Jpn. J. Cancer Res. 82, 716–723, June 1991

Interleukin-2-inducible Killer Activity and Its Regulation by Blood Monocytes from Autologous Lymphocytes of Lung Cancer Patients

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The ability of blood lymphocytes of newly diagnosed lung cancer patients to respond to interleukin 2 (IL-2) to become IL-2-activated killer (LAK) cells and its regulation by autologous monocytes were examined. LAK activity was measured by ^{51}Cr release assay. The abilities of lymphocytes among blood mononuclear cells (MNC) of subjects of different ages without malignancies to generate LAK activity against NK-cell resistant Daudi cells and lung adenocarcinoma (PC-9) cells were very similar. The LAK activity of blood MNC of lung cancer patients was also nearly the same as that of blood MNC of control subjects. There was no significant difference in IL-2-inducible LAK activity between MNC of patients with small cell lung cancer (SCLC) and those of patients with non-SCLC. Monocytes and lymphocytes were separated from blood MNC on a one-step Percoll gradient. Monocytes of lung cancer patients were found to augment *in vitro* induction of LAK activity by IL-2 of autologous blood lymphocytes. In contrast, endotoxin-stimulated monocytes suppressed LAK induction of autologous lymphocytes of cancer patients. These findings suggest that administration of IL-2 and LAK cells induced *in vitro* may be of benefit in the treatment of lung cancer.

Key words: Interleukin 2 — Lymphocyte — Monocyte — Lung cancer

In vitro cultivation of unprimed lymphocytes for 4 to 6 days with interleukin 2 (IL-2) results in the generation of nonspecific cytotoxic cells, named lymphokine-activated killer (LAK) cells, with a wide target spectrum including lung cancer cells.[1,2] There is encouraging evidence that systemic administration of IL-2 results in partial regression of terminal stage cancer.[3,4] But, in patients with various cancers, the presence of a growing cancer has been shown to be associated with alteration in the response of lymphocytes to IL-2 for development of LAK activity.[5–7] For example, in patients with malignant melanoma, LAK expression is impaired.[5,7] On the other hand, induction of LAK activity has been reported to be regulated by various cellular and humoral factors.[8–12] Recently, we demonstrated that human monocyte-macrophages of healthy donors up- or down-regulated induction of LAK activity depending on their state.[13–15] Little is known, however, about the effect of a tumor burden on regulation by monocyte-macrophages of induction of human LAK activity from autologous lymphocytes.

Lung cancer, which frequently occurs in aged individuals, has been found to be associated with alteration in host immunocompetence.[16–24] There is a close association of aging and decrease in immune competence.[25,26] So it is important to examine the effect of aging on induction of LAK activity from lymphocytes of lung cancer patients in comparison with that in lymphocytes of control subjects without malignancies. Patients with small-cell lung cancer (SCLC) have been found to have impaired immune functions,[17–19] and a worse prognosis than those with non-SCLC,[27] suggesting that the defensive activity in patients with lung cancer depends on the histological type of cancer. In the present work, we found that the IL-2 induced LAK activity of mononuclear cells (MNC) from patients with lung cancer was similar to that induced in MNC from control subjects without malignancies, except that LAK induction was impaired in patients in terminal stages. We also found that, depending on their activation state, blood monocytes of lung cancer patients caused up- or down-regulation of LAK induction in autologous lymphocytes.

Abbreviations: BRM, biological response modifier; FBS, fetal bovine serum; IL-2, interleukin 2; LAK, lymphokine (IL-2)-activated killer; MNC, mononuclear cells; NK, natural killer; non-SCLC, non-small cell lung cancer.

MATERIALS AND METHODS

Subjects The subjects examined were 56 lung cancer patients, 37 to 77 years old (mean, 64.5 years), in Tokushima University Hospital who had not received any conventional anticancer therapy. Lung cancer was diagnosed by either histological examinations of tissue specimens or cytologic examination of sputum, or specimens obtained by bronchial brushing, lymph node biopsy, lung aspiration, or thoracotomy. The stage of

Table I. Data on the 107 Control Subjects and 56 Lung Cancer Patients without Anticancer Therapy Studied

Classification		No. of patients		No. of controls
		Male	Female	
Histological types				
SCLC		14	1	
non-SCLC:	Squamous cell carcinoma	19	3	
	Adenocarcinoma	13	1	
	Large cell carcinoma	5	0	
Age in years	–49	5	1	40
	50–59	4	1	13
	60–69	25	1	16
	70–79	12	2	25
	80–	5	0	13

lung cancer was graded according to the tumor-node-metastasis classification system (Union Internationale Contre le Cancer, 1987). Clinical staging was determined according to the criteria of the Japanese Lung Cancer Association. The clinical characteristics of the patients are listed in Table I. The five patients with SCLC all had extensive disease. According to criteria for immunological studies of aged subjects,[28] 107 control subjects of various ages with no signs of malignancies, infections or general debility and no abnormal chest X-ray findings were selected. These subjects were used for studies on the effect of aging on LAK induction by IL-2 *in vitro*.

Reagents Fetal bovine serum (FBS) was purchased from Gibco Laboratories (Grand Island, NY). Recombinant interleukin 2 (IL-2) was prepared at 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. All the reagents were free of endotoxins as determined by the *Limulus* amebocyte lysate assay (sensitivity limit, 0.1 ng/ml). Lipopolysaccharide (LPS: *E. coli* 055:B5) was obtained from Difco Laboratories, Detroit, MI.

Cell culture A human Burkitt lymphoma cell (Daudi) line was purchased from the American Type Culture Collection (ATCC), Rockville, MD. A human lung adenocarcinoma (PC-9) cell line was kindly supplied by Dr. Y. Hayata (Tokyo Medical College, Tokyo). These cells, which were free of mycoplasma, were maintained as stationary suspension cultures in RPMI 1640 medium supplemented with 5% heat-treated FBS, designated as CRPMI 1640. LAK cell-mediated cytotoxicity assays were performed in CRPMI 1640 medium, when the cultured target cells were in the exponential growth phase.

Isolation of human peripheral blood lymphocytes and monocytes MNC were separated from heparinized venous blood diluted 2-fold with PBS in lymphocyte separation medium (LSM, Litton Bionetics, Kensington, MD). For examination of the regulatory effect of monocytes on LAK induction by IL-2 from autologous blood lymphocytes, lymphocytes and monocytes were separated from the MNC samples on a one-step, discontinuous Percoll gradient, as described previously.[24, 29] The purity of the lymphocytes was >99%. The population enriched in monocytes (82 to 90% as determined by morphology and nonspecific esterase staining) was suspended in CRPMI 1640 medium and adjusted to 5×10^5 monocytes/ml. Monocytes (10^4/well) were added to each well of a 96-well, flat-bottomed Microtest III plate (Falcon Plastics, Oxnard, CA). The monocytes were allowed to adhere for 1–2 h at 37°C, and then non-adherent cells were removed by washing the plates 3 times with warmed RPMI 1640 medium. The purity of the monocytes was >99% as assessed in terms of their morphology, examination with monoclonal anti-monocyte antibody (OKM3) conjugated with FITC, and measurement of nonspecific esterase.

Induction and assay of LAK activity LAK activity was assayed by measuring ^{51}Cr release as described in detail previously.[10, 11, 14] Briefly, blood MNC (1×10^5 per well) were added to wells of flat-bottomed, Microtest III plates and incubated in the presence or absence of IL-2 at 37°C under 5% CO_2 in air for 4 days. The cultures were thoroughly washed and their cytotoxicity against ^{51}Cr-labeled 10^4 Daudi (Burkitt lymphoma) cells was measured at various effector/target (E/T) cell ratios. To examine monocyte-mediated regulation of LAK induction, blood lymphocytes with or without an optimal concentration of IL-2 (1 U/ml) were added to cultures with or without monolayers of autologous monocytes. Preliminary experiments indicated that at this time there was no difference in the numbers of IL-2-treated and untreated lymphocytes (data not shown). After incubation for 4 h, the supernatants (0.1 ml per well) were harvested by brief centrifugation at 1,500 rpm, and their radioactivities 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 with different target cells ranged from 5 to 15% (total lysis).

Statistical analysis The statistical significance of differences in values for groups was analyzed by using Student's *t* test.

RESULTS

LAK activity of blood MNC of subjects without malignancies First, we examined the effect of age on LAK expression induced by IL-2 from blood MNC of control subjects without malignancies. For this, peripheral blood MNC samples from control subjects without malignancies were incubated for 4 days in medium alone (control) or with an optimal concentration of recombinant human IL-2 (1 U/ml), and then the LAK activity of MNC was assayed by measuring ^{51}Cr release from Daudi or PC-9 target cells at various E/T ratios. The ability of blood MNC to generate LAK activity in response to IL-2 was examined in 107 control subjects without malignancy. The subjects were divided into various age groups and the mean (±SD) for each group was calculated. There was no significant difference in the cytotoxic activities of MNC of different age groups incubated in medium alone (data not shown). As shown in Fig. 1, the LAK activity of MNC of elderly control subjects without malignancies was similar to that of MNC of young adults. Similarly, there was no age-dependent change in the induction of LAK activity by IL-2 in MNC of the lung cancer patients and control subjects without malignancies (Fig. 2).

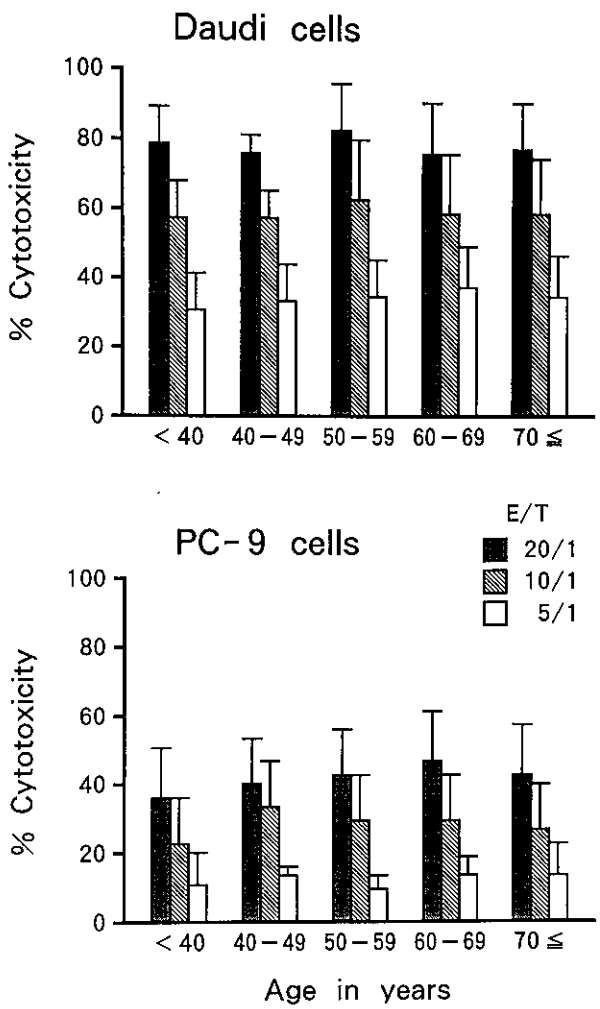

Fig. 1. IL-2-activated killer (LAK) activity of blood MNC of control subjects without malignancies. Columns and bars show means and SDs.

LAK activity of blood MNC of patients with different histological types of lung cancer Next, we compared the LAK activities of MNC of patients with different histological types of lung cancer. For this, blood MNC were incubated for 4 days in medium with or without IL-2 (1 U/ml) and then their LAK activity against NK-resistant Daudi cells at an E/T ratio of 20:1 was measured as described in "Materials and Methods." The results are shown in Table II. Untreated MNC of patients with non-SCLC (squamous cell carcinoma, adenocarcinoma or large cell carcinoma) showed slightly elevated levels of cytotoxic activity. When activated with an optimal concentration of IL-2 (1 U/ml), MNC from patients with lung cancer generated almost the same LAK activity as those from control subjects (69.3±16.2 vs. 75.6±8.7). On classification of the 56 patients into two groups: those with SCLC and those with non-SCLC (squamous cell carcinoma, adenocarcinoma and large cell carcinoma), blood MNC of the group with SCLC were found to

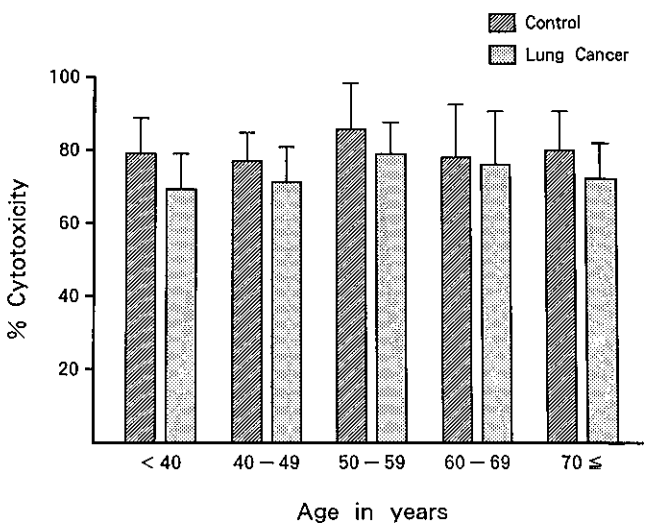

Fig. 2. Comparison of LAK activities of blood MNC of lung cancer patients (▩) of different ages with those of control subjects (▨). Blood MNC of control subjects and lung cancer patients were incubated for 4 days in medium with 1 U/ml of IL-2 before assay of LAK activity against Daudi cells at an E/T ratio of 20:1 as described in "Materials and Methods." Bars show SD of the mean.

Table II. LAK Activities of Peripheral Blood MNC of Control Subjects and Patients with Lung Cancer

Malignant disease	No.	% Cytotoxicity against Daudi cells	
		Medium only	IL-2 (1 U/ml)
None	107	6.4±4.0[a]	75.6±8.7
Lung cancer patients	56	13.7±12.1	69.3±16.2
Squamous cell carcinoma	23	14.7±15.9	73.0±12.3
Adenocarcinoma	13	15.6±11.9	66.2±22.4
Large cell carcinoma	15	14.3±2.0	79.5±11.3
Small cell carcinoma	5	8.2±7.7	61.8±13.9

a) Values are means±SD for the indicated numbers of subjects.

Table III. LAK Activities in Lung Cancer Patients at Different Stages

Factor		No. of cases	% Cytotoxicity against Daudi cells		
			E/T ratio		
			20:1	10:1	5:1
Clinical stage	I	5	80.1±15.0[a]	57.5±10.3	28.6±5.5
	II	6	65.4±29.2	54.4±22.4	37.7±3.5
	III	27	68.3±13.5	49.6±15.4	29.6±8.0
	IV	18	67.2±19.5	47.3±12.6	26.5±12.8
T-factor	1	5	74.4±17.5	52.6±12.3	28.9±12.6
	2	26	71.6±14.2	48.6±14.6	27.6±12.0
	3	14	72.5±8.9	51.6±13.6	25.8±23.6
	4	11	56.5±22.3[b]	40.6±21.6	25.6±2.6
N-factor	0	7	75.5±22.7	55.7±26.9	32.7±26.5
	1	11	69.2±13.1	49.5±12.5	30.2±9.2
	2	33	67.4±16.9	54.8±15.3	30.2±14.5
	3	5	59.5±15.5	30.9±4.8[c]	16.1±1.6[d]
M-factor	0	35	68.6±15.8	44.0±16.9	27.5±13.5
	1	21	64.5±17.5	52.7±16.9	30.4±15.2

a) Mean±SD for the indicated numbers of subjects.
b) Significantly different ($P<0.05$) from the values for patients showing T-factor 3.
c) Significantly different ($P<0.05$) from the values for patients showing N-0 to N-2.
d) Significantly different ($P<0.05$) from the values for patients showing N-1 and N-2.

generate slightly, but not significantly, less LAK activity than MNC from patients with non-SCLC (squamous cell and large cell carcinomas).

Effect of the stage of lung cancer on LAK expression by MNC We examined whether LAK induction was influenced by various factors such as the clinical stage or TNM classification of lung cancer. For this, the 56 lung cancer patients were classified according to these criteria. As shown in Table III, there was no significant difference in the induction of LAK expression by IL-2 in blood MNC from lung cancer patients of various clinical stages. Next, the patients were classified according to the TMN classification. Patients with T-factor 4 had significantly less LAK activity than the other T-factor groups as measured on Daudi cells at E/T ratios of 20:1 and 10:1. Similarly, MNC of patients with N-factor 3 showed lower ability to generate LAK activity than those with N-factor 0 to 2. Interestingly, M-factor did not influence the ability of MNC to become LAK cells in response to IL-2.

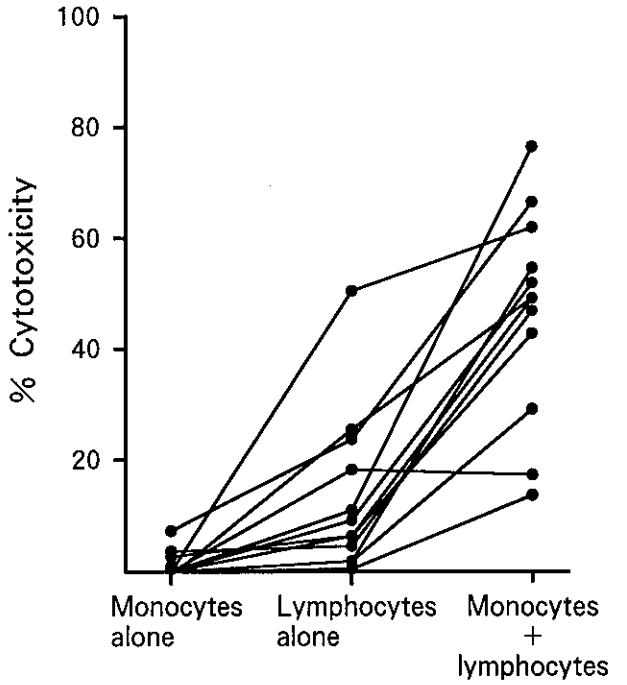

Fig. 3. Up-regulation of LAK induction by monocytes from autologous blood lymphocytes of lung cancer patients.

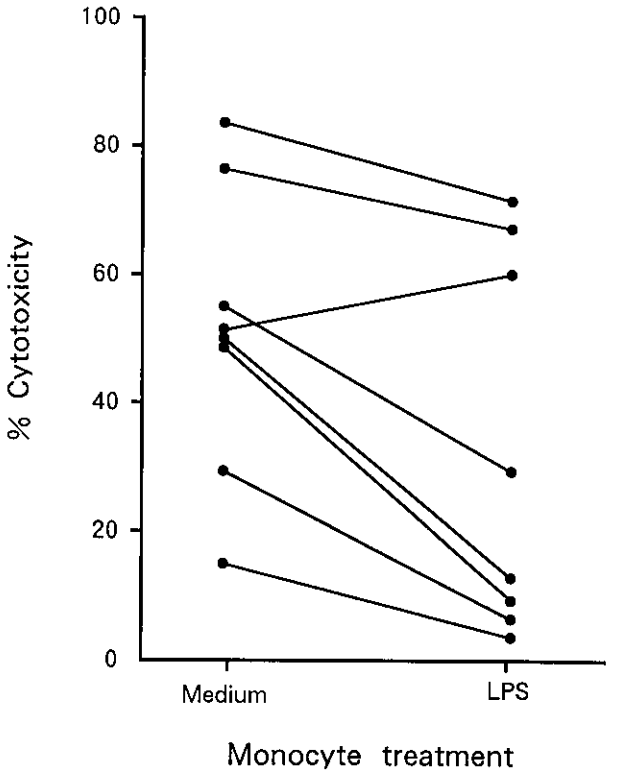

Fig. 4. Down-regulation of LAK induction by LPS-treated monocytes from autologous blood lymphocytes of lung cancer patients.

Regulation by blood monocytes of LAK induction from autologous lymphocytes of lung cancer patients Human monocyte-macrophages of healthy donors were previously found to up- or down-regulate induction of LAK activity by IL-2, depending on their functional states.[13-15] Therefore, we examined whether blood monocytes from lung cancer patients regulated the induction of LAK activity by IL-2 from autologous lymphocytes. Blood monocytes (2×10^4) from 12 patients were plated for 2 h, and then gently washed twice. Autologous blood lymphocytes (10^5) plus IL-2 (1 U/ml) were then added to the cultures. Four days later, the cells were washed and tested for LAK activity on Daudi cells at an E/T ratio of 10:1. No significant cytotoxic activity was seen in cultures of lymphocytes and/or monocytes that had been incubated for 4 days in medium alone (data not shown). As shown in Fig. 3, the LAK activities of IL-2-stimulated lymphocytes were variably cytotoxic, and the activities of those of 11 of 12 patients were increased significantly by addition of autologous blood monocytes at a lymphocyte/monocyte ratio of 10:2.

LAK induction from lymphocytes of normal donors was previously found to be down-regulated by LPS-activated monocytes.[13] Therefore, in a parallel experiment we examined whether monocytes activated with LPS affected LAK induction from autologous lymphocytes of lung cancer patients. For this, blood lymphocytes (10^5) and monocytes (4×10^4) of lung cancer patients at a ratio of 10:4 were incubated for 4 days in medium with IL-2 (1 U/ml) in the absence or presence of LPS (1 μg/ml), and then LAK activity against Daudi cells was measured at an E/T ratio of 10:1. As shown in Fig. 4, addition of LPS suppressed LAK induction in cells from 7 of 8 patients.

DISCUSSION

In this work we found that blood MNC from newly diagnosed lung cancer patients generated similar LAK activity to that of MNC from control individuals without malignancies, and that freshly isolated monocytes of lung cancer patients up- or down-regulated *in vitro* induction of LAK activity from autologous blood lymphocytes, depending on their activation state.

For establishment of an effective protocol for immunotherapy of lung cancer patients with IL-2 and LAK cells, at least two problems have to be solved. One is the effect of aging on the ability of blood lymphocytes to generate

LAK activity, because in humans carcinogenesis occurs most frequently in elderly individuals. Aging has been found to be associated with decreases in characteristics of cellular immunity such as IL-2 production and proliferation in response to IL-2.[25,26] Interestingly, the abilities of blood lymphocytes in MNC to generate LAK activity against Daudi cells and PC-9 lung cancer cells were very similar in all age groups tested of adults without malignancies (Fig. 1). The second problem is whether the presence of lung cancer affects induction of LAK activity by IL-2, because failure of lymphocytes to respond to IL-2 would restrict their role in cell-mediated cancer cell destruction *in vivo*. In the present study we found that lymphocytes in MNC of lung cancer patients could generate LAK activity in response to an optimal concentration of IL-2 *in vitro*. Moreover, LAK expression by MNC of lung cancer patients was not influenced by the age of the patients (Fig. 2), suggesting that treatment with IL-2 may be effective even in elderly patients with lung cancer.

Lung cancer is frequently associated with alterations in immunological functions, such as impaired lymphocyte responses to mitogens,[18,19] decrease in NK activity,[23] antibody-dependent cytotoxicity,[16] decreased ability to produce IL-2[21] and impaired monocyte functions.[22,24] But, these alterations appear to depend on the type of lung cancer and stage of diseases examined. We found that after incubation with an optimal concentration of IL-2, blood MNC of patients with SCLC showed quite similar LAK activity to those of patients with non-SCLC. Moreover, the ability to generate LAK activity was not impaired even in patients with stage IV lung cancer. But, MNC of patients with T-factor 4 generated significantly less LAK activity than those of patients with T-factor 3 (Table III). MNC of lung cancer patients with N-3 also generated less LAK activity in response to IL-2 than those of patients with N-1 or N-2. Interestingly, no correlation was found between LAK activity and the M-factor in lung cancer patients. Thus, the ability of MNC to generate LAK activity appears to be impaired only in lung cancer patients in terminal stages (T4 and N3), though the mechanism of the impaired LAK induction is unknown. Histologically, patients with SCLC showed slightly, but not significantly, less LAK activity than those with non-SCLC (squamous cell and large cell carcinomas). In general, SCLC patients have a worse prognosis than those with non-SCLC.[27] This suppression is probably related to the fact that SCLC grows rapidly and has a high metastatic potential.

LAK induction by IL-2 from lymphocytes was previously found to be up- or down-regulated by various humoral and cellular factors.[8–12] For example, we found that blood monocytes obtained from healthy donors up-regulated LAK induction by IL-2 through cell-to-cell contact, but LAK induction was suppressed by the supernatants obtained from cultures of alveolar macrophages and monocytes which had been activated with endotoxin.[13–15] Recently we found that this LAK-suppressive factor is a new monokine (MW, 68 kd; pI 6.8), which is different from TGF-β (S. Sone *et al.*, submitted for publication). It is important from the therapeutic point of view to examine whether malignancies are associated with alteration in the regulatory role of blood monocytes, because in several malignancies, such as melanoma and colon cancer, depletion of monocytes from blood MNC resulted in significant augmentation of LAK induction by IL-2 *in vitro*.[5,7] This was not the case in our study, however, because purified blood lymphocytes of lung cancer patients alone responded to IL-2 *in vitro* to generate LAK activity, while addition of autologous monocytes to cultures of lymphocytes plus IL-2 resulted in a significant increase in LAK induction (Fig. 3), indicating that even in the presence of lung cancer, blood monocytes may up-regulate *in vitro* LAK expression by lymphocytes induced by IL-2. Under the same experimental conditions, monocytes of almost all the patients suppressed LAK induction when activated with LPS, as seen in normal donors.[13,14]

These studies indicated that, at least *in vitro*, IL-2 could activate blood lymphocytes of patients with lung cancer, even in stage IV, to generate LAK activity. Indeed, clinical studies in humans have shown partial eradication of primary and metastatic pulmonary tumors.[4,30] Tumor-infiltrating lymphocytes (TIL) obtained from melanomas were found to be far more cytotoxic than blood LAK cells,[30] but TIL isolated from patients with primary lung cancer appeared to have less ability to kill autologous and allogeneic tumor cells than peripheral blood LAK cells,[31,32] suggesting that peripheral blood LAK cells should be more effective than TIL for adoptive immunotherapy of lung cancer. Thus, administration of IL-2 and LAK cells induced *in vitro* may be beneficial for treatment of lung cancer in combination with conventional anticancer therapy.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Health and Welfare of Japan and from the Osaka Foundation for Promotion of Clinical Immunology.

(Received December 19, 1990/Accepted March 12, 1991)

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