen, shall be taken to confirm this issue.

We fractionated bulk peptides eluted from the KE-4 tumor cell line by reverse-phase HPLC (Fig. 3*A*). Each of these fractions was pulsed to the KE-4 EBV-BCL, followed by measurement of their susceptibility to the KE-4 CTL. The result of one representative experiment was shown (Fig. 3*B*). One distinct HPLC fraction (fraction 23) displayed biological activity. Although fraction 16 dimly possessed the biological activity in repeated experiments, its biological significance is unclear. Similar fractions were also obtained from the KE-3 (HLA A2/A24) cell line and were used for the same experiments, but there were no fractions showing the biological activity (data not shown). Allogeneic (HLA A24$^+$) EBV-BCL failed to present any peptide fractions of the KE-4 tumor cells to the KE-4 CTL (data not shown).

VA-13 fibroblast cells were transfected with *HLA A2601* cDNA and were used as target cells after pulsing peptide fractions from the KE-4 tumor cells. The KE-4 CTL lysed VA-13 cells transfected with *HLA A2601* cDNA when pulsed by peptide fraction 23 (13% specific lysis at an effector:target cell ratio of 20). In contrast, the KE-4 CTL did not lyse either VA-13 cells transfected with *HLA A2601* without pulsing any peptide (2% lysis) or those pulsed by a peptide other than fraction 23 (2% lysis).

Experiments using a panel of tumor cell lines and *HLA A2601* cDNA transfection studies suggest that the antigen recognized by the KE-4 CTL is distributed in the SCC of at least two different organs (esophagus and lung). The HLA A26 molecule is expressed by approximately 20% of Japanese, 13.5% of Blacks in Cape Town, South Africa, and 16.6% of Caucasians in Yugoslavia (15). The subtype of HLA A26 found most frequently is A2601 in Japanese.[4] Therefore, substantial numbers of esophageal and lung cancer patients may be suitable candidates for specific immunotherapy by an antigen recognized by the KE-4 CTL. Thus, KE-4 CTL will be a useful tool in identifying a gene encoding its antigen.

In summary, this study showed the existence of HLA A2601-restricted CTL precursors recognizing a peptide antigen expressed on the SCC in PBMC of a patient with esophageal cancer. This information is important for a better understanding of immune recognition of autologous tumor cells in cancer patients with SCC.

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