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The Induction of Cytotoxic T Lymphocytes against HLA-A Locus-matched Lung Adenocarcinoma in Patients with Non-small Cell Lung Cancer

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To induce cytotoxic T lymphocytes (CTL) against non-small cell lung cancer (NSCLC) efficiently, the induction of CTL was attempted using HLA-A locus-shared allogeneic NSCLC cells. T cells derived from either tumor tissue specimens or the regional lymph nodes of patients with NSCLC were stimulated twice or three times with an HLA-A2/A24-positive NSCLC cell line (PC-9), and thereafter the cytotoxic activity was examined by ^{51}Cr-release assay. In patients with HLA-A24/adenocarcinoma, anti-PC-9 cytotoxicity was induced in all 6 patients tested. Anti-PC-9 cytotoxicity was induced in 2 out of 5 patients with HLA-A2 (A24$^-$)/adenocarcinoma, in 2 out of 4 patients with HLA-A24/squamous cell carcinoma, and 1 of 2 patients with HLA-A2/squamous cell carcinoma. The cytotoxic activity was observed to kill PC-9 selectively, not other NSCLC lines, and the activity was substantially blocked by anti-MHC class I antibody, but not by anti-MHC class II antibody. The PC-9-specific CTL produced γ-interferon in response to autologous tumor cells. These results indicated that the anti-PC-9 cytotoxicity was mediated by cytotoxic T lymphocytes that may recognize the T cell epitope(s) shared and presented by HLA-A2 and/or HLA-A24-positive NSCLC.

Key words: Non-small cell lung cancer — Tumor-infiltrating lymphocyte — Lymph node lymphocyte — Cytotoxic T lymphocyte — MHC class I

The majority of patients with non-small cell lung cancer (NSCLC) die within a few years when the disease is unresectable, and chemotherapy or radiotherapy brings little benefit as regards the prolongation of survival.[1,2] Therefore, a new therapeutic modality is needed for NSCLC. Immunotherapy has been performed using biologic response modifiers[3,4] or cytokine-activated lymphocytes,[5,6] but little or no therapeutic advantage has been found. It is known that anti-tumor T cell response exists in patients at an early stage of the disease,[7–9] though the mechanism of the recognition system by tumor-reactive T cells has not been clarified yet, since it is hard to prepare an adequate number of fresh tumor cells of NSCLC in order to induce autologous cytotoxic T lymphocytes (CTLs). Recent investigations of malignant melanoma have revealed the existence of melanoma-associated antigens, such as the products of the *MAGE* gene family,[10–12] tyrosinase,[13] MART-1/Melan-A,[14] and gp100,[15] which provide CTL epitopes in the context of MHC class I.[16–19] This evidence means that the tumor-associated antigens as recognized by CTL are shared among patients with an identical HLA haplotype. Based on those findings, in this study we attempted to induce CTL against an allogeneic lung adenocarcinoma line, PC-9 (HLA-A2/A24), from T cells derived from tumor-infiltrating lymphocytes (TIL) and/or regional lymph node lymphocytes (RLNL) of NSCLC patients possessing HLA-A2 or HLA-A24.

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MATERIALS AND METHODS

Culture medium (CM) CM consisted of RPMI1640 (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL), 10 m*M* HEPES, 100 units/ml penicillin G and 100 mg/ml streptomycin sulfate.

Preparation of TIL and autologous tumor cells TIL and autologous tumor cells were prepared as described previously.[20] Briefly, fresh tumor tissue was excised from surgical specimens and then minced into small pieces with scissors. This minced tissue was placed in a flask with a mixture of 0.1 mg/ml DNase type I, 1 mg/ml collagenase type IV, and 0.5 mg/ml hyaluronidase type V (all from Sigma Chemical Co., St. Louis, MO) in RPMI 1640 and then stirred at room temperature for 2 to 4 h. The resultant cell suspension was washed in Hanks' balanced salt solution (HBSS) and subjected to Ficoll-Hypaque gradient (LSM, Organon Teknika, Durham, NC) centrifugation (1,000*g*, 30 min). The interface was collected, suspended in CM, and subsequently subjected

to two-layered (75% and 100%) Ficoll-Hypaque discontinuous density gradient centrifugation at 1,000*g* for 20 min. Cells from the 100% interface and 75% interface were used as TIL and tumor cells, respectively. TIL and tumor cells were also obtained from either malignant pleural effusion or ascites using the above discontinuous gradient centrifugation method.

Preparation of RLNL Macroscopically non-metastatic regional lymph nodes were obtained at the time of surgery. Each lymph node was divided into two parts for the histological diagnosis and for this study. The latter part of each lymph node was squeezed between a pair of glass slides in HBSS and then passed through a gauze filter. The cells were washed twice with HBSS and resuspended in CM. Approximately 5 to 10% of macrophages were removed by plastic adherence, and non-adherent cells were used as RLNL.

Tumor cell lines NSCLC cell lines (PC-9, Sq-1, and A549) were obtained from Dr. Kyogo Itoh (Kurume University, Kurume), who also determined the genotype of the HLA-A locus of these lines. PC-9 is an HLA-A2/A24, -B7/B51 positive adenocarcinoma line, Sq-1 is an HLA-A11/24, -B52/B62 positive squamous cell line, and A549 is an HLA-A26/A30 positive adenocarcinoma line. QG56,[7] an HLA-A26 positive lung squamous cell carcinoma line, was also used as a stimulator.

Culture of lymphocytes TIL and RLNL obtained as described above were stimulated with solid-phase anti-CD3 mAb (Ortho Pharmaceutical Corporation, Raritan, NJ) for 48 h and expanded in CM containing 50 IU/ml recombinant interleukin-2 (Takeda Chemical Industry, Osaka) for 10 to 14 days.[21] Subsequently, TIL/RLNL were stimulated with irradiated allogeneic tumor cell lines weekly for 2 to 4 weeks.

Serotyping of cultured T cells The expanded TIL/LNL were subjected to MHC class I typing, performed by SRL Co., Tokyo.

Monoclonal antibodies (mAbs) Anti-CD4 mAb (phycoerythrin (PE)-conjugated) and anti-CD8 mAb (FITC-conjugated) were purchased from Becton & Dickinson (Mountain View, CA). The culture supernatants of ATCC HB82 and ATCC HB95 (American Type Cell Culture, Rockville, MD) were used as a source for anti-HLA DR mAb (L227) and anti-HLA A, B, C (W6/32), respectively. mAb for the extracellular domain of erbB2 (TA-1) was purchased from Oncogene Science, Uniondale, NY. Anti-ICAM-1 mAb and anti-B7-1 mAb were obtained from Becton & Dickinson, and anti-B7.2 mAb was purchased from PharMigen, San Diego, CA.

Flow cytometry For the direct single or two-color analysis, cells (5×10^5) were incubated for 30 min at 4°C with PE and/or FITC-conjugated mAb or murine control antibodies. In indirect staining, the cells were incubated with the first mAb, or murine IgG (control) in the staining medium (HBSS with 5% fetal calf serum) for 30 min at 4°C. After washing, the cells were stained with FITC-conjugated anti-mouse IgG. The stained cells were fixed with 1% paraformaldehyde and then kept at 4°C until analysis. A fluorescence analysis was performed using a flow cytometer.

Cytotoxicity assay The cytotoxicity of tumor-stimulated lymphocytes against tumor cells was examined by a standard ^{51}Cr-release cytotoxicity assay. The tumor targets were labeled for 1 h at 37°C with ^{51}Cr and washed. The target cells (5×10^3) were incubated with 2.5×10^4 to 1×10^5 effector cells (effector/target ratio=5 to 20) in 200 ml of CM in a 96-well round-bottomed microtiter plate for 4 h at 37°C. The supernatant (100 ml) was collected and the samples were counted in a gamma counter. The percent specific lysis was evaluated by measuring the experimental minus spontaneous divided by the maximum minus spontaneous ^{51}Cr release.

Blocking of cytotoxic activity by mAb The target cells were incubated with 1/10-diluted supernatant of L227-producing hybridoma or BB7.2-producing hybridoma for 30 min at 4°C after labeling with chromium. The mAb was added to the effector cells and target cells at 1/4-dilution, and a 4 h ^{51}Cr-release assay was performed as described above.

Detection of γ-interferon (γ-IFN) by enzyme-linked immunosorbent assay (ELISA) The PC-9-stimulated lymphocytes (10^6/ml) were cocultured with autologous tumor cells (10^5/ml) for 18 h, and the amount of γ-IFN in the supernatant was measured using a Human Interferon Gamma ELISA Test Kit (Gibco BRL) according to the instruction manual.

RESULTS

Induction of PC-9-specific cytotoxicity in T cells RLNL and/or TIL were obtained from a total of 19 patients, and expanded 50- to 100-fold for 10 to 14 days by *in vitro* culture with solid-phase anti-CD3 mAb and low-dose interleukin-2 in all cases. The results of serotyping of the cultured T cells revealed that HLA-A2 and HLA-A24 were the major haplotypes of the HLA-A locus in these patients (52.6% and 57.9%, respectively) (Table I). Nineteen patients (94.7%) were found to be positive for either HLA-A2 or HLA-A24. Therefore, CTL induction was tested using HLA-A2/A24-positive PC-9 in all cases. The results are shown in Table I. In all 6 cases with HLA-A24/adenocarcinoma, anti-PC-9 cytotoxicity was induced. Anti-PC-9 cytotoxicity was induced in 2 out of 4 patients with HLA-A24/squamous cell carcinoma, 2 out of 5 patients with HLA-A2/adenocarcinoma, and 1 of 2 patients with HLA-A2/squamous cell carcinoma. In case 18 (HLA-A11/A31 negative squamous cell carcinoma), the induction of cytotoxicity against PC-9 failed.

Table I. Induction of Cytotoxicity against an HLA-A2/A24 Positive Lung Adenocarcinoma Cell Line (PC-9)

Case	MHC class I			Cell type[a]	Cell source	Cytotoxicity induction[b]
	A	B	Cw			
1	24	35/48		Adeno	TIL/RLNL	S
2	9/24	5/52	3	Adeno	TIL	S
3	11/24	54/75	1/3	Adeno	TIL	S
4	24/31	7/52	7	Adeno	TIL/RLNL	S
5	2/24	52/54	1	Adeno	RLNL	S
6	2/24	62/75	3/4	Adeno	RLNL	S
7	2/11	7/54	1/7	Adeno	RLNL	S
8	2/26	60/70		Adeno	TIL/RLNL	U
9	2/31	5/51		Adeno	TIL	U
10	2/33	44/51		Adeno	TIL	S
11	2/31	35/56	3/4	Adeno	TIL	U
12	24	61	3/7	Squamous	RLNL	S
13	24	35	3	Squamous	TIL	U
14	24	7/35		Squamous	TIL	U
15	9/24	5/52		Squamous	TIL	S
16	2	35/52	3	Squamous	TIL/RLNL	S
17	2/26	39/61		Squamous	TIL	U
18	11/31	51/62		Squamous	RLNL	U
19	2/24	52/61		Poor dif. carcinoma[c]	RLNL	S

a) Adeno, adenocarcinoma; Squamous, squamous cell carcinoma.
b) S, successful for induction of cytotoxicity; U, unsuccessful for induction of cytotoxicity.
c) Cell type was not definitely determined due to poor differentiation.

Table II. Profile of PC-9-stimulated Lymphocytes

Case	Surface molecule			Cytotoxicity (E/T ratio) (%)	% inhibition by anti-class I[a] (%)
	$CD3^+$ (%)	CD4/CD8	$CD3^-$ $CD56^+$ (%)		
1 (TIL)	88.8	0.12	11.6	57.1 (5 : 1)	40.0
1 (RLNL)	99.3	0.03	8.46	25.0 (5 : 1)	54.0
2 (TIL)	76.1	0.98	23.4	41.8 (5 : 1)	43.9
3 (TIL)	96.4	0.34	1.5	35.5 (5 : 1)	25.4
4 (TIL)	91.1	2.35	1.2	30.7 (5 : 1)	33.3
5 (RLNL)	97.9	0.13	0.2	21.5 (20 : 1)	79.0
6 (RLNL)	ND[b]	ND	ND	82.6 (20 : 1)	22.3
7 (RLNL)	83.8	0.16	12.8	84.3 (20 : 1)	24.8
10 (TIL)	90.0	0.67	5.3	24.9 (5 : 1)	24.0
12 (RLNL)	96.4	0.37	2.7	73.7 (20 : 1)	32.7
15 (TIL)	84.0	0.37	11.5	53.6 (20 : 1)	35.2
16(TIL)	90.8	0.17	5.4	25.6 (5 : 1)	46.1
16 (TLNL)	98.4	0.08	1.0	52.9 (5 : 1)	28.2
19 (RLNL)	98.0	0.06	1.3	77.3 (20 : 1)	35.8

a) % inhibition was calculated as follows: (cytotoxicity without anti-class I)/(cytotoxicity with anti-class I) × 100.
b) ND, not done.

In case 19 (HLA-A2/poorly differentiated carcinoma), PC-9 cytotoxicity was induced. No shared HLA-B locus or -C locus was observed in the patients for whom the induction of cytotoxicity succeeded.

Characterization of CTL against PC-9 The phenotypic profile and cytotoxicity of the lymphocytes with anti-PC-9 cytotoxicity are presented in Table II. $CD3^+$ cells were always the major population (76.1%–99.3%), though

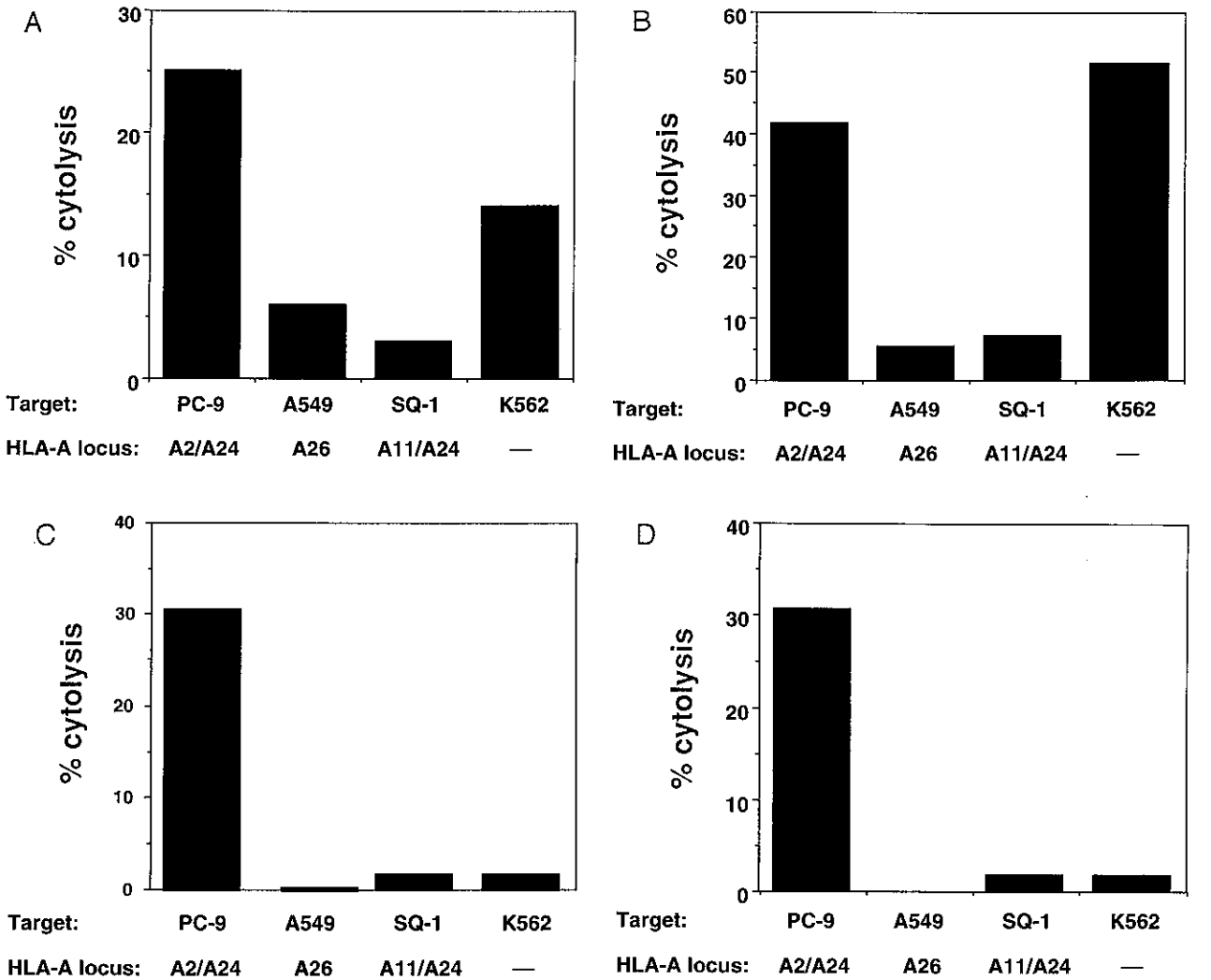

Fig. 1. Cytotoxicity of lymphocytes stimulated with PC-9. TIL or RLNL from lung cancer patients were expanded by solid-phase anti-CD3 antibody and low-dose rIL-2, followed by multiple stimulation with irradiated PC-9, then examined for cytotoxic activity against tumor targets by ^{51}Cr-release assay at an effector/target ratio of 5/1. Four representative cases are shown. A, Case 1: HLA-A24-positive adenocarcinoma; B, Case 2: HLA-A9/A24-positive adenocarcinoma; C, Case 4: HLA-A24/A31-positive adenocarcinoma; D, Case 10: HLA-A2/A33-positive adenocarcinoma. Case numbers are identical to those in Table II.

the ratio of CD4/CD8 varied among the cases (0.03–2.35). $CD3^-$ $CD56^+$ cells, which exert non-specific cytotoxicity, were a major population in case 2 although they were only a minor population in others. Anti-PC-9 cytotoxic activities of four representative cases are shown in Fig. 1. All of the PC-9-stimulated lymphocytes exhibited selective cytotoxicity against PC-9 (Fig. 1, A, B, C and D) at an E/T ratio of 5 : 1. None of them killed A549 (an HLA-A26/30-positive adenocarcinoma) or SQ-1 (an HLA-A11/A24-positive squamous cell carcinoma). In the PC-9-stimulated lymphocytes derived from case 2, non-specific killing against K562 (NK-sensitive) was also observed, as well as against PC-9 (Fig. 1B). This could be explained by the fact that the lymphocytes prepared from case 2 included a relatively high proportion of $CD3^-$ $CD56^+$ cells (23.4%).

Blocking effect of anti-MHC class I mAb To test the MHC class I restriction of the anti-PC9 cytotoxicity, anti-MHC class I (HLA-A, B, C) mAb was added during the cytotoxicity assay. The anti-PC-9 cytolytic activity of the PC-9-stimulated lymphocytes derived from case 2 was substantially blocked by anti-MHC class

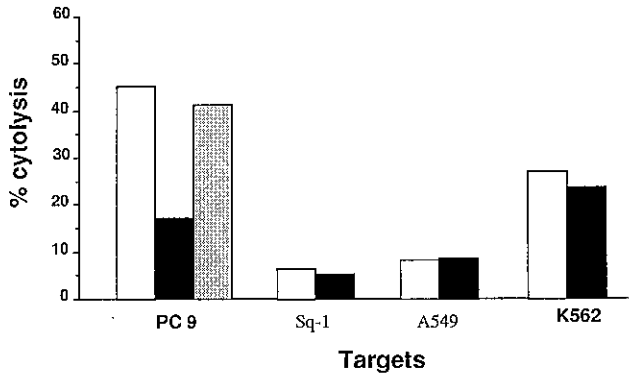

Fig. 2. Blocking effect of anti-MHC class I antibody on the anti-PC-9. Cytotoxicity assay was performed in the absence or presence of anti-MHC antibody. Representative data from case 2 are shown. Open bars: medium; closed bars: with anti-HLA-A, B, C mAb; and shaded bar: with anti-HLA-DR mAb.

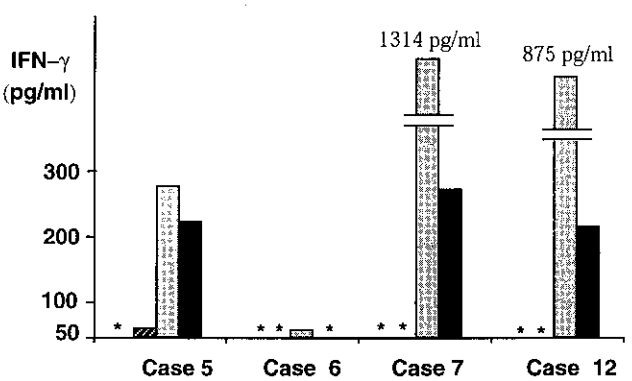

Fig. 3. Production of γ-IFN by anti-PC-9 CTL. The anti-PC-9 CTL derived from 4 cases were co-cultured with or without PC-9 or autologous tumor cells for 18 h, and γ-IFN in the supernatant of the mixed culture was measured by ELISA. Open bars: autologous tumor cells only; hatched bars: CTL only; shaded bars: CTL/PC-9; closed bars: CTL/autologous tumor cells. PC-9 only: <50 pg/ml. $*$ <50 pg/ml.

I mAb, but not by anti-HLA-DR mAb (Fig. 2). However, the cytolytic activity against K562 was not abrogated by the addition of anti-HLA class I mAb. Similar results were observed in all experiments on anti-PC-9 cytotoxicity. The percentage inhibition of CTL activity by anti-HLA class I mAb ranged from 22.3% to 79.0% (Table II). These results indicate that the anti-PC-9 cytotoxicity was mediated by tumor-specific CTL. However, the blocking effect of anti-MHC class I mAb was always incomplete in multiple experiments among all the cases (Table II), even if the concentration of the mAb was increased. Specific but non-MHC restricted recognition of PC-9 might also exist in the cytotoxic response.

Table III. Induction of CTL against Lung Cancer by HLA A-locus/cell Type-matched Allogeneic Tumor Cell Lines

HLA A-locus shared by patients	Cell type of patients[a)]	Cell line used as stimulator (HLA A-locus)	Success rate of CTL induction (%)
2 and/or 24	Adeno	PC-9 (A2/A24)	72.7 (8/11)
24	Squamous	SQ-1 (A11/A24)	0 (0/4)
26	Adeno	A549 (A26)	0 (0/1)
26	Squamous	QG56 (A26)	0 (0/1)

a) Adeno, adenocarcinoma; Squamous, squamous cell carcinoma.

Response of anti-PC-9 CTL against autologous tumor cells Anti-PC-9 CTL derived from 4 cases were examined for anti-autologous tumor cell cytotoxicity, but without success, owing to a low uptake of ^{51}Cr by the tumor cells. Therefore, γ-IFN production by the anti-PC-9 CTL in response to autologous tumor cells was examined. The CTL produced substantial amounts of γ-IFN when cocultured with autologous tumor cells as well as with PC-9 (Fig. 3). From these results, the CTL against PC-9 also respond to autologous tumor cells, resulting in the production of γ-IFN, which implies that these CTL may recognize the T cell epitope(s) shared and presented by HLA-A24 and/or HLA-A2-positive NSCLC.

Induction of CTL using HLA-A locus/cell type-shared cell lines To investigate the stimulatory function of other available tumor cell lines such as Sq-1, A-549 or QG56, the TIL/RLNL derived from 15 cases were subjected to CTL induction against those cell lines in an HLA-A locus/cell type-matched setting. Although the induction of CTL against PC-9 was consistently successful (8 of 11 cases) with HLA-A2 and/or 24 and adenocarcinoma, it was unsuccessful in all the cases using other cell lines as stimulators (Table III).

Characterization of lung cancer cell lines used for stimulation of lymphocytes From the above results, PC-9 was apparently a reliable stimulator of T lymphocytes in the induction of CTL. To investigate the differences of the stimulatory potential of such cell lines to T cells, the cell surface molecules of the tumor cell lines used (A549, QG-56, Sq-1 and PC-9) were analyzed by flow cytometry. MHC class I expression was higher in PC-9 than in the others, and only PC-9 expressed a substantial level of HLA-DR. High-level expression of intercellular adhesion molecule (ICAM)-1 was detected on PC-9. QG56 also expressed ICAM-1, while A549 and Sq-1 did not. These results thus indicate that PC-9 is an excellent

antigen presenter. B7-1/B7-2 are known to be co-stimulatory molecules during antigen recognition by T cells[22]; however, none of the tumor lines tested showed any expression of these molecules.

DISCUSSION

Human lung cancer cells are recognized by host T lymphocytes,[15–17, 20] but *in vitro* stimulation with tumor cells is essential to detect the tumor-directed response of such T cells.[21] However, one continuing dilemma is that an adequate number of autologous tumor cells for multiple stimulation of T cells cannot always be prepared because of the low success rate of the tumor cell culture. In this study, we therefore tried to induce NSCLC-specific CTL using HLA-A locus/cell type-matched allogeneic tumor cell lines to resolve the above problem, based on the hypothesis that MHC class I-restricted shared antigens exist in lung cancer. As a result, allogeneic CTL were effectively induced from TIL/RLNL, especially in the HLA-A24-matched setting (6 out of 6 cases with adenocarcinoma and 2 out of 4 cases with squamous cell carcinoma). These findings indicated that PC-9 is useful for inducing lung cancer-specific CTL, and thus that HLA-A24-restricted antigens are shared by both cell types. In the patients with HLA-A2 but not -A24, anti-PC-9 CTL could also be generated in 2 out of 5 cases with adenocarcinoma and 1 out of 2 cases with squamous cell carcinoma. It appears that PC-9-derived antigens are multiple and cross-reactive among the cell types.

We can not completely rule out the possibility that the CTL response in this study was a response against allogeneic MHC molecules. However, we considered that the allogeneic response made little or no contribution to the overall CTL response for the following reasons. (a) There was no shared HLA-B or -C locus among the patients in whom the induction of CTL was successful (Table II). (b) Anti-PC-9 CTL produced γ-IFN in response to autologous tumor cells. The cytokine production in response to autologous tumor cells was at a low level compared to that of PC-9. This was considered to be due to the poor viability of autologous tumor cells, and to the higher expression of adhesion molecules, such as ICAM-1, on PC-9. (c) Anti-PC-9 CTL derived from some cases also exhibited HLA class I (HLA-A2/A24 and B7/B51)-restricted killing of another lung cancer cell line, 1-87 (HLA-A2/A11, B46/B54, and Cw1), which shares only HLA-A2 with PC-9 (Fujie *et al.*, unpublished data). To resolve this issue fully, we are trying to establish clones of the anti-PC-9 CTL.

The remarkable difference of characteristics between PC-9 and other NSCLC lines (SQ-1, A549 and QG56) thus seemed to be related to a higher expression level of MHC molecules and ICAM-1 in PC-9. This might mean that PC-9 has a high potential for antigen presentation, and thus PC-9 might stimulate the T cells very effectively through co-stimulatory molecules, as well as signaling via the MHC/antigen complex.

Anti-PC-9 CTL were also induced in several HLA-A locus-matched, but cell type-unmatched patients. This raises two possibilities. One is that PC-9 might present other shared antigens restricted by an HLA-B or -C locus as reported in melanoma.[11, 12] The other is that non-MHC-restricted response might be involved in the anti-PC-9 CTL response, since PC-9 expresses a high level of MUC1, which is a non-MHC-restricted tumor antigen expressed in many kinds of human tumors,[23, 24] and the cytolytic activity against PC-9 cannot be completely blocked by an anti-MHC class I antibody. Examination of this issue is in progress.

HLA-A2 and -A24 are common haplotypes of the HLA-A locus in Japanese (approximately 40% and 60%, respectively),[25] and our present results on the serotyping of MHC class I of Japanese NSCLC patients conformed to this pattern. Lung cancer has become the most frequent cause of death from malignancy in males, and its frequency is considered likely to increase in Japan.[26] Based on the above findings, HLA-A2/24-restricted antigens, which induce specific CTL against NSCLC, are expected to be ideal target molecules for specific immunotherapy in lung cancer patients possessing HLA-A2/A 24. Studies to identify acid-eluted antigens of PC-9 are in progress.

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