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Generation and Expansion of Lymphokine-activated Killer Cells from Lymph Node Lymphocytes in Human Lung Cancer

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Abstract—*We cultured lymph node lymphocytes (LNL) from lung cancer patients in the presence of recombinant interleukin 2 (rIL2). The ability of LNL to respond to rIL2 was not affected by the advance of cancer stage when tested for proliferation and for lymphokine-activated killer (LAK) activity. The LAK activity of LNL was comparable to that of the corresponding peripheral blood lymphocytes. The rIL2-induced proliferation of macrophage-depleted LNL was augmented by the reconstitution with autologous alveolar macrophages while the LAK activity was not affected. However, macrophage-reconstituted LNL expanded rapidly and reached higher cell densities and exhibited a significantly lower LAK activity than macrophage-depleted LNL. The diminished LAK activity in macrophage-reconstituted LNL were markedly augmented by the subculture at a low cell density. From these results, we conclude that LNL can be a good material for the postoperative LAK therapy and that macrophage is useful in culture of LAK cells.*

INTRODUCTION

LUNG CANCER is now one of the main causes of deaths due to cancer. It is due partly to the advanced stage of cancer at the time of surgery and to the lack of effective postoperative adjuvant therapies. The adoptive transfer of lymphokine-activated killer (LAK) cells is a new approach to cancer treatment and has been encouraged in the treatment of advanced metastatic cancer [1]. We are planning 'postoperative adjuvant LAK therapy' for advanced stages of lung cancer. Essentially, this therapy necessitates a large source of lymphocytes and *in vitro* culture with recombinant interleukin 2 (rIL2). We feel that regional lymph nodes removed during surgery are a good source of lymphocyte since more than 10^9 lymphocytes can be obtained without any special technique for cell separation.

Much effort has been made to clarify the mechanism of rIL2-induced proliferation [2, 3] and of the LAK phenomenon [4–10]. Recently, it has been reported that peripheral blood monocytes could augment rIL2-induced proliferation of peripheral blood lymphocytes (PBL) [11, 12]. However, it is not clear whether macrophages/monocytes influence the generation of LAK activity. To clarify this issue might be helpful for the optimum cultivation of LAK cells.

In this study, we evaluated regional lymph node lymphocytes obtained from lung cancer patients as a source of LAK cells and further investigated the role of macrophages in the culture of these cells.

MATERIALS AND METHODS

Culture media

Complete culture medium (CM) consisted of RPMI 1640 (Nissui Seiyaku Co., Ltd., Tokyo, Japan) supplemented with 20 mM hepes, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated pooled human AB serum.

Preparation of lymph node lymphocytes (LNL)

Macroscopically non-metastatic regional lymph nodes (hilar and mediastinal lymph nodes) were obtained at the time of surgical operations for lung cancer. Each lymph node was divided into two parts for histological diagnosis and for this study. The latter part of lymph nodes was squeezed between a pair of slides in Hank's balanced salt solution (HBSS) and passed through a gauze filter. The cells were washed two times with HBSS and resuspended in CM. The yielded cells were more than 90% of

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lymphocytes, 5.2–9.9% of macrophages and a very small percentage (less than 0.5%) of polymorphonuclear cells. Viability was usually more than 90%, as determined by trypan blue dye exclusion.

Depletion of macrophages

Approximately 10^7 LNL were incubated in 10 cm plastic dishes for 1 h at 37°C in 5% CO_2. Non-adherent cells were harvested and incubated in plastic dishes for another 1 h. After these serial plastic-adherence procedures, non-adherent cells were collected and resuspended in CM. The yielded cells contained less than 1.5% non-specific esterase positive cells, which will be hereafter referred to as macrophage-depleted LNL.

Preparation of alveolar macrophages

Non-tumor-bearing segments of resected lungs were irrigated with 400 ml of PBS through a Nélaton catheter. The lavage cells were washed three times with HBSS and resuspended in CM. This cell suspension was placed in a plastic dish and incubated at 37°C in 5% CO_2. After 1 h incubation, non-adherent cells were removed by gently washing three times with HBSS. Adherent cells were detached from the dish by a jet stream of HBSS through a 27-gauge needle. More than 98% of the yielded cells were macrophages, as assessed by non-specific esterase staining.

PBL

PBL were isolated from heparinized blood samples, obtained prior to surgical operations, on Ficoll-Hypaque gradients (LSM, Litton Bionetics, Kensington, MD). They were depleted of monocytes by adherence in plastic dishes for 2 h at 37°C and cultured under the same conditions as LNL for generation of LAK cells as described below.

rIL2

Human rIL2 was provided by Takeda Chemical Industries, Ltd., Osaka, Japan. The specific activity was 3.5×10^4 units/mg protein as determined by the ability to maintain NKC-3 cells [13]. When the Biological Response Modifiers Program standard was used, it was 1.2×10^7 units/mg of protein. Usually, 4 units (U)/ml of this rIL2 can induce LAK activity *in vitro*.

Lymphocyte proliferation assay

Macrophage-depleted LNL, 1×10^5/well, were seeded in 96-well flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) and cultured in 5% CO_2 at 37°C for 72 h with various amounts of rIL2. In the macrophage reconstitution experiment, various numbers of autologous alveolar macrophages were added into each well and cultured in otherwise the same conditions as described above. Before addition to the wells, alveolar macrophages were incubated with mitomycin C (40 μg/m) for 30 min at 37°C and washed three times with HBSS. Five hours before termination of culture, each well was pulsed with 0.4 μCi [^{3}H]thymidine (Amersham, Buckinghamshire, U.K.). Cells in each well were harvested onto glass-fiber filters with a multichannel automated cell harvester (Labo Mash No. LM-101; Labo Science Co., Ltd., Tokyo, Japan). Incorporation of [^{3}H]thymidine was assessed with an Aloka Scintillation System 1000 (Aloka Co., Ltd., Tokyo, Japan). Data are reported as mean dpm of triplicate cultures. Standard deviation of triplicates was always less than 15%.

LNL culture for generation of LAK cells

Macrophage-depleted LNL at a concentration of 5×10^5/ml (3×10^6/culture) were cultured in CM containing rIL2 (4 U/ml) with or without monolayered autologous alveolar macrophages for 4–8 days in 5% CO_2 at 37°C. The cultures were refed with fresh CM containing rIL2 (4 U/ml) on day 4. At the end of the culture, the cells were harvested and washed three times with HBSS. The cells were counted and used as effector cells for cytotoxicity assays.

Tumor targets

Two human tumor cell lines were used as targets: QG-56, a lung cancer cell line; K-562, a myeloid leukemia cell line [14]. Fresh tumor cells (termed 326) were prepared from the surgical specimen of a lung cancer patient, histologically diagnosed as adenocarcinoma, with the method described by Grimm and Wilson [15].

Cytolysis assay

Cytotoxic activity was assessed by a 4 h ^{51}Cr release assay as previously described [16]. Briefly, target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 h at 37°C and then washed three times with CM. Target cells (5×10^3/well) were added to various numbers of effector cells in 96-well round-bottomed microtiter plates (Nunc, Roskilde, Denmark). After 4 h incubation at 37°C, 5% CO_2, the culture supernatants were harvested and counted with a gamma counter. Maximal and spontaneous ^{51}Cr release were obtained by incubation of target cells with 0.1 N hydrochloric acid and medium alone, respectively. All determinations were made in triplicate and percent specific cytotoxic activity was calculated according to the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{experimental (cpm)} - \text{spontaneous (cpm)}}{\text{maximum (cpm)} - \text{spontaneous (cpm)}} \times 100.$$

Lytic units (LU) were calculated according to the formula of Pross *et al.* [17]. One lytic unit was defined as the number of effector cells needed to lyse 30% of the targets. Data are reported as LU/10^6 effector cells.

FACS analysis

Cytofluorographic analysis of cell populations cultured with rIL2 was performed with FITC-conjugated monoclonal antibodies to CD3 (Leu4), CD4 (Leu3a), CD8 (Leu2a), and CD16 (Leu11a) (Becton Dickinson, Mountain View, CA). Cells (5×10^5) were incubated for 30 min at 4°C with each of the above-mentioned antibodies. The stained cells were washed three times with PBS and examined for fluorescence with a FACS 440 (Becton Dickinson). The FACS 440 was set to analyze only viable lymphocytes by a 0° light scatter gate. Data analysis was done by counting 10^4 cells.

Statistics

The statistical significance of the data was determined by Student's *t* test.

RESULTS

Influence of the advance of cancer stage on the rIL2-induced response of LNL

We first cultured macrophage-depleted LNL obtained from 21 lung cancer patients with rIL2 (4 U/ml) and assayed the proliferative response. The 21 patients consisted of 10 stage I and 11 stage III when the tumor-nodes-metastasis classification system (Union International Contre Cancer, 1978) was used for staging of the disease. As shown in Table 1, there was no significant difference in the rIL2-induced proliferation between stage I and stage III, although the magnitude of [^{3}H]thymidine incorporation varied among all the patients. Concurrently we assayed LAK activity on day 4 of culture and Table 1 also shows the results. The LAK activity in stage III was comparable to that in stage I when tested against QG-56. From these results, it is suggested that the ability of LNL to respond to rIL2 is not affected by the advance of cancer stage.

Comparison of LAK activity between LNL and PBL

Macrophage/monocyte-depleted LNL and autologous PBL were cultured for 8 days under identical conditions and tested for the LAK activity. The results from seven cases are summarized in Table 2. The LAK activity of LNL were quantitatively similar to that of PBL against both QG-56 and K-562 targets.

Dissociative effects of macrophages on proliferation and LAK activity

Next we investigated the role of macrophage in culture of LAK cells to establish the optimum culture system for LAK cells. In the preliminary experiments, macrophage-depleted LNL were significantly less responsive to rIL2 than whole LNL when tested for 72 h [^{3}H]thymidine incorporation (9693 ± 4283.4 dpm vs. 26599 ± 11496.6 dpm, $n = 9$, $P < 0.01$). Then we performed the reconstitution of macrophage-depleted LNL with autologous alveolar macrophages. As shown in Table 3, rIL2-induced proliferation was augmented with the increased number of macrophages added. In the next experiment, macrophage-depleted LNL with or without 10% autologous alveolar macrophages were cultured with rIL2 (4 U/ml) and tested for proliferative response and LAK activity. The results are shown in Table 4. In spite of the existence of a clear difference in the proliferative response, there was no difference in the LAK activity on day 4 between macrophage-depleted LNL and macrophage-reconstituted LNL. In all of the cases examined, the number of cells recovered on day 4 were one half to two thirds of the initial number of cells in macrophage-depleted LNL and the same as the initial number in macrophage-reconstituted LNL (data not shown). To further clarify the effect of macrophage, we assayed LAK activity on day 8. At this time, the number of macrophage-reconstituted LNL was about three-fold more than that of macro-

Table 1. Influence of cancer stage on the rIL2-response of lymph node lymphocytes (LNL)

	72 h [^{3}H]thymidine incorporation (dpm)		LAK activity (day 4)
	IL2 (−)	IL2 (+)	against QG-56 (LU/10^6 cells)
Stage I	336 ± 71.2	10,364 ± 4824.4 ($n = 10$)	29.7 ± 4.9 ($n = 7$)
Stage III	334 ± 96.2	9513 ± 4303.2* ($n = 11$)	26.1 ± 7.5* ($n = 8$)

*Not significant stage III vs. stage I).

*Table 2. Comparison of LAK activity (day 8) between LNL and autologous PBL**

Effector	LAK activity (LU/10^6 cells) QG-56	K-562
LNL (n = 7)	63.4 ± 24.0†	189.0 ± 47.1†
PBL (n = 7)	69.3 ± 21.9	216.2 ± 40.7

*Macrophage/monocyte-depleted LNL and PBL from seven patients were cultured for 8 days under identical conditions.
†Not significant (LNL vs. PBL).

phage-depleted LNL which was approximately the same as the initial number (Table 5). On the other hand, the LAK activity of macrophage-reconstituted LNL was significantly lower than that of macrophage-depleted LNL in all of the patients examined (Table 5).

Effect of subculturing at a lower cell density on LAK activity

The cell concentrations on day 8 were 1.5–2.5 × 10^6/ml in macrophage-reconstituted LNL, while 0.5–0.7 × 10^6/ml in macrophage-depleted LNL. The lower LAK activity in macrophage-reconstituted LNL may be attributed to higher cell density. To clarify this possibility, we subcultured LNL, cultured with rIL2 (4 U/ml) for 8 days, at a concentration of 5 × 10^5/ml with rIL2 (4 U/ml) and assayed the LAK activity on day 2 after subculture. At the time of subculture, macrophages were removed. Table 6 shows the results from two individual patients, in which the cell densities on day 8 were respectively 1.8 × 10^6/ml and 1.7 × 10^6/ml. As shown in Table 6, the LAK activity in macrophage-reconstituted LNL was markedly augmented by subculture at low cell density.

Phenotypic analysis of LNL cultured with rIL2

To investigate the influence of macrophages on cell populations in LNL, we examined the surface markers of cultured LNL. LNL from five patients (the same as shown in Table 5) were cultured with rIL2 (4 U/ml) for 8 days, and their surface phenotypes were analyzed with a flow cytofluorometry. Before culture, LNL contained 11.8 ± 4.4% of Leu2a-, 63.2 ± 5.9% of Leu3a-, 75.8 ± 4.4% of Leu4-, and 3.5 ± 2.8% of Leu11a-positive cells. On day 8, the histograms with the number of cells on the y axis and the cell size on the x axis formed one peak, and the peak shifted to the right (larger size) compared to that before culture. This finding was consistent with the microscopical finding of blastic changes of LNL. As shown in Table 7, the majority of LNL cultured with rIL2 were Leu4-positive. In macrophage-depleted LNL, Leu2a-positive cells were more dominant than Leu3a-positive cells. As compared with macrophage-depleted LNL, macrophage-reconstituted LNL contained a slightly smaller percentage of Leu2a-positive cells and a much larger percentage of Leu3a-positive cells. Because the number of cells recovered from macrophage-reconstituted LNL was about three-fold more than that from macrophage-depleted LNL, these results indicated that both Leu2a-positive and Leu3a-positive cells were more proliferative by addition of macrophages and that this effect of macrophages was more evident in Leu3a-positive cells than in Leu2a-positive cells.

Effect of macrophages on long term culture of LAK cells

LNL were cultured with rIL2 over 8 days by subculturing at a cell density of 5 × 10^5/ml as needed. In macrophage-reconstituted cultures, autologous alveolar macrophages were newly added at the time of subculturing, which had been cryopreserved at −80°C on preparation. Table 8 shows

Table 3. Effect of addition of macrophages on the rIL2-induced proliferative response of macrophage-depleted LNL

	[^{3}H]thymidine incorporation (dpm)* Exp. 1	Exp. 2	Exp. 3
Adherent cell-depleted LNL	5752	1351	11,754
with 5% macrophages	9618	4810	23,361
with 10% macrophages	13,227	8368	27,263
with 20% macrophages	16,998	12,472	32,528
with 40% macrophages	22,144	12,120	35,520
Macrophages alone†	39	313	152

*Macrophage-depleted lymph node lymphocytes (LNL) from three individual patients were cultured with rIL2 (4 U/ml) and various numbers of autologous alveolar macrophages for 72 h. Without rIL2, the values of [^{3}H]thymidine incorporation were all less than 500 dpm.
†The number of macrophages (4 × 10^4/well) was comparable to 40% macrophages described above.

Table 4. Influence of macrophages on rIL2 (4 U/ml)-induced proliferation and generation of LAK activity

Donor	Cells	72 h [^{3}H]thymidine incorporation (dpm) IL2 (−)	IL2 (+)	LAK activity (day 4)* QG-56	K-562
1	Macrophage-depleted	239	9316	24.4	71.4
	With 10% macrophage	244	22,075	22.0	66.5
2	Macrophage-depleted	264	11,754	15.9	47.6
	With 10% macrophage	296	27,263	17.5	52.0
3	Macrophage-depleted	391	3869	20.0	52.6
	With 10% macrophage	376	7623	21.3	58.8

*Data are reported as LU/10^6 cells.

Table 5. Comparison of LAK activity on day 8 between macrophage-depleted LNL and macrophage-reconstituted LNL

Donor	Cells	Number* of cells recovered	LAK activity (LU/10^6 cells) QG-56	K-562	326
1	Macrophage-depleted	3.0×10^6	90.9	333.3	ND†
	With 10% macrophage	9.3×10^6	26.3	125.0	ND
2	Macrophage-depleted	3.0×10^6	76.9	ND	29.4
	With 10% macrophage	10.2×10^6	33.8	ND	13.7
3	Macrophage-depleted	3.8×10^6	71.4	ND	23.2
	With 10% macrophage	10.1×10^6	49.0	ND	13.2
4	Macrophage-depleted	3.3×10^6	23.0	105.3	ND
	With 10% macrophage	15.0×10^6	8.2	51.3	ND
5	Macrophage-depleted	4.4×10^6	30.3	153.8	ND
	With 10% macrophage	12.4×10^6	11.4	83.3	ND

*Initial number of cells = 3×10^6/culture.
†Not done.

Table 6. Effect of subcultures at a low cell density on the LAK activity in macrophage-reconstituted LNL

Exp.	Time of assay	LAK activity* QG-56	326
1	Before†	19.6	6.7
	After‡	71.4	23.8
2	Before	61.7	ND
	After	120.5	ND

*Data are reported as LU/10^6 cells.
†Macrophage-reconstituted LNL were cultured with rIL2 (4 U/ml) for 8 days and tested for cytotoxicity.
‡Macrophage-reconstituted LNL, cultured for 8 days, were subcultured at a concentration of 5×10^5/ml. Two days after subculture, they were tested for cytotoxicity.
ND = not done.

proliferative and cytolytic profiles of three representative LNL cultures. LNL expanded well in the presence of macrophages but not in the absence of them. In LAK activity, however, there was no remarkable difference between LNL with and without macrophages. These results suggest that the presence of macrophages in rIL2-culture of LNL is necessary for cellular expansion but not for the maintainance of LAK activity.

DISCUSSION

To improve the postoperative survival of advanced lung cancer, we are planning the postoperative adjuvant LAK therapy. In this preparatory study, we selected LNL for a source of lymphocytes, because regional lymph nodes are necessarily removed at surgery and supply a number of lymphocytes. This study demonstrated that the rIL2-response of LNL for proliferation or LAK generation was not affectd by the advance of cancer and that macrophage could augment the proliferative response to rIL2 of LNL but did not directly affect the generation of LAK activity.

Macrophage/monocyte-dependent augmentation of rIL2-induced proliferation is indisputable on the basis of our observation and others' [11, 12]. However its mechanism is controversial. Lakhanpal *et al.* [11] observed that human recombinant interleukin 1 (rIL1) did not substitute for monocytes and that preincubation of peripheral blood mononuclear cells (PBM) with anti-HLA-DR framework mono-

Table 7. Phenotype analysis of LNL cultured with rIL2 for 8 days

Donor*	Cells	Surface marker (%) Leu2a (CD8)	Leu3a (CD4)	Leu4 (CD3)	Leu11a (CD16)
1	Macrophage-depleted	30.0	26.5	71.5	0
	With 10% macrophage	24.2	44.7	77.5	0
2	Macrophage-depleted	33.8	17.5	52.2	21.9
	With 10% macrophage	33.5	36.5	66.6	10.6
3	Macrophage-depleted	38.9	20.9	64.5	0
	With 10% macrophage	31.8	37.3	73.6	0
4	Macrophage-depleted	46.5	26.9	83.5	0
	With 10% macrophage	42.6	42.6	89.0	0
5	Macrophage-depleted	56.2	19.7	75.2	12.2
	With 10% macrophage	50.0	33.3	82.1	6.0

*Each number of donor corresponds to the number shown in Table 5.

clonal antibody inhibited rIL2-induced proliferation. They concluded that rIL2-induced proliferation of PBM was a result of an autologous mixed lymphocyte reaction. On the other hand, Roosnek *et al.* [12] observed that the culture supernatant from lipopolysaccharide (LPS)-stimulated monocytes could substitute for monocytes and thought that the activity of monocyte supernatant was possibly attributed to IL1. In our hands, macrophage supernatant could only partially reconstitute the macrophage-dependent augmentative effect but recombinant human interleukin 1 (Genzyme, Boston, MA) up to the concentration of 100 U/ml did not exhibit any effects (data not shown). We think that both direct contact with macrophages and their soluble factors are involved in the macrophage-dependent augmentation of rIL2-induced proliferation of lymphocytes.

On the other hand, recent studies have shown that macrophages/monocytes inhibited the induction of LAK cells [18, 19]. Further, Nii *et al.* [20] reported that monocytes up- or down-regulated LAK cell induction, depending on their functional state. But all those studies gave no attention to the proliferative states of cultures. It is important to take account of the idea that macrophages/monocytes can augment IL2-induced proliferation of lymphocytes, because the 'identity' of culture conditions between fast- and slow-growing cultures might not be assured. In this study, we observed that macrophage-reconstituted LNL proliferated far better but carried a significantly lower LAK activity than macrophage-depleted LNL on day 8 of culture with rIL2. However, it is unlikely that macrophages directly suppressed the generation of LAK activity since LAK activity of macrophage-reconstituted LNL was comparable to that of macrophage-depleted LNL on day 4. The diminished LAK activity of macrophage-reconstituted LNL was markedly enhanced by subculturing at a lower cellular density. Hoyer *et al.* [21] reported that LAK induction was inhibited at high cell concentrations (higher than 1×10^6/ml) in the presence of monocytes. The significance of this phenomenon may exist in the

Table 8. Expansion of LAK cells with autologous alveolar macrophages

Donor	Cells	Total days in culture	Total-fold expansion	LAK activity* GG-56	K-562
1	Macrophage-depleted	18 days	0.7	15.2	100.0
	With 10% macrophage	18 days	38.9	21.3	111.1
2	Macrophage-depleted	19 days	5.4	13.3	60.0
	With 10% macrophage	19 days	229.1	24.3	90.9
3	Macrophage-depleted	14 days	1.1	20.4	76.9
	With 10% macrophage	14 days	18.4	16.1	71.4

*After culture in the presence of rIL2 with or without macrophages for the indicated periods by subculturing as needed, LNL were further subcultured at a density of 5×10^5/ml without macrophages and tested for LAK activity after two days. Data are reported as LU/10^6 cells.

different way of IL2 to be utilized for proliferation and for generation of LAK activity, because macrophage/monocytes augment IL2-induced proliferation but do not LAK induction. It is thought to be necessary for effective induction of LAK activity to better clarify the significance of cell concentrations and of macrophages/monocytes in culture with rIL2.

We did not address ourselves to the characterization of LAK effector cells in LNL in this study. It is still controversial about LAK effector and precursor cells. Grimm *et al.* [4] first suggested that LAK effector cells in PBL did express the cytotoxic T cell marker (CD3 and CD8). Later Itoh *et al.* [6] reported that Leu11a-positive lymphocytes with NK activity were precursors of LAK cells in PBL. More recent studies [22–24] suggested that LAK activity was not confined to any single subpopulation both at a precursor and an effector level although a Leu11-positive NK-cell population played the major part of LAK activity in PBL. Previously, we reported that LNL had a markedly lower NK activity than PBL in lung cancer patients and that the LAK activity of LNL cultured with purified IL2 was not affected by the treatment with Leu11a antibody and complement [25]. Moreover, in this phenotypic analysis of LNL cultured with rIL2, LNL from three out of five patients examined did not contain Leu11a-positive cells (Table 7). But all these LNL bore the LAK activity comparable to the LNL containing Leu11a-positive cells from two other patients. Therefore we think that Leu11a-positive NK cells are the minor population in LAK activity of LNL.

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