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Autologous Tumor-specific Cytotoxic T Lymphocytes in a Patient with Lung Adenocarcinoma: Implications of the Shared Antigens Expressed in HLA-A24 Lung Cancer Cells

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Human lung adenocarcinoma-specific cytotoxic T lymphocytes (CTL) were generated by multiple stimulations with autologous tumor cells (named A110L) from regional lymph node lymphocytes and tumor-infiltrating lymphocytes expanded by solid-phase anti-CD3 monoclonal antibody (mAb) and recombinant interleukin-2. The CTL lysed A110L but failed to kill either autologous B lymphocytes immortalized by the Epstein-Barr virus or K562. The killing activity of the CTL against autologous A110L was inhibited by anti-MHC class I mAb (W6/32), but not by anti-MHC class II mAb. The CTL produced interferon-γ and GM-CSF in response to A110L and the production was completely blocked by the addition of anti-MHC class I mAb. The HLA type of the CTL was HLA-A2/A24, B52/B54, Cw1/–. Allele-specific deletion of HLA-A2 molecules was observed in A110L by staining with anti-HLA-A2 mAb. A partial blocking effect on the cytokine production from the CTL was also obtained with anti-CD8, and anti-HLA-A24 mAbs, but not with anti-MHC class II, anti-CD4 and anti-HLA-A2 mAbs. To analyze further the mechanism of antigen recognition by the CTL, the cross reactivity of the CTL against several HLA-A locus-matched (HLA-A24+) and mismatched allogeneic tumor cells (HLA-A24–) was investigated. The A110L-specific CTL showed a weak but significant cytotoxicity against some HLA-A24 positive lung cancer cell lines, such as Sq-1 (HLA-A11/A24, squamous cell carcinoma) and PC-9 (HLA-A2/A24, adenocarcinoma), but failed to kill HLA-A locus-mismatched allogeneic tumors. This cross reactivity of the CTL against Sq-1 and PC-9 was blocked by anti-MHC class I mAb. These results thus demonstrate that shared common tumor antigens might exist among lung cancer cells in the context of HLA-A24.

Key words: CTL — Lung cancer — HLA-A24 — Shared tumor antigen — Regional lymph node

Since the first discovery of tumor antigens recognized by cytotoxic T lymphocytes (CTL) in human melanoma,[1)] several tumor antigens have been identified.[2–5)] These studies have led to a better understanding of the specific recognition of tumor cells by CTL. More recently, a novel specific immunotherapy against malignancy using tumor-specific peptide antigen and dendritic cells has also been applied and some benefit has been reported.[6)]

Lung cancer has become the most frequent cause of death in Japanese males. Although surgical resection is still the only effective therapy,[7, 8)] about 30% of all patients die from lung cancer within 5 years after operation even in the case of stage I disease. We previously reported that tumor-reactive T cells exist in the tumor tissue,[9, 10)] pleural cavity,[11)] and regional lymph nodes (unpublished data) in lung cancer patients. However, the mechanism of the tumor recognition by such T cells remains unclear. It was also recently reported that the recognition of lung cancer cells by CTL was restricted by such MHC class I molecules as HLA-Aw68,[12)] HLA-A2.[13)] Among the various HLA-A locus haplotypes, HLA-A24 has a frequency of approximately 60% in the Japanese.[14)] If HLA-A24-restricted common tumor antigens are identified in lung cancer cells, cancer-specific immunotherapies should be applicable to a large number of Japanese with lung cancer. It is thus necessary to investigate antigen-specific immunotherapy against lung cancer to demonstrate the existence of a shared antigen(s) recognized by CTL among lung cancer cells. In the present study, we induced lung cancer-specific CTL in order to analyze the possible existence of a shared common antigen(s) in lung cancer cells.

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 tumor-infiltrating lymphocytes (TIL) and autologous tumor cells The specimens were obtained from a 61-year-old man with lung adenocarcinoma at the time of surgery. The HLA serotype of the patient was HLA-A2/A24, B52/B54, Cw1/– by the serological microcytotoxicity assay (as described below). TIL and autologous tumor cells were prepared as described previously.[9, 10)] Briefly,

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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, St. Louis, MO) in RPMI 1640 and then stirred at room temperature for 2 to 4 h. The resultant cell suspension was washed in HBSS, applied to a Ficoll-Hypaque gradient (LSM, Organon Teknika, Durham, NC) and centrifuged (1000*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 1000*g* for 20 min. The cells from the 100% interface and 75% interface were used as TIL and tumor cells, respectively. An autologous tumor cell line (named A110L) was generated from the above preparation and has been maintained for >9 months as a monolayer culture by serial passages in RPMI 1640 with 10% fetal calf serum (FCS).

Preparation of regional lymph node lymphocytes (RLNL) Regional lymph nodes from lung cancer patients were obtained at the time of surgery. Each lymph node was divided into two parts for 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.[15)]

Preparation of peripheral blood lymphocytes (PBL) Heparinized peripheral blood was obtained from the patient at the time of surgery. The PBL were prepared from blood samples by the use of Ficoll-Hypaque gradients (LSM; Organon Teknika, Durham, NC).[15)]

Tumor cell lines Non-small cell lung cancer cell lines (PC-9, Sq-1, A549 and RERF-LC-AI) were kindly donated by Dr. Kyogo Itoh, Kurume University, Kurume, and the genotypes of the HLA-A locus of the cell lines were determined by them.[16)] PC-9 is an HLA-A2/A24-positive adenocarcinoma line, Sq-1 is an HLA-A11/24-positive squamous cell carcinoma line, A549 is an HLA-A26/A30-positive adenocarcinoma line and RERF-LC-AI is an HLA-A24-positive squamous cell carcinoma line. A110L, A112L, A803L (lung adenocarcinoma) and A609HN (squamous cell carcinoma obtained from metastatic lung tumor in a patient with head and neck cancer) were established in our laboratory and their HLA genotypes were identified either by Dr. Kyogo Itoh, Kurume University, Kurume, or Shionogi Co., Tokyo, as shown in Table II. K562 is an erythroleukemia cell line lacking MHC class I expression on the cell surface and is sensitive to NK cells. Raji is a Burkitt lymphoma line and is sensitive to lymphokine-activated killer cells.

Induction of autologous tumor (A110L)-specific CTL TIL and RLNL obtained as described above were stimulated with solid-phase anti-CD3 monoclonal antibody (mAb) (Ortho Pharmaceutical Corporation; Raritan, NJ) for 48 h and expanded in CM containing 50 unit/ml recombinant interleukin-2 (rIL-2) (kindly donated by Takeda Chemical Industries, Osaka) for 14 days.[17)] The expanded lymphocytes were aliquoted and cryopreserved in –130°C. Subsequently, the cryopreserved TIL/RLNL was rapidly thawed and then stimulated *in vitro* with irradiated (10000 rad) cultured A110L weekly at a tumor-to-lymphocyte ratio of 1:10 in CM with 50 unit/ml rIL-2 for more than 3 weeks.[18)]

HLA serotyping of patients The peripheral blood was obtained from patients with lung cancer and was subjected to MHC class I serotyping (performed by SRL Co., Tokyo).

mAb FITC-conjugated Nu-T3 (anti-CD3), Nu-TH/I (anti-CD4) and Nu-B2 (anti-CD20) were purchased from Nichirei Corp., Tokyo. PE-conjugated Leu-19 (anti-CD56) and Leu-2a (anti-CD8) were purchased from Becton & Dickinson, Mountain View, CA for phenotypic analysis. The culture supernatants of ATCC HB-145 (IVA12; anti-HLA-DR, DP, DQ mAb), ATCC HB-95 (W6/32; anti-HLA-A, B, C), HB-82 (BB7.2; anti-HLA-A2), HB-164 (A11.1; anti-HLA-A24 and anti-HLA-A11), CRL-8002 (OKT 4; anti-CD4), CRL-8014 (OKT 8; anti-CD8) were used as sources for blocking mAbs (American Type Cell Culture, Rockville, MD).

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 either the first mAb or murine IgG (control) in the staining medium (HBSS with 5% FCS) for 30 min at 4°C. They were washed and stained with FITC-conjugated anti-mouse IgG. The stained cells were fixed with 1% paraformaldehyde and then kept at 4°C until analysis. Fluorescence analysis was performed using a flow cytometer.[15, 18)]

Cytotoxicity assay The cytotoxicity of CTL 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 indicated effector cells (effector/target ratio = 5/1 to 80/1) in 200 μl of CM in a 96-well round-bottomed microtiter plate for 4 h at 37°C. The supernatant (100 μl) 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 CTL activity by mAb The ^{51}Cr-labeled target cells were preincubated for 1 h at 37°C with 1/2-diluted supernatant of hybridomas. The mAb was added to the effector cells and target cells in 1/4-dilution at the final concentration, and a 4-h ^{51}Cr-release assay was performed as described above.[18)]

Detection of interferon-γ (IFN-γ) and granulocyte macrophage-colony stimulating factor (GM-CSF) by enzyme-linked immunosorbent assay (ELISA) The CTL (10^6/ml) induced by the above method were cocultured

with autologous tumor cells (AT) (10^5/ml) for 24 h, and the amount of IFN-γ in the supernatant was measured using a Human Interferon Gamma ELISA Test Kit (Gibco BRL, Grand Island, NY or Amersham International plc, England) according to the instruction manual. In the mAb blocking assay, 1/4-diluted supernatant of hybridoma was added to the coculture of the CTL and AT.[18] The mAb was confirmed not to affect the cytokine assay in preliminary experiments.

RESULTS

Induction of CTL Freshly isolated RLNL and TIL from a surgical specimen were first expanded by stimulation with solid-phase anti-CD3 mAb, followed by a low dose of rIL-2 (50 unit/ml) for 14 days, then these lymphocytes were stimulated with irradiated A110L as described in detail in "Materials and Methods." RLNL without tumor stimulation showed no cytotoxicity against A110L (data not shown). A low level of cytotoxicity against A110L (15.8% lysis at an E/T ratio of 20/1) was observed after two stimulations of RLNL with A110L. The CTL activity against A110L was first compared among TIL, RLNL and PBL after 3 successive tumor stimulations. As shown in Fig. 1a, successful CTL induction was observed in both TIL and RLNL (70.5% and 35.3% lysis respectively, at an E/T ratio of 20/1), but the induction was modest in PBL (10.8% of lysis) after three autologous tumor stimulations. These results may be ascribed to differences in the frequencies of CTL precursors in TIL, RLNL and PBL. The cytolytic activity was inhibited by the addition of anti-MHC class I mAb (Fig. 1b). The CTL generated from TIL showed no cytotoxicity against autologous B cells immortalized by the Epstein-Barr virus (EBV) (Fig. 1c). To analyze the specificity of the CTL, a cold target inhibition assay was performed. AT unlabeled with ^{51}Cr (cold target) inhibited the CTL activity against AT in a dose-dependent manner, but unlabeled K562, which does not express MHC class I molecules, exhibited no inhibitory effect (Fig 2). Because a large number of lymphocytes could be obtained from the regional lymph nodes but not from the tumor tissue, CTL were mainly generated from RLNL in the following experiments.

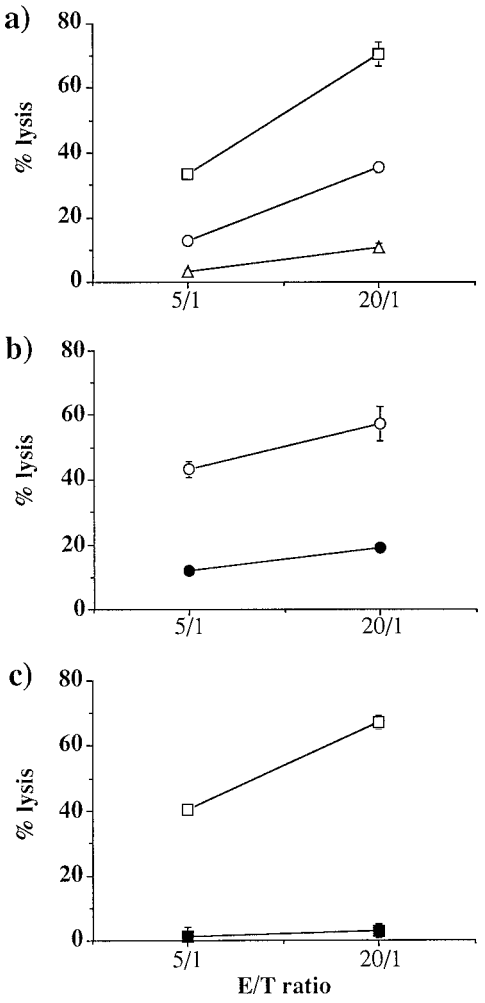

Fig. 1. Induction of AT-specific CTL from TIL and RLNL in a patient with lung adenocarcinoma. RLNL (a, b), TIL (a, c) and PBL (a) were stimulated with irradiated AT in the presence of 50 unit/ml IL-2 as described in detail in "Materials and Methods." The cytolytic activity was assessed by a standard 4-h ^{51}Cr-release assay. a, CTL activities generated from TIL, RLNL and PBL against AT are indicated. □ TIL, ○ RLNL, △ PBL. b, The CTL activity from RLNL was inhibited by the addition of anti-MHC class I mAb. ○ without mAb, ● with anti-MHC class I mAb. c, The CTL lysed AT, but not autologous EB virus-transformed B cells. □ AT, ■ auto-EBV-B.

Specific cytokine production from CTL in response to AT The CTL generated from RLNL were assayed for cytokine production in response to stimulation by A110L. The production of IFN-γ and GM-CSF was induced by AT stimulation of the CTL and was completely inhibited by the addition of anti-MHC class I mAb, but not by anti-MHC class II mAb (Table I). No cytokine production was detected in either the culture supernatant of the unstimulated CTL or that of irradiated A110L alone. The HLA-A haplotype of the patient was HLA-A2/A24, as described in "Materials and Methods." Interestingly, however, A110L expressed no HLA-A2 on the cell surface as judged from the flow cytometry analysis, although EBV-B from the patient expressed HLA-A2, as reported by Hoshino *et al.*[19] in other cancer cell lines. Allele-specific deletion of HLA-A2 of A110L was confirmed by genotypic examination (Table II). To analyze further the recognition mechanisms of the CTL against A110L, blocking assays with anti-MHC class I, MHC class II, HLA-A2, HLA-A24, CD4 and CD8 mAbs

were performed as shown in Fig. 3. Anti-HLA-A24 mAb partially inhibited the IFN-γ production from the CTL (generated from TIL), whereas anti-MHC class I mAb, but not anti-MHC class II mAb (control Ab), completely blocked the production (Fig. 3, Table I). An inhibitory effect on IFN-γ production was also observed upon the addition of anti-CD8 mAb, but not anti-CD4 mAb. These results indicated that CD8+ cells were responsible for the CTL activity and that the recognition by the CTL was restricted by MHC class I molecules, presumably HLA-A24.

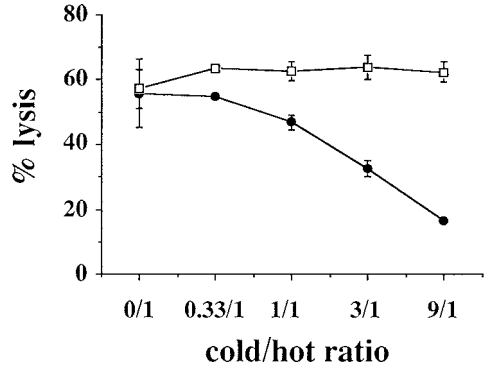

Fig. 2. Specific cytotoxicity of the CTL against AT. Cold targets (K562, AT) were added in the cytotoxic assay of the AT-specific CTL. Cold/hot target ratio is indicated. CTL were generated from RLNL by multiple stimulations with AT. □ K562, ● AT.

Cross reaction of the CTL against HLA-A locus-matched allogeneic cancer cell lines The patient's lymphocytes were found to express HLA-A2/A24, B52/B54, Cw1/– by serological microcytotoxicity assay. From the results in Fig. 3, the CTL recognized A110L in an HLA-A24 restricted manner. To investigate whether common tumor antigens are expressed in allogeneic lung cancer cell lines, several allogeneic cancer cell lines were used as targets for the cytotoxicity assay. The HLA types of these cell lines are shown in Table II. As shown in Fig. 4, the CTL lysed autologous A110L in an MHC class I-restricted manner and also lysed HLA-A24-positive Sq-1 (HLA-A11/A24) and PC-9 (HLA-A2/A24) to lower but still substantial extents (more than 10% lysis at an E/T ratio of 20/1). The cytotoxic activities against RERF-LC-AI (HLA-A24-positive lung squamous cell carcinoma) and A609HN (HLA-A2/A24-positive head and neck cancer) were modest (less than 10% lysis). On the other hand, HLA-A24-negative tumor cell lines were not lysed at all by the CTL.

DISCUSSION

Recent remarkable progress in the field of tumor immunology has helped us to understand the mechanisms of the tumor-host relationship and may lead eventually to the development of novel specific immunotherapy for malignancies. In malignant melanoma, therapeutic trials of vaccination with peptides recognized by CTL are in progress in several institutes since the identification of the MAGE family, MART-1 or tyrosinase and their epitope peptides.[20)]

Table I. MHC Class I Restricted Cytokine Production from CTL Stimulated with AT

Effector	AT stimulation	Antibody	IFN-γ (pg/ml)	GM-CSF (pg/ml)
+	+	–	595	596
+	+	anti-class I	<50	<10
+	+	anti-class II	456	434
+	–	–	<50	14
–	+	–	<50	<10

Table II. HLA Class I Types of Cancer Cell Lines Tested

Cell line	Origin	Histology	A-locus	B-locus	C-locus
A110L (AT)	lung	adeno.	2402/	5201/	Cw1202/
PC-9	lung	adeno.	0206/2402	0702/5502	Cw0303/Cw0702
Sq-1	lung	squamous	1101/2402	1501/5201	Cw0401/Cw1202
A549	lung	adeno.	2603/3001	1801/44031	Cw1203/Cw1601
RERF-LC-AI	lung	squamous	2402/	5201/	Cw1202/
A803L	lung	adeno.	3101/3302	44031/5101	Cw1402/Cw1403
A112L	lung	adeno.	1101/		
A609HN	head and neck	squamous	2/24	61/51	Cw3/

In this study, lung cancer-specific CTL were generated from RLNL and TIL after several stimulations with AT following expansion with anti-CD3 mAb and a low dose of IL-2. More than 30% cytotoxicity (E/T ratio = 20:1) was always obtained after 3 stimulations by autologous tumor cells (A110L). The induced CTL activities were always higher in TIL than RLNL. This may be because the frequency of CTL precursors is higher in TIL than in RLNL.

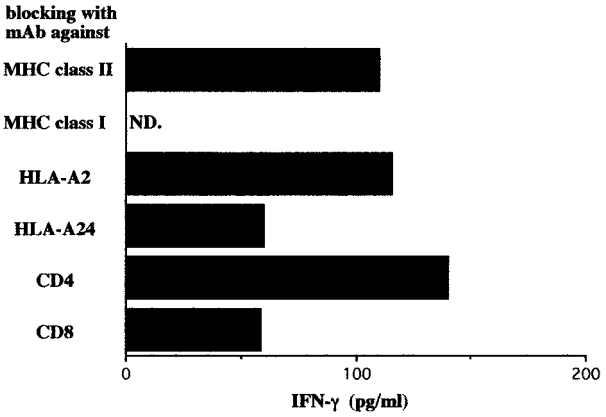

Fig. 3. The blocking effects of mAbs on the CTL activity. The CTL generated from TIL were co-cultured with irradiated AT in the presence of the indicated mAbs for 24 h. The supernatant of the mixed culture were collected and assayed for interferon-γ (IFN-γ). ND, not detected.

From the result of the cold target inhibition assay (Fig. 2), the cytolytic activity of the CTL was specific for AT. This cytolytic activity was partially inhibited by treatment with anti-MHC class I mAb (W6/32). The production of such cytokines as GM-CSF and IFN-γ from the CTL stimulated with autologous A110L was also abrogated by anti-MHC class I mAb (Table I).

The blocking effect of anti-MHC class I mAb was always incomplete in a cytotoxicity assay for CTL, whereas it was almost complete in the assay for cytokine release by CTL (Figs. 1 and 4). This difference of the inhibitory effect by anti-MHC class I mAb between cytolytic activity and cytokine production might be due to heterogeneity of the CTL productions. The CTL consisted of 70–80% CD8+, 10% CD4+, and 10% CD3-CD56+ populations. The CTL obtained were thus heterogeneous populations. The MHC class I non-restricted cytolytic activity against A110L might be mediated by non-T cells (CD3– population, such as NK cells) or by T cells which show MHC class I non-restricted cytotoxicity, like the CTL for MUC-1.[21, 22] The effectors which exert these non-specific cytotoxic activities might not produce such cytokines as GM-CSF and IFN-γ in response to autologous A110L. Therefore, the assay for cytokine release may be more sensitive than a cytotoxicity assay to detect the MHC class I-restricted specific reaction of the CTL.

In this study, the CTL generated against autologous A110L (HLA-A24+) recognized Sq-1 (HLA-A24+) as well as PC-9 (HLA-A24+), thus indicating the existence of a shared common antigen(s) among those tumor cells al-

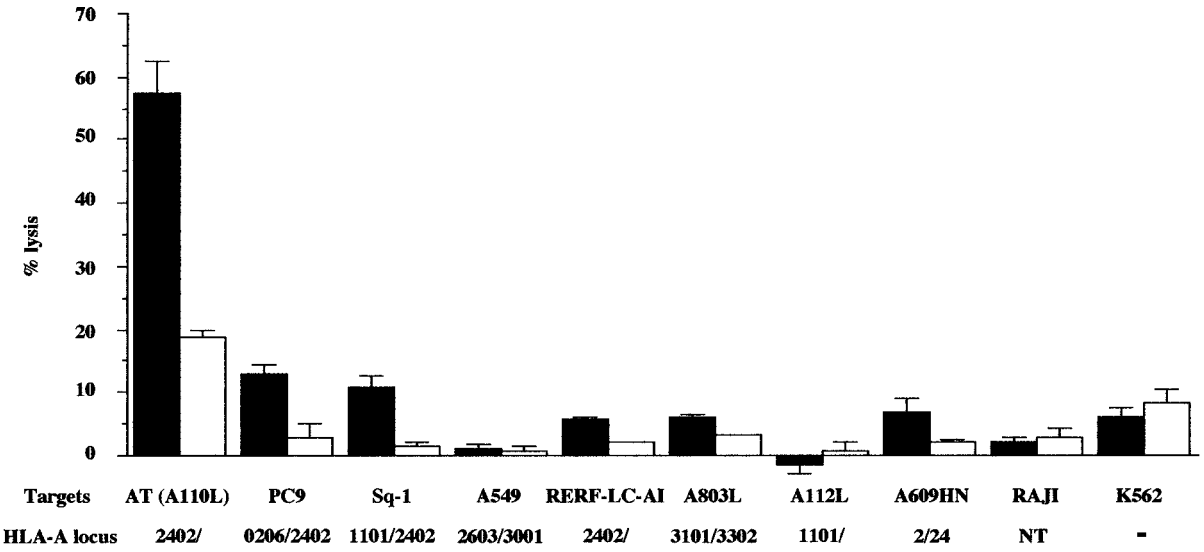

Fig. 4. Cross-reactive cytotoxicity against HLA-A locus-matched allogeneic cancer cell lines. Cytolytic activity of AT-specific CTL generated from RLNL were assayed against HLA-A locus-matched and mismatched allogeneic cell lines. Targets and their HLA-A types are indicated. MHC class I blocking of the cytotoxicity was also analyzed as described in detail in "Materials and Methods." NT, not tested. ■ without mAb, □ with anti-MHC class I mAb.

though these cross reactivities of the CTL were relatively low. There are two possible reasons for the low CTL-activity against allogeneic tumor cells (Sq-1, PC-9); one possibility is that a minor population in the autologous tumor-specific CTL might recognize the common antigen(s) on allogeneic tumor cells and A110L, and the other is that small populations of Sq-1 or PC9 express a shared antigen(s) recognized by the CTL. To settle the above issue of the heterogeneity of both CTL and tumor cells, further clonal analyses of the CTL and tumor cells will be needed, and are in progress in our laboratory. The results may clarify the exact mechanisms involved. The CTL showed no cross-reactivity against HLA-A24+ RERF-LC-AI and A609HN. Weak or absent expression of antigenic peptides on these HLA-A24+ cell lines may be responsible for the failure of recognition by the CTL.

Tumor antigens recognized by CTL in the context of HLA-A2 were reported in melanoma (MAGE-3,[23] MART-1/Melan-A,[4] and gp100[5]) and in ovarian cancer (*erb*B-2).[13] Regarding HLA-A24, the products of a tyrosinase gene,[3] *p15*[24] and a mutated β-catenin[25] were present in the context of HLA-A24 in melanoma. We previously reported that an *erb*B-2 peptide was recognized by the CTL from patients with ovarian cancer and lung cancer in the context of HLA-A2.[13] As shown in Fig. 4, mAb to HLA-A24 inhibited the response of the CTL to A110L, as did the addition of anti-MHC class I mAb. These results thus suggest that the antigen(s) recognized by the CTL existed in the context of HLA-A24. This is the first report to show that HLA-A24-restricted CTL could be generated from a patient with lung cancer. The inhibitory effect of anti-HLA-A24 mAb on CTL activity was, however, incomplete as compared with that by anti-MHC class I mAb. The reason for this might be the existence of some other restriction element, such as an HLA-B or HLA-C locus. This question should be addressed in further investigations. We thus paid close attention to *erb*B-2 peptides, which can bind to HLA-A24,[26] as common Ag candidates because high expression of *erb*B-2 was observed in A110L, as well as PC9 and Sq-1 (data not shown).

The results presented here are considered to be significant since the HLA-A24-positive population is quite high among Japanese, as mentioned previously. The incidence of HLA-A24-positives among patients with lung cancer was more than 60% based on our findings in 100 patients (unpublished data). Such specific immunotherapies as adoptive transfer of CTL and vaccination using shared common tumor antigen(s) for lung cancer patients with HLA-A24 may therefore be applicable, if appropriate antigens can be identified in the future.

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