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Effect of recombinant interleukin 2 (R-IL2) on in vivo growth of murine myeloma X5563

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Summary. The present study deals with the effect of recombinant interleukin 2 (R-IL2) on in vivo growth of murine myeloma X5563. Administration of R-IL2 (5×10^4 J.U./mouse per day) s. c. starting 1 day after X5563 inoculation i.d. had a marginal effect on the growth of X5563, and all the mice repeatedly given R-IL2 from day 1 to day 17 died. However, daily administration of R-IL2 starting 7 days after the tumor inoculation was highly effective and significantly lengthened survival time compared with the control mice injected with vehicle alone. About 50% of the treated mice were completely cured, and survived for more than a month after the therapy ceased. In a representative experiment, where the growth of X5563 was slow because of the small number of inoculated tumor cells, all the mice ($n=6$) given R-IL2 from day 11 to day 23 showed complete cure of the established X5563 solid tumor. These mice showed in vivo protective immunity and in vitro cytotoxic T cell responses to X5563 tumor antigens. Histologically, a large number of macrophages and lymphocytes had infiltrated the area around the necrotic X5563 tumor mass in the mice which had received R-IL2 therapy. These results suggest that repeated injections of R-IL2 at the local site after tumor development can augment antitumor immunological responses and subsequently induce tumor regression.

Introduction

Since interleukin 2 (IL2) was originally described as a humoral factor required for continual proliferation of activated T cell clones [12, 13, 21], various biological activities of this lymphokine have been reported. It augments natural killer (NK) activity [18, 23], generates alloantigen-specific cytotoxic T lymphocytes (CTL) [17, 27] and induces other lymphokines such as interferon-gamma [6] and a B cell growth factor [19]. Recently, attention has been paid to the possible immunotherapy of cancer with this substance, and IL2 has been reported to be extremely effective in augmenting the efficacy of IL2-dependent long-term cultured T lymphocytes combined with cyclophosphamide in adoptive immunochemotherapy [2]. Rosenberg et al. have reported that spleen cells and peripheral blood mononuclear leukocytes cultured with IL2 for as little as 2 days generate cells designated as lymphokine-activated killer (LAK) cells in mice [25] and humans [15, 16]; they display lytic activity against fresh syngeneic NK-resistant tumor cells as well as NK-sensitive cells. The Rosenberg group have demonstrated that the adoptive transfer of LAK cells inhibited pulmonary metastases of B16 melanoma without the prior administration of cyclophosphamide or other immunosuppressants [20]. It was also reported that supernatants from concanavalin A-stimulated rat spleen cell cultures containing IL2 inhibited the growth of a transplantable 3-methylcholanthrene-induced sarcoma in syngeneic mice [1]. However, the IL2 used in these experiments was crude or semipurified, and therefore the possibility that other contaminating factors were responsible for the antitumor activity cannot be ruled out. This problem has been solved using recent DNA-recombination techniques. Taniguchi et al. established the cloning and expression of a human IL2-complementary DNA and the cDNA could encode biologically active IL2 [26]. The availability of highly purified recombinant IL2 in large quantities makes it possible to evaluate the in vivo antitumor effectiveness of this lymphokine more extensively. Rosenberg et al. have reported that adoptive immunotherapy with recombinant IL2-induced LAK cells was effective in inhibiting the metastases of established pulmonary sarcoma [22]. Recently, they reported that systemic administration of high doses of recombinant IL2 also could inhibit the pulmonary metastases of MCA-105, MCA-106 sarcomas and B16 melanoma, and the growth of established palpable s. c. MCA-105 sarcomas [24]. In this work, we examined the therapeutic effect of recombinant human IL2 (R-IL2) alone in C3H/HeN mice against syngeneic myeloma X5563 and found that multiple s. c. injections of R-IL2 starting 7 or 11 days after tumor inoculation was highly effective, leading to complete cure of some mice. Moreover, the cured mice showed in vivo protective immunity and in vitro cytotoxic T cell responses to X5563 tumor antigens.

Materials and methods

Mice. Female C3H/HeN mice were purchased from Charles River Japan Inc. and used at 7–10 weeks of age.

Tumors. X5563 myeloma and MH134 hepatoma which were kindly supplied by Prof. Hamaoka (Osaka University, Osaka, Japan), both derived from the C3H/He strain and maintained by i. p. passage into syngeneic C3H/HeN mice in the ascitic form, were utilized.

IL2. The recombinant human IL2 (R-IL2) (sp. act. $1-2 \times 10^7$ J. U./mg protein: Biogen, Geneva, Switzerland)

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was adjusted to 2.5×10^5 J. U./ml with the vehicle, i.e., phosphate-buffered saline solution (PBS, pH 7.2) containing 0.0083 % human serum albumin. R-IL2 was assayed by ^{3}H-thymidine incorporation of the R-IL2-dependent CTLL-2 cell line obtained from Biogen. Units of R-IL2 are defined as the reciprocal of the dilution that induced 50% of the maximum ^{3}H-thymidine incorporation by CTLL-2 cells and adjusted to Jurkat Units (J. U.) of the reference human Jurkat-derived natural IL2 standard (BRMP. ISDP-84) obtained from the National Cancer Institute.

Therapy with R-IL2. Groups of mice inoculated i. d. with 10^5 to 10^6 X5563 tumor cells were given vehicle or 5×10^4 J. U. R-IL2 in 0.2 ml of PBS per mouse per day s. c. starting 1 day or 7–11 days after tumor inoculation. Tumor growth was measured and expressed as the geometric mean of the tumor diameter with the standard error. In some experiments, therapeutic effect was expressed as a percentage of inhibition of control tumor diameter according to the following formula:

$$1 - \frac{\text{Tumor diameter of mice treated with R-IL2}}{\text{Tumor diameter of control (nontreated) mice}}.$$

Survival was determined at 50 days after tumor inoculation.

In vivo tumor-neutralizing assay. Spleen cells (1×10^7) from normal or X5563-cured mice (by R-IL2 therapy) were admixed with 1×10^5 viable tumor cells and injected i. d. into normal syngeneic mice. Tumor growth was expressed as the mean tumor diameter in groups of four mice.

In vitro sensitization of cytotoxic effectors and cytotoxicity assay. The method for cytotoxicity assay was essentially the same as described by Fujiwara et al. [9, 11]. Briefly, 5×10^6 spleen cells from normal or X5563-bearing mice treated with or without R-IL2 were sensitized with 1×10^5 X5563 tumor cells treated with mitomycin C (100 µg/ml, 37 °C, 60 min). Then effector cells generated after 5 days of culture were assayed with ^{51}Cr-labeled X5563 tumor target cells in a ^{51}Cr-release assay for 4 h.

Treatment of spleen cells with antibody plus complement. Spleen cells (10^7/ml) from X5563-cured mice given R-IL2 therapy were incubated at room temperature for 30 min with monoclonal anti-Thy 1.2 antibody (CEDARLANE, Hornby, Ontari, Canada) at a dilution of 1:200. Then, cells were washed and incubated at 37 °C for 60 min with Low-Tox-M rabbit complement (CEDARLANE) at a final dilution of 1:10. Cells were washed 3 times with Hanks balanced salt solution and used as responding cells for CTL induction.

Histological examination. X5563 tumors inoculated i.d. were removed from each mouse given therapy with the vehicle or R-IL2. The tumors were fixed in 10% buffered-formalin, embedded in paraffin and sectioned using conventional methods. Hematoxylin- and eosin-stained sections were examined under light microscopy.

Results

1. *Inhibitory effect of R-IL2 on in vivo growth of X5563 myeloma*

C3H/HeN mice were i. d. inoculated with 1×10^6 viable X5563 tumor cells. Control mice (Group-1) were given the vehicle s. c. starting 1 day after X5563 inoculation in both

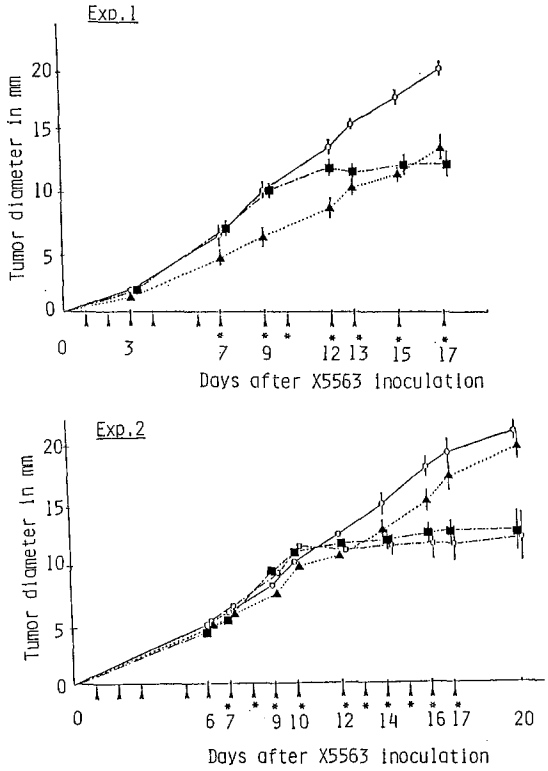

Fig. 1. Inhibitory effect of R-IL2 on in vivo growth of murine myeloma X5563. C3H/NeN mice were inoculated i. d. with; 10^6 viable X5563 tumor cells and R-IL2 administration (5×10^4 J. U./mouse) was started 1 day later (Group-2: ▲--------▲) or 7 days later s. c. on the same side (Group 3: ■—·—·—■) or the opposite side (Group-4: □—·—·—□) of the trunk to the tumor inoculation. Control mice (Group-1: ○————○) were injected with vehicle from 1 day after tumor inoculation. Tumor growth was measured and expressed as the geometric mean of the tumor diameter with the standard error. In both Exp. 1 and Exp. 2, Groups 1 and 2 were given vehicle or R-IL2 on the day (↑) and Groups 3 and 4 were injected on the day (*)

Exps. 1 and 2. R-IL2 (5×10^4 J. U./mouse) was administered s. c. to mice on the same side as tumor inoculation starting 1 day (Group-2) or 7 days (Group-3) after tumor inoculation. Group-3 was injected with R-IL2 7 and 10 times in Exp. 1 and Exp. 2, respectively. The antitumor effect of R-IL2 was expressed by the tumor diameter. As shown in Fig. 1, together with results of Exps. 1 and 2, Group-2 displayed marginal, if any, growth inhibition of X5563 tumor compared with Group-1. There was no significant difference in survival time between Groups 1 and 2 (survival days: 23 ± 3, 22 ± 5 (Exp. 1), 21 ± 1, 25 ± 3 (Exp. 2)). However, tumor growth in Group-3, which received R-IL2 treatment 7 days after the tumor inoculation, was effectively inhibited after the third or fourth dose of R-IL2. These mice showed significantly prolonged survival times in both Exp. 1 (42 ± 10) and Exp. 2 (42 ± 11), and complete cures occurred in 50% (3/6) in Exp. 1 and 60% (3/5) in Exp. 2. In the X5563-cured mice given R-IL2 therapy, X5563 tumor cells were macroscopically and microscopically not detected at the tumor inoculation site or metastatic organs including spleen, lymph nodes at 50 days after tumor injection. Furthermore, similar potent inhibition of growth of X5563 was observed in Group-4 (41 ± 12), where the mice had been injected with R-IL2 s. c. on the opposite side of the trunk to tumor inoculation.

2. Complete cure of X5563-bearing mice given multiple injections of R-IL2

Repeated injections of R-IL2 at the local site after tumor development were highly effective in inhibiting tumor growth and led to complete tumor regression in several experiments. As shown in Fig. 2, where the growth of X5563 was slow because of the small number of tumor cells inoculated (1×10^5), all the mice given R-IL2 s. c. 11 times from day 11 to day 22 showed tumor regression and became completely cured. Histologically, little growth of viable tumor cells was observed and granular tissue had formed at the periphery of the necrotic tumor. A large number of macrophages and lymphocytes had infiltrated the area around the necrotic tumor mass (Fig. 3b). In contrast, tumors grew progressively in the vehicle-treated mice. Histologically, remarkable growth of viable tumor cells was observed, and infiltration of macrophages and lymphocytes was not seen (Fig. 3a).

3. Anti-X5563 immune responses in the mice cured by R-IL2 therapy

Immune resistance to X5563 myeloma can be induced in C3H/He mice by i. d. inoculation of viable X5563 tumor cells, followed by surgical resection of the tumor. The immunity is mediated by tumor-specific T cells as assessed by tumor-neutralizing tests [8, 28]. In the present study, we examined the antitumor immune responses in the cured mice shown in Fig. 2 by examining the generation of anti-X5563 tumor-neutralizing activity and CTL responses to X5563 tumor cells. The representative data are shown in Figs. 4

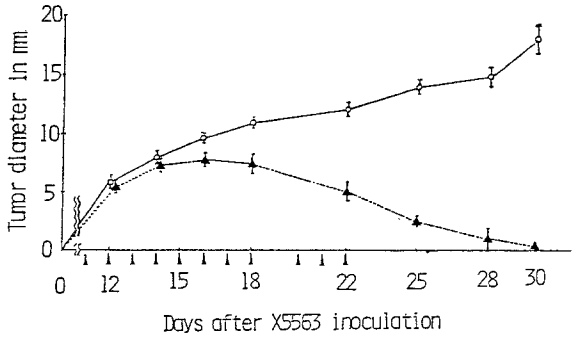

Fig. 2. Complete cure of X5563-bearing mice given R-IL2 s. c. from day 11 to day 22 after tumor inoculation. C3H/HeN mice were inoculated i. d. with 1×10^5 viable X5563 tumor cells and administration of vehicle (O———O) or R-IL2 (5×10^4 J. U./mouse) (▲------▲) was started 11 days later (total 11 times). Tumor growth was measured and expressed as the geometric mean of the tumor diameter with the standard error

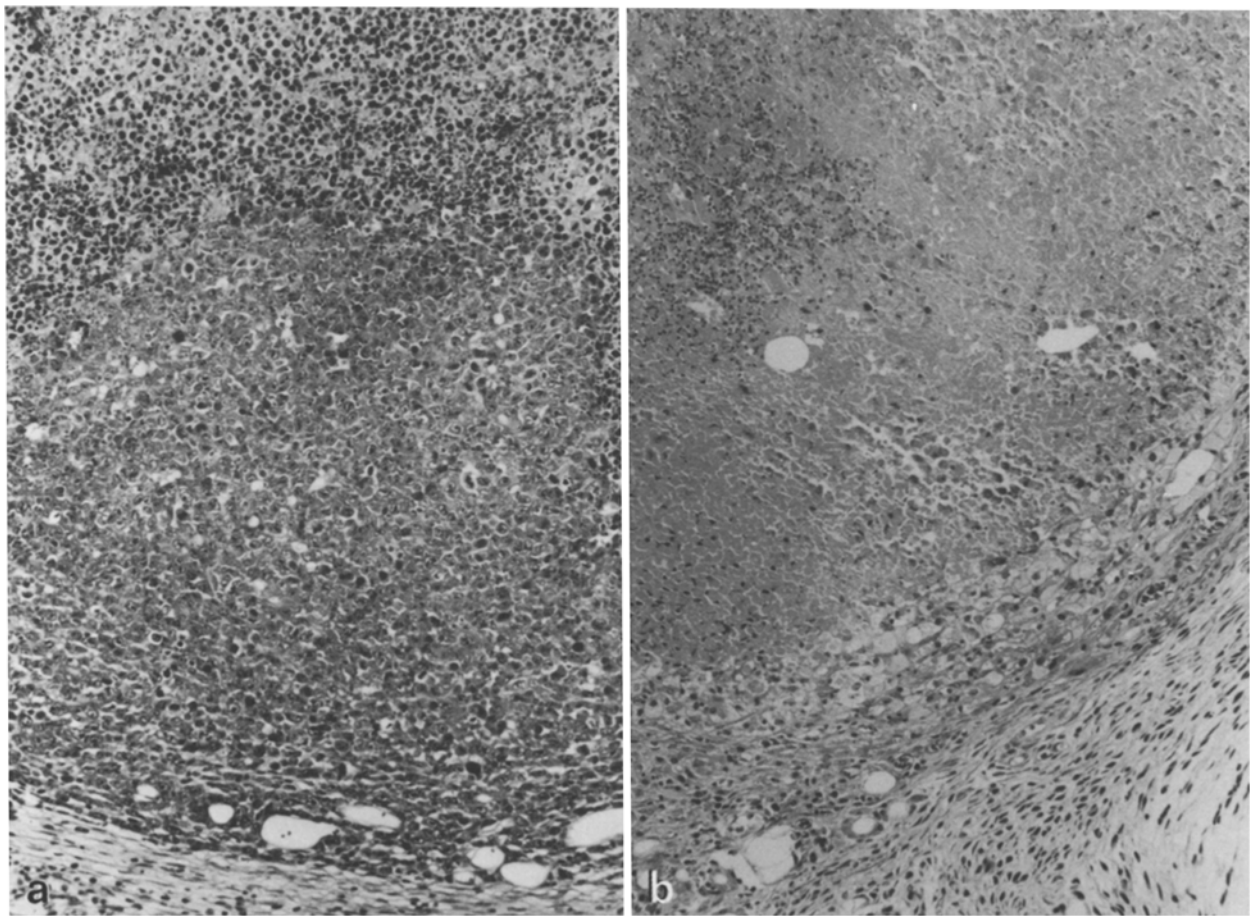

Fig. 3. C3H/HeN mice had viable X5563 tumor cells implanted i. d., then were repeatedly injected with vehicle or R-IL2 11 times from day 11 to day 22 (see Fig. 2 for details). The X5563 tumor mass was removed from each of the mice at 25 days after tumor inoculation. Histological appearance of tumor tissue in mice injected with vehicle (a) or R-IL2 (b). (Hematoxylin and eosin, original magnification $\times 125$)

and 5 and Table 1. The results in Fig. 4 show that tumor-neutralizing activity was observed with the spleen cells of these mice against X5563 but not against MH134, which indicated that the in vivo protection generated was tumor-specific. Furthermore, CTL response to X5563 tumor cells was ascertained in these mice. The spleen cells of the cured mice were restimulated in vitro with mitomycin C-treated X5563 tumor cells and their cytotoxic activity was assayed after 5 days of culture using ^{51}Cr-labeled X5563 tumor target cells. As shown in Fig. 5, anti-X5563 CTL were generated in spleen cells of the X5563-regressed (growth inhibition of 80%) and -cured (nonpalpable) mice by R-IL2 treatment at 25 days after tumor inoculation, but not in those of normal and X5563-progressive mice treated with the vehicle. Similar CTL were also generated from spleen cells in X5563-cured (nonpalpable) mice at 41 days after tumor inoculation (data not shown). The results in Table 1 show that anti-CTL responses were tumor-specific and were almost abrogated after treatment of spleen cells with monoclonal anti-Thy 1.2 antibody plus complement. We confirmed these results in other experiments (data not shown).

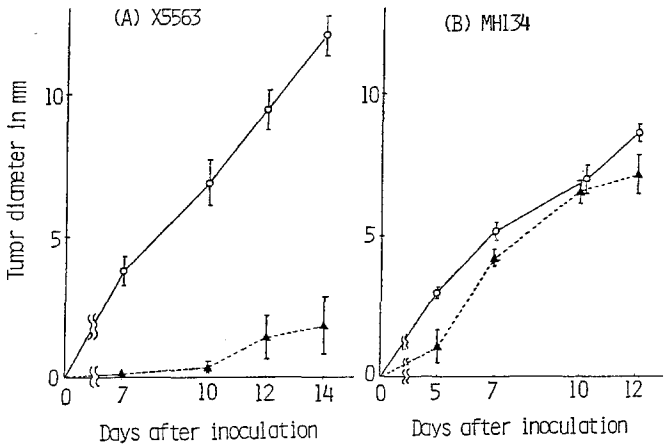

Fig. 4. In vivo tumor-neutralizing activity against X5563 and MH134 in spleen cells of X5563-cured mice receiving R-IL2 therapy (see Fig. 2 for details). 10^7spleen cells from normal (O———O) or X5563-cured (nonpalpable) mice given R-IL2 therapy at 25 days after the tumor inoculation (▲------▲) were admixed with 10^5 viable X5563 tumor cells (A) or MH134 tumor cells (B) and injected i. d. into syngeneic mice. Tumor growth was measured and expressed as the geometric mean of the tumor diameter with the standard error

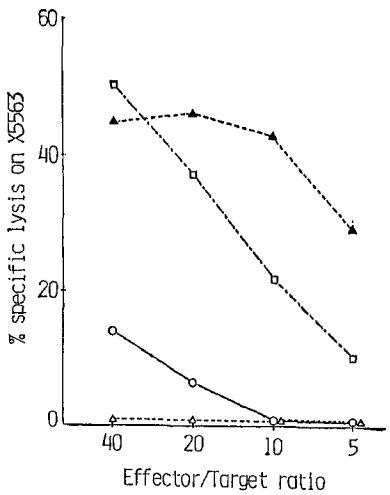

Fig. 5. Anti-X5563 CTL induction from spleen cells in X5563-regressed and-cured mice given R-IL2 therapy (see Fig. 2 for details). 5×10^6 spleen cells from normal mice (O———O), X5563-bearing mice injected with vehicle (△-----△), X5563-regressed (80% inhibition) mice (□—-—□) and X5563-cured (nonpalpable) mice (▲———▲) given R-IL2 therapy at 25 days after X5563 inoculation, were sensitized with 10^5 mitomycin C-treated X5563 cells and effector cells generated after 5 days of culture were assayed with ^{51}Cr-labeled X5563 tumor target cells at the effector:target ratio shown

Table 1. Effect of treatment of X5563-cured mouse spleen cells with monoclonal anti-Thy 1.2 antibody plus complement on the generation of anti-X5563 CTL response

Speen cells[a] from	CTL Response[b] % specific lysis on		
	X5563 40:1	X5563 20:1	MH134 40:1
Normal	5.6	2.0	0
X5563-cured (untreated)	48.5	40.3	0
X5563-cured (C alone)	50.1	40.4	ND[c]
X5563-cured (anti-Thy 1.2+C)	5.2	3.4	ND

[a] Spleen cells from X5563-cured mice given R-IL2 therapy were treated with anti-Thy 1.2 antibody plus complement (C)
[b] Spleen cells were in vitro sensitized with X5563 tumor cells. Effector cells generated after 5 days of culture were assayed with X5563 tumor target cells at effector: target ratio shown
[c] Not done

Discussion

Recently, many papers have reported successful adoptive immunotherapy of cancers with killer cells generated in in vitro cultures of lymphocytes with IL2 [2, 4, 5, 20, 22]. Administration of IL2 together with transfer of the IL2-induced killer cells would be more effective than cell transfer alone, probably due to prolonged in vivo maintenance of the killer cells transferred [3]. The in vivo antitumor activity of IL2 alone remains to be clarified, even though it is known that in vivo administration of this lymphokine can augment NK activity [18, 23]. The present study showed that repeated injections of R-IL2 alone produced potent suppression of the in vivo growth of syngeneic myeloma cells, X5563, in C3H/HeN mice. This finding is consistent with the recently published paper by Rosenberg et al. [24] which reported a marked suppressive effect of multiple doses of recombinant IL2 on the in vivo growth of s. c. inoculated MCA-105 sarcoma and pulmonary metastases of B16 melanoma, MCA-105 and MCA-106 sarcomas. Although the mechanism of such an in vivo action of recombinant IL2 is not clear, there are two possible explanations: activation of nonspecific killer cells and induction of tumor-specifc cytotoxic T lymphocytes. The possibility of potentiation of nonspecific killer cells comes from the well-documented phenomenon that normal mouse lymphocytes cultured with IL2 for as little as 2 days develop lytic activity against various types of tumor cells including NK-resistant and NK-sensitive tumor cells [15, 16, 25]. The cells responsible for the killing have been described as

LAK cells of the Thy 1^+ and Lyt 1^-2^+ phenotypes [25]. These cells are also known to lyse syngeneic fresh tumors [25]. Rosenberg et al. [24] attributed the in vivo antitumor activity of recombinant IL2 alone to the generation of LAK cells. In our experiments, however, in vitro culture of mouse spleen cells for 2 to 4 days in the presence of R-IL2 (50 J. U./ml) generated cytotoxic cells of the NK-like phenotype, i.e., Thy 1^- and asialo GM_1^+, which display a wide target cell spectrum (manuscript in preparation). Similar killer activity was also induced in peritoneal exudate cells and peripheral blood lymphocytes of C3H/HeN mice treated in vitro with R-IL2. However, X5563 cells were hardly lysed by such R-IL2 activated nonspecific killer cells under our test conditions. These results raise an alternative possibility, that antitumor activity of R-IL2 against X5563 myeloma may be due to induction of X5563-specific immunity. Histological observation showed remarkable interstitial infiltration of macrophages and lymphocytes around the necrotic X5563 tumor mass in the mice injected with R-IL2 but not in those injected with the vehicle alone; this also favors the second possibility, though the infiltrated cells have not yet been identified. Further support comes from the production of tumor-specific protection against reinoculation of tumor cells in mice which showed complete regression of a primary tumor after R-IL2 treatment. Also X5563-specific cell-mediated cytotoxicity was obtained when spleen cells from the cured mice were restimulated in vitro with mitomycin C-treated X5563 cells. Recently, the role of Lyt $1^+2^-3^-$ T lymphocytes (helper/DTH effector subset) in tumor regression has been estimated to be more important than that of Lyt $1^-2^+3^+$ cells (killer/suppressor subset) [11, 14]. Fujiwara et al. [10] have established that enhanced helper T cell activity to the TNP hapten conjugated in situ with X5563 cells can augment effector T cells specific to X5563 cells through T-T interaction in C3H/HeN mice. Also, Forni et al. [7] have suggested that radioresistant functions of Thy1^+, Lyt 1^+2^- and of asialo GM_1^+ cells were independently involved in lymphokine-activated tumor inhibition. Therefore, the cellular mechanism by which R-IL2 causes tumor regression need to be analyzed. One important finding of the present study is that the effectiveness of R-IL2 differed depending on the time of its administration. Treatment with R-IL2 starting 1 day after tumor inoculation exerted little effect on the tumor growth, while starting 7 or 11 days later produced potent suppression leading to complete cure in approximately half of the animals tested. A similar time-dependent difference was shown by Rosenberg et al. [24]. As for the failure of R-IL2 treatment starting shortly after tumor inoculation, two mechanisms are proposed. One is that R-IL2 injected soon after tumor inoculation produces antibodies capable of neutralizing R-IL2 given later, and consequently allows tumor growth. Our preliminary experiment demonstrated that a single dose of R-IL2 (5×10^4 J. U.) was sufficient to raise the detectable amount of antibodies 10 days later (unpublished). The other possible mechanism is that administration of R-IL2 before establishment of the priming of X5563-specific T lymphocytes leads to nonspecific expansion of T lymphocytes and therefore inefficient induction of X5563-specific T cells. R-IL2 augments proliferation of X5563-specific T lymphocytes when given after the antigenic priming has already been produced. Both mechanisms are probably operative. Whatever the action mechanism may be, our finding that injections of R-IL2 alone produce marked tumor regression in the mouse offers hope for the possible therapy of human cancers with this lymphokine.

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