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Lymphokine-activated Killer Induction and Its Regulation by Macrophages in Malignant Pleural Effusions

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Mononuclear cells (MNC) from pleural effusions and peripheral blood of 18 patients with primary lung cancer with malignant pleural effusion were studied. Pleural and blood MNC generated lymphokine-activated killer (LAK) activity similarly when cultured for 4 days with an optimal concentration of interleukin 2 (IL-2). Highly purified lymphocytes (>98%) and monocyte-macrophages (>90%) were isolated by discontinuous Percoll gradient centrifugation from pleural and blood MNC. Pleural macrophages, as well as blood monocytes, showed significant augmenting effects on *in vitro* LAK cell induction from pleural and blood lymphocytes by IL-2. During daily intrapleural administration of IL-2, significant induction of LAK activity *in vivo* was observed after 3 days, but then this LAK activity in pleural MNC decreased almost to zero by day 15. Daily injections of IL-2 resulted in reduction in the up-regulation of LAK induction by pleural macrophages and also in increase in the levels of soluble IL-2 receptors in pleural effusions. These findings indicate that *in vivo* LAK induction of lymphocytes in malignant effusions by IL-2 may be regulated by macrophages in the effusions.

Key words: Pleural effusion — Interleukin 2 — Macrophage — Lymphokine-activated killer cell

Nonspecific antitumor cells with a wide target spectrum can be induced without antigenic stimulation by *in vitro* culture of unprimed lymphocytes for 4 to 6 days with lymphokines including interleukin 2 (IL-2[4]).[1-3] These cells, referred to as lymphokine-activated killer (LAK) cells, destroy various fresh autologous and allogeneic tumor cells.[3-5] There is accumulating evidence that expression of LAK activity induced by IL-2 can be regulated by humoral and cellular factors.[5,6] We recently demonstrated that human monocyte-macrophages up- or down-regulated induction of LAK activity depending on their functional state.[7-9] Moreover, expression of LAK activity was found to be up- or down-regulated by various cytokines such as type I interferon (α and β),[10] type II interferon (γ),[4] tumor necrosis factor α (TNFα)[11] and IL-1.[12] These findings raise the question of whether the LAK activity induced by IL-2 in tumor-growing sites can be regulated by humoral or cellular factors.

Malignant pleurisy, seen frequently in patients with lung cancer, is of particular immunological interest, because pleural exudate cells are expected to be important in defense of the host against invading cancer cells. In fact, intrapleural administration of biological response modifiers (BRMs) such as bacterial preparations[13,14] were effective in controlling malignant pleural effusion. Recently, daily intrapleural instillation of IL-2 was found to result in disappearance of malignant cells.[15] These findings, together with the fact that both effctor cells and tumor cells coexist in a defined cavity raise the question of whether MNC in malignant pleural effusions can generate highly effective LAK activity in response to *in vitro* and *in situ* activation with IL-2. It is also important to examine the regulatory roles in LAK induction of humoral and cellular factors present in malignant pleural effusions. In the present study, we found that 1) LAK cells could be induced by IL-2 *in vitro* and *in vivo* from pleural MNC, and 2) expression of LAK activity by IL-2 was regulated by monocyte-macrophages.

MATERIALS AND METHODS

Subjects Studies were made on 18 patients with primary lung cancer with malignant pleural effusions before conventional anticancer therapy. They were all in-patients in Tokushima University Hospital, Tokushima, and ranged in age from 46 to 78 years old. On histological examination, 11 adenocarcinomas, 3 squamous cell carcinomas, 3 large cell carcinomas and one small cell carcinoma were found in these patients. The patients had received no previous treatment against pleural effusion, but 4 patients

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[4] Abbreviations: FBS, fetal bovine serum; IL-2, interleukin 2; IL-2R, IL-2 receptor; LAK, lymphokine-activated killer; MNC, mononuclear cells; NK, natural killer; PBS, phosphate-buffered saline.

(pts. 5, 8, 10 and 11) received local IL-2 therapy after obtaining written informed consent from their families.

Reagents Fetal bovine serum (FBS) was purchased from M.A. Bioproducts, Walkersville, MD. Recombinant human IL-2 was kindly provided by Takeda Pharmaceutical Co. (Osaka), and had a specific activity of 3.5×10^4 U/mg as assayed on IL-2-dependent murine NKC3 cells.[16]

Cytological examinations of pleural effusions Serial samples of pleural effusions were centrifuged for analysis of cell components after May-Giemsa staining. At least 300 cells were counted and classified as cancer cells, macrophages, lymphocytes/lymphoblasts, neutrophils, eosinophils, and others (histiocytes and mesothelial cells).

Cell cultures A human Burkitt lymphoma cell (Daudi) line was purchased from the American Type Culture Collection (ATCC), Rockville, MD. These cells were maintained as stationary suspension cultures in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamycin, designated as CRPMI 1640, at 37°C in a humidified atmosphere of 5% CO_2 in air.

Isolation of blood monocytes and pleural macrophages MNC were separated from heparinized venous blood by Ficoll-Hypaque centrifugation. Then lymphocytes (>98 %) and monocytes (>90%) were separated from the MNC by a one-step Percoll gradient method.[17] The cells were then washed twice, and resuspended in CRPMI-1640 medium at appropriate concentrations. In a parallel experiment, specimens of pleural effusions were obtained by puncture or by inserting a catheter into the pleural cavity of the patients. After centrifugation at 1000 rpm for 10 min, the effusion fluid was stored at −70°C, while the cells were washed with RPMI-1640 medium, and layered on a Ficoll-Hypaque gradient. After centrifugation at 400*g* for 20 min, MNC were collected from the interface and washed twice with RPMI-1640 medium. These effusion cells consisted of 52.1 ± 5.9 (SE) % lymphocytes, 32.9 ± 6.0 (SE) % macrophages and 12.7 ± 4.6 (SE) % others (including cancer cells). The washed MNC from the effusion fluid were examined for generation of LAK activity. The viability of these MNC was more than 95% as judged by the trypan blue exclusion test. For examination of the regulatory function of macrophages in the pleural effusion, pleural MNC including 1×10^4 macrophages per 0.2 ml were plated for 2 h in wells of a Microtest III plate (Falcon, Oxnard, CA) and then nonadherent cells were removed by gentle washing. More than 98% of the resultant adherent cells were macrophages as judged from their morphology and nonspecific esterase staining. In several cases, lymphocytes (>95%) and macrophages (>85%) were separated from the MNC by one step Percoll gradient methods. In preliminary experiments, no difference was found in the regulatory functions of macrophages isolated by these two different methods.

Measurement of soluble IL-2 receptors (R) Pleural effusions were analyzed to determine the level of IL-2R by fluorescence sandwich enzyme-linked immunosorbent assay (FS-ELISA) as described by Honda *et al.*[18] Briefly, microplates were coated with monoclonal antibody, H48, and aliquots of diluted samples were added, followed by biotinylated monoclonal antibody, HA26. All wells then received streptoavidin-β-galactosidase. 4-Methylumbelliferyl-β-D-galactoside was added, and the absorbance was measured with a fluorescence microplate reader with excitation and emission wavelengths of 360 nm and 450 nm, respectively. The culture supernatant of human peripheral MNC stimulated with PHA was assigned an activity of 1000 U/ml of IL-2R, and the absorbance values at 450 nm determined by ELISA of serial two-fold dilutions of the supernatant were used as references. For calculation of IL-2R activity (U/ml), absorbance values of the test wells were compared with the reference curve.

LAK activity assay LAK activity was measured in terms of ^{51}Cr release as described in detail previously.[7,9] Briefly, for inducing LAK activity, precursor cells with or without IL-2 were added to wells of a flat-bottomed Microtest III plate, and incubated at 37°C under 5% CO_2 in humidified air for 4 days. Unless otherwise described, 1 U/ml of IL-2 was used to induce LAK activity from pleural effusion or blood MNC, as we had previously found that 1 U/ml of IL-2 was optimal for induction of maximal LAK activity from blood lymphocytes.[7,9] No significant difference was seen in the number of cells after culture with or without IL-2 for 4 days. The cytotoxicities of these cultured cells and freshly isolated pleural MNC against ^{51}Cr-labeled Daudi cells (10^4) were measured at various effector/target (E/T) ratios. Coculture of effector and target cells was terminated after 4 h, and the radioactivities of the supernatants (0.1 ml per well) separated by brief centrifugation at 65*g*, were determined in a gamma counter. The percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = 100 \times \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}}$$

The spontaneous release observed was about 10% (7–13% of total lysis).

Administration of IL-2 IL-2 was injected into the pleural cavity through a silicon coated, 500 mm long, 7 French catheter (Hanako Medical Co., Tokyo) at a daily dose of 1000 U/person for 14 days. Administration of antipyretics against fever and temporary drainage to avoid increase of pleural effusions were performed as conservative therapy.

Statistical analysis The statistical significance of differences between test groups was analyzed by the use of Student's t test.

RESULTS

***In vitro* induction of LAK activity from MNC in malignant effusion** First, we compared the LAK activities of MNC obtained from malignant pleural effusions with those of blood MNC. Pleural and blood MNC obtained simultaneously from the same patient were incubated for 4 days with or without an optimal concentration of rIL-2 (1 U/ml) and then examined by LAK-mediated cytotoxicity assay. The results obtained with the 18 lung cancer patients tested are shown in Fig. 1. The blood and pleural MNC of all but one of the patients were not cytotoxic to NK-resistant Daudi cells when incubated with them for 4 days in medium without IL-2. Signifi-

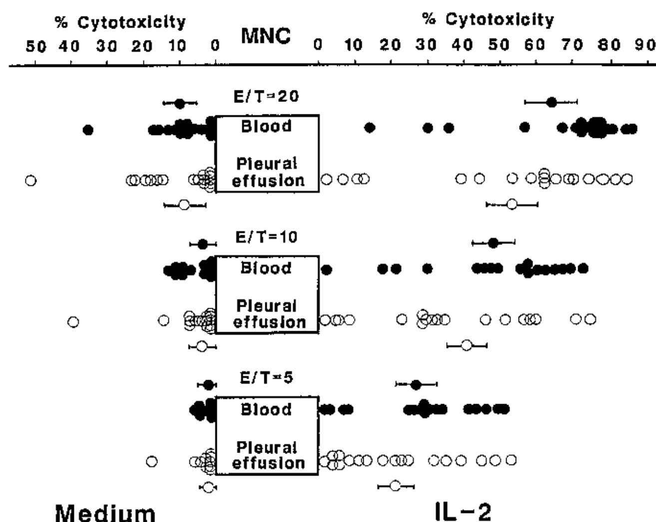

Fig. 1. *In vitro* induction by IL-2 of LAK activity of MNC of pleural effusion (○) and blood (●) of 18 patients with malignant pleurisy. MNC isolated from pleural effusion and blood of the same patient were incubated for 4 days in medium with or without 1 U/ml of IL-2 before assay of LAK activity against 1×10^4 labeled Daudi cells at E/T ratios of 20:1, 10:1 and 5:1 as described in "Materials and Methods." Bars show means±SE.

Table I. Up-regulation of LAK Induction by Monocytes and Pleural Macrophages

Patient	Percent cytotoxicity against Daudi cells[a]						
	Lymphocytes alone	Lymphocytes and blood monocytes		Lymphocytes and pleural macrophages		Blood monocytes	Pleural macrophages
Set 1. Blood lymphocytes							
4	9.5±2.6[b]	39.7±4.0[c]	(418)[d]	39.8±4.0[c]	(419)	0.2±0.7	1.5±0.6
5	32.5±4.7	45.6±3.4[c]	(140)	43.1±1.8[c]	(133)	0.6±0.6	1.7±0.3
6	11.5±0.5	34.5±0.5[c]	(300)	78.3±5.5[c]	(681)	0	4.4±4.3
9	2.0±1.4	6.6±1.3[c]	(330)	29.7±4.1[c]	(1485)	0	0.1±0.4
10	6.8±3.1	52.3±3.2[c]	(769)	61.7±3.1[c]	(907)	6.1±1.6	0.4±1.1
17	8.4±1.8	12.2±2.5	(145)	19.8±2.8[c]	(236)	0	0.4±0.3
18	25.8±3.2	30.6±1.4	(119)	33.7±1.6[c]	(131)	2.2±1.0	0.9±0.7
Set 2. Pleural effusion lymphocytes							
6	9.1±0.8	33.0±3.2[c]	(362)	37.9±1.8[c]	(416)	0	5.6±1.1
9	16.3±1.3	29.7±4.1[c]	(182)	2.6±1.0[c]	(16)	0	0.1±0.4
10	8.8±4.6	50.9±6.9[c]	(578)	57.3±2.6[c]	(651)	6.1±1.6	0.4±1.1
14	24.6±4.8	36.5±2.7[c]	(148)	35.8±2.9[c]	(146)	0.2±0.7	1.5±0.6
17	4.2±1.0	7.7±1.1	(183)	12.0±1.7[c]	(286)	0	0.4±0.3
18	31.4±4.2	67.3±7.7[c]	(214)	55.0±4.7[c]	(175)	2.2±1.0	0.9±0.7

a) Blood or pleural effusion lymphocytes (10^5) were incubated for 4 days in medium containing IL-2 (1 U/ml) with or without 10^4 monocytes or macrophages before assay of LAK activity against 10^4 labeled Daudi cells.
b) Mean±SD for triplicate cultures.
c) Significant difference from value for lymphocytes alone ($P<0.05$).
d) Values in parentheses indicate % increase in LAK activity compared with that with lymphocytes alone.

cantly high LAK activity was induced in the blood MNC of almost all the patients and in the pleural MNC of all 18 patients, by incubation with an optimal concentration of IL-2 (1 U/ml). The LAK activities of MNC in malignant pleural effusions of the respective patients were almost as high as those of their blood MNC.

Effect of pleural effusion macrophages on induction of LAK activity Next, we examined whether pleural effusion macrophages obtained from malignant effusions had any effect on the induction of LAK activity by IL-2, as human monocyte-macrophages were previously found to regulate induction of LAK activity by IL-2.[7–9] Pleural macrophages and blood monocytes of the same patient were plated for 2 h, and washed gently, twice. Then blood or pleural lymphocytes plus IL-2 (1U/ml) were added to the cultures. Four days later, the cells were washed and tested for LAK activity on Daudi cells. As shown in Table I, the LAK activities of lymphocytes obtained from blood and pleural effusions were variably cytotoxic, and their activities were increased significantly by addition of blood monocytes at a lymphocyte/monocyte ratio of 10:1. Under the same experimental conditions, pleural macrophages also had a marked effect in augmenting the induction of LAK activity by IL-2 not only of blood lymphocytes, but also of lymphocytes from pleural effusion. Thus, pleural macrophages may upregulate LAK induction by IL-2 in pleural effusions.

***In vivo* induction of pleural LAK activity by intrapleural administration of IL-2** Lymphocytes in effusions were reported to consist largely of CD3+ cells.[19] We also examined the phenotypes of cells present in malignant pleural effusions of four lung cancer patients. Before therapy these patients had ranges of 66.5–84.5% CD3+ cells (mean, 79.1), 55.4–73.3% CD4+ cells (mean, 64.0), 5.1–33.3% CD8+ cells (mean, 15.3), 3.9–19.9% CD16+ cells (mean, 10.9) and 4.5–15.3% Leu-7+ cells

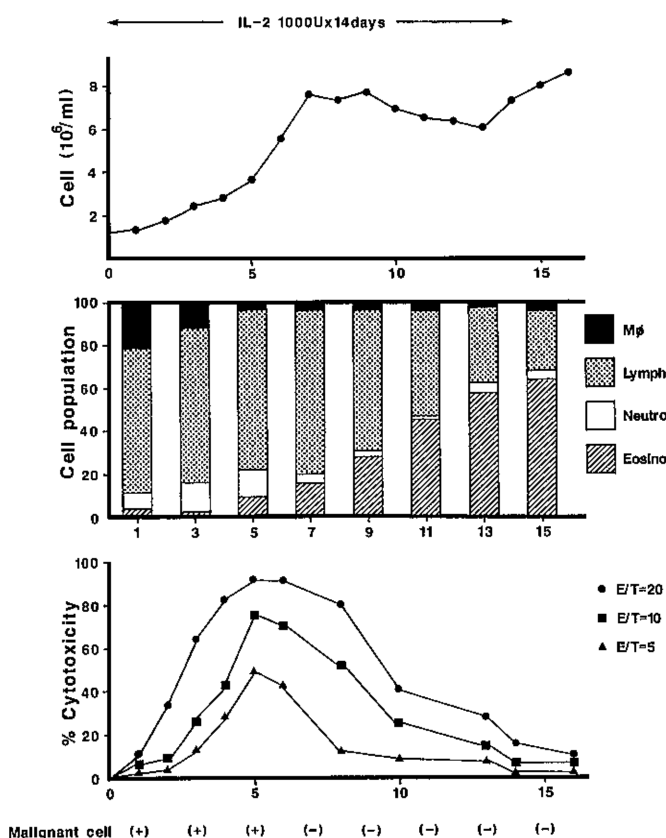

Fig. 2. Effects of intrapleural administration of IL-2 into a lung cancer patient (pt. 8) with malignant pleural effusion. IL-2 (1000 U) was injected intrapleurally for 14 days.

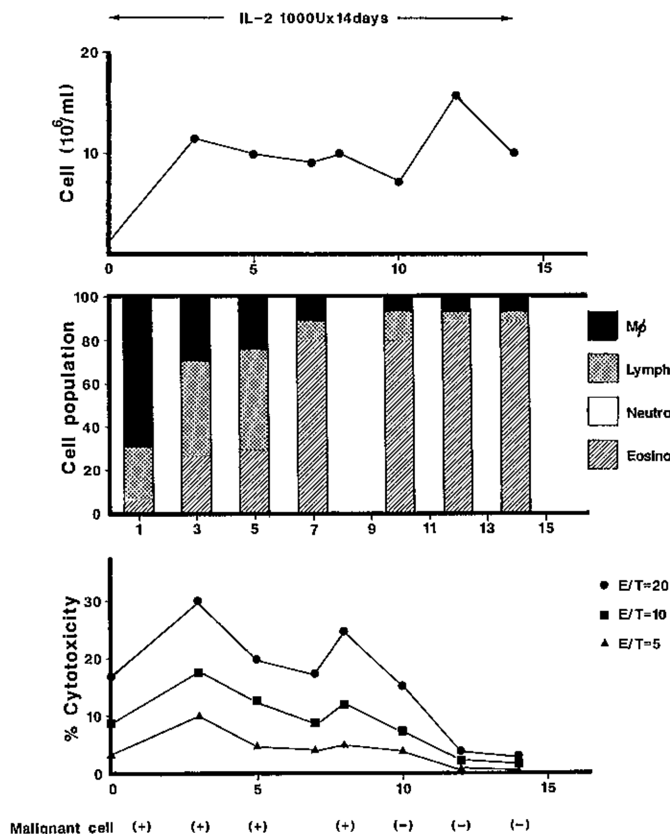

Fig. 3. Effects of intrapleural administration of IL-2 into a lung cancer patient (pt. 10) with malignant pleural effusion. Samples of pleural effusion were obtained serially during therapy with IL-2 (1000 U) and their LAK activity was measured.

(mean 9.8). During daily intrapleural injections of IL-2 (1000 U/day) for 14 days, the lymphocytes in pleural effusions of three of these patients consisted of 82.8–83.7% CD3+ (mean, 83.4), 73.9–77.0% CD4+ (mean, 75.4), 8.6–16.5% CD8+ (mean, 12.4), 1.1–7.1% CD16+ (mean, 3.7) and 1.2–7.9% Leu-7 (mean, 3.7) on day 7, and of 42.4–86.4% CD3+ cells (mean, 69.3), 33.8–66.4% CD4+ cells (mean, 52.7), 14.6–28.7% CD8+ cells (mean, 22.7), 6.3–8.1% CD16+ cells (mean, 7.2) and 3.0–7.7% Leu-7+ cells (mean, 5.0) on day 14.

Next, we measured the LAK activity of pleural effusion MNC obtained before and serially during local IL-2 therapy. Four lung cancer patients (pts. 5, 8, 10 and 11) with malignant effusions received daily intrapleural injections of 1000 U/body of IL-2. Time-courses of changes in cell number, cell populations and LAK activity of pleural effusion cells in two of these patients are shown in Fig. 2 (pt. 8) and Fig. 3 (pt. 10). The cell number gradually increased during the therapy, with an early increase in neutrophils (pt. 8), then enlargement of the lymphocytes population, and late eosinophilia (up to 80%). In both cases, LAK activity against Daudi cells, measured immediately after harvesting the MNC from pleural effusions without any stimulation with IL-2, gradually increased in the first 3 days of IL-2 injections, reached a maximum on day 3 or day 5, and remained high for the next 4 to 5 days. Cancer cells completely disappeared from the pleural effusion within 1 week when the LAK activity was high. But from 10 days after the start of IL-2 administration, the LAK activity gradually decreased in spite of daily IL-2 administration, and disappeared almost completely within 2 weeks, We examined whether this rapid loss of LAK activity was due to disappearance of the up-regulating properties of macrophages in the effusions. We examined the reguratory effect of autologous blood monocytes and pleural macrophages on *in vitro* induction of LAK activity by IL-2 from pleural effusion lymphocytes before and during the local IL-2 therapy. Representative results (pt. 10) of two patients examined are given in Fig. 4. Lymphocytes were separated from pleural effusion MNC obtained serially from the patients before and during the therapy. These lymphocytes (10^5) mixed with or without blood monocytes (10^4) or pleural effusion macrophages (10^4) were treated for 4 days in medium with IL-2 (1 U/ml) before assay of their LAK activities against 1×10^4 Daudi cells at an E/T ratio of 10:1. Blood monocytes and pleural effusion macrophages, whether or not they were treated with IL-2, were not cytotoxic to Daudi cells. The LAK activity induced by IL-2 from pleural effusion lymphocytes obtained serially during the therapy gradually decreased, but the addition of monocytes or pleural macrophages to the lymphocytes in the presence of IL-2 (1 U/ml) resulted in a significant increase in *in vitro* induction of LAK activity of the lymphocytes within 10 days. But, from 10 days after the start of daily IL-2 instillation, the up-regulating properties of pleural macrophages significantly decreased as compared to those of blood monocytes. We also examined whether the disappearance of LAK activity was due to production of

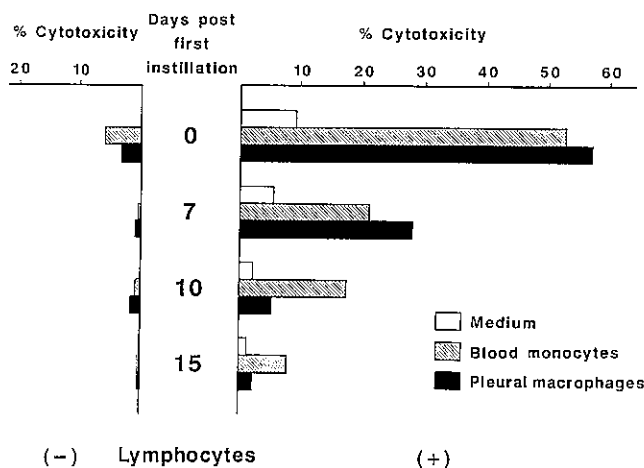

Fig. 4. *In vitro* induction of LAK activity from pleural effusion lymphocytes and its regulation by blood monocytes and pleural macrophages of lung cancer patient (pt. 10) before and during local therapy with IL-2.

Table II. Soluble IL-2R Levels in Serial Samples of Effusions Obtained during Intrapleural Administration of IL-2

Days after start of daily IL-2 injections[a]	Soluble IL-2R[b] (U/ml)		
	Pt. 8	Pt. 10	Pt. 11
Pretreatment	25	0	0
1	63	51	0
3	ND[c]	19	7
5	95	27	53
7	96	55	71
10	208	116	172
12	171	181	282
14	205	144	397
17	124	195	263

a) Lung cancer patients with malignant pleurisy received daily intrapleural injections of rIL-2 (1000 U/day) for 14 days. Samples of pleural effusion were obtained on the indicated days of treatment.
b) The levels of soluble IL-2R in pleural effusions were measured by FS-ELISA as described in "Materials and Methods."
c) ND, not done.

soluble IL-2R. For this purpose, samples of pleural effusion were obtained serially during IL-2 therapy. The results (Table II) showed that the level of soluble IL-2R was increased significantly in the pleural effusions collected on days 5 to 7 of local IL-2 therapy.

DISCUSSION

The present study demonstrated that high LAK activity could be generated in cells of pleural effusions *in vitro* and *in vivo* and that pleural macrophages in the pleural effusions up-regulated the induction of LAK activity by IL-2.

Cells in malignant pleural effusions are of great interest because they may contain a biologically significant antitumor effector population. Indeed, there are reports that on local administration of BRMs such as streptococcal preparations[14] or *Nocardia rubra* cell wall skeleton,[13] NK cells and macrophages were generated as antitumor effector cells to prevent malignant pleural effusion. IL-2 was also found to generate LAK cells cytotoxic to autologous and allogeneic tumor cells.[3-5] Recently, Yasumoto *et al.*[15] reported that IL-2 was effective in controlling malignant pleural effusion of lung cancer patients when injected into the pleural space. In the present study we found no significant difference in the inductions of LAK activity by IL-2 in lymphocytes of pleural effusions and the blood. These findings suggest that LAK precursors in malignant pleural effusions may be therapeutically useful, as cells harvested from malignant pleural effusions of lung cancer patients can be allowed to grow *in vitro* in the presence of IL-2 in the hope of generating tumor-reactive populations of lymphocytes for adoptive immunotherapy.

There is accumulating evidence that induction of LAK activity by IL-2 may be regulated by humoral and cellular factors. For example, LAK expression was found to be regulated by monocyte-macrophages obtained from peripheral blood or solid tumors.[20-23] We found previously that blood monocytes freshly isolated from healthy donors could up-regulate LAK induction, whereas endotoxin-activated blood monocytes or alveolar macrophages inhibited the expression of LAK activity,[7-9] indicating that up- or down-regulation of LAK induction by macrophages is largely dependent on their functional state. Interestingly, the present findings showed that macrophages isolated from malignant pleural effusions before IL-2 therapy augmented the expression of IL-2-induced LAK activity in lymphocytes of blood and effusions, like that of blood monocytes (Table I), thus suggesting a possible role of tumor-associated macrophages in the *in situ* expression of LAK activity.

Daily intrapleural injections of IL-2 into patients with malignant effusions induced LAK activity in the pleural effusions, but after 10 days the LAK activity gradually decreased, disappearing completely 15 days after the start of injections (Figs. 2 and 3). Moreover, we found that when lymphocytes separated from pleural MNC obtained serially during local IL-2 therapy were restimulated for 4 days with optimal concentration of IL-2 *in vitro*, the LAK activity gradually decreased after 7 days (Fig. 4), suggesting a rapid decay of the anti-tumor properties of the LAK cells. On the other hand, the previous demonstration of the existence of a suppressive factor responsible for impaired expression of LAK activity in cancer patients,[6] raised the question of whether the disappearance of LAK activity of pleural MNC after 10 days could be due to the generation of a suppressor factor and/or cells. Many factors might contribute to the disappearance of LAK activity. For example, one possibility is that the rapid loss of the LAK activity during IL-2 therapy was due to disappearance of macrophages in the effusions responsible for up-regulation of LAK induction, since matured macrophages were recently found to be susceptible to cytotoxicity by LAK cells.[24,25] Indeed, the present findings showed that the up-regulatory properties of the effusion macrophages significantly decreased after 10 days. Another possibility is that it was due to the increase of soluble IL-2R in the pleural cavity. This possibility is consistent with reports that soluble IL-2R was released in large amounts from activated human lymphoid cells,[26] and appeared in the circulation in an early stage of IL-2 treatment.[27] In fact, we also found that the level of soluble IL-2R increased during daily IL-2 injections into the pleural cavity (Table II). It is also likely that the appearance of suppressor cells or some other cytokine(s) such as IL-4[28] or transforming growth factor-β (TGF-β)[29] in the effusions might be involved in *in situ* down-regulation of LAK induction. These potential mechanisms responsible for the disappearance of pleural LAK activity are under investigation.

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