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Suppression of pulmonary tumour metastasis in mice by recombinant human interleukin-2: role of asialo GM1-positive cells

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SUMMARY

Recombinant human interleukin-2 (rIL-2) suppressed metastatic tumour colony formation in the lungs of C57BL/6 mice bearing Lewis lung carcinoma (3LL). In tumour-bearing mice given rIL-2, non-specific killer cells that were cytotoxic not only against natural killer-sensitive YAC-1 cells but also against 3LL cells in an *in vitro* ^{51}Cr-release assay were concomitantly induced as tumour metastasis was suppressed. These non-specific killer cells were mostly removed by treatment with anti-Thy 1.2 or anti-asialo GM1 antibody plus complement (C) *in vitro* but not with anti-Lyt 1.2 or anti-Lyt 2.2 plus C, indicating that they were positive for Thy 1 and asialo GM1 but not for Lyt 1 and Lyt 2. In order to explore the mechanism by which rIL-2 suppressed tumour metastasis, we examined the clearance of intravenously injected ^{51}Cr-labelled 3LL cells in the lungs of mice given rIL-2. The rate of tumour cell clearance was increased. This enhanced clearance was almost completely removed by injecting anti-asialo GM1 antibody. In addition, the injection of anti-asialo GM1 antibody also depleted most of the non-specific killer cells induced by administering rIL-2. These results indicate that asialo GM1-positive cells are not only cytotoxic *in vitro* but also play a critical role in the clearance of 3LL cells in the lungs *in vivo*. Our results indicate that asialo GM1-positive cells play an important role as anti-metastatic effector cells in suppressing the metastasis of 3LL cells in mice given rIL-2.

INTRODUCTION

Interleukin-2 (IL-2), a lymphokine, transmits a signal for the proliferation and/or differentiation of T, B, and natural killer (NK) cells via binding with specific cell membrane receptors. Through this action, IL-2 plays a critical role, at least *in vitro*, in a variety of immune reactions (Morgan, Ruscetti & Gallo, 1976; Suzuki *et al.*, 1983; Tsudo, Uchiyama & Uchino, 1984; Naruo *et al.*, 1985). Therefore, it has been hoped that IL-2 would be therapeutically useful to augment the immune responses of patients suffering from certain immunodeficiencies or malignant neoplasma. However, because only limited amounts of IL-2 can be obtained from cultured T cells, it has been very difficult to test its effect *in vivo* using sufficient amounts. Recently, Taniguchi *et al.* (1983) succeeded in cloning IL-2 cDNA from a human T-cell line, Jurkat, for the first time. We also cloned IL-2 cDNA from human peripheral blood leuckocytes and subsequently succeeded in producing purified recombinant human IL-2 (rIL-2) on a large scale (Hinuma *et al.*, 1982; Kato *et al.*, 1985b; Yamada *et al.*, 1986). This rIL-2 has comparable biological activities to natural IL-2 *in vitro* (Kato *et al.*, 1985a; Naruo *et al.*, 1985). Therefore, we have been able to test the effect of IL-2 *in vivo* using adequate amounts.

Although studies reporting the effect of IL-2 *in vivo* have been even fewer (Donohue *et al.*, 1984; Lotze *et al.*, 1985) than those reporting its effect *in vitro*, Rosenberg *et al.* (1985) recently reported that repeatedly administering a high dose of rIL-2 results in the suppression of some tumour metastases in mice. They also demonstrated that the administration induces non-specific killer cells, often referred to as lymphokine-activated killer (LAK) cells, *in vivo* in mice (Chang, Hyatt & Rosenberg, 1984; Rosenberg *et al.*, 1985). However, the precise nature of these cells induced *in vivo* remains unclear in detail, and there is no direct evidence to suggest that the suppression of the tumour metastases is attributable to these cells, as IL-2 affects a variety of immune responses. We have examined several characteristics, including a cell surface phenotype, of non-specific killer cells induced *in vivo* in normal mice by rIL-2 (Hinuma *et al.*, 1986). In this paper, we demonstrate that non-specific killer cells having the same phenotypes as those induced in normal mice are induced in tumour-bearing mice by administering rIL-2. We also demonstrate that the same cell phenotype plays an important role in clearing tumour cells in the lungs of mice given rIL-2.

Abbreviations: C, complement; E/T, effector/target; FCS, fetal calf serum; IL-2, interleukin-2; rIL-2, recombinant human interleukin-2; i.v., intravenously; LAK, lymphokine-activated killer; 3LL, Lewis lung carcinoma; NK, natural killer; NRS, normal rabbit serum; s.c., subcutaneously.

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MATERIALS AND METHODS

Mice

Specific pathogen-free female C57BL/6 mice (H-2^b), 8 weeks old, were purchased from Japan Charles River Co. Ltd. (Atsugi, Kanagawa, Japan) and were used when they were 9 – 10 weeks old. All of the experiments conducted *in vivo* were carried out under the specific pathogen-free barrier system.

Tumours

YAC-1 cells, Moloney leukaemia virus-induced lymphoma cells (H-2^a), were passaged by culture at 37° in RPMI-1640 (Flow Laboratories, Irvine, Ayrshire, U.K.) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mg/ml $NaHCO_3$, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid, and 10% fetal calf serum (FCS) (RPMI-1640 medium containing FCS). A high metastatic line of Lewis lung carcinoma (3LL) (H-2^b) was generously given to us by Dr S. Fujimoto (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo) in 1984. 3LL cells were passaged *in vivo* as a solid tumour in C57BL/6 mice by subcutaneously transplanting small fragments of the tumour mass; if used for isotope labelling, they were transferred to culture *in vitro* to prepare single cell suspensions.

rIL-2

The purification and biochemical characterization of rIL-2 was detailed elsewhere (Kato *et al.*, 1985a; Yamada *et al.*, 1986). Briefly, the purified rIL-2 was apparently homogeneous both on sodium dodecyl sulphate–polyacrylamide gel electrophoresis and reverse-phased high-performance liquid chromatography. Its specific activity was $1{\cdot}2 \times 10^7$ units/mg, based on the Biological Response Modifier Program reference reagent human IL-2 (Jurkat) of the National Cancer Institute (U.S.A.). It had comparable biological activities to purified natural IL-2 on the basis of protein weight *in vitro* (Naruo *et al.*, 1985).

Assay for tumour growth and metastasis

Small fragments of the mass of 3LL cells were transplanted subcutaneously (s.c.) into C57BL/6 mice. Three to seven days afterwards, mice having similar sized tumour masses were selected and used for the experiments. Eighteen to nineteen days after the tumour was transplanted, the mice were killed, the tumours were weighed, and the pulmonary tumour colonies were counted; the number was determined by counting all observable surface colonies.

Administration of rIL-2

rIl-2 was dissolved in saline containing 5% normal mouse serum, and 100 μl of the solution were injected s.c. into C57BL/6 mice as a bolus. Usually, 10 μg/mouse of rIL-2 were administered into mice daily for about 10 days, because this protocol for administering rIL-2 was found to be sufficient to manifest the inhibitory effect of rIL-2 on the metastasis of 3LL cells (K. Ootsu *et al.*, unpublished data). The solution without rIL-2 (vehicle) was injected in the same manner and served as a control.

In vitro *treatment with antibody and complement (C)*

Spleens aseptically removed from C57BL/6 mice were pressed through gauze to prepare a single cell suspension in serum-free RPMI-1640 medium. After the cells were washed twice with the same medium, they were resuspended at 2×10^7 cells/ml in RPMI-1640 medium containing 10% FCS. Mouse monoclonal anti-Thy 1·2, anti-Lyt 1·2, anti-Lyt 2·2 (Cedarlane Laboratories Ltd, Ontario, Canada) and rabbit polyclonal anti-asialo GM1 (Wako Pure Chemical Industries Ltd, Osaka, Japan) antibodies were used at a final dilution of 1:500, 1:500, 1:100 and 1:50, respectively. Low-Tox-M rabbit complement (C) (Cedarlane Laboratories Ltd) was used at a final dilution of 1:10. Antibody and C were added to the spleen cell suspension (2 ml) in Linbro 24-well microplates (Difco Laboratories Inc., McLean, VA), which were then incubated at 37° under humidified atmosphere of 5% CO_2 in air for at least 2 hr. After they had been incubated, the cells were harvested and washed twice with serum-free RPMI-1640 medium. They were resuspended in RPMI-1640 medium containing 10% FCS, and viable cells, determined by a trypan blue dye exclusion test, were adjusted to appropriate concentrations.

In vitro ^{51}*Cr-release assay*

The tumour target cells were labelled according to the method of Kumagai *et al.* (1982). These were incubated, 2×10^6 in 100 μl of a solution containing 0·1 mCi of $Na_2{}^{51}CrO_4$ (Dai-ichi Radioisotope Laboratory, Tokyo, Japan) at 37° for 1 hr. After being washed once with RPMI-1640 medium containing 10% FCS, the cells were resuspended in the same medium and incubated for another 1 hr at 37° to ensure a lower spontaneous release. Then, the cells were washed twice and adjusted to 2×10^5/ml in RPMI-1640 medium containing 10% FCS.

In order to determine killer activity of spleen cells against tumour target cells, the tumour cell suspension (50 μl) and spleen cell suspension (150 μl) were added to each well of round-bottomed 96-well microplates (Nunc Intermed, Roskilde, Denmark) and incubated at 37° for 4 hr under a humidified atmosphere of 5% CO_2 in air. Then, the culture supernatant was harvested and its radioactivity measured in a gamma counter. Percentage specific ^{51}Cr-release was determined by the formula:

$$^{51}\text{Cr-release (\%)} = \frac{(\text{experimental release} - \text{spontaneous release})}{\text{total count} - \text{spontaneous release}} \times 100.$$

The assay was performed in duplicate and the data were expressed as mean values; the standard error of each sample was usually less than 2%.

In vivo *clearance of tumour cells*

3LL cells were labelled with ^{51}Cr as described above. These labelled cells were suspended at $2{\cdot}5 \times 10^6$ cells/ml in serum-free RPMI-1640 medium, and 200 μl of the suspension were injected intravenously (i.v.) via the tail vein of C57BL/6 mice. Three or twenty-four hours later, the mice were killed by cervical dislocation, and the lung, liver, and spleen of each one were collected. The radioactivity of each organ was measured in the gamma counter. Recovery of radioactivity was determined as a percentage of total radioactivity of injected ^{51}Cr-labelled 3LL cells. The data are expressed as mean ± SD.

In some experiments, 200 μl of rabbit polyclonal anti-asialo GM1 antibody solution were injected i.v. twice via the tail vein of C57BL/6 mice both 1 day and 2 days before the labelled tumour cells were injected; normal rabbit serum (NRS) was

injected in the same manner as a control. In these experiments, two mice that were not injected with ^{51}Cr-labelled tumour cells were killed at the same time as the 3LL-injected mice to analyse tumour cell clearance, and killer activity in the spleen cells was determined by the *in vitro* ^{51}Cr-release assay.

Statistics

Statistical analyses were performed by Student's *t*-test.

RESULTS

Suppression of tumour metastasis in mice by rIL-2

Table 1 shows a representative result of the effect of rIL-2 on growth and metastasis of 3LL cells. Although transplanted 3LL cells were growing in mice given rIL-2 and in those given the vehicle, the growth of cells in the former was significantly inhibited compared with that in the latter. Tumour colony formation in the lungs was also reduced by rIL-2, and this effect seemed to be more profound than that on tumour growth: test/control (T/C) values of lung colonies were always less than those of the tumour weights.

Non-specific killer cells: generation by rIL-2 and cell surface phenotypes

We have observed that repeated administration of rIL-2 greatly enhances non-specific killer activities in spleen cells against a variety of tumour cells, including 3LL cells, in normal C57BL/6 mice (Hinuma *et al.*, 1986). Therefore, we expected that the same non-specific killer cells might be induced in 3LL-bearing mice given rIL-2, and that the generation of these cells might be responsible for suppressing the growth or metastasis of tumour cells. As expected, non-specific killer activities in spleen cells

Table 1. Effect of rIL-2 on growth and metastasis of 3LL cells in mice

Agent*	No. of mice	Tumour weight (mg)†	T/C (%)‡	No. of colonies in lungs†	T/C (%)‡
None	10	2649 ± 814		59 ± 27	
Vehicle	5	2792 ± 574	100	49 ± 27	100
rIL-2	5	967 ± 618§	35	6 ± 5§	12

* One-hundred microlitres of a solution containing rIL-2 (10 μg/mouse) were s.c. administered to C57BL/6 mice daily for 9 days, starting on Day 3 after 3LL cells were transplanted on Day 0. The solution without rIL-2 (vehicle) was injected in the same manner and served as a control.

† Tumour weight and metastatic colonies in lungs were determined on Day 18 after tumour transplantation. Data were expressed as mean ± SD.

‡ Test/control (T/C) values were expressed as a percentage of a control (group given the vehicle).

§ $P < 0.01$ as compared with a control.

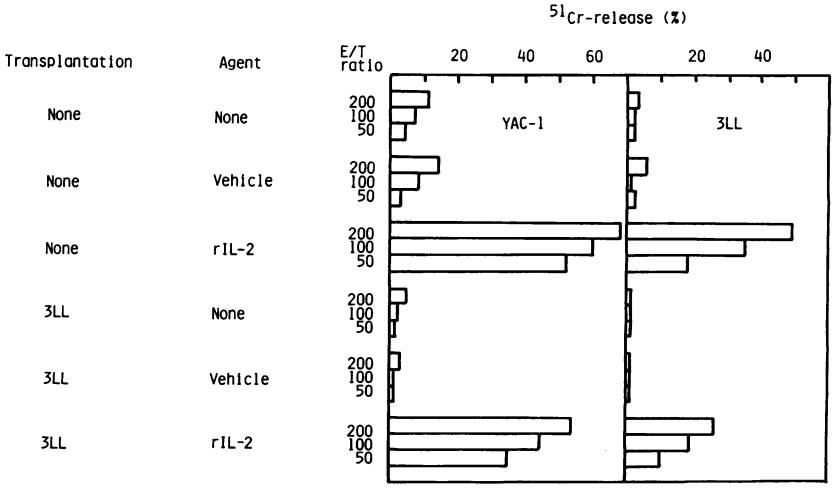

Figure 1. Non-specific killer activities in spleen cells of normal and 3LL-bearing mice after the administration of rIL-2. rIL-2 (10 μg/mouse) was administered s.c. to normal C57BL/6 mice or 3LL-bearing mice (starting 6 days after the transplantation of 3LL cells) daily for 15 days. The solution, which did not contain rIL-2 (vehicle), was injected into mice in the same manner and served as a control. Twenty-four hours after the last dose of rIL-2 or vehicle, the spleen cells of each group of mice (five mice/group) were pooled and their killer activities against YAC-1 (left panel) and 3LL cells (right panel) were determined in a 4-hr ^{51}Cr-release assay.

against YAC-1 and 3LL cells were greatly enhanced by rIL-2 in 3LL-bearing and normal mice (Fig. 1). However, non-specific killer activities against both targets were lower in 3LL-bearing mice given rIL-2 than in normal mice given rIL-2. The reduction of non-specific killer activity in 3LL-bearing mice was consistently observed against both targets at similar rates in repeated experiments (in Fig. 1, killer activity against 3LL cells seemed to be reduced more than that against YAC-1 cells in 3LL-bearing mice given rIL-2, but this is because killer activity in spleen cells of normal mice given rIL-2 reached a plateau). The number of spleen cells in normal mice was about doubled by rIL-2, but such a drastic increase was not observed in 3LL-bearing mice, although their spleens were originally enlarged by the transplantation of tumour cells.

Treatment with various antibodies plus C clearly showed that the enhanced non-specific killer activities in spleen cells of 3LL-bearing mice were mostly removed by treatment with anti-Thy 1.2 or anti-asialo GM1 plus C, but were not removed with anti-Lyt 1.2 or anti-Lyt 2.2 plus C (Fig. 2). These responses were very similar to those of non-specific killer cells induced by rIL-2 in normal mice, but clearly different from those of resident NK cells, which were not as susceptible to treatment with anti-Thy 1.2 plus C as were the non-specific killer cells (Hinuma *et al.*, 1986).

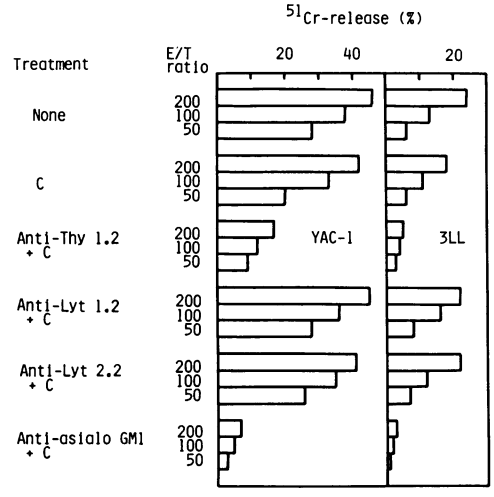

Figure 2. Cell surface phenotypes of non-specific killer cells of 3LL-bearing mice induced by rIL-2. Spleen cells of 3LL-bearing mice given rIL-2 (see legend for Fig. 1) were treated with the indicated antibodies plus complement (C) at 37° for 2 hr. After they had been washed, these cells were adjusted to appropriate numbers in viable cells and their killer activities against YAC-1 (left panel) and 3LL cells (right panel) were determined in a 4-hr ^{51}Cr-release assay.

Tumour cell clearance in mice given rIL-2

In order to explore further the mechanism of suppression of tumour metastasis, we examined tumour cell clearance in mice given rIL-2 (Table 2). Three hours after ^{51}Cr-labelled 3LL cells were injected, the radioactivity recovered in the lungs of mice given rIL-2 was significantly lower than that of control mice, whereas that in livers was slightly higher. Significant change was not detected in the spleen. Although the recovery of radioactivity in the lungs was decreased markedly in both control mice and those given rIL-2 after 24 hr, the recovered radioactivity in the lungs of mice given rIL-2 was slightly but significantly lower than that of control mice. Radioactivity recovered in livers and spleens was almost unchanged after 24 hr, but significantly lower in mice given rIL-2 than in control mice.

Effect of anti-asialo GM1 on the tumour cell clearance in mice given rIL-2

Non-specific killer activities in spleen cells induced by rIL-2 were mostly removed by treatment with anti-Thy 1·2 or anti-asialo GM1 plus C *in vitro* (Fig. 2). Therefore, we examined

Table 2. Effect of rIL-2 on tumour cell clearance in various organs

Agent*	Time after tumour injection (hr)†	Recovery of radioactivity (%)† Lung	Liver	Spleen
None	3	21·9 (±3·2)	16·2 (±1·5)	1·6 (±0·3)
	24	3·4 (±0·6)	20·5 (±2·8)	2·5 (±0·6)
Vehicle	3	27·3 (±1·7)	17·9 (±1·2)	1·7 (±0·3)
	24	4·0 (±0·7)	22·3 (±1·4)	2·5 (±0·6)
rIL-2	3	9·1 (±3·1)‡	21·5 (±1·8)‡	1·7 (±0·2)
	24	2·4 (±0·2)‡	19·8 (±1·3)§	1·9 (±0·3)§

* One-hundred microlitres of a solution containing rIL-2 (10 μg/mouse) were s.c. injected into normal C57BL/6 mice daily for 10 days. The solution without rIL-2 (vehicle) was injected in the same manner and served as a control.

† Two-hundred microlitres of suspension of ^{51}Cr-labelled 3LL cells ($2·5 \times 10^6$ cells/ml) were injected via the tail vein of each mouse 3 hr after the last dose of rIL-2 or vehicle. Total radioactivity of injected ^{51}Cr-labelled 3LL cells was 248,631 c.p.m. Each group consisted of five mice and data were expressed as mean ± SD.

‡ $P < 0·01$, § $P < 0·05$; as compared with a control.

Table 3. Effect of anti-asialo GM1 antibody on the tumour cell clearance in mice given rIL-2

Agent*	Injection†	Recovery of radioactivity (%)‡		
		Lung	Liver	Spleen
None	None	17·5 (±2·0)	18·1 (±1·2)	1·6 (±0·2)
Vehicle	None	25·0 (±5·0)	18·7 (±2·2)	1·8 (±0·2)
Vehicle	NRS	21·2 (±2·4)	18·3 (±2·4)	2·1 (±0·3)
Vehicle	Anti-asialo GM1	38·4 (±3·1)§	16·4 (±0·8)	2·1 (±0·2)
rIL-2	None	9·6 (±1·3)	23·7 (±1·5)	1·7 (±0·1)
rIL-2	NRS	7·1 (±0·9)	24·0 (±1·6)	2·1 (±0·1)
rIL-2	Anti-asialo GM1	33·1 (±1·1)§	20·0 (±0·7)§	2·6 (±0·2)¶

* One-hundred microlitres of a solution containing rIL-2 (10 μg/mouse) were s.c. injected into normal 57BL/6 mice daily for 10 days. The solution without rIL-2 (vehicle) was injected in the same manner and served as a control.

† Two-hundred microlitres of anti-asialo GM1 antibody were i.v. injected twice via the tail vein of mice both 1 day and 2 days before tumour injection. Normal rabbit serum (NRS) was injected in the same manner and served as a control.

‡ Two-hundred microlitres of suspension of ^{51}Cr-labelled 3LL cells ($2·5 \times 10^6$ cells/ml) were injected via the tail vein of each mouse 3 hr after the last dose of rIL-2 or vehicle. Total radioactivity of injected ^{51}Cr-labelled 3LL cells was 200, 254 c.p.m. Recovery of radioactivity was determined 3 hr after tumour injection.

§ $P < 0·01$, ¶ $P < 0·05$; as compared with a control.

whether the injection of these antibodies affected the rate of tumour cell clearance in mice given rIL-2 (Table 3). In this experiment, recovery of radioactivity was determined 3 hr after ^{51}Cr-labelled tumour cells were injected. When mice given the vehicle were previously injected with anti-asialo GM1 antibody, radioactivity recovered in the lungs was increased from 21·2 to 38·4% without significant changes in the liver and spleen. The recovery of radioactivity in the lungs was suppressed by rIL-2. However, the suppression was almost completely removed by injecting anti-asialo GM1 antibody: 7·1% of the recovered radioactivity was greatly increased to 33·1% by injecting this antibody. It is noteworthy that this percentage was very near that in mice given vehicle after anti-asialo GM1 antibody was injected. On the other hand, radioactivity recovered in the liver was slightly decreased and that in the spleen were slightly increased by injecting the antibody.

At the same time, we examined non-specific killer activities in the spleen cells of these mice by the *in vitro* ^{51}Cr-release assay (Fig. 3). The non-specific killer activities in spleen cells of recipient mice against YAC-1 and 3LL cells were drastically depleted by anti-asialo GM1 antibody, regardless of whether the mice had been given rIL-2 or not. In this experiment, injecting normal rabbit serum to mice given rIL-2 seemed to affect the killer activity *in vitro*, but this observation was not reproducible in other experiments (data not shown).

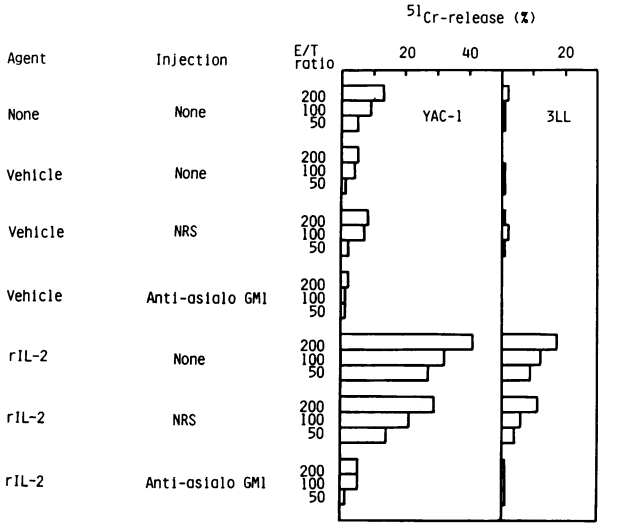

Figure 3. Effect of injecting anti-asialo GM1 antibody on non-specific killer activities in spleen cells of normal mice and those given rIL-2. Two mice that were not injected with ^{51}Cr-labelled 3LL cells were killed at the same time as mice injected with ^{51}Cr-labelled 3LL cells to determine the recovery of radioactivity s(see legend for Table 3), and then spleen cells were pooled and their killer activities against YAC-1 (left panel) and 3LL cells (right panel) were determined in a ^{51}Cr-release assay.

DISCUSSION

We have shown that rIL-2 suppressed tumour metastasis in 3LL-bearing mice; killer cells, which lyse 3LL cells *in vitro*, are concomitantly induced. Induction of killer cells by rIL-2 was consistently observed in both normal and 3LL-bearing C57BL/6 mice, and furthermore in a different strain of mice transplanted with different tumour cells (data not shown); these killer cells commonly had the ability to lyse a variety of tumour target cells, including not only YAC-1 and 3LL cells but also mastcytoma and ovarian carcinoma cells, and the target spectrum was almost the same whether they were induced in normal mice or in those bearing tumours (data not shown).

Therefore, it seems that the majority of these killer cells are non-specific killer cells rather than typical antigen-specific killer T cells. These cells differed from killer T cells on the basis of cell surface phenotypes, as will be discussed later. Rosenberg *et al.* (1985) recently reported a similar phenomenon using different tumour cells: rIL-2 suppresses tumour metastasis and generates non-specific killer cells *in vivo*. In this study, we further clarified the cell surface phenotype of these non-specific killer cells and demonstrated that cells having the same phenotype played an important role as effector cells in tumour cell clearance in lungs.

Our analyses on cell surface phenotypes revealed that non-specific killer cells induced in 3LL-bearing mice by rIL-2 are positive for Thy 1 and asialo GM1 but not for Lyt 1 and Lyt 2. Although these non-specific killer cells are cytotoxic against both YAC-1 (NK-sensitive) and 3LL cells in the *in vitro* ^{51}Cr-release assay, the cytotoxicity against the latter is always lower than that against the former. These results suggest that these non-specific killer cells belong to the same lineage as NK rather than T cells (Kumagai *et al.*, 1982), although they are clearly different from resident NK cells in some respects. Non-specific killer cells possessing almost the same characteristics are induced in normal mice by rIL-2. In addition, non-specific killer cells partially sharing common characteristics are also induced by the culture of spleen cells with rIL-2 *in vitro* (Hinuma *et al.*, 1986).

As tumour metastasis consists of complex processes (Fidler, Gerstein & Hart, 1978), analyses of anti-metastatic effector cells are not easy. Nevertheless, recent studies are gradually revealing each process and the role of anti-metastatic effector cells. The rate of *in vivo* tumour cell clearance is thought to correlate with a certain metastatic process of tumour cells, and NK cells are one of the effector cells (Wiltrout *et al.*, 1983, 1985a; Barlozzari *et al.*, 1985). Therefore, we also examined the rate of tumour cell clearance in mice to analyse further the mechanism of suppression of tumour metastasis by rIL-2. In the course of this study, the recovery of radioactivity in the lungs after ^{51}Cr-labelled 3LL cells were injected was found to be far greater than that of leukaemia cells reported by other investigators (Wiltrout *et al.*, 1983), suggesting that 3LL cells have a high affinity for lungs; in fact these tumour cells are known to form metastatic colonies specifically in the lungs. When it was determined at 3 or 24 hr after ^{51}Cr-labelled 3LL cells were injected, the radioactivity recovered in lungs was reduced by rIL-2. Tumour colony formation in the lungs was also significantly reduced when determined about 2 weeks later (data not shown). These results indicate that tumour cell clearance in the lungs is enhanced by rIL-2, and that this enhanced clearance is closely related to the suppression of the metastatic tumour colony formation. On the other hand, radioactivity recovered in the liver was slightly and transiently increased by rIL-2 and was substantially different from that in the lung; it has been reported that the use of ^{51}Cr-labelled tumour cells is inadequate to assess tumour migration into the liver but not the lung, although the precise reason is unclear (Wiltrout *et al.*, 1983). Radioactivity recovered in the spleen was substantially lower than that in the lung or liver, suggesting that 3LL cells have a low affinity for the spleen; in contrast, it has been reported that injected normal spleen cells migrate largely to the spleen (Wiltrout *et al.*, 1983).

A number of investigators have demonstrated that anti-asialo GM1 antibody specifically depletes asialo GM1-positive cells *in vivo*, usually resident NK cells; as a result, the growth or metastasis of some tumours is drastically increased in mice and rats (Habu *et al.*, 1981; Wiltrout *et al.*, 1983; Barlozzari *et al.*, 1985; Wiltrout *et al.*, 1985a). As non-specific killer cells induced in mice given rIL-2 were positive for Thy 1 and asialo GM1, we also tried to inject anti-asialo GM1 antibody into the mice to determine whether these non-specific killer cells acted as anti-metastatic effector cells *in vivo*. The injection of asialo GM1 antibody almost completely removed the enhanced tumour cell clearance in the lungs of mice given rIL-2. It was noteworthy that this removal was more profound than that expected if merely resident NK cells were removed: the injection of anti-asialo GM1 antibody was thought to deplete not only resident NK cells but also rIL-2-induced non-specific killer cells. In fact, the *in vitro* ^{51}Cr-release assay showed that most of the non-specific killer cells induced by rIL-2 were depleted by injecting anti-asialo GM1 antibody. In addition, the results suggest the possibility that precursor cells as well as the effector cells of non-specific cytotoxicity were positive for asialo GM1 as the antibody was injected before the tumour cells. These results obtained *in vivo*, taken together with the above results obtained *in vitro*, in which non-specific killer cells induced by rIL-2 were positive for asialo GM1, suggest that asialo GM1-positive non-specific killer cells play a critical role in enhancing tumour cell clearance in the lungs of mice given rIL-2. However, we could not rule out the possibility that some asialo GM1-positive cells other than non-specific killer cells defined by the *in vitro* ^{51}Cr-release assay acted in the *in vivo* tumour cell clearance, because Wiltrout *et al.* have reported that murine-activated macrophages express asialo GM1 antigen on their surfaces (Wiltrout *et al.*, 1985b), although the same authors have reported that administering anti-asialo GM1 antibody at a dose similar to that used here does not affect cytotoxic function of macrophages *in vivo* (Wiltrout *et al.*, 1985a). We injected monoclonal anti-Thy 1.2 antibody that could eliminate Thy 1-positive cells *in vivo*, however, mice given rIL-2, but not control mice, were very susceptible to this antibody and died within several hours of receiving the injection; the reason is unknown. Therefore, we could not confirm our conclusion using this antibody.

It has been reported that non-specific killer cells, the so-called LAK cells, induced *in vitro* by IL-2 act as anti-metastatic effector cells against some tumour cells when they are transferred into mice (Mule *et al.*, 1984; Mazumder & Rosenberg, 1984). We have deomonstrated here that asialo GM1-positive cells in mice given rIL-2 are not only cytotoxic *in vitro*, but also act as effector cells of tumour cell clearance in the lungs. These results suggest that asialo GM1-positive cells act as anti-metastatic effector cells in certain metastatic process of 3LL cells. In any case, understanding the anti-tumour mechanism of rIL-2 in the mouse may be important in the successful use of this drug in human subjects.

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REFERENCES

Barlozzari T., Leonhardt J., Wiltrout R.H., Herberman R.B. &

REYONALDS C. W. (1985) Direct evidence for the role of LGL in the inhibition of experimental tumor metastasis. *J. Immunol.* **134,** 2783.

CHANG A.E., HYATT C.L. & ROSENBERG S.A. (1984) Systemic administration of recombinant human interleukin-2 in mice. *J. biol. Resp. Modif.* **3,** 561.

DONOHUE J.H., LOTZE M.T., ROBB R.J., ROSENSTEIN M., BRAZIEL R.M., JUFFE E.S. & ROSENBERG S.A. (1984) *In vivo* administration of purified Jurkat-derived interleukin 2 in mice. *Cancer Res.* **44,** 1380.

FIDLER I., GERSTEIN D. & HART I. (1978) The biology of cancer invasion and metastasis. *Adv. Cancer Res.* **28,** 149.

HABU S., FUKUI H., SHIRAHAMA K., KASAI K., NAGAI Y., OKUMURA K. & TAKAOKI N. (1981) *In vivo* effect of anti-asialo GM1. I. Reduction of NK activity and enhancement of transplanted tumor growth in nude mice. *J. Immunol.* **127,** 34.

HINUMA S., NARUO K., SHIHO M. & TSUKAMOTO K. (1986) Characteristics of murine non-specific killer cells induced *in vivo* by recombinant human interleukin-2. *Immunology*, **59,** 251.

HINUMA S., ONDA K., NARUO K., ICHIMORI Y, KOYAMA M. & TSUKAMOTO K. (1982) Translation of interleukin 2 mRNA from human peripheral blood leukocytes in *Xenopus* oocytes. *Biochem. biophys. Res. Commun.* **109,** 363.

KATO K., NARUO K., KOYAMA K., KAWAHARA K., HINUMA S., TADA H., SUGINO H., & TSUKAMOTO K. (1985a) Purification and partial sequence analysis of human interleukin-2 derived from peripheral blood leukocytes. *Biochem. biophys. Res. Commun.* **127,** 182.

KATO K., YAMADA T., KAWAHARA K., ONDA H., ASANO T., SUGINO H. & KAKINUMA A. (1985b) Purification and characterization of recombinant human interleukin-2. *Biochem. biophys. Res Commun.* **130,** 692.

KUMAGAI K., ITOH K., SUZUKI R., HINUMA S. & SAITO F. (1982) Studies of murine large granular lymphocytes. I. Identification as effector cells in NK and K cytotoxicities. *J. Immunol.* **129,** 388.

LOTZE M.T., FRANA L.W., SHARROW S.O., ROBB R.J. & ROSENBERG S.A. (1985) *In vivo* administration of purified human interleukin 2. I. Half-life and immunologic effects of the Jurkat cell line-derived interleukin 2. *J. Immunol.* **134,** 157.

MAZUMDER A. & ROSENBERG S.A. (1984) Successful immunotherapy of natural killer-presistant established pulmonary melanoma metastasis by the intravenous adoptive transfer of syngeneic lymphocytes activated *in vitro* by interleukin 2. *J. exp. Med.* **159,** 495.

MORGAN D.J, RUSCETTI F.W. & GALLO R.C. (1976) Selective *in vitro* growth of T lymphocytes from human bone marrows. *Science*, **193,** 1007.

MULE J.J., SHU S., SCHWARZ S.L. & ROSENBERG S.A. (1984) Adoptive immunotherapy of established pulmonary metastasis with LAK cells and recombinant interleukin-2. *Science*, **225,** 1487.

NARUO K., HINUMA S., KATO K., KOYAMA M., TADA H., SHIHO O. & TSUKAMOTO K. (1985) Comparison of the biological properties of purified natural and recombinant human interleukin-2. *Biochem. biophys. Res. Commun.* **128,** 257.

ROSENBERG S.A., MULE J.J., SPIESS P.J., REICHERT C.M. & SCHWARZ S.L. (1985) Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2. *J. exp. Med.* **161,** 1169.

SUZUKI R., HANDA K., ITOH K. & KUMAGAI K. (1983) Natural killer (NK) cells as a responder to interleukin 2 (IL 2). I. Proliferative response and establishment of cloned cells. *J. Immunol.* **130,** 981.

TANIGUCHI T., MATSUI T., FUJITA T., TAKAOKA C., KASHIMA N., YOSHIMOTO R. & HAMURO J. (1983) Structure and expression of a cloned cDNA for human interleukin-2. *Nature (Lond.)*, **302,** 305.

TSUDO M., UCHIYAMA T. & UCHINO H. (1984) Expression of Tac antigen on activated normal human B cells. *J. exp. Med.* **160,** 612.

WILTROUT R.H., GORELIK E., BRUNDA M.J, HOLDEN H.T. & HERBERMAN R.B. (1983) Assessment of *in vivo* natural antitumor resistance and lymphocyte migration in mice: comparison of ^{125}I-iododeoxyuridine with ^{111}indium-oxine and ^{51}chromium as cell labels. *Cancer Immunol. Immunother.* **14,** 172.

WILTROUT R.H., HERBERMAN R.B., ZANG S., CHIRIGOS M.A., ORTALDO J.R., GREEN K.M. & TALMADGE J.E. (1985a) Role of organ-associated NK cells in decreased formation of experimental metastases in lung and liver. *J. Immunol.* **134,** 4267.

WILTROUT R.H., SANTONI A., PETERSON E.S., KNOTT D.C., OVERTON W.R., HERBERMAN R.B. & HOLDEN H.T. (1985b) Reativity of anti-asialo GM1 serum with tumoricidal and non-tumoricidal mouse macrophages. *J. Leukocyte Biol.* **37,** 597.

YAMADA T., KATO K., KAWAHARA K. & NISHIMURA O. (1986) Separation of recombinant human interleukin-2 and methionyl interleukin-2 produced in *Escherichia coli. Biochem. biophys. Res Commun.* **135,** 837.