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Detection and characterization of anti-tumour effector cells in Meth-A-bearing mice treated with recombinant human interleukin 2

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SUMMARY

When Meth-A fibrosarcoma-bearing BALB/c mice were injected subcutaneously with 10 μg of recombinant human interleukin 2 (rIL-2) once a day for 10 days, tumour growth inhibition was in the range of 22–31% of that of the control animals. Anti-tumour effector cells against Meth-A were detected in the spleen cells of the tumour-bearing BALB/c mice injected with rIL-2, using a modified Winn-type neutralization assay with the auxiliary injection of rIL-2. To induce the strongest anti-tumour activity in this assay system, the following were necessary: 1) the effector cells were derived from tumour-bearing BALB/c mice; 2) the donors of the effector cells were injected with rIL-2; 3) the recipient mice in the Winn assay were auxiliarily injected with rIL-2 (a modified Winn assay). The anti-tumour effector activity detected in the modified Winn assay was inhibited by treatment with anti-CD8 or anti-asialo GM1 antibodies plus complement (C), but not completely. We supposed that at least two kinds of anti-Meth-A effector cells with different surface antigens, positive for CD8 and asialo GM1 antigens, were induced in the Meth-A-bearing BALB/c mice injected with rIL-2; these populations seemed to function independently and at least partly as anti-tumour effector cells in this tumour-host system. These spleen cells showed *in vitro* cytotoxicity against Meth-A cells, which are resistant to NK cells, if the activity was measured in a 24 h ^{51}Cr-release assay in the presence of rIL-2.

Keywords interleukin 2 anti-cancer Winn assay effector cell

INTRODUCTION

Interleukin 2 (IL-2), discovered by Morgan, Ruscetti & Gallo (1976), is a lymphokine produced by T cells, and the gene coding for human IL-2 was cloned from the Jurkat cell line and sequenced by Taniguchi *et al.* (1983). The anti-tumour activity of IL-2 has been described (Bubenick *et al.*, 1983; Chang, Hyatt & Rosenberg, 1984; Mazumder & Rosenberg, 1984; Rosenberg *et al.*, 1985; Hinuma *et al.*, 1987). Rosenberg's group and our group reported that non-specific killer cells were generated in normal mice injected with rIL-2 (Chang *et al.*, 1984; Rosenberg *et al.*, 1985; Hinuma *et al.*, 1986). Furthermore, Mazumder & Rosenberg (1984) observed that multiple administration of high doses of rIL-2 into the peritoneal cavity of tumour-bearing mice reduced the number of visible pulmonary tumour colonies. Hinuma *et al.* (1987) also reported the anti-metastatic effect of rIL-2 using Lewis lung carcinoma, which is relatively sensitive to non-specific killer cells. In that report, it was suggested that the anti-metastatic effect was caused by non-specific killer cells, positive for asialo GM1 antigen, that were induced by rIL-2 in the host. But the anti-tumour mechanism induced *in vivo* by rIL-2 against other tumours that are resistant to non-specific killer cells still remains unclear in detail.

In this paper, we describe a modified Winn assay system to analyse the IL-2-induced anti-tumour effector cells against Meth-A fibrosarcoma, which is resistant to NK cells. We also describe two kinds of anti-tumour effector cell populations with different surface antigens that are induced by rIL-2 in the host; and each population takes part in the suppression of tumour growth in this tumour-host system, although other populations may also act in some ways as effector cells.

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MATERIALS AND METHODS

Mice

Specific-pathogen-free female BALB/c (H-2^d) mice were obtained from Charles River Japan, Inc. (Atsugi, Kanagawa, Japan). They were 9–10 weeks old when each experiment was started.

Tumour cells

A methylcholanthrene-induced fibrosarcoma, Meth-A (H-2^d), was passaged *in vivo* as an ascitic form in BALB/c mice.

Moloney virus-induced lymphoma cells, YAC-1 (H-2^a), were cultured at 37°C in RPMI-1640 medium (Flow Labs, Irvine, U.K.) supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin, 2 mg/ml $NaHCO_3$, 25 mM *N*-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, and 10% heat-inactivated fetal calf serum (FCS) (RPMI-1640/10% FCS).

Preparation of rIL-2

The production of rIL-2 in *E. coli* and its purification have been presented in detail elsewhere (Kato *et al.*, 1985). The specific activity of the purified rIL-2 was $3{\cdot}5 \times 10^4$ units/mg in our laboratory unit (Hinuma *et al.*, 1982). It corresponded to $1{\cdot}2 \times 10^7$ units/mg, calculated on the basis of the Biological Response Modifiers Program reference reagent human IL-2 (Jurkat) of National Cancer Institute (USA). The purity was more than 99% and contained an undetectable level of endotoxin (less than 0·001%). Various biological activities of the rIL-2 *in vitro* were comparable to those of natural IL-2 (nIL-2) on the basis of protein weight (Naruo *et al.*, 1985).

In-vivo administration of rIL-2

Purified rIL-2 was dissolved in saline containing 5% normal mouse serum (vehicle). One hundred microlitres of the solution containing 10 μg of rIL-2 were injected subcutaneously (s.c.) into mice once a day for 10–15 days. As a control, a solution of vehicle was injected in the same manner.

A modified Winn assay

The inhibition of tumour growth *in vivo* by spleen cells of normal or tumour-bearing mice injected with or without rIL-2 was assayed by a modified Winn-type neutralization assay system (Fig. 1).

Treatment with antibody and complement (C)

Monoclonal mouse anti-Thy 1.2, anti-CD5 (anti-Lyt 1.2), and anti-CD8 (anti-Lyt 2.2) antibodies in ascites (Cedarlane Laboratories, Ltd., Ontario, Canada) were used at final dilutions of 1:500, 1:500 and 1:100 respectively. Polyclonal rabbit anti-asialo GM1 antibody (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used at a final dilution of 1:50. As a C source, Low-Tox-M rabbit complement (Cedarlane Laboratories, Ltd.) was used at a final dilution of 1:10. Spleen cells were suspended at 2×10^7 cells/ml in RPMI-1640/10% FCS. Antibody and C were simultaneously added to the cell suspension and incubated at 37°C for 2 h. After the incubation, the cells were collected and washed twice with serum-free RPMI-1640 medium. After the treatment with antibodies plus C, the number of viable cells was determined by the trypan blue dye exclusion test, and this number was used as the number of effector cells in a Winn assay.

Chromium release assay

The method of this assay has been presented in detail elsewhere (Hinuma *et al.*, 1986). Briefly, target cells, labelled with $Na_2{}^{51}CrO_4$ (Dai-ichi Radioisotope Laboratory, Tokyo, Japan), and effector cells were added to each well of round-bottomed 96-well microplates (Nunc Intermed, Roskilde, Denmark) and incubated for 4 or 24 h under a humidified atmosphere of 5% CO_2 in air. Then an aliquot of the culture supernatant was harvested and its radioactivity was counted. Percentage specific ^{51}Cr-release was determined by the following formula:

$$\text{Specific } {}^{51}\text{Cr-release (\%)} = \frac{(\text{Experimental release} - \text{Spontaneous release})}{(\text{Total count} - \text{Spontaneous release})} \times 100$$

Statistical analysis

The paired Student's *t*-test or the Wilcoxon rank sum test was used to determine any significant difference from the appropriate groups.

RESULTS

Retardation of tumour growth by injection of rIL-2

Recombinant human interleukin 2 inhibited the growth of Meth-A fibrosarcoma (Table 1). Mice bearing Meth-A were injected s.c. with 10 μg of rIL-2 once a day for 10 days starting on day 7 after the tumour transplantation. The tumour growth was significantly retarded and the suppression of tumour weight was in the range of 22–31% of that of the vehicle-treated control animals ($P < 0{\cdot}01$). However, these multiple administrations of rIL-2 could cure no Meth-A-bearing mice in these experiments.

Detection of anti-tumour effector cells in the tumour-bearing mice injected with rIL-2

To detect the anti-tumour effector cells against Meth-A in the tumour-bearing mice injected with rIL-2, we designed a modified Winn assay system (Fig. 1). The representative results of the assay are shown in Table 2. The strongest anti-tumour effector activity was observed when spleen cells from the tumour-bearing mice injected with rIL-2 were used as effector cells and the rIL-2 was injected auxiliarily once a day for 5 days (Exp.1; T/C=4%: $P < 0{\cdot}01$, Exp.2; T/C=8%: $P < 0{\cdot}01$). For example, when the spleen cells of normal BALB/c mice injected with rIL-2 were used as effector cells, the anti-tumour effector activity was weaker than that of the spleen cells of tumour-bearing BALB/c mice injected with rIL-2 (Exp.2; T/C=34% vs. 8%: $P < 0{\cdot}05$).

Table 1. Effect of rIL-2 injection on the growth of Meth-A fibrosarcoma*

Experiment No.	Treatment	No. of mice	Tumour weight (g)	T/C‡ (%)
1	none	5	3·46±0·65†	
	vehicle	5	3·17±0·82	100
	rIL-2	19	0·98±0·29	31§
2	none	3	2·66±0·82	
	vehicle	3	3·58±0·16	100
	rIL-2	25	0·78±0·42	22§

* Meth-A cells (1×10^6) were inoculated s.c. into female BALB/c mice on day 0. The tumour-bearing mice were injected s.c. with 10 μg of rIL-2 or vehicle once a day for 10 days starting on day 7. On the next day after the last injection of rIL-2 (day 17), the tumours were cut off and weighed (mean ± standard deviation).

† Results expressed as mean ± s.d.

‡ rIL-2 treated/vehicle treated.

§ $P < 0{\cdot}01$, as determined using the paired Student's *t*-test, compared to the vehicle-treated group.

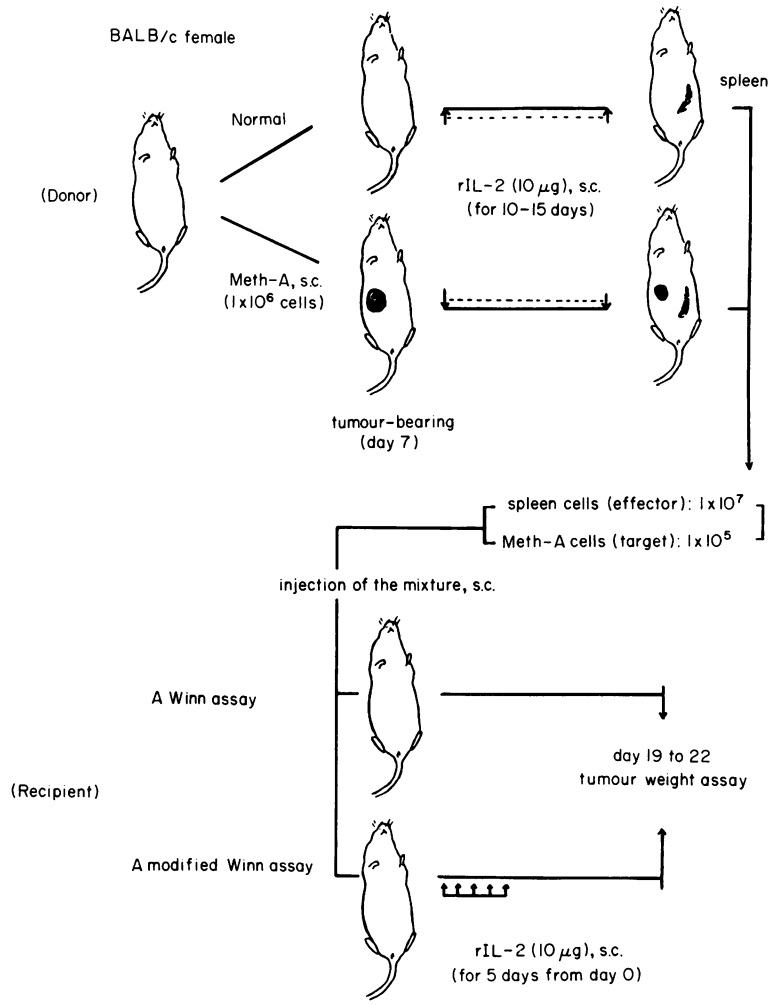

Fig. 1. A modified Winn assay. From the seventh day after the subcutaneous Meth-A inoculation (1×10^6 cells/mouse), the Meth-A-bearing BALB/c mice were injected s.c. with 10 μg of rIL-2 once a day for 10–15 days, at sites separated from growing-tumour. This schedule was found to effectively suppress the growth of Meth-A (K. Ootsu *et al.*, unpublished results) and to stimulate NK activity in spleen cells of normal mice (Hinuma *et al.*, 1986). The spleen cells of normal or Meth-A-bearing BALB/c mice injected with or without rIL-2 were used as effector cells. In the study to characterize the phenotypes of effector cells, the spleen cells were treated with various kinds of antibodies plus C as described later. The effector cells and target Meth-A cells were mixed, and the resulting cell suspensions were washed with serum-free RPMI-1640 medium and resuspended in the same medium. One-hundred microlitres of the mixed cell suspension consisting of 1×10^7 effector cells and 1×10^5 Meth-A cells were injected s.c. into a normal BALB/c mouse on day 0 (a Winn assay). In addition, some of the recipient mice were auxiliarily injected s.c. with 10 μg of rIL-2 once a day from day 0 to day 4, at sites separated from the injection site of tumour cells (a modified Winn assay). The tumours were weighed on days 19 to 22.

Characterization of the effector cells against Meth-A using the modified Winn assay

To characterize the phenotypes of the anti-tumour effector cells detected in the modified Winn assay, the spleen cells of tumour-bearing mice injected with rIL-2 for 13 days were treated with anti-Thy 1.2, anti-CD5, anti-CD8 and anti-asialo GM1 antibodies plus C; then their anti-tumour effector activities were measured in the modified Winn assay. The effector activity was inhibited by treatment with anti-CD8 or anti-asialo GM1 antibodies plus C, but not completely (Fig. 2). The NK activity of Meth-A-bearing BALB/c mice injected with rIL-2 was completely abolished by treatment with anti-asialo GM1 antibody plus C, but not with anti-CD8 antibody plus C (data not shown). We suppose that at least two kinds of anti-Meth-A effector cells with different surface antigens, positive for CD8 and asialo GM1, were induced in the Meth-A-bearing BALB/c mice injected with rIL-2; these populations seemed to function independently *in vivo* and at least partly as anti-tumour effector cells against Meth-A.

Cytotoxic activity of the effector cells against YAC-1 and Meth-A in vitro

The spleen cells of the Meth-A-bearing mice injected with rIL-2 were cytotoxic against NK cell-sensitive YAC-1 cells, using a 4 h ^{51}Cr-release assay (data not shown). However, the spleen cells of the Meth-A-bearing mice injected with rIL-2 were not cytotoxic against NK cell-resistant Meth-A cells in a regular 4 h ^{51}Cr-release assay, but they showed cytotoxic activity if the activity was examined in a 24 h ^{51}Cr-release assay in the presence of rIL-2 (Fig. 3 and Table 3). Spleen cells from normal mice treated with

Table 2. Detection of anti-tumour effector activity against Meth-A in the spleen cells of Meth-A-bearing BALB/c mice injected with rIL-2

Exp. No.	Donor	Treatment of donor*	Treatment of recipient†	Tumour weight‡ (g)	T/C§ (%)
1	—	—	none	6·03±1·11	100
			rIL-2	4·92±1·17	82
	normal mice	none	none	5·67±1·45	94
			rIL-2	4·18±0·97	69**
		none	none	4·80±2·42	80
			rIL-2	4·23±1·90	70
	tumour-bearing mice¶	vehicle	none	7·13±1·53	118
			rIL-2	4·80±1·07	80
		rIL-2	none	3·71±1·23	62**
			rIL-2	0·23±0·33	4††
2	—	—	none	3·10±2·08	100
	normal mice	none	rIL-2	2·17±1·51	70
		vehicle	rIL-2	2·81±0·68	91
		rIL-2	rIL-2	1·05±0·83	34**
	tumour-bearing mice¶	none	rIL-2	4·23±0·81	136
		vehicle	rIL-2	3·53±0·97	114
		rIL-2	rIL-2	0·25±0·54	8††,‡‡

* The donors of effector cells were injected s.c. with or without rIL-2 (10 μg/mouse) once a day for 13 days (Exp.1) or 15 days (Exp.2).

† The recipients in a modified Winn assay were injected s.c. with none (a Winn assay), vehicle or 10 μg/mouse of rIL-2 (a modified Winn assay) once a day for 5 days from the day of cell inoculation.

‡ The tumours of five recipients were weighed on day 22 (Exp.1) or day 19 (Exp.2) after cell inoculation. Mean ± s.d. of five tumour weights.

§ Test/control

¶ BALB/c mice were injected s.c. with the Meth-A firbrosarcoma cells (1×10^6 cells/mouse) on day 0 and the injection of rIL-2 was started seven days after the inoculation.

** $P < 0.05$, as determined using the paired Student's *t*-test, compared to the control group.

†† $P < 0.01$, as determined using the paired Student's *t*-test, compared to the control group.

‡‡ $P < 0.05$, as determined using the Wilcoxon rank sum test, compared to the lane 4 group (the donors of effector cells were rIL-2-treated normal mice and the recipients were treated with rIL-2).

rIL-2 also had the cytotoxic activity against Meth-A cells by a 24 h ^{51}Cr-release assay in the presence of rIL-2 (Table 3). However, the cytotoxic activity of spleen cells from normal mice treated with rIL-2 was much weaker than that of spleen cells from tumour-bearing mice treated with rIL-2. The spleen cells of tumour-bearing mice, tumour-bearing mice injected with vehicle, and normal mice were not cytotoxic against Meth-A in a 24 h ^{51}Cr-release assay even in the presence of rIL-2 (Table 3). These *in vitro* results are fully compatible with the *in vivo* results mentioned before in which the effector cell activity of Meth-A-bearing BALB/c mice injected with rIL-2 was stronger than that of normal BALB/c mice injected with rIL-2, when the recipient mice in the Winn assay were auxiliarily injected with rIL-2 (Table 2). Therefore, it was confirmed that there were cytotoxic cells against Meth-A in the spleen cells of the tumour-bearing mice injected with rIL-2, and that in order to show them *in vitro*, they needed to be tested for 24 h in the presence of rIL-2.

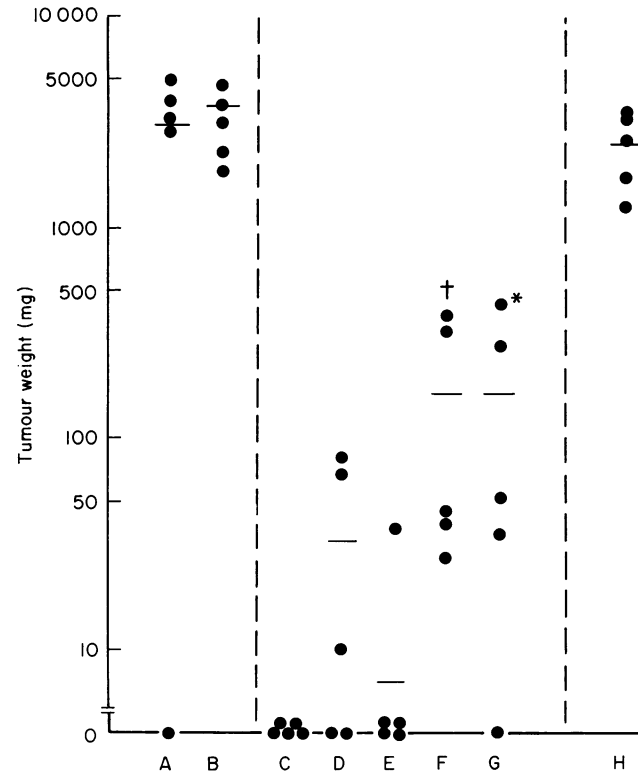

Fig. 2. Effect of various antibodies plus complement treatment on the effector cell activity in a modified Winn assay. The effector cells from Meth-A-bearing BALB/c mice injected with rIL-2 were prepared as shown in Fig. 1. They were not treated (A, B) or treated with complement (C), anti-Thy 1.2 antibody + complement (D), anti-CD5 antibody + complement (E), anti-CD8 antibody + complement (F) or anti-asialo GM1 antibody + complement (G). Then these effector cells (1×10^7 viable cells/50 μl) were mixed with the target cells (Meth-A; 1×10^5 cells/50 μl), and the mixture was injected into recipient BALB/c mice. The recipient mice were non-treated (A), injected with vehicle (B) or with rIL-2 (10 μg/mouse) once a day for 5 days from the day of inoculation (C to G). As a control, the Meth-A fibrosarcoma cells (1×10^5 cells/100 μl) without effector cells were injected into normal BALB/c mice without auxiliary injection of rIL-2 (H). On day 21 after the inoculation of effector and target cells, the tumours were weighed. * $P < 0.05$, † $P < 0.005$, as determined using the Wilcoxon rank sum test, compared with the group C.

DISCUSSION

In the study reported here, we showed that rIL-2 inhibits the growth *in vivo* of Meth-A fibrosarcoma which is resistant to NK cells (Table 1). Furthermore, anti-tumour effector cells were detected using a modified Winn assay system.

The spleen cells of normal BALB/c mice injected with rIL-2 had anti-tumour activity in a Winn assay when rIL-2 was auxiliarily injected, but the activity was weaker than that of Meth-A-bearing mice injected with rIL-2 (Table 2). We previously reported that non-specific killer cells, which were positive for asialo GM1 and Thy 1 antigens and detected in a regular 4 h ^{51}Cr-release assay, were induced in normal mice injected with rIL-2 (Hinuma *et al.*, 1986). The augmentation of such activity was also observed in the tumour-bearing mice injected with rIL-2, as in normal mice (data not shown). However, these effector cells were not cytotoxic against NK-

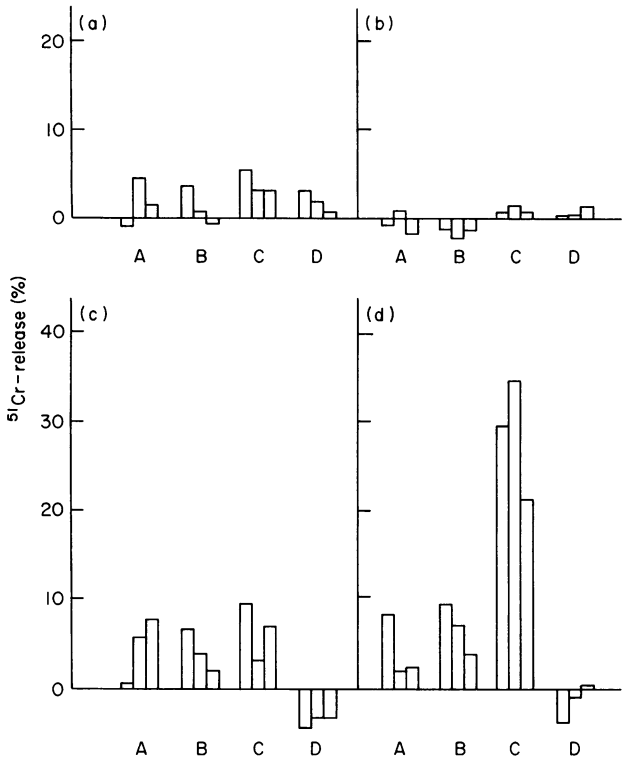

Fig. 3. Cytotoxic activity of effector cells against Meth-A fibrosarcoma cells *in vitro*. The effector cells were derived from non-treated (A), vehicle-injected (B), and rIL-2-injected Meth-A-bearing BALB/c mice (10 μg/mouse of rIL-2 was injected s.c. once a day for 13 days) (C), and from normal BALB/c mice (D). The cytotoxic activities against Meth-A *in vitro* were examined using a ^{51}Cr-release assay in the absence (a and c), or in the presence (b and d) of rIL-2 (100 ng/ml). After 4 h (a and b) or 24 h (c and d) incubation, the radioactivity of the supernatants was counted. E/T ratio was 200, 100 and 50 from the left to the right respectively in each group.

Table 3. Cytotoxic activity of effector cells against Meth-A fibrosarcoma cells *in vitro*

Donor mice	Drug†	4 h* rIL-2 −	4 h* rIL-2 +	24 h* rIL-2 −	24 h* rIL-2 +
normal	none	−1·5	0·1	1·1	1·3
mice	vehicle	−1·0	0·7	0·3	2·3
	rIL-2	−1·2	0·2	1·7	4·6
tumour-	none	0·2	−0·6	2·2	0·7
bearing	vehicle	0·2	0·2	2·8	1·6
mice	rIL-2	−0·7	1·1	0·4	18·0

Results expressed percentage ^{51}Cr-release.

* A 4 h or 24 h ^{51}Cr-release assay in the presence or absence of rIL-2 (100 μg/ml).

† Normal or tumour-bearing BALB/c mice were injected s.c. with or without rIL-2 (10 μg/mouse) once a day for 10 days. Tumour-bearing BALB/c mice were injected s.c. with the Meth-A firbrosarcoma cells (1×10^6 cells/mouse) on the 7th day before the starting day of the drugs treatment.

cell-resistant Meth-A cells under a regular 4 h ^{51}Cr-release assay. It is noteworthy that the cytotoxic cells against Meth-A cells were detected only when they were assayed for 24 h in the presence of rIL-2 (Fig. 3 and Table 3). The need of rIL-2 *in vitro* to show cytotoxicity against Meth-A cells seems to have some relevance to the need of auxiliary rIL-2 for the maximum *in vivo* effector activity. Spleen cells from normal mice treated with rIL-2 also had the cytotoxic activity against Meth-A cells by a 24 h ^{51}Cr-release assay in the presence of rIL-2 (Table 3). This cytotoxic activity was weaker than that of spleen cells from tumour-bearing mice treated with rIL-2. These *in vitro* results are also compatible with the *in vivo* results that show effector cell activity of Meth-A-bearing BALB/c mice injected with rIL-2 was stronger than that of normal BALB/c mice injected with rIL-2.

The treatment of anti-asialo GM1 antibody plus C completely abolished the NK-like non-specific killer activity (data not shown), but the anti-tumour activity was only slightly abolished by this treatment (Fig. 2). Therefore, we supposed that anti-tumour effector cells other than NK-like, asialo GM1$^+$, non-specific killer cells might be induced in the tumour-bearing mice injected with rIL-2. Besides the treatment of anti-asialo GM1 antibody plus C, the treatment of anti-CD8 antibody plus C also partially abolished the neutralization activity in the modified Winn-type assay. So, we supposed that CD8$^+$ effector cells, as well as asialo GM1$^+$ effector cells, might be induced in the tumour-bearing mice by rIL-2. Furthermore, it is possible that CD8$^+$ cells were also cytotoxic to Meth-A cells when they were incubated in the presence of IL-2 for 24 h. It is supposed that the CD8$^+$ and/or asialo GM1$^+$ anti-tumour effector cells detected in our system might be cytotoxic against Meth-A cells in the presence of rIL-2 *in vivo*. We are interested in the issue of whether or not the CD8$^+$ cells are specific killer cells. This issue is unclear at present, and we are going to investigate this point in future.

Forni, Giovarelli & Santoni (1985) reported that multiple local injections of low doses of IL-2 where lymphocytes from tumour-bearing mice contact neoplastic cells led to very efficient tumour inhibition. They used the spleen cells of non-treated tumour-bearing mice as effector cells, and IL-2 was injected daily for 10 days at the challenge sites in a Winn assay. They evaluated the anti-tumour effector activity on the basis of tumour takes, while we evaluated it on the basis of tumour weights as well as tumour takes. They suggested that Thy 1.2$^+$, CD5$^+$, CD8$^-$ and asialo GM1$^+$ cells independently worked as effector cells in their experimental system. Our experimental system differed from theirs in that we used the spleen cells of tumour-bearing mice injected with rIL-2 as effector cells, and IL-2 was injected daily for 5 days at the sites separated from that of the growing tumour. Anyway, we did not observe CD5$^+$ cells as anti-tumour effector cells in our system. Nakajima *et al.* (1985) also reported that CD5$^+$ cells play a role in inhibiting tumour growth. They suggested that CD5$^+$ CD8$^-$ cells recognized the tumour-associated antigens to release macrophage activating factor: macrophages activated by this factor had anti-tumour activity. Various kinds of effector cell might play roles in inhibiting tumour growth, but their roles and effective stages might differ depending on the tumour-host relation (Berendt & North, 1980; North, 1982; Mills & North, 1983; Evans, 1986).

Therefore we do not rule out $CD5^+$ cells as anti-tumour effector cells.

The sequential treatments of (anti-Thy 1.2 antibody plus C) and (anti-asialo GM1 antibody plus C) or (anti-CD8 antibody plus C) and (anti-asialo GM1 antibody plus C), abolished the anti-tumour activity more than each single treatment, but the effect was not complete (data not shown). Adherent cells also act in some ways as effector cells against Meth-A in our system (data not shown), although the exact role of these effector cells remains to be clarified in detail.

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