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The Difference in Surface Phenotypes Between Cytotoxic Lymphocytes Induced *In Vivo* by Systemic Administration of Human Recombinant Interleukin-2 and Lymphokine Activated Killer Cells Induced *In Vitro*

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Abstract—*The cytotoxicity of spleen lymphocytes of C57BL/6 mice is augmented both by incubation of spleen lymphocytes with human recombinant interleukin-2 (rIL-2)* in vitro *(LAK cell) and by systemic administration of high doses of rIL-2 into C57BL/6 mice for more than 3 consecutive days. In this study, the precursors and effectors of LAK cells and the cytotoxic cells induced by systemic administration of rIL-2 were characterized by using anti-asialoGM1 and anti-Thy1.2 antibody. The* in vitro *induced LAK cells were demonstrated to be derived partly from asialoGM1 negative cells and the cytotoxicities of LAK cells induced* in vitro *with rIL-2 were partially resistant to lysis by anti-asialoGM1 antibody plus complement in comparison with NK cells. Contrary to this, the cytotoxicities of spleen lymphocytes of C57BL/6 mice pretreated with anti-asialoGM1 antibody were not augmented by* in vivo *injection of rIL-2. In addition, the cytotoxic activities of spleen lymphocytes, augmented by systemic administration of rIL-2, were completely suppressed by anti-asialoGM1 antibody and complement* in vitro.

These findings indicate that the cytotoxic lymphocytes induced in vivo *by systemic administration of rIL-2 are different from* in vitro *induced LAK cells and have the same surface phenotype as NK cells.*

INTRODUCTION

It is well known that lymphokine activated killer (LAK) cells can be induced in the absence of antigen by culturing nonprimed lymphocytes with interleukin-2. These cells have a high degree of anti-tumor reactivity, as shown *in vitro* by the capacity to lyze a wide variety of fresh noncultured tumor cells including NK resistant cells, but they do not lyze normal cells [1–3]. Mule *et al.* [4, 5] reported that the combined administration of human recombinant interleukin-2 (rIL-2) and LAK cells can effectively suppress the artificial pulmonary metastasis of B-16 melanoma cells. Furthermore, LAK cells may also play a role in the immune surveillance against spontaneous neoplasms. On the other hand, Rosenberg *et al.* [6] demonstrated that the systemic administration of rIL-2 alone in high doses leads to the regression of established tumors in mice. In a previous study, we demonstrated that more cytotoxic cells were generated in spleen cells of C57BL/6 mice by multiple injections of high doses of rIL-2 [7, 8].

In this study, by using anti-asialoGM1 antibody and anti-Thy1.2 antibody, we examined the difference in surface phenotypes between cytotoxic lymphocytes induced *in vivo* by systemic administration of human rIL-2 and LAK cells obtained *in vitro* by incubation with rIL-2.

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MATERIALS AND METHODS

1. *Mice*

Specific pathogen-free male C57BL/6 mice were purchased from Sizuoka Experimental Animal

Institute (Sizuoka). The mice were housed under specific pathogen-free (SPF) conditions and used at the age of 6–10 weeks.

2. *Tumor cells*

YAC-1, a Moloney virus-induced lymphoma of A/Sn mice, and B-16 wild and F-10 melanoma cells which are syngeneic to C57BL/6 mice were used for cytotoxicity assay. These cell lines were maintained *in vitro* in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (RPMI-FCS). B-16 and F-10 cell suspensions were obtained by trypsinization of monolayer cultures before each experiment.

3. *Human recombinant interleukin-2*

Lyophilized human rIL-2 (Biogen S.A., Switzerland and Shionogi Pharmaceutical Company, Osaka) was kept at −20°C and reconstituted with balanced salt solution (BSS) at an appropriate concentration immediately before experiment. The titer of rIL-2 was evaluated in terms of the [^{3}H]thymidine incorporation of CTLL-2 cells according to the methods of Gillis *et al.* [9] and was expressed in international units.

4. *Antibody*

Lyophilized anti-asialoGM1 antiserum (Wako Pure Chemical Industries, Osaka) was reconstituted with BSS and kept at −20°C. As reported previously, anti-asialoGM1 antibody can eliminate completely NK activity *in vitro* at a dilution of 1:200 in the presence of guinea-pig complement (1:20) [10]. For the *in vivo* experiment 0.3 ml (500 μg) of anti-asialoGM1 antibody was administered i.v. into the tail vein of C57BL/6 mice [11].

Lyophilized anti-Thy1.2 monoclonal antibody (Cedarlane Laboratories, U.S.A.) was stored at −20°C and was reconstituted with BSS before experiments. This antibody can deplete a cell population of Thy1.2 positive lymphocytes *in vitro* at a dilution of 1:20 in the presence of Cedarlane Low Toxic Rabbit Complement (Cedarline Laboratories, U.S.A.) (1:6).

5. *Spleen cells*

The spleens of C57BL/6 mice were cut into small pieces with scissors and then squeezed through a 45-mesh stainless steel sieve with Eagle's minimum essential medium (MEM). Erythrocytes were removed by exposure by hypo-osmotic shock for 10 s. After centrifugation, the nucleated spleen cells were suspended in RPMI-FCS at a concentration of 1×10^7/ml. Aliquots (15 ml) of spleen cell suspension were placed in Falcon 3003 plastic dishes (Falcon Labware Div., Becton Dickinson Co., Oxnard, CA) and incubated in an atmosphere of humid 5% CO_2 and 95% air at 37°C for 1 h. Each culture was then washed three times with Eagle's MEM, and nonadherent spleen lymphocytes were collected and suspended in RPMI-FCS at an appropriate concentration. The fresh spleen lymphocytes obtained from normal non-treated mice were used for the determination of NK activity.

6. In vitro *induction of LAK cells*

For the induction of LAK cells *in vitro*, spleen lymphocytes (3×10^6/ml) were incubated with human rIL-2 in a final volume of 1 ml in Corning 25820 flat-bottomed 24-well plates (well diameter 16 mm) (Corning Glass Works, New York). To determine the effect of the concentration of human rIL-2 and the time of incubation with human rIL-2 on LAK cell induction, spleen lymphocytes were incubated with 1×10 to 1×10^4 units of human rIL-2 for 72 h, or with 1×10^3 units of human rIL-2 for 1–7 days.

7. In vivo *augmentation of the cytotoxicity of spleen lymphocytes*

For the *in vivo* augmentation of spleen lymphocyte cytotoxicity, C57BL/6 mice were injected with human rIL-2 (1×10^5 units) either 7, 5, 3, 2 or 1 day before experiments, or for 3, 5, 7 or 10 consecutive days before experiments.

8. *Labeling of tumor cells*

Target cells (2.5×10^6/0.25 ml) were incubated with 0.25 ml of 125 μCi of $Na_2{}^{51}CrO_4$ (Japan Radioisotope Association, Tokyo) for 60 min, and washed twice with 15 ml of MEM and once with 15 ml of RPMI-FCS to remove unbound ^{51}Cr. Finally, the cells were suspended at a concentration of 10^5/ml in RPMI-FCS.

9. *Lysis with antibodies*

Spleen cells were suspended at a concentration of 1×10^7/ml in RPMI-FCS containing the antibodies at a final dilution of 1:20. Cells to be treated with complement only were incubated in culture medium. After 45 min incubation at 4°C, Low Toxic Rabbit Complement was added at a final dilution of 1:6, and the cells were incubated for 1 h at 37°C. After 1 h incubation, the cells were washed twice with MEM, and resuspended in 1 ml of RPMI-FCS.

10. *Cytotoxicity assay*

For the determination of the cytotoxicity of spleen lymphocytes, 0.1 ml quantities of the target cell suspension (10^5 ml) were mixed with 0.1 ml of the spleen lymphocyte suspension (2.5×10^6/ml, 5×10^6/ml or 10×10^6/ml) in 96-well V-bottomed microtiter plates (Linbro Scientific Co., Hamden, CT), which produced final effector/target (E/T)

ratios of 25/l, 50/l and 100/l, respectively. The plates were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37°C for 5 h. After incubation, all the plates were centrifuged at 400 ***g*** for 10 min, then 0.1 ml of the supernatant from each well was removed and its radioactivity was counted in an auto γ-counter. Spontaneous target cell release of ^{51}Cr was determined from the supernatant of the target cells cultured without effector cells. The maximum release of ^{51}Cr was obtained by five cycles of freezing and thawing in a dry-ice/alcohol mixture and hot water. Triplicate cultures were used throughout. The percentage cytotoxicity was calculated as:

$$100 \times \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}$$

In the Results section, only the cytotoxicities at the E/T ratio 100:1 are presented.

RESULTS

1. *Kinetics of the induction of LAK cells*

For the induction of LAK cells *in vitro*, spleen lymphocytes were incubated with human rIL-2. To determine the effect of the concentration of human rIL-2 on the induction of LAK cells, spleen lymphocytes were incubated with 1 × 10 to 1 × 10^4 units of human rIL-2 for 72 h. As shown in Table 1, without incubation with rIL-2, the cytotoxicities of lymphocytes almost disappeared even against YAC-1 cells during 3 days. The cytotoxicities of LAK cells against YAC-1 and B-16 melanoma cells increased linearly with increasing concentration of human rIL-2 up to 10^3 units, beyond which further augmentation of the cytotoxicity was not seen.

Table 1. In vitro *induction of lymphokine activated killer cells (LAK) by incubation with various concentrations of human rIL-2*

amount of rIL-2(unit)	cytotoxicity③(%) target cells YAC-1	B-16	F-10
0①	2.6±1.3[a]	0	0
10	18.9±2.2[b]	4.5±0.7	0
10^2	33.2±5.9[c]	9.6±2.8[g]	0
10^3	45.3±5.3[d]	15.8±2.7[h]	4.3(n=2)
10^4	45.0±4.9[e]	17.2±3.8[i]	7.3(n=2)
NK②	24.0±0.9[f]	2.3±0.4[j]	0

1 Spleen lymphocytes of C57BL/6 mice (3 × 10^6/ml) were incubated with various concentrations of rIL-2 for 3 days.

2 NK activity was measured in fresh spleen lymphocytes from normal non-treated mice.

3 The cytotoxicity was evaluated against YAC-1, B-16 and F-10 cells at an E/T ratio of 100:1. Statistical significance: c vs. d, c vs. f, g vs. h are not significant; a vs. b and h vs. j are $P < 0.01$; d vs. f is $P < 0.02$; g vs. j is $P < 0.05$.

In order to determine the effect of a period of incubation with human rIL-2 on LAK cell induction, spleen lymphocytes were incubated with 1 × 10^3 units of human rIL-2 for 1–7 days. As shown in Table 2, augmentation of the cytotoxicities of spleen lymphocytes against YAC-1 and B-16 melanoma cells began 1 day after the beginning of incubation, was maintained until 5 days, and fell thereafter. Based on these results, spleen lymphocytes were incubated with 1 × 10^3 units of human rIL-2 for 3 days for the induction of so-called LAK cells.

Table 2. In vitro *induction of lymphokine activated killer cells (LAK) by incubation with human rIL-2 for various times*

co-culture periods (day)	cytotoxicity③(%) target cells YAC-1	B-16	F-10
1①	33.8±3.9[a]	20.9±1.0[d]	7.6±3.6
3	34.4±2.8[b]	20.8±3.6[e]	8.9±4.3
5	31.0(n=2)	13.0(n=2)	10.1(n=2)
7	27.5	7.3	12.1
NK②	15.4±0.8[c]	3.0±0.6[f]	1.5(n=2)

1 Spleen lymphocytes of C57BL/6 mice (3 × 10^6/ml) were incubated with 1 × 10^3 unit of rIL-2 for various times.

2 NK activity was measured in fresh spleen lymphocytes from normal non-treated mice.

3 The cytotoxicity was evaluated against YAC-1, B-16 and F-10 cells at an E/T ratio of 100:1. Statistical significance: a vs. b and d vs. e are not significant; b vs. c is $P < 0.01$; e vs. f is $P < 0.02$; d vs. f is $P < 0.001$.

2. *Kinetics of the* in vivo *augmentation of cytotoxicities of spleen lymphocytes against YAC-1 and B-16 melanoma cells*

We have previously examined the kinetics of augmentation of the cytotoxicities of spleen lymphocytes by several schedules of administration of human rIL-2 [8]. As reported, the cytotoxicities of spleen lymphocytes against YAC-1 and B-16 cells were significantly elevated 1 day after a single i.p. or s.c. injection of 1 × 10^5 units of human rIL-2, but they returned to pretreatment levels 2 days later. Much greater elevations of cytotoxicities of spleen lymphocytes against YAC-1 and B-16 melanoma cells were observed when mice were injected s.c. with 1 × 10^5 units of human rIL-2 for 3–10 consecutive days. Table 3 shows the effect of doses of human rIL-2 on the augmentation of cytotoxicities in spleen lymphocytes. C57BL/6 mice injected s.c. with 1 × 10^3 to 2 × 10^5 units of human rIL-2 for 3 consecutive days. The cytotoxicities of spleen lymphocytes against YAC-1 and B-16 cells increased linearly and reached peak levels when mice were injected with 1 × 10^5 units of human rIL-2 for 3 consecutive days.

Table 3. In vivo *augmentation of killing activity of spleen lymphocytes of C57BL/6 mice by multiple s.c. injection of various doses of rIL-2*

doses of rIL-2	No. of animals	cytotoxicity (%) target cells YAC-1	B-16	F-10
1×10^3	4	44.2±2.3	15.3±2.1	1.3±1.3
1×10^4	4	55.1±3.2[a]	22.9±2.7[c]	2.7±1.2
1×10^5	4	64.2±2.5[b]	34.2±2.0[d]	11.7±1.9
2×10^5	4	59.9±3.4	27.9±2.2	11.1±2.1
NK	4	20.7±1.2	4.0±3.0	0

C57BL/6 mice were injected s.c. with 1×10^3 to 2×10^5 units of rIL-2 for 3 consecutive days before the experiments. The control group was injected with normal saline. Cytotoxicities of spleen lymphocytes were evaluated against YAC-1, B-16 and F-10 cells at E/T ratios of 100:1. Statistical significance: a vs. b is not significant; c vs. d is $P < 0.05$.

3. *Characterization of the precursors of LAK cells* (*Fig.* 1)

Spleen lymphocytes from normal untreated mice or mice pretreated with anti-asialoGM1 antibody were incubated with 1×10^3 units of human rIL-2 for 3 days. As described previously, the NK activities of spleen lymphocytes 7 days after administration of anti-asialoGM1 antibody were significantly decreased (A). There were no significant cytotoxic responses when spleen lymphocytes were cultured with RPMI-FCS alone (B). Generation of LAK activity was seen when normal spleen lymphocytes were incubated with optimal dose of human rIL-2 (C). Contrary to this, spleen lymphocytes from mice treated with anti-asialoGM1 antibody generated a significant but reduced level of cytotoxicity (D).

These results indicate that at least a proportion of the precursors of LAK cells are asialoGM1 negative cells. In contrast, further examination is necessary to determine whether asialoGM1 positive cells are precursors or immuno-regulatory ancillary cells for the induction of LAK cells.

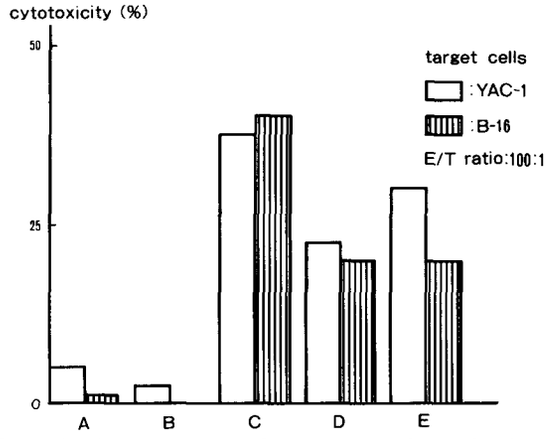


Fig. 1. In vitro *augmentation of killing activity of spleen lymphocytes from C57BL/6 mice pretreated with anti-asialoGM1 antibody by incubation with human rIL-2. NK activity of spleen lymphocytes 7 days after administration of anti-asialoGM1 antibody* (*A*). *Spleen lymphocytes from normal untreated mice were incubated with medium alone* (*B*) *or* 1×10^3 *units of human rIL-2* (*C*) *respectively for* 3 *days. Spleen lymphocytes from mice administered with anti-asialoGM1 antibody* 7 *days before sacrifice were incubated with* 1×10^3 *units of human rIL-2 for* 3 *days* (*D*). *NK activity of normal untreated mice* (*E*).

4. *Characterization of the effectors of LAK cells* (*Table* 4)

After 3 days of culturing, the LAK cells induced by human rIL-2 were subjected to lysis by anti-asialoGM1 antibody or anti-Thy1.2 antibody plus complement to determine their serological phenotypes. The NK activity was almost completely removed by the treatment with anti-asialoGM1 antibody plus complement, but only decreased by approx. 50% by the treatment with anti-Thy1.2 antibody plus complement. On the other hand, the cytotoxicities of LAK cells against B-16 and F-10 melanoma cells were decreased from 23.3% and 14.6% to 16.5% and 7.6% respectively, by the treatment with anti-Thy1.2 antibody plus complement, and more than half of the LAK activities remained after the treatment with anti-asialoGM1 antibody plus complement.

These results suggest that the LAK cells are not classic NK cells.

5. *Characterization of the cytotoxic cells induced by* in vivo *administration of human rIL-2*

To characterize the precursors of the cytotoxic cells induced by *in vivo* administration of human rIL-2, mice administered with anti-asialoGM1 antibody 7 days before the experiment were injected with human rIL-2 for 3 consecutive days before the experiment (Table 5). Augmentation of the cytotoxicities of spleen lymphocytes was not seen in

Table 4. *Characterization by antibody treatment of the effectors of NK, LAK and cytotoxic cells induced by systemic administration of rIL-2*

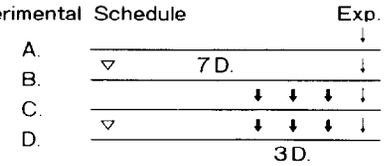
effector cells	treatment	No. of animals	cytotoxicity (%) target cells YAC-1	B-16	F-10
NK cells	A	3	10.8±1.1[a]	3.5±0.9[j]	N.T.
	B	3	5.9±0.6[b]	1.5±0.3[k]	N.T.
	C	3	0.1±0.1[c]	0	N.T.
LAK cells	A	3	34.1±1.3[d]	23.3±0.5[l]	14.6±2.5[r]
	B	3	24.5±2.0[e]	16.5±1.4[m]	7.6±0.8[s]
	C	3	17.1±1.0[f]	14.5±1.3[n]	8.7±0.9[t]
cytotoxic cells induced in vivo	A	3	33.7±1.4[g]	13.9±0.5[o]	N.T.
	B	3	22.6±0.5[h]	10.9±0.5[p]	N.T.
	C	3	1.0±0.8[i]	0.5±0.5[q]	N.T.

Normal untreated spleen cells (NK cells), LAK cells and cytotoxic cells induced by systemic administration of human rIL-2 were treated with complement alone (A), anti-Thy1.2 antibody plus complement (B) or anti-asialoGM1 plus complement (C). Each type of cytotoxicity was evaluated against YAC-1, B-16 and F-10 cells at E/T ratios of 100:1. NT.; not tested. Statistical significance: j vs. h, m vs. n, r vs. s and r vs. t are not significant; a vs. b, d vs. e, e vs. f and o vs. p are $P < 0.05$; l vs. m is $P < 0.02$; a vs. c, d vs. f, g vs. h and i vs. n are $P < 0.01$; g vs. i, h vs. i, o vs. q and p vs. q are $P < 0.001$.

Table 5. In vivo *augmentation of killing activity of spleen lymphocytes of C57BL/6 mice pretreated with anti-asialoGM1 antibody by multiple injection of rIL-2*

	No. of animals	cytotoxicity (%) target cells YAC-1	B-16
A	5	29.8±2.6	14.9±1.5
B	5	1.2±0.3	0.2±0.1
C	5	40.9±1.1	22.1±1.3
D	5	5.1±0.9	2.2±0.3

Experimental Schedule



▽: Anti-asialoGM1 antibody was administered i.v. 7 days before experiment.

↓: Human rIL-2 was injected subcutaneously for 3 consecutive days before experiment.

Cytotoxicities of spleen lymphocytes were evaluated against YAC-1 and B-16 cells at E/T ratios of 100:1.

the mice injected with anti-asialoGM1 antibody 7 days before experiment. This result indicates that asialoGM1 positive NK-like cells constituted the most probable precursors.

Experiments were performed to characterize the effectors of the cytotoxic cells induced by *in vivo* administration of human rIL-2. Spleen lymphocytes from normal untreated mice or mice administered with human rIL-2 for 3 consecutive days before experiments were subjected to lysis by anti-asialoGM1 antibody or anti-Thy1.2 antibody plus complement to determine their serological phenotypes (Table 4). The NK activities and the cytotoxic activities induced by *in vivo* administration of human rIL-2 were almost completely eliminated by *in vitro* treatment with anti-asialoGM1 antibody plus complement. On the other hand, both cytotoxicities of lymphocytes were only partially susceptible to the lysis by anti-Thy1.2 antibody plus complement. From these results, it is suggested that the cytotoxicity of spleen lymphocytes induced by *in vivo* administration of human rIL-2 is dependent on the activity of asialoGM1 positive NK-like cells.

DISCUSSION

IL-2 is a potent immunoregulatory lymphokine which has a wide variety of *in vitro* and *in vivo* effects [1–3, 12–15]. The incubation of normal lymphoid cells with IL-2 leads to the generation of lymphokine activated killer (LAK) cells. Rosenberg *et al.* have shown that the adoptive transfer of these *in vitro* induced LAK cells plus IL-2 can mediate the regression of established pulmonary metastasis from a variety of murine tumors [4, 5, 16]. Contrary to this, we demonstrated in our previous study that the cytotoxicity of spleen lymphocytes against YAC-1 cells, as well as syngeneic B-16 and F-10 melanoma cells, is augmented not only by incubation of spleen lymphocytes with human rIL-2 *in vitro* but also by *in vivo* injection of high dose of rIL-2 into C57BL/6 mice for more than 3 consecutive days [7, 8]. Furthermore, we demonstrated that the adoptive transfer of these activated lymphocytes induced *in vivo* by injection of rIL-2 plus rIL-2 can also reduce the number of pulmonary metastasis of B-16 melanoma cells inoculated i.v. into C57BL/6 mice immunosuppressed by injection of anticancer agents [7].

The present studies were undertaken to investigate the surface phenotypes of the precursors and the effectors of LAK cells and cytotoxic cells induced *in vivo* by injection of rIL-2. NK activity was partially suppressed by the treatment with anti-Thy1.2 antibody plus complement although it was completely abrogated by anti-asialoGM1 antibody plus complement. This evidence suggests that NK cells are composed of asialoGM1 positive cells, and that they are weakly positive or that some of them are positive for Thy1.2. The cytotoxicities of lymphocytes against YAC-1 cells and B-16 melanoma cells, completely suppressed by *in vivo* administration of anti-asialoGM1 antibody, were partially recovered by incubating spleen cells with rIL-2 *in vitro*. These findings indicate that the precursors of LAK cells are derived partly from asialoGM1 negative cells. More than half of *in vitro* induced LAK activities remained by the treatment with either anti-asialoGM1 antibody or anti-Thy1.2 antibody plus complement. These results suggest that (1) LAK cells are composed of cell populations with different surface phenotypes and (2) LAK cells are relatively resistant to the lysis by anti-asialoGM1 antibody plus complement compared to NK cells. On the other hand, the cytotoxicities of spleen lymphocytes of C57BL/6 mice pretreated with anti-asialoGM1 antibody were not augmented by *in vivo* injection of high dose of rIL-2 into C57BL/6 mice for 3 consecutive days. Furthermore, the cytotoxic activities in spleen lymphocytes, augmented by *in vivo* injection of high dose of rIL-2 for 7 consecutive days, were almost completely suppressed by *in vitro* treatment with anti-asialoGM1 antibody plus complement. These findings indicate that the cytotoxicities of spleen lymphocytes induced by *in vivo* injection of rIL-2 are dependent on activated NK cells.

Ettinghausen *et al.* [17] reported that the treatment of mice with rIL-2 *in vivo* produced not only proliferation of lymphocytes but also the generation of LAK cells bearing Thy1 marker on their surface. In our present study, the surface phenotypes of cytotoxic cells induced *in vivo* by the treatment of mice with rIL-2 were similar to NK cells. Thus the cytotoxic cells induced *in vivo* by injection of rIL-2 are different from so-called LAK cells induced *in vitro* by incubation of spleen cells with rIL-2. However, in

our previous study, we showed that the adoptive transfer of these *in vivo* induced cytotoxic lymphocytes plus rIL-2 as well as *in vitro* derived LAK cells plus rIL-2 can mediate the suppression of pulmonary metastasis of B-16 cells inoculated i.v. into C57BL/6 mice immuno-suppressed by injection of anticancer agents [7].

The recent availability of a large amount of purified rIL-2 has opened the possibility of IL-2 therapy in humans [18]. It is important to realize that the systemic administration of rIL-2 mediates the induction of cytotoxic lymphocytes but not LAK cells. Such cytotoxic lymphocytes may be useful for the treatment of metastasis of malignancies independently of so-called LAK cells.

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