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Effects of Interleukin-12 on the Induction of Cytotoxic T Lymphocytes from the Regional Lymph Node Lymphocytes of Patients with Lung Adenocarcinoma

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Lung cancer-specific cytotoxic T lymphocytes (CTL) were induced by repeated stimulations of regional lymph node lymphocytes (RLNL) in lung cancer patients with either autologous or HLA-A-locus-matched tumor cells. To investigate the effect of interleukin-12 (IL-12), IL-12 was added during the stimulation of RLNL from HLA A24 / adenocarcinoma patients with either autologous tumor cells or HLA A24-positive adenocarcinoma cells (PC-9) in combination with, or instead of interleukin-2 (IL-2), and then the cytotoxic activity, cytokine production and populations of the lymphocyte subsets were examined. The addition of IL-12, or the substitution of IL-2 by IL-12 was found to enhance the cytotoxic activity and the cytokine production (IFN-γ, GM-CSF) of the CTL as compared with IL-2 alone. The cytotoxic activity and cytokine production were both partially inhibited by anti-MHC-class I monoclonal antibody. The CTL thus induced by IL-12 had a higher proportion of $CD3^+/CD56^+$ cells than the CTL induced with IL-2 alone. The positively selected $CD8^+/CD56^-$ lymphocytes showed PC-9-specific cytotoxic activity, because the population did not show any cytotoxicity to K562 or A549 (HLA-A26/A30). However, the $CD3^+/CD56^+$ lymphocytes were cytotoxic to both PC-9 and K562. In conclusion, IL-12 is considered to be a useful cytokine for both the induction of lung-cancer specific CTL and the augmentation of non-MHC-restricted cytotoxicity against tumor cells, and may be applicable for adoptive immunotherapy using CTL.

Key words: Regional lymph node lymphocytes — Interleukin-12 — Cytotoxic T lymphocytes — Lung cancer

Interleukin 12 (IL-12) has been proven to be a potential anti-tumor cytokine in mice bearing a variety of malignancies through a number of biological activities, such as stimulation of natural killer (NK) or T cells to produce interferon-γ (IFN-γ), stimulation of Th1 reactivity, and enhancement of cytotoxic T lymphocyte (CTL) response.[1–4] We previously made a functional analysis of the regional lymph node lymphocytes (RLNL) of human lung cancer activated by interleukin-2 (IL-2) and IL-12, and as a result, IL-12 and suboptimal doses of IL-2 were found to enhance synergistically the non-specific cytotoxic activity and IFN-γ synthesis of RLNL.[5] We recently reported that tumor-reactive T cells exist in RLNL as well as in tumor tissue,[6] and induce lung cancer-specific CTL from RLNL or tumor-infiltrating T cells of lung cancer by multiple stimulations with autologous tumor cells or histocompatibility leukocyte antigen (HLA)-A locus-matched tumor cells.[7] In this study, we evaluated the effects of IL-12 on the induction of CTL against autologous tumors and an HLA A locus-shared allogeneic lung cancer cell line.

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MATERIALS AND METHODS

Preparation of RLNL Regional lymph nodes (hilar and mediastinal lymph nodes), which were macroscopically non-metastatic, were obtained at the time of surgery for lung cancer. The preparation of RLNL has been described in another paper.[7] The cells were subjected to Ficoll-Hypaque gradient (LSM, Litton Bionetics, Kensington, MD) centrifugation (1000*g*, 30 min). The interface was collected and the resultant cells were suspended in complete culture medium (CM) as RLNL. The CM consisted of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 20 m*M* Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum. Serological MHC class I typing of RLNL was performed by means of a microtoxicity assay. The RLNL with HLA-A 24 molecules were used for induction of HLA-A-locus-matched allogeneic tumor-specific CTL. The characteristics of the patients are shown in Table I.

Reagents Human recombinant IL-2 was kindly provided by Takeda Chemical Industries, Ltd., Osaka. The specific activity of IL-2 was 0.98×10^7 IU/ mg protein. Human recombinant IL-12 was kindly provided by Dr. M. Gately

Table I. Profile of the Patients with Lung Cancer

	Case 1	Case 2	Case 3	Case 4
Age	61	58	73	46
Gender	M[b)]	F	M	M
Histology	adenocarcinoma	adenocarcinoma	adenocarcinoma	adenocarcinoma
TNM classification[a)]	$T_3N_2M_1$	$T_1N_0M_0$	$T_1N_0M_0$	$T_3N_0M_0$
HLA A locus	2/ 24	24	11 / 24	24 / 31

a) Pathological diagnosis.
b) M, male; F, female.

(Hoffmann-La Roche, Nutley, NJ). The IL-12 activity was 4.17×10^8 units /mg. IL-2 was used at a concentration of 50 IU/ml while IL-12 was used at a concentration of 50 U/ml.

Tumor cell line The lung cancer cell lines (PC-9 and Sq-1) 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 his group. PC-9 is an HLA-A2 and -A24 positive lung adenocarcinoma cell line,[8)] A549 is an HLA-A26 and -A30 positive lung adenocarcinoma cell line, and Sq-1 is an HLA-A11 and -A24 positive lung squamous cell carcinoma line. A110L was established from an HLA-A2 and -A24 positive patient with lung adenocarcinoma (case 1 in this study). K562 had been established from a human chronic myelogenous leukemia.

Generation and culture of CTL Four million RLNL were incubated in 2 ml of CM in 24-well plates (Nunc, Roskilde, Denmark), in which each well was precoated with solid-phase anti-CD3 monoclonal antibody (mAb) for 48 h at 37°C in a 5% CO_2 atmosphere. IL-2 was routinely added every three days, and each culture was scaled-up to maintain a cell density of 1 to 2×10^6/ml. The lymphocytes were expanded up to 50-fold at 10–14 culture days. At this time, the cells were harvested, washed and re-suspended with irradiated (5000 rad) autologous tumor cells or irradiated HLA-A locus shared allogeneic tumor cells (PC-9) at a 10 : 1 lymphocyte : tumor cell ratio for 2 days in CM containing IL-2 (50 IU) and/or IL-12 (50 U/ml). Thereafter, the lymphocytes and tumor cells (stimulator) were washed again and cultured for 5 more days in CM containing IL-2. This stimulation with tumor cells was repeated 2 or 3 times.

Cytotoxicity assay The cytotoxic activity was assessed by a 4-h ^{51}Cr release assay, as previously described.[7)] The target cells used were A110L, PC-9 and A549 which had been established from a human lung cancer. The target cells were labeled with 0.1 mCi of $Na_2{}^{51}CrO_4$ (Japan Atomic Energy Research Institute, Ibaragi) for 60 min at 37°C. The ^{51}Cr-labeled target cells (5×10^3/well) were added to three concentrations of effector cells (2.5×10^4/well, 5×10^4/well, 1×10^5/well) in 96-well, round-bottomed microplates (Nunc). The maximum and spontaneous ^{51}Cr release values were obtained by incubating the target cells with 1% Triton-X (Sigma Chemical Co., St. Louis, MO) and medium alone, respectively. All determinations were made in triplicate, and the cytotoxic activity was calculated as previously described.[7)]

Blocking of CTL activities Target cells were incubated with a 1/10 diluted supernatant of W6/32 (anti-HLA-A, B, C mAb)-producing hybridoma cells or L227 (anti-HLA-DR mAb)-producing hybridoma for 30 min prior to plating in 96-well plates.

Flow cytometry The immunofluorescence staining of RLNL has been described previously.[7)] A two-color analysis was performed using an EPICS XL (Coulter Electronics, Hialeah, FL), after staining of the cells with fluorescein isothiocyanate-conjugated and phycoerythrin-conjugated mAb. The mAbs used were NU-T3 (anti-CD3), NU-T H/I (anti-CD4) (Nichirei Co., Tokyo), Leu-2a (anti-CD8), Leu-19 (anti-CD56) (Becton-Dickinson, Mountain View, CA) and isotype-matched control mAbs (FITC- or PE-conjugated IgG1, FITC-conjugated IgG2a or IgG2b) (Caltag Laboratories, South San Francisco, CA). Data analysis was done for 1×10^4 viable cells.

Isolation of the lymphocyte subsets Subpopulations of RLNL were isolated by magnetic immunoselection using Dynabeads (Dynal A. S., Norway). This separation method allowed us to isolate a more than 98% negative binding population for each mAb (anti-CD4, anti-CD8, anti-CD56). The positively selected population demonstrated more than 90% positive binding for each mAb (anti-CD8, anti-CD56). The positively selected populations were used after overnight culture to rule out any possible effect of mAb binding.

Measurement of lymphokine production by enzyme-linked immunosorbent assay (ELISA) The quantitative determinations of IFN-γ and granulocyte macrophage colony-stimulating factor (GM-CSF) in the culture supernatant were performed using a commercial ELISA kit (Quantikine R & D Systems, Minneapolis, MN). Standard curves were generated for each set of samples assayed.

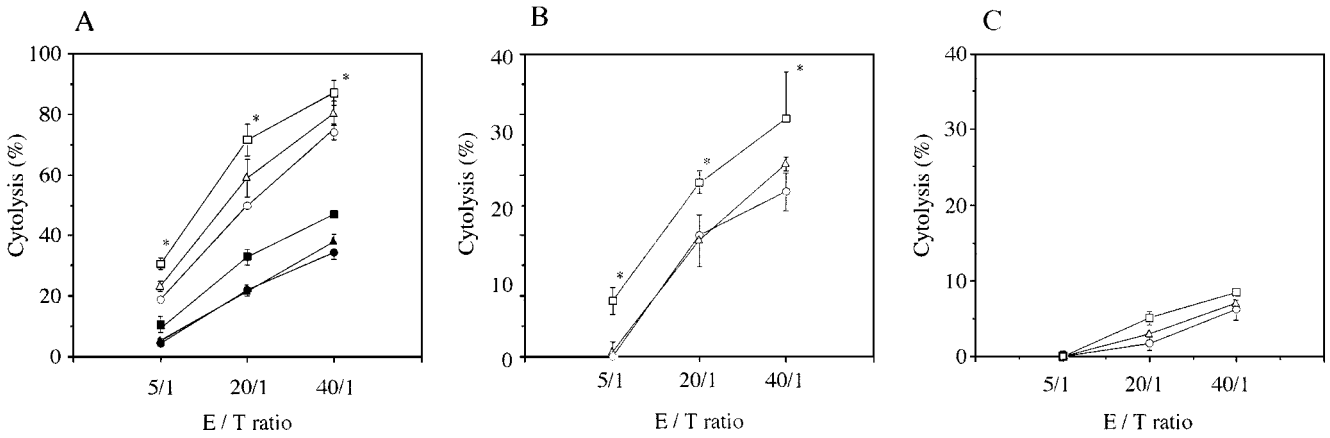

Fig. 1. The effect of IL-12 on the cytotoxic activity of RLNL stimulated by autologous tumor cells (A110L). The RLNL was stimulated with irradiated autologous tumor cells twice at an interval of one week in CM containing IL-2 and/or IL-12. The cytotoxicity against autologous tumor (A110L) (A) and allogeneic tumor cells (PC-9, B; A549, C) was examined. Open squares, IL-2+IL-12; open triangles, IL-12; open circles, IL-2; closed squares, IL-2+IL-12 with anti-HLA class I mAb; closed triangles, IL-12 with anti-HLA class I mAb; closed circles, IL-2 with anti-HLA class I mAb. The values are expressed as the mean±SD. * The cytotoxic activity was more significantly enhanced by IL-2 and IL-12 than by either CTL induced with IL-12 alone or IL-2 alone (P<0.05).

All standards and samples were averaged based on duplicate readings.

Statistics The statistical analyses were performed by using Student's *t* test. Differences in the means were considered to be significant when the *P* value did not exceed 0.05.

RESULTS

The effect of IL-12 on CTL activity In case 1, CTL activity was induced from RLNL by twice-weekly stimulations with autologous tumor cells (A110L) in the presence of IL-2 (50 IU/ml) as shown in Fig. 1. When the CTL was induced using IL-12 with/without IL-2, the cytotoxic activity against autologous tumor cells was increased (Fig. 1A). The autologous tumor-specific CTL also showed cytotoxicity against HLA-A locus-shared allogeneic tumor cells (PC-9: HLA-A2/24) and this cytotoxic activity was also enhanced by addition of IL-12 (Fig. 1B). However, the autologous tumor-specific CTL lacked cytotoxicity against an HLA-mismatched cell line (A549: HLA-A26/30) and the cytotoxicity was always less than 10% (Fig. 1C).

In all 7 patients with lung adenocarcinoma expressing HLA-A2 and/or A24, we succeeded in inducing MHC-restricted cytotoxicity against PC-9 (HLA-A locus-shared lung adenocarcinoma cell line) by multiple tumor stimulations in the presence of IL-2 (50 IU/ml).[7] In 3 cases (cases 2–4), the effects of IL-12 on the CTL induction

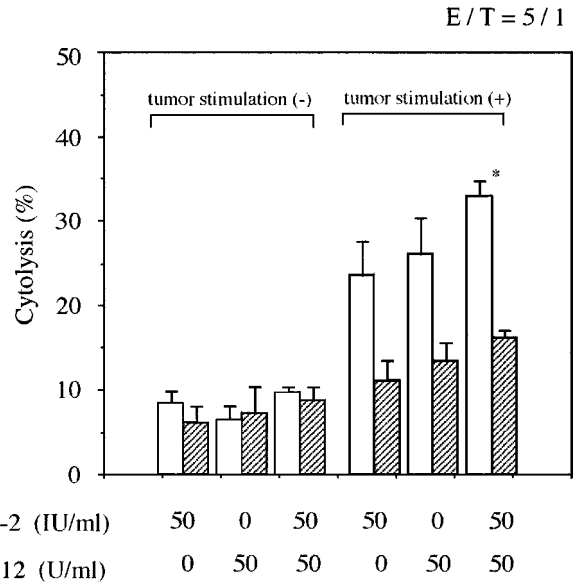

Fig. 2. The effect of IL-12 on the cytotoxic activity of RLNL stimulated by an HLA A locus-shared allogeneic tumor (PC-9). The data are the mean cytotoxic activity of the 3 cases at an E/T ratio of 5:1, as a percentage. The cytotoxic activity of RLNL cultured with IL-2 alone was lower against PC-9 than that of RLNL stimulated with IL-2+IL-12. Open bars, without any mAb; hatched bars, with anti-MHC class I mAb. The values are expressed as the mean±SE. * The cytotoxic activity was more significantly enhanced by the combination of IL-2 and IL-12 than by IL-2 alone (P< 0.05).

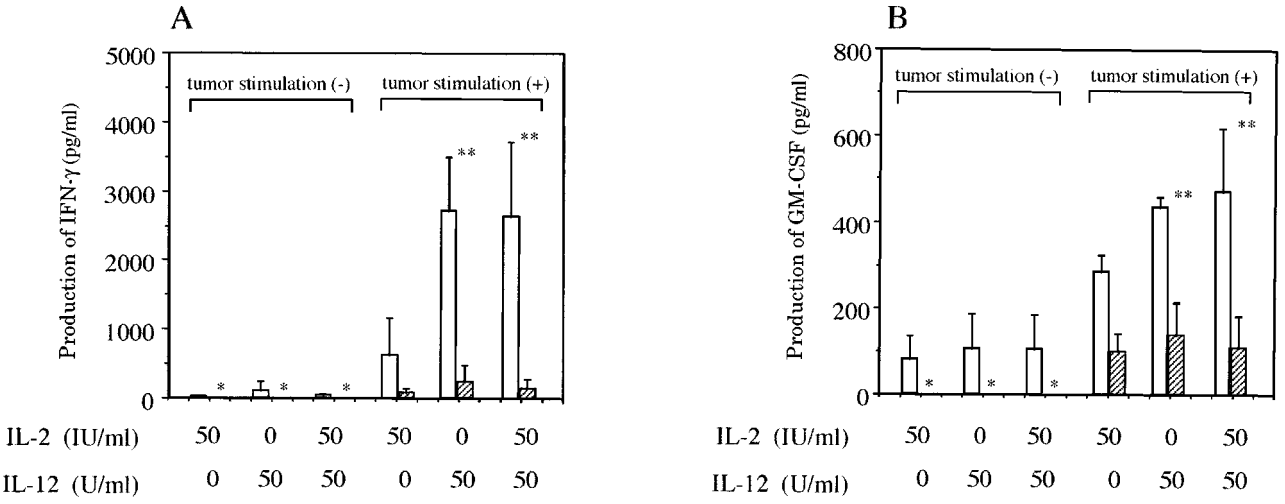

Fig. 3. The effect of IL-12 on cytokine productions by the CTL. The CTL induced were re-stimulated with irradiated tumor cells, and productions of IFN-γ (A) and GM-CSF (B) were examined. The data are the mean cytokine productions of the 4 cases, as a percentage. Open bars, without any mAb; hatched bars, with anti-MHC class I mAb. The values are expressed as the mean±SD. ∗ No blocking with MHC class I mAb was performed. ∗∗ The cytokine production was more significantly enhanced by the presence of IL-12 with/without IL-2 than by IL-2 alone, in an HLA class I-restricted manner ($P< 0.05$).

Table II. Phenotypic Analysis of Tumor-stimulated RLNL

IL-2	IL-12	*n*	$CD3^+$	$CD4^+$	$CD8^+$	$CD3^+CD56^+$	
50[a)]	0[b)]	4	95.6±2.9[c)]	31.9±19.7	63.9±17.7	16.1±6.2	*
0	50	4	96.4±2.2	26.6±12.6	68.4±10.9	24.6±10.5	*
50	50	4	96.4±1.5	29.0±16.9	67.1±14.6	22.6±8.0	

a) IU/ml.
b) U/ml.
c) Percentage of positive cells (mean±SD).
∗ $P<0.05$, significant difference according to Student's *t* test.

were examined. The CTL activity against PC-9 was enhanced by the use of IL-12 with/without IL-2 during tumor stimulation (Fig. 2). This augmented CTL activity was partially blocked by the addition of anti-MHC class I mAb. When the RLNL were not stimulated by tumor cells, no effect of IL-12 was observed (Fig. 2). However, the PC-9-specific CTL always showed less than 10% cytotoxicity against the HLA-mismatched cell line (A549).

The effect of IL-12 on cytokine production by the CTL The induced CTL were re-stimulated with irradiated tumor cells, and the production of IFN-γ and GM-CSF was examined. Significant amounts of IFN-γ were released from the CTL induced in the presence of IL-2 alone, but the CTL induced in the presence of IL-12 with/without IL-2 produced greater amounts of IFN-γ than the CTL induced in the presence of IL-2 alone in all cases tested (Fig. 3A). The production of IFN-γ was inhibited by anti-MHC class I mAb, but not by anti-MHC class II mAb (data not shown). GM-CSF was also produced by the CTL in response to tumor cells. Similarly to the case of IFN-γ production, the CTL induced in the presence of IL-12 produced a larger amount of GM-CSF than the CTL induced in the presence of IL-2 alone (Fig. 3B).

The effect of IL-12 on the lymphocyte population of the CTL A flow-cytometric analysis showed more than 90% of the CTL to be $CD3^+$ cells (Table II). When IL-12 was used at the time of tumor stimulation, the proportion of $CD3^+/CD56^+$ cells was significantly increased. Additional staining proved that more than 90% of the $CD56^+$ cells were $CD8^+$ cells (data not shown). There was no difference in the proportion of $CD4^+$ cells and $CD8^+$ cells among the cultures with IL-2 and/or IL-12, except for case 2, in whom the $CD4^+$ cells were decreased and the $CD8^+$ cells were increased by using IL-12.

An analysis of effector cells exerting CTL activity To identify the main populations exerting CTL activity, $CD4^+$, $CD8^+$ or $CD56^+$ lymphocytes were depleted from the anti-PC-9 CTL induced in the presence of both IL-2 and IL-12, by magnetic immunoselection, and the PC-9-specific cytotoxic activity and cytokine production were examined. When the $CD4^+$ lymphocytes were depleted, the cytotoxic activity against PC-9 was increased (Fig. 4). However, the cytotoxic activity against PC-9 was decreased by the depletion of $CD8^+$ or $CD56^+$ lymphocytes. The depletion of $CD8^+$ or $CD56^+$ cells also abrogated the production of IFN-γ and GM-CSF (Fig. 5). The $CD8^+$ or $CD56^+$ lymphocytes were positively selected from the CTL by magnetic immunoselection and the cytotoxic activity was examined (Fig. 6). The $CD8^+/CD56^-$ lymphocytes showed PC-9-specific cytotoxic activity, because the population did not show any cytotoxicity against K562 or A549. However, the $CD3^+/CD56^+$ lymphocytes also showed cytotoxicity against both PC-9 and K562. These results thus indicated that the $CD8^+/CD56^-$ lymphocytes can mediate MHC-restricted CTL activity, while the $CD3^+/CD56^+$ lymphocytes showed a non-MHC-restricted cytotoxicity against tumor cells.

DISCUSSION

A continuing dilemma is the inability to induce tumor-specific CTL stably in patients with lung cancer, even though autologous tumor-reactive T cells exist in various types of tumor tissue and the regional lymph nodes.[6] We were recently able to induce lung cancer-specific CTL from the RLNL of lung cancer patients by initial stimulation with an immobilized anti-CD3 mAb followed by repeated stimulations with tumor cells in the presence of a low concentration of recombinant IL-2 (50 IU/ml).[7] In the present study, we evaluated the effect of IL-12 at the induction phase of lung cancer-specific CTL. The coexistence of IL-12 during tumor stimulations for CTL induction enhanced an MHC class I-restricted cytotoxicity

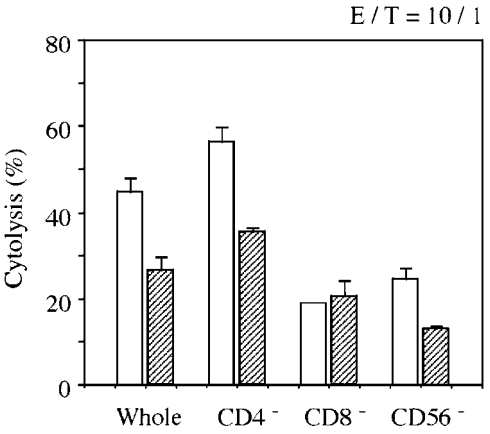

Fig. 4. The cytotoxic activities of negatively selected populations from the CTL. After stimulation with irradiated PC-9, the CTL were depleted of $CD4^+$, $CD8^+$ or $CD56^+$ lymphocytes by magnetic immunoselection and then examined for cytotoxic activity against PC-9 in the presence or absence of anti-MHC class I. When $CD4^+$ lymphocytes were depleted, the cytotoxic activity against PC-9 was augmented. Open bars, without any mAb; hatched bars, with anti-MHC class I mAb. The values are expressed as the mean±SD.

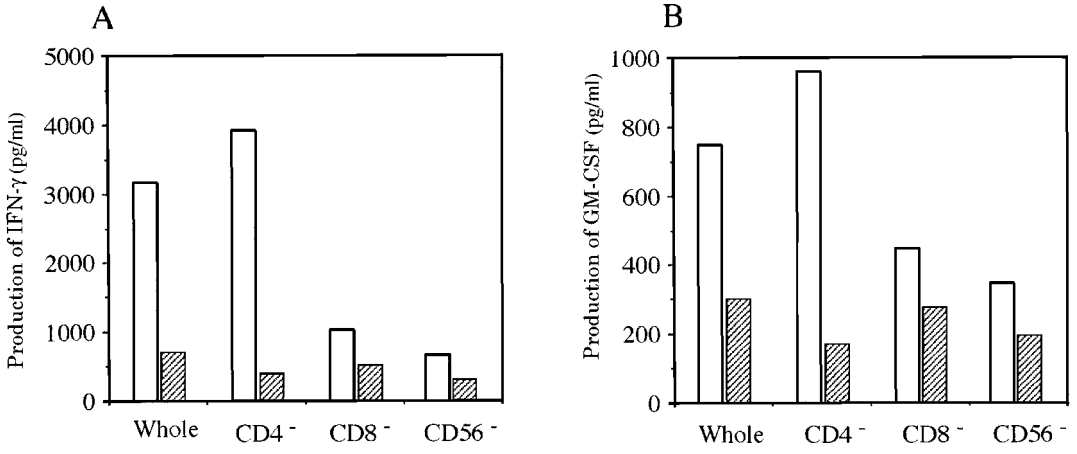

Fig. 5. The cytokine productions of negatively selected populations from the CTL. After stimulation with PC-9, the CTL were depleted of $CD4^+$, $CD8^+$ or $CD56^+$ lymphocytes by magnetic immunoselection and then co-cultured with PC-9 in the presence or absence of anti-MHC mAb. IFN-γ (A) and GM-CSF (B) of the culture supernatant were measured by ELISA. Open bars, without any mAb; hatched bars, with anti-MHC class I mAb.

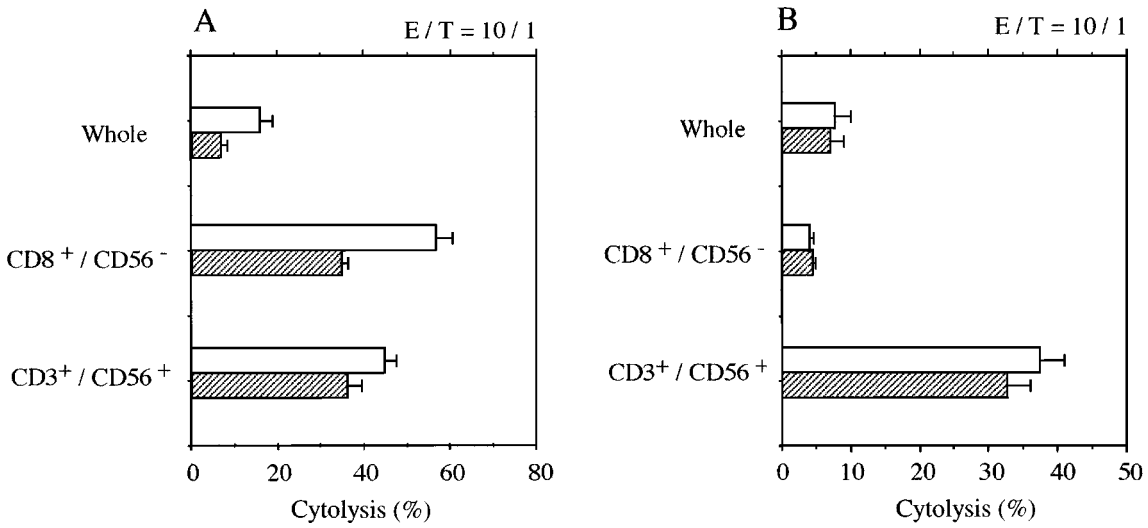

Fig. 6. The cytotoxic activities of positively selected populations from the CTL. After stimulation with irradiated PC-9, The $CD8^+$ or $CD56^+$ lymphocytes were positively selected from the CTL by magnetic immunoselection and then examined for cytotoxic activity against PC-9 (A) and K562 (B). Positively selected populations were used after overnight culture to rule out any possible effect of mAb binding. Open bars, without any mAb; hatched bars, with anti-MHC class I mAb. The values are expressed as the mean±SD.

against lung cancer cells, and the production of INF-γ and GM-CSF in response to lung cancer cells.

A phenotypic analysis of CTL induced with IL-12 revealed increased proportions of $CD3^+$, $CD8^+$ and $CD56^+$ lymphocytes. Schmidt-Wolf *et al*. reported that the non-MHC-restricted cytotoxic activity of $CD3^+/CD56^+$ lymphocytes was lower than that of $CD3^-/CD56^+$ NK cells, but higher than that of $CD3^+/CD56^-$ lymphocytes.[9] However, to our knowledge, the cytotoxic activity of $CD3^+/CD56^+$ of cultured RLNL has not previously been investigated. Our results indicate that $CD3^+$, $CD8^+$, and $CD56^+$ lymphocytes play an important role in the anticancer effect of the CTL, because both the cytotoxic activity and the cytokine production were inhibited by the depletion of $CD8^+$ or $CD56^+$ lymphocytes. Therefore, the effect of IL-12 on the CTL activity may be mediated by an expanded population of $CD8^+/CD56^+$ lymphocytes. Recently, Takeda *et al*. reported that TNK cells (NK 1.1^{high} TCR^{int} cells) play an important role in tumor rejection in IL-12-injected mice.[10] In humans, no definite TNK population has yet been found, but $CD3^+/CD56^+$ cells are most likely one of the counterparts, and they proliferate in response to IL-12.[11] We thus performed a positive selection and found that $CD3^+/CD56^+$ cells showed NK-like (non-MHC-restricted cytotoxicity) functions. Fig. 4 and Fig. 6 showed that IL-12 augmented both MHC-restricted cytotoxicity mediated by $CD8^+/CD56^-$ lymphocytes and non-MHC-restricted cytotoxicity mediated by $CD3^+/CD56^+$ lymphocytes. This finding has favorable implications for adoptive immunotherapy for lung cancer, because one of the problems in CTL immunotherapy is that the expression of MHC class I molecules often decreases in human lung cancer.[12]

Recent studies have identified tumor-associated antigens that are bound to MHC class I molecules in human melanoma,[13–15] but these have not yet been identified in other human solid tumors. In our study, the autologous tumor-specific CTL (case 1; HLA-A2/A24) also showed cytotoxicity against HLA A24-positive allogeneic tumor cells (PC-9, Sq-1) in an MHC-class I restricted manner. We therefore postulate that there is a shared antigen of lung cancer which is restricted by HLA-A24. We attempted to induce HLA-A24 restricted allogeneic tumor (PC-9)-specific CTL derived from RLNL of HLA-A24-positive lung cancer patients. CTL against PC-9 were effectively induced in HLA-A-24-positive patients with lung cancer patients (as shown in Table I). The utilization of HLA-A locus-shared tumor cell lines should overcome one of the problems in the induction of CTL, that is, the difficulty in establishing tumor cell lines to be used for multiple stimulations for the activation of CTL. In the present study, the CTL activity was augmented by using IL-12 instead of, or in combination with, IL-2 during tumor stimulation of the anti-CD3-activated and IL-2-expanded RLNL from HLA A24-positive patients. The utilization of IL-12 at the induction phase of lung cancer-specific CTL may thus help to upgrade the conventional method with anti-CD3 mAb and IL-2, and thereby be very helpful in facilitating adoptive immunotherapy against cancer.

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