Induction of Killer Cells from Lymphocytes in Pleural Effusion of Advanced Lung Cancer Patients

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We analyzed the phenotype and cytotoxic ability of pleural exudative lymphocytes (PLEL) which were obtained from 18 advanced lung cancer patients. Freshly isolated PLEL were mainly CD4(+) T cells and had weak natural killer, autologous tumor killing and lymphokine-activated killer activities. After cultivation of PLEL with interleukin-2, cytotoxicity of PLEL against autologous tumor cells was increased at 2 weeks, but it was remarkably reduced at 4 weeks. When PLEL were stimulated by mitomycin C-treated autologous tumor cells during culture, autologous tumor killing activity of PLEL was significantly enhanced even after 4 weeks of cultivation. Cold target inhibition analysis and binding inhibition assays using monoclonal antibodies indicated that autologous tumor stimulation could induce major histocompatibility complex class I restricted cytotoxic T lymphocytes specific for autologous tumor cells in some cases.

Key words: Lung cancer — Pleural exudative lymphocytes — Cytotoxic T lymphocytes — Autologous tumor stimulation

The intensity of T cell infiltration into tumor tissues was found to be correlated very well with the prognosis of cancer patients.¹,² Furthermore, we previously reported that tumor-infiltrating T cells played a crucial role in the rejection of inoculated tumor growth in rodent systems.³-⁶ However, the use of TIL⁴ for adoptive immunotherapy of cancer patients was hampered by the limited availability of TIL.

Rosenberg et al.⁷ reported that LAK/IL-2 therapy has arrested tumor growth and tumor progression in some patients. However, the incidence of objective responses (complete and partial) appeared to vary with the histologic types of cancer. The good responses were seen in patients with renal cell cancer, melanoma and colorectal cancer.⁷ However, lung cancer patients exhibited very poor response to LAK/IL-2 therapy. Rabinowich et al.⁸ reported that TIL separated from resected lung of lung cancer patients had very low cytotoxic activity against autologous tumors, though cultivation of TIL with IL-2 could increase the cytotoxic activity against autologous tumors. Because it was very difficult to obtain large numbers of TIL from resected lung tissues, we have separated lymphocytes from carcinomatous pleural effusion. We have also separated tumor cells from carcinomatous pleural effusion and established tumor cell lines.

These tumor cell lines were used for the stimulation of autologous lymphocytes. Lymphocytes obtained from carcinomatous pleural effusions in conjunction with autologous tumor cell lines may provide a better means for the generation of CTL.

MATERIALS AND METHODS

Patients Pleural effusion was collected from 18 advanced lung cancer patients at the Department of Internal Medicine, Section 3, Sapporo Medical College and Minami Ichijo Hospital, Sapporo. Histological diagnosis revealed that 15 patients had adenocarcinoma, two patients had epidermoid carcinoma and one patient had small cell carcinoma (Table I). Ten male and eight female patients ranged in age from 33 to 79 years.

Collection of carcinomatous pleural effusion Specimens of pleural effusion (500–2000 ml) were obtained from the patients at the time of diagnosis, when these patients had not received any anticancer agents. The effusion was then centrifuged at 400g for 10 min at 20°C. The pellet was washed with sterile PBS and then layered on a Ficoll-Hypaque cushion (Pharmacia, Discataway, NJ). After centrifugation at 400g for 30 min, tumor cells and mononuclear cells were collected from the interface and washed twice in PBS. The washed effusion cells were then suspended in RPMI 1640 medium containing 5×10⁻³ M 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal calf serum (referred to as complete medium). The percentage of tumor cells ranged from 5 to 90% as judged by morphological examination of Wright Giemsa-stained cytocentrifuged preparations.
Separation of tumor cells from pleural exudative lymphocytes

Tumor cells were separated from mononuclear cells by centrifugation on discontinuous Percoll density gradients as described previously by Blanchard et al., with minor modifications. Complete medium and Percoll (Pharmacia) were adjusted to 285 mOs mol/kg H2O with distilled water and 10× concentrated PBS, respectively. Four different concentrations of Percoll in medium were prepared (30, 40, 50 and 70%) and 3.5 ml of each concentration was layered into 15 ml plastic conical tubes. Then 1×10^6 effusion cells were loaded on top of each gradient and the gradients were centrifuged at 550g for 30 min. Cells banding at each layer were collected, washed and suspended in complete medium. Cells obtained from Fraction 0 (at the interface of medium and 30% Percoll) were 80-95% tumor cells and were subsequently cultured for establishment of tumor cell lines. Five autologous tumor cell lines were established as described in Table I. Fraction 1 (at the interface of 30 and 40% Percoll) was a mixture of tumor cells, mesothelial cells and macrophages. Fraction 2 (at the interface of 40 and 50% Percoll) contained typically 40-60% large granular lymphocytes with the remainder consisting of small lymphocytes and contaminating macrophages or tumor cells at less than 20%. The majority of small lymphocytes was concentrated at the interface of 50 and 70% Percoll (Fraction 3; typically this fraction contained 80-90% small lymphocytes) and red blood cells were found in the pellet. To further remove macrophages from lymphocytes, fractions 2 and 3 were pooled and 5×10^6 cells were incubated for 60 min at 37°C on plastic Petri dishes. Nonadherent cells were removed by gentle washing of plates with warm complete medium three times and were used as PLEL. The viability of cells in all fractions was greater than 90% as judged from the ability to exclude trypan blue.

Activation and stimulation of PLEL

PLEL were incubated at a density of 1×10^6 cells/ml in complete medium with 2 U/ml of human recombinant IL-2 (supplied by Takeda Chemical Industries, Osaka). Using established cell lines, ATS was carried out in tissue culture flasks once a week for the indicated number of weeks, in which 1×10^6 PLEL and 1×10^6 mitomycin C-treated (50 μg/ml for 60 min at 37°C) autologous tumor cells were cultured in the presence of 2 U/ml human recombinant IL-2 in a total volume of 10 ml. The in vitro-stimulated cells were harvested at appropriate times, washed, resuspended in complete medium and used as cultured PLEL with ATS.

Allogeneic tumor cell lines

K562 (NK-sensitive) and Daudi (LAK-sensitive) cell lines were used in cytotoxicity assays. HMC-1 (breast cancer line), LC8, LC18, LC27, LC51, LC59, LC76, LC81 and LC84 (lung cancer lines) were established in our laboratory. 59, 76 and 81 were established from adenocarcinoma, LC8 from adenosquamous cell carcinoma, LC27 from large cell carcinoma and LC84 from epidermoid carcinoma of the lung.

Detection of PLEL surface markers

Surface markers of PLEL were detected by indirect immunofluorescence using saturating amounts of MAbs, OKT3 (CD3), OKT4 (CD4), OKT8 (CD8), Leu1A, Leu1D, Leu1R and B1 (CD20). The first three MAbs were purchased from Ortho Pharmaceutical Co., Raritan, N.J. Leu reagents were obtained from Becton Dickinson, Mountain View, CA, and B1 from Coulter, Hialeah, FL.

Measurement of cytotoxicity

The cytotoxicity was measured by using 12-h [Cr] release assays against autologous tumor, K562 and Daudi cells. Target cells were labeled with 100 μCi of sodium [51Cr] chromate (Amersham Corp.) and were cultured for 3 h in 1.0 ml of medium at 37°C. The cells were washed five times with medium and 1×10^6 target cells in 0.1 ml of complete medium were seeded into U-bottomed microtiter plates. Thereafter, 0.1 ml of effector cell suspension at the predetermined cell number was added, and the plates were centrifuged at 200g for 10 min. After 12 h incubation at 37°C, 0.1 ml aliquots of culture supernatants were harvested and counted with a liquid scintillation counter (Packard autogamma scintillation spectrometer). The percentage of lysis was determined as % specific lysis=(experimental release – spontaneous release)×100/(maximal release– spontaneous release). To determine maximal release, 0.1 ml of 1% Nonidet P-40 (Nakara Chemical Co., Kyoto) was added to appropriate wells. Spontaneous release was assessed by incubation of target cells with complete medium alone. Spontaneous release was below 20% of maximal release in all experiments. All determinations were made in triplicate and the data are expressed as the mean values. A two-tailed Student's t test was used to determine the significance of differences between experimental and control groups. In a separate experiment, cold target inhibition assay was carried out. Autologous tumor lines were used as hot targets, and allogeneic and autologous tumor lines, were used as cold targets. Stimulated PLEL (4-week cultivation) were used as effector cells in this cytotoxicity assay. Ten thousand hot target cells in 0.08 ml were seeded into microtiter plates and then 5×10^5 cold target cells (in 0.08 ml) that had been treated with mitomycin C (50 μg/ml for 60 min at 37°C) were seeded. Thereafter, 0.08 ml of effector cell suspension (5×10^4) was added. After incubation, culture supernatants were harvested and counted. In another experiment, the effects of the MAbs OKT3, OKT4, OKT8, HH-1 and OKDR on the cytotoxicity of stimulated PLEL (4-week cultivation) were assessed. MAbs HH-1 and OKDR react against the framework portions of the human MHC class I and II products.

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respectively. HH-1 was made in our laboratory and OKDR was purchased from Ortho. Stimulated PLEL were treated with saturating amounts of MAbs OKT3, OKT4 and OKT8 at 4°C for 60 min. The cells were washed twice with PBS, and then used as effector cells in the cytotoxicity assays against autologous tumor lines. HH-1 and OKDR were used to pretreat autologous tumor lines. Autologous tumor lines treated with saturating amounts of these MAbs were washed twice with PBS, and then were used as target cells in the cytotoxicity assays.

RESULTS

Characterization of established lung cancer lines

Tumor cells were separated from pleural effusion and subsequently cultured. Lung cancer lines were established in 5 out of 18 cases. The expression of MHC class I antigen on tumor cells as well as the NK susceptibility of tumor cells was examined (Table I). Four out of 5 lines expressed MHC class I antigen, while the other did not. However, all cell lines were resistant to NK cells (% cytotoxicity: case 1, 1.2±3.5%; case 3, 2.4±4.7%; case 4, -2.8±5.7%; case 11, 4.7±6.9%; case 15, 5.9±6.7%; mean; 15.1±11.2%). The population of Leulla, LeuIL2R or Bl was very small (Leulla, 4.6±4.5%; LeuIL2R, 1.8±1.2%; Bl, 5.1±4.0%). These results show that the majority of PLEL are CD4-positive T cells at the time of isolation.

Table I. Profiles of Advanced Lung Cancer Patients

| Case | Age | Sex | Histology | Autologous tumor line |
|------|-----|-----|-----------|-----------------------|
| 1    | 51  | F   | adenoca.  | established<sup>a</sup> |
| 2    | 51  | F   | small cell ca. | not established |
| 3    | 33  | M   | adenoca.  | established<sup>a</sup> |
| 4    | 79  | M   | adenoca.  | established<sup>a</sup> |
| 5    | 76  | F   | adenoca.  | not established |
| 6    | 48  | F   | adenoca.  | not established |
| 7    | 65  | F   | adenoca.  | not established |
| 8    | 58  | F   | adenoca.  | not established |
| 9    | 73  | M   | epidermoid ca. | not established |
| 10   | 42  | M   | adenoca.  | not established |
| 11   | 68  | M   | adenoca.  | established<sup>a</sup> |
| 12   | 66  | M   | adenoca.  | not established |
| 13   | 47  | M   | adenoca.  | not established |
| 14   | 57  | M   | adenoca.  | not established |
| 15   | 66  | M   | epidermoid ca. | established<sup>a</sup> |
| 16   | 78  | F   | adenoca.  | not established |
| 17   | 57  | M   | adenoca.  | not established |
| 18   | 47  | F   | adenoca.  | not established |

<sup>a</sup> F, female; M, male.

<sup>b</sup> These lines were shown to express MHC class I antigen by the use of anti-MHC class I antigen antibody, HH-1.

<sup>c</sup> These lines were resistant to NK cells.

Surface markers of PLEL. PLEL were separated from pleural effusion. Freshly isolated PLEL were tested for surface markers using MAbs. The results are shown in Fig. 1. OKT3-positive cells ranged from 55.3 to 90.3% (mean; 72.7±10.8%). OKT4-positive cells ranged from 36.6 to 72.1% (mean; 52.1±15.0%), while OKT8-positive cells ranged from 5.9 to 56.7% (mean; 21.3±11.5%). The population of Leulla, LeuIL2R or Bl was very small (Leulla, 4.6±4.5%; LeuIL2R, 1.8±1.2%; Bl, 5.1±4.0%). These results show that the majority of PLEL are CD4-positive T cells at the time of isolation.

PLEL separated from pleural effusion were subsequently cultured in the presence of IL-2 for the indicated number of weeks. Surface markers of these PLEL were detected by indirect immunofluorescence using MAbs (Fig. 1). Populations of cultured PLEL were compared to those of the original PLEL. The OKT3, OKT8 and LeuIL2R-positive cells increased markedly in 2- or 4-week PLEL, compared to those of freshly isolated PLEL (OKT3: 2 weeks, 89.4±4.9%; 4 weeks, 85.0±9.0%; OKT8: 2 weeks, 41.7±10.4%; 4 weeks, 36.7±8.0%; LeuIL2R: 2 weeks, 21.5±7.0%; 4 weeks, 18.9±9.2%). The OKT4, Bl- and Leulla-positive populations did not change significantly.

PLEL were also cultured for 4 weeks with repeated stimulation by autologous tumor lines as described in "Materials and Methods." Surface markers of these PLEL were tested. As shown in Fig. 1, the OKT3-positive population of cultured PLEL with ATS was 89.1±6.9%. This value was almost the same as that without ATS. In most cases, the stimulated PLEL population tended to have an increased proportion of the OKT8-positive phenotype (43.2±10.8%) with a corresponding decrease in the proportion of OKT8-positive phenotype (49.3±7.8%) as compared with those of 4-week-cultured PLEL without ATS. However, the difference between the two groups was found to be statistically insignificant.

Cytotoxicity of PLEL. PLEL were also tested for lytic activity against autologous tumor, K562 and Daudi cells at various effector:target (E/T) ratios.

First, we assessed kinetics of the cytotoxic activity of PLEL obtained from the patients' pleural cavities (Fig. 2). Freshly isolated PLEL were cultured in the presence of IL-2. PLEL were recovered at 0, 2, and 4 week(s) after initiation of culture and were used in cytotoxicity assays as effector cells.

Freshly isolated PLEL exhibited very weak autologous tumor killing activity (ranged from -1.5 to 25.8%; mean; 5.0±7.0%) as well as NK (ranged from -9.3 to 40.2%; mean; 15.1±11.2%) and LAK activities (ranged from -12.6 to 16.7%; mean; 7.0±7.7%). In contrast, after a 2-week cultivation, PLEL exhibited strong NK as well as LAK activity as compared to freshly isolated
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Fig. 1. Phenotypic analysis of pleural exudative lymphocytes (PLEL). PLEL were cultured in the presence of IL-2 at 0, 2 and 4 week(s) after initiation of culture with (○) or without autologous tumor stimulation (●) as described in “Materials and Methods.” These lymphocytes were stained with OKT3 (a), OKT4 (b), OKT8 (c), B1 (d), Leulla (e) and LeuIL2R (f).

Fig. 2. Cytotoxicity of pleural exudative lymphocytes (PLEL). PLEL were cultured in the presence of IL-2 at 0, 2 and 4 week(s) after initiation of culture. These lymphocytes were used in cytotoxicity assays as effector cells against K562 (a), Daudi (b) and cryopreserved autologous tumor cells (c) (E/T ratio = 50:1).
Table II. Cytotoxicity of Pleural Exudative Lymphocytes

| Target          | Week(s) after initial culture | 1         | 3         | Case | 4         | 11        | 15         |
|-----------------|------------------------------|----------|----------|------|----------|----------|----------|
| K562            | 0                            | 20.3± 4.9| 13.2± 5.1| nd   | 20.3± 6.6| 8.4± 4.1  |
|                 | 2 (without ATS)              | 62.3± 5.9| nd       | 44.7± 8.9| 81.7± 5.1| 27.4± 1.2 |
|                 | 2 (with ATS)                 | 70.1± 7.2| nd       | 40.7± 9.1| 72.1± 6.3| 40.5± 8.7 |
|                 | 4 (without ATS)              | 72.3± 10.9| nd     | 44.3± 7.1| 29.0± 1.4| 72.3± 4.3 |
|                 | 4 (with ATS)                 | 67.1± 9.1| nd       | 40.4± 6.7| 17.8± 2.3| 66.7± 4.1 |
| Daudi           | 0                            | 18.5± 5.2| 1.3± 1.9 | 8.7± 4.1| −3.7± 12.5| 8.4± 4.1  |
|                 | 2 (without ATS)              | 63.2± 10.8| 74.8± 10.1| 44.7± 8.9| 81.7± 5.1| 33.0± 5.3 |
|                 | 2 (with ATS)                 | 65.1± 9.9| 72.3± 5.1| 23.1± 7.2| 50.1± 7.2| 34.1± 3.7 |
|                 | 4 (without ATS)              | 73.2± 5.7| 54.9± 5.7| 46.6± 3.0| 26.2± 3.0| 61.7± 5.1 |
|                 | 4 (with ATS)                 | 68.3± 5.2| 40.4± 6.7| 48.1± 5.1| 19.2± 1.9| 62.5± 2.9 |
| Autologous      | 0                            | 5.3± 3.1 | 1.2± 4.5 | 2.2± 3.3| 5.3± 2.9 | −1.5± 3.9 |
| tumor           | 2 (without ATS)              | 29.3± 4.3| 18.3± 5.9| 27.8± 5.9| 62.0± 5.9| 15.5± 0.6 |
|                 | 2 (with ATS)                 | 32.1± 5.2| 35.1± 7.2| 29.3± 10.2| 60.1± 12.1| 21.3± 3.2 |
|                 | 4 (without ATS)              | 9.3± 5.7 | 10.1± 5.9| 17.8± 2.9| 27.2± 3.0| 22.1± 5.1 |
|                 | 4 (with ATS)                 | 55.3± 7.4| 61.3± 5.9| 57.1± 2.9| 68.1± 2.7| 31.3± 3.1 |

a) Pleural exudative lymphocytes were cultured in the presence of IL-2 with or without autologous tumor stimulation (ATS), and were used in cytotoxicity assays against K562, Daudi and autologous tumor cells (established lines) as effector cells (E/T ratio=50:1).
b) Values represent the mean of % specific lysis± SE.
c) nd; not determined.

Fig. 3. Representative dose-response curves in cytotoxic assays. PLEL were separated from carcinomatous pleural effusion. Freshly isolated PLEL (□), 2-week-cultured PLEL without autologous tumor stimulation (ATS) (△), 4-week-cultured-PLEL without ATS (○) and 4-week-cultured-PLEL with ATS (●) were used as effector cells. Cytotoxicity was assessed by in vitro 51Cr release assay against K562 (a), Daudi (b) and cryopreserved autologous tumor cells (c) at various effector:target ratios. Bars represent SE.

PLEL. NK activity ranged from 27.4 to 93.6% (mean; 63.2± 18.1%) and LAK activity ranged from 15.8 to 87.0% (mean; 52.8± 20.2%). Cytotoxic ability of PLEL against autologous tumor cells also increased after a 2-week cultivation. However, the degree of autologous tumor killing activity is not comparable to that of NK or
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Table III. Cold Target Inhibition Assays on the Lysis of Autologous Tumors

| Cold target               | Case 1 | Case 3 | Case 4 | Case 11 | Case 15 |
|---------------------------|--------|--------|--------|---------|---------|
| Autologous tumor          | 92.1 ±3.8<sup>a)</sup> | 90.7 ±4.2 | 95.1 ±5.6 | 86.1 ±7.7 | 82.7 ±4.6 |
| Autologous lymphoblasts   | 3.1 ±1.7 | 3.1 ±2.1 | 2.8 ±1.4 | 1.8 ±1.2 | 1.9 ±1.3 |
| K562                      | 8.1 ±3.4 | 7.2 ±2.1 | 44.5 ±4.6 | 28.1 ±3.3 | 24.1 ±3.9 |
| Daudi                     | 2.7 ±2.4 | 2.1 ±3.1 | 7.9 ±1.8 | 25.7 ±4.1 | 7.6 ±3.1 |
| HMC-1                     | 3.1 ±2.0 | 2.4 ±1.8 | 61.3 ±7.1 | 19.1 ±4.2 | 5.1 ±3.2 |
| LC8                       | 2.3 ±3.1 | 2.3 ±2.9 | nd<sup>d)</sup> | nd | nd |
| LC18                      | —<sup>d)</sup> | 3.1 ±3.5 | 1.8 ±1.7 | 15.1 ±2.5 | 2.9 ±1.8 |
| LC27                      | 1.9 ±1.8 | 8.1 ±2.0 | 13.1 ±5.1 | nd | 30.1 ±4.1 |
| LC51                      | nd | — | 4.6 ±2.2 | nd | 35.8 ±3.1 |
| LC59                      | nd | nd | — | nd | nd |
| LC76                      | nd | nd | nd | 33.1 ±3.2 | 37.3 ±4.6 |
| LC81                      | nd | nd | nd | — | nd |
| LC84                      | nd | nd | nd | 9.9 ±2.1 | — |

<sup>a)</sup> Stimulated PLEL were used as effector cells against autologous tumors (established lines) at an E/T of 50:1 with various cold targets as described in "Materials and Methods." Cold-to-hot ratios were 50:1.

<sup>b)</sup> Values represent the mean of % inhibition ± SE.

<sup>c)</sup> nd: not determined.

<sup>d)</sup> -: autologous tumor cells.

Table IV. Effects of MAbs on the Cytotoxic Activity of Stimulated PLEL against Autologous Tumors

| Pretreated with | Case 1 | Case 3 | Case 4 | Case 11 | Case 15 |
|-----------------|--------|--------|--------|---------|---------|
| No treatment    | 55.6 ±9.2<sup>a)</sup> | 52.1 ±10.4 | 67.3 ±6.3 | 66.1 ±6.3 | 54.1 ±5.1 |
| OKT3            | 35.1 ±3.1<sup>a)</sup> | 32.1 ±6.1<sup>a)</sup> | 68.3 ±3.9 | 60.3 ±5.7 | 58.1 ±4.0 |
| OKT4            | 52.1 ±4.1 | 56.3 ±2.9 | 67.4 ±7.7 | 66.4 ±3.2 | 57.4 ±3.4 |
| OKT8            | 25.1 ±4.2<sup>a)</sup> | 15.1 ±8.1<sup>a)</sup> | 64.7 ±7.9 | 63.9 ±5.4 | 58.8 ±5.6 |
| HH-1            | 26.1 ±3.1<sup>a)</sup> | 27.1 ±6.0<sup>a)</sup> | 68.1 ±4.0 | 58.9 ±10.6 | 50.1 ±12.9 |
| OKDR            | 55.1 ±6.3 | 50.1 ±5.1 | 64.2 ±9.8 | 69.3 ±3.0 | 57.0 ±3.2 |

<sup>a)</sup> PLEL were cultured with mitomycin C-treated autologous tumor cells for 4 weeks in the presence of IL-2. These stimulated PLEL were treated with saturating amounts of MAbs OKT3, OKT4 and OKT8 at 4°C for 60 min and were used as effector cells in the cytotoxicity assays against autologous tumor cells. HH-1 and OKDR MAbs were used to treat autologous tumor cells (target cells) at 4°C for 60 min and then the cytotoxicity assays were performed at an effector/target ratio of 50:1.

<sup>b)</sup> Values represent the mean of % specific lysis ± SE.

<sup>c)</sup> Denotes % specific lysis significantly different from that of untreated cells (P<0.01).

LAK activity. After a 4-week cultivation, NK as well as LAK activity was maintained. However, the cytotoxic ability of PLEL against autologous tumor cells decreased significantly except in case 15 (33.5 ±13.8% after 2-week cultivation and 16.1 ±9.0% after 4-week cultivation).

PLEL were cultured with mitomycin C-treated autologous tumor cells in the presence of IL-2 as described in "Materials and Methods." These cells were recovered at 2 and 4 weeks, and were used in cytotoxicity assays as effector cells. The cytotoxic ability of cultured PLEL with ATS was compared to that of cultured PLEL without ATS (cases 1, 3, 4, 11 and 15). The results are shown in Table II. After 2-week cultivation with ATS, PLEL exhibited strong NK as well as LAK activity, equivalent to those exhibited by 2-week-cultured PLEL without ATS. After a 2-week cultivation with ATS, the cytotoxic ability of PLEL against autologous tumor cells was very similar to that exhibited by 2-week-cultured PLEL without ATS. However, it should be noted that even after a 4-week-cultivation with ATS, PLEL exhibited strong
cytotoxicity against autologous tumor cells whereas PLEL did not show significant cytotoxicity after a 4-week cultivation without ATS.

We have also examined the cytotoxic ability of PLEL against autologous tumor, K562 and Daudi cells by using various E/T ratios. Representative results are shown using PLEL obtained from case 1 patient in Fig. 3. Cytotoxic ability was clearly dose-dependent.

**Cold target inhibition assay** Our results demonstrate that repeated autologous tumor stimulation greatly enhanced the autologous tumor killing activity of PLEL. Cold target inhibition assays were carried out in order to assess whether cytotoxicity induced by repeated ATS was specific for autologous tumor cells.

Cold target inhibition analysis clearly demonstrated that cytotoxicity was highly specific for autologous tumor cells in case 1 as well as case 3, whereas the cytotoxicity was not specific for autologous tumor cells in cases 4, 11 and 15, since cytotoxicity was inhibited by K562 as well as other allogenic tumor cells (Table III). **Mechanisms of cytotoxicity against autologous tumor**

We further studied the mechanism of cytotoxicity against autologous tumors, using MAbS, OKT3, OKT4, OKT8, HH-1 and OKDR. As shown in Table IV, the cytotoxicity of stimulated PLEL obtained from two patients (cases 1 and 3) was blocked by pretreatment of stimulated PLEL with OKT3 and OKT8, but not OKT4, and was also inhibited by pretreatment of target cells with anti-human MHC class I antigen antibody, HH-1, but not anti-human MHC class II antigen antibody, OKDR. In contrast, the cytotoxicity of stimulated PLEL which were obtained from the other patients (cases 4, 11 and 15), was not blocked by pretreatment with any of the MAbS mentioned above.

**DISCUSSION**

Lung cancer is one of the most frequent human cancers. However, the prognosis of most lung cancer patients is very poor despite recent advances in surgery, radiation and chemotherapy. Chemotherapy has so far had little impact on the natural course of non-small cell lung cancers. 13, 14)

Several groups have begun the in vivo use of biological response modifiers with lung cancers. These studies examined the effects of adjuvant immunotherapy, either by Bacillus Calmette-Guérin or Corynebacterium parvum immunizations or by systemic administration of retinoids or tumor-associated antigen polypeptide purified from lung cancer cell membrane, on the survival of lung cancer patients. 15-17) These adjuvant immunotherapies of lung cancer have not yet been found to be of major benefit in terms of the survival of patients with this malignancy. Therefore, new therapeutic approaches are necessary for human lung cancer. The use of IL-2 in the therapy of cancer has been under intensive investigation. Rosenberg et al. 7) reported that LAK/IL-2 therapy generated from autologous peripheral blood has arrested tumor growth for certain cancer patients. In animal tumor models, IL-2-expanded TIL were shown to be from 50 to 100 times more potent on a per cell basis than LAK cells in mediating the rejection of established pulmonary metastases. 18) The systemic administration of IL-2-expanded TIL obtained from resected surgical specimens is a new approach to the adoptive immunotherapy of cancer. 19) However, TIL were difficult to obtain from surgical specimens of lung cancer patients. Our recent observations demonstrated that TIL as well as peritoneal exudative lymphocytes obtained from peritoneal cavity of tumor-sensitized animals exhibited strong cytotoxic activity against syngeneic tumor cells. 20) Therefore, we studied the functional characterization of PLEL which were obtained from pleural cavities of advanced lung cancer patients since these lymphocytes may be a good source for adoptive immunotherapy.

The phenotype of PLEL obtained from advanced lung cancers had the following characteristics. a) The majority of PLEL was CD4-positive T cells at time of isolation. b) Cultured PLEL had an increased proportion of CD8-positive T cells with a corresponding increase in the proportion of CD3-positive T cells.

Some investigators have reported that TIL obtained from human cancer tissues are predominantly CD8-positive T cells at time of isolation. 8, 21, 22) PLEL of advanced lung cancer are phenotypically different from TIL. The phenotype of lung cancer PLEL is closely similar to that of lung cancer PBL. 30) The difference between phenotypes of TIL and PLEL might depend on the staging of lung cancer or PLEL might be contaminated by peripheral blood, because carcinomatous pleural effusions are sometimes bloody. Although PLEL were phenotypically distinct from TIL, it should be pointed out that the cytotoxicity against autologous tumor cells could be induced by ATS. Moreover, MHC class I restricted CTL was induced from PLEL by ATS in 2 out of 5 advanced lung cancer patients (cases 1 and 3). On the basis of all these results, it is conceivable that PLEL contain pre-CTL that can be differentiated into mature CTL upon autologous tumor stimulation. Therefore, PLEL provide a better source of CTL.

Our results also demonstrated that strong cytotoxicity against autologous tumors could be induced by ATS in all five cases, although MHC class I restricted CTL could not be induced in 3 out of 5 cases. The failure to generate MHC class I restricted CTL in case 4 may be explained by the fact that target tumor cells did not express MHC class I antigen. However, the cell surface phenotype and proliferative activity of PLEL after ATS in cases 11 and
suppressor T cells were generated in cases 11 and 15. Alternatively the generation of functionally active helper T cells is impaired in cases 11 and 15 for some reason. It is well known that helper factors produced by helper T cells are involved in the differentiation and generation of CTL.\(^4\)\(^5\)\(^22\) In this regard, a study is in progress in our laboratory to test if CTL can be induced by addition of exogenous mixed lymphocyte culture supernatant, which may contain various factors for CTL generation.

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