Jpn. J. Cancer Res. (Gann), 79, 965–972 ; August, 1988

Effect of Recombinant Human Interleukin 2 on the Growth of a BALB/c Sarcoma Induced by Moloney Murine Sarcoma Virus

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The effect of *in vivo* administration of recombinant interleukin 2 (rIL2) on the growth of a primary female BALB/c sarcoma induced by Moloney murine sarcoma virus (M-MSV) was studied. Although low-dose administration of (6,000 JU/mouse × 14 days) rIL2 had no effect on the growth of the tumors, high-dose (15,000–80,000 JU/mouse × 14 days) intraperitoneal inoculation of rIL2 induced tumor regression, dose-dependently. Tumors in mice which received 80,000 JU/mouse/day of rIL2 regressed completely 2 weeks after the initiation of treatment. The survival rates of the treated groups were significantly higher than those of the control group. A time course experiment disclosed that the effect of rIL2 was restricted only to the group in which rIL2 treatment started 8 days after the inoculation of M-MSV. The cytotoxic activity of regional lymph node lymphocytes from rIL2-treated mice was demonstrated against primary culture of M-MSV-induced sarcoma but not against syngeneic tumor induced by methylcholanthrene (Meth A). The effect of rIL2 was partially blocked by the administration of anti-IL2 receptor antibody. Immunohistochemical examination revealed that infiltration of Thy1.2$^+$Lyt1$^+$2$^-$ (helper/inducer subset) lymphocytes into the tumor tissue was prominent in mice which received high-dose rIL2. The results indicated that IL2 induced regression of M-MSV-induced sarcoma mainly through activation of IL2-receptor-positive helper T cells in the tumor tissues and of killer cells in the draining lymph nodes.

Key words: Interleukin 2 — Tumor regression — Moloney murine sarcoma virus

There has been great interest in adoptive immunotherapy following the discovery of T cell growth factor (interleukin 2; IL2), since its clinical application was made possible by the production of recombinant type IL2.[1] The results of applications of rIL2 to animal models encouraged us to proceed further to adoptive immunotherapy.[2–8] Although IL2 therapy is effective in mediating anti-tumor immunity in some cases, severe side effects[4, 9] and discrepancies between clinical studies and animal experiments prompted us to study animal models which closely approximate human models.

Progressor Moloney murine sarcoma virus (M-MSV) induces tumors at the site of inoculation which eventually kill the host animal. This primary tumor system is superior to transplanted tumor systems, since primary tumors have a close affinity for surrounding tissues, abundant blood supply, and a relatively slow growth rate, all of which permit the immunocompetent cells to penetrate deeply into the solid tumor mass. In the present study, using this progressor M-MSV, we investigated the effect of *in vivo* administration of rIL2 on the growth of virus-induced primary tumors.

MATERIALS AND METHODS

Mice Six- to eight-week-old female BALB/c mice were obtained from the Shizuoka Agricultural Cooperative for Laboratory Animals, Shizuoka.

Induction of Sarcomas The progressor M-MSV (#239) was kindly provided by Dr. A. F. Gazder, National Cancer Institute, Bethesda, Maryland, USA. Amounts of 5×10^4 focus-forming units (FFU) of progressor M-MSV in 0.1 ml of saline were inoculated into the hind leg muscle of the mouse. As stated elsewhere,[10–12] since the growth of M-MSV-induced sarcomas is closely related to

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the source of the virus, as well as the strain, sex, and age of the mouse, the same lots of virus preparation (progressor M-MSV) and 6- to 8-week-old female BALB/c mice were used.

Transplanted Tumors Methylcholanthrene-induced BALB/c fibrosarcoma (Meth A) was kindly provided by Dr. Y. Hashimoto, Department of Hygienic Chemistry, Pharmaceutical Institute, Tohoku University, and 2×10^6 Meth A cells were inoculated into a hind leg of a BALB/c mouse.

Measurement of Tumor Size The mean diameter of tumor-bearing legs was measured by means of a slide caliper 2 to 3 times a week. Tumor size was calculated by subtracting the mean diameter of normal legs (5.5 mm) from the mean diameter of the tumor-bearing legs. The mean tumor size was thus calculated for each group (10 mice/group). When an animal died, the last available measurement of tumor size was used in the calculation.

rIL2 and Anti-IL2 Receptor Antibody Recombinant human IL2 was kindly provided by Shionogi Pharmaceutical Co., Osaka; it was dissolved in saline at concentrations of 3.0 to 40.0×10^4 JU/ml, and 0.2 ml of rIL2 solution was inoculated intraperitoneally (ip) into the experimental groups. Two-tenths milliliter of saline was given ip as a control. Anti-IL2 receptor monoclonal antibody (Boehringer Mannheim Biochemica) was also dissolved in saline and inoculated ip.

Preparation of Tumor Cells Tumor cells from M-MSV-induced primary sarcoma were used as the target cells of the *in vitro* cytotoxicity test of lymphocytes from tumor-bearing mice. Tumor tissues of M-MSV-induced sarcoma were removed aseptically, minced, and suspended in RPMI-1640 medium (GIBCO: Grand Island Biological Co., NY) containing 2000 U/ml of Dyspase (Godo Shusei Co. Ltd.), 0.5% collagenase and 10% heat-inactivated fetal bovine serum (FBS: GIBCO). After incubation at 37° in a 5% CO_2 atmosphere for 2 hr under gentle stirring by a magnetic stirrer, cell debris was removed by filtration through a nylon mesh. Cells were then washed by cold centrifugation at 400*g* for 10 min 3 times, suspended in McCoy's 5a medium (GIBCO) containing penicillin, streptomycin, HEPES buffer and MEM nonessential amino acids (complete medium) at a concentration of 10^6 cells/ml, transferred to other flasks, and incubated for 7 days until a tumor cell monolayer covered the entire surface of the culture flasks.

Preparation of Lymphocytes The methods for the preparation of lymphocytes were described elsewhere.[12, 13] Briefly, the regional lymph nodes and spleens of tumor-bearing mice were removed aseptically at 7, 10, 12, 15, and 20 days after the inoculation of M-MSV. Lymph nodes and spleens were minced, suspended in Hanks' balanced salt solution (HBSS), washed 3 times, suspended in complete medium containing 5% FBS at 10^7 cells/ml and used as effector cells in cytotoxicity tests.

Cytotoxicity Test Four-hour ^{51}Cr-release test was utilized to determine the *in vitro* cytotoxicity of regional lymph node and spleen lymphocytes against syngeneic M-MSV-induced sarcoma and Meth A tumor cells. One million lymphocytes and 10^4 ^{51}Cr-labeled target cells were put in a well of a microtiter plate (N 1482, NUNC, Denmark), and incubated at 37° for 4 hr in 5% CO_2 atmosphere. After centrifugation at 900*g* for 10 min, the radioactivity of 0.1 ml of supernatant from each well was counted with an ultra-gamma counter. The following formula was used for the calculation of percent cytotoxicity.

Percent cytotoxicity = [cpm (test) − cpm (medium control)]/[cpm(maximum control) − cpm (medium control)] × 100, where the maximum and medium control were determined by using 1*N* HCl and medium alone, respectively, instead of the effector lymphocytes. All tests were performed in triplicate.

Histological and Immunohistochemical Examinations Tumors of the mice in each group of the dose-response study were studied histologically and immunohistochemically. Every 3 to 4 days after the inoculation of M-MSV, tumors were resected and one half was fixed, and stained with hematoxylin and eosin for histological examination. The other half was fixed in PLP and frozen in liquid nitrogen. Four-micrometer-thick sections were cut on a cryostat and dried in air after successive 15 min blockings with avidin D and biotin solution (Becton Dickinson Co.). Cryostat sections were incubated with biotinized anti-Thy1.2, Lyt1, and Lyt2 monoclonal antibody at 37° overnight. After extensive washing with PBS buffer, sections were incubated with avidin D-horseradish peroxidase for 30 min and with diaminobenzidine for 10 min, and counterstained with methyl green.

Dose-response Study of IL2 on the Growth of M-MSV-induced Sarcoma Fifty mice bearing tumors of 9 to 12 mm in diameter among 80 mice inoculated with M-MSV 7 days previously were divided into 5 groups. Mice of groups A–D received 6,000, 15,000, 40,000, 80,000 JU/mouse/day of rIL2 dissolved in 0.2 ml saline from day 8 to 22, respectively. Mice of group E received 0.2 ml saline alone as a control.

Time Course Study Ten mice in each group received 80,000 JU/mouse/day of rIL2 for 7 days at different time intervals: groups A, B, C, and D on days 1–7, 8–14, 15–21, and 22–28 respectively.

Statistics Significance of differences was calculated by using Student's *t*-test except for the survival rate, which was calculated by applying the generalized Wilcoxson test.

Results

Effect of rIL2 on the Growth of M-MSV-induced Sarcoma (Dose-response Study) Although the tumor sizes of the mice in groups A and B did not differ from that of group E (control), those of groups C and D were significantly smaller than that of group E (C, $P<0.01$; D, $P<0.001$). The tumor sizes were inversely proportional to the IL2 doses, that is, the higher the dose of IL2, the smaller the tumor size. In particular, tumors in mice receiving 80,000 JU/mouse of rIL2 (group D) had completely regressed 2 weeks after the initiation of treatment (Fig. 1). As for survival, no mouse of the control group survived

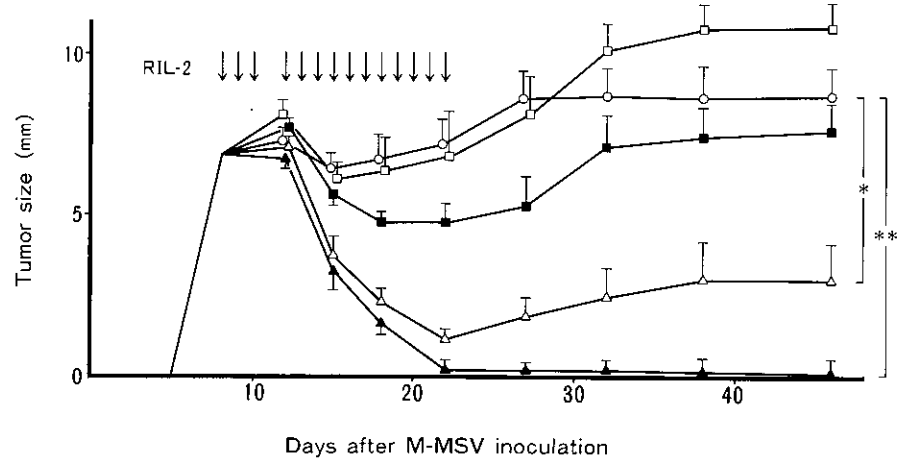

Fig. 1. Effect of rIL2 on the growth of M-MSV induced BALB/c sarcoma (dose-response study). Seven days after the inoculation of M-MSV, 50 BALB/c mice bearing progressively growing tumors (9–12 mm in diameter) were randomly divided equally into 5 groups. Group E (○) received 0.2 ml/mouse of saline as the control. Group A (□) received 6,000 JU/mouse/day, group B (■) 15,000 JU/mouse/day, group C (△) 40,000 JU/mouse/day, group D (▲) 80,000 JU/mouse/day of rIL2. Arrows indicate the day of IL2 (or saline) administration. The bars indicate the SE. *, $P<0.01$; **, $P<0.001$.

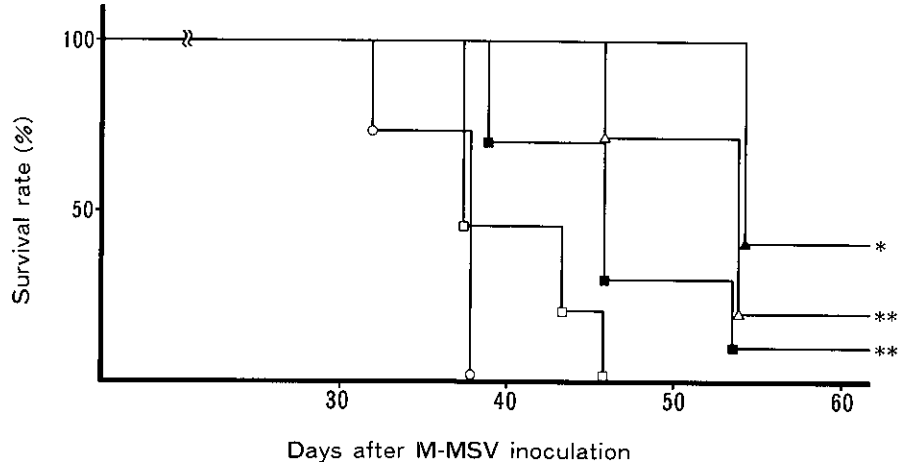

Fig. 2. Survival rate. See Fig. 1 for details. (*, **, ***, $P<0.001$)

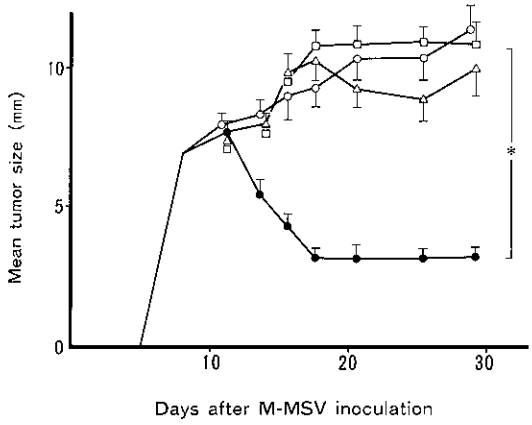

Fig. 3. Effect of rIL2 on the growth of M-MSV-induced BALB/c sarcoma (time course study). Eighty thousand JU/mouse/day of rIL2 was administered for 7 days from day 1 (group A, ○), day 8 (group B, ●), day 15 (group C, △), and day 22 (group D, □). *, $P<0.01$.

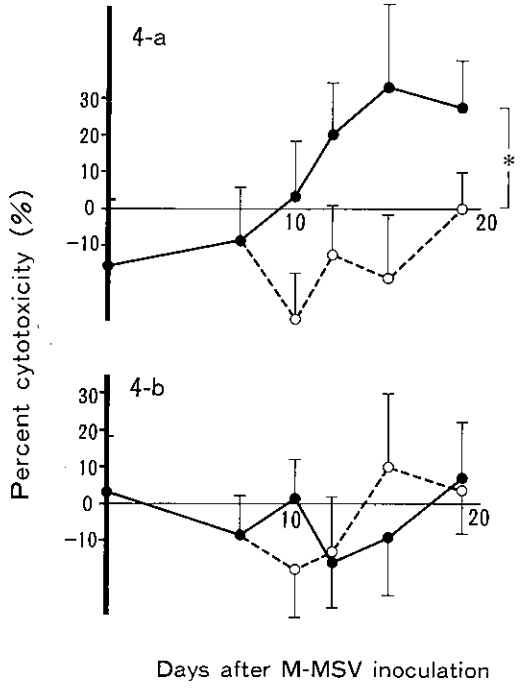

Fig. 4. Cytotoxicity test of regional lymph node lymphocytes from M-MSV-induced tumor-bearing mice against M-MSV-induced sarcoma cells (Fig. 4-a) and against Meth A tumor cells (Fig. 4-b). The E:T ratio was 100:1. *, $P<0.05$.

beyond 38 days after virus inoculation, while those of groups B, C and D survived longer than 50 days. The survival rate (Fig. 2) of the latter was significantly higher than those of the former ($P<0.001$).

Time Course Study The effect of the time of rIL 2 inoculation on the growth of the M-MSV induced sarcoma was studied (Fig. 3). The effect of rIL2 was restricted only to the group which received rIL2 from days 8 to 14 (group B) ($P<0.01$).

Cytotoxicity Test To examine the effect of IL2 on the cytotoxic activity of lymphocytes, we determined the cytotoxic activities of lymphocytes from regional lymph nodes and spleens of tumor-bearing mice against M-MSV-induced sarcoma and also against syngeneic Meth A tumors. The cytotoxic activities of lymphocytes from regional lymph nodes of mice inoculated with rIL2 increased gradually from 3 days after the initiation of the treatment and reached a maximum at day 16 (Fig. 4-a), while those of control groups failed to show any cytotoxic activity. To determine the specificity of the cytotoxic activity, the syngeneic Meth A tumor was used as target cells (Fig. 4-b). Highly cytotoxic lymphocytes against M-MSV-induced tumor cells did not

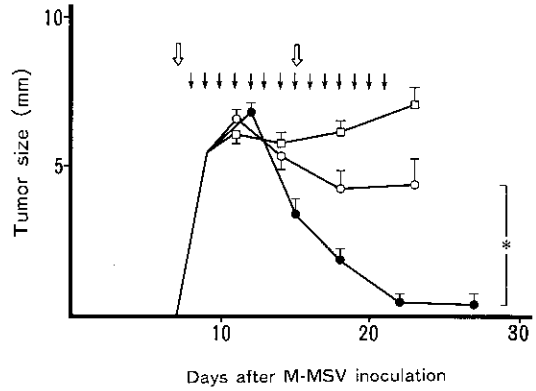

Fig. 5. Effect of anti-IL2 receptor antibody on IL2-induced regression of the sarcoma. Five microgram/mouse of anti-IL2 receptor antibody was administered ip on day 7 and day 15, with or without rIL2 inoculation. Outlined arrows show the days of anti-IL2R antibody administration. Thin arrows show the days of rIL2 administration. Group A (IL2 alone, ●), group B (anti-IL2 antibody+rIL2, ○), control (anti-IL2R antibody+saline, □). *, $P<0.01$.

show cytotoxicity against antigenically distinct Meth A tumors. Spleen cells of these groups did not show any activity in this experiment (data not shown).

Effect of Anti-IL2 Receptor Antibody on IL2-induced Regression of the Sarcoma To determine whether the effect of IL2 on the growth of M-MSV sarcoma is mediated by lymphocytes activated by IL2 or is due to direct effects on tumor cells, 5 μg/mouse of anti-IL2 receptor antibody was administered ip on days 7 and 15 with (group B) or without (group A) rIL2 inoculation. The effect of IL2 was partially (64%) inhibited by the administration of anti-IL2 receptor antibody (Fig. 5) ($P<0.01$).

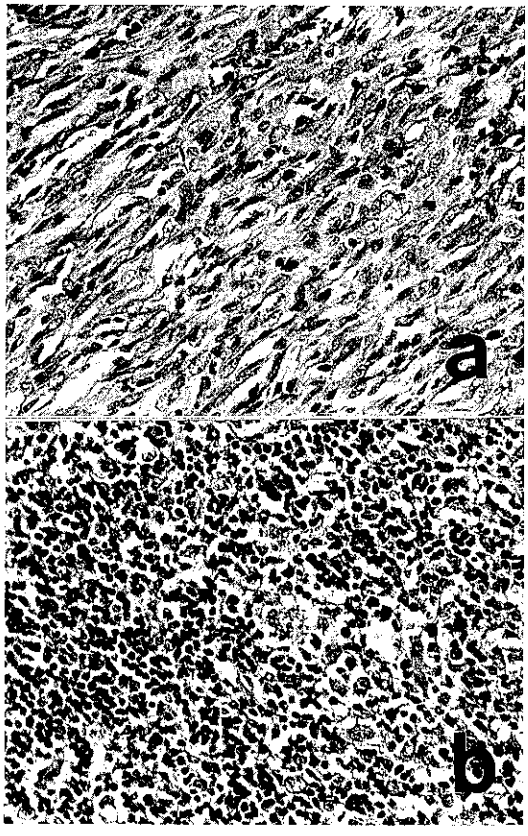

Fig. 6. Six-week-old female BALB/c mice were inoculated with 5×10^4 FFU of M-MSV im and treated with 8×10^4 JU/mouse/day of rIL2 or 0.2 ml of saline as a control for 14 days from day 8. Histological examination was performed every 3 to 5 days after inoculation of the virus. The figure shows tumor tissue 21 days after virus inoculation. (a) Saline, (b) rIL2. (Hematoxylin and eosin $\times200$)

Histological and Immunohistochemical Examinations Histological examination was performed every 3 to 4 days after the inoculation of the virus to study the effect of IL2. Although the group receiving only M-MSV exhibited a typical pattern of tumor growth (Fig. 6-a), the tumors of the group which received 80,000 JU/mouse/day of rIL2 for 14 days from day 8 exhibited lymphocytic infiltration soon after the initiation of treatment (Fig. 6-b). Immunohistochemical examinations with Thy1.2, Lyt1, and Lyt2 monoclonal antibody demonstrated that the phenotypes of lymphocytes infiltrating into tumors are $Thy1.2^+$ and $Lyt1^+2^-$ (helper/inducer subset).

Discussion

The present study clearly demonstrated the effect of rIL2 on the growth of M-MSV-induced sarcoma. The effect of rIL2 was dose-dependent, since the groups of mice receiving low doses (6,000–15,000 JU) of IL2 failed to show a remarkable effect on the tumor growth, although the groups of mice that received high doses (40,000–80,000 JU) of IL2 showed significant regression of tumors and a better survival compared with that of the control group.

Histological and immunohistochemical examination indicated marked $Thy1.2^+Lyt1^+2^-$ lymphocyte infiltration into tumor tissues starting soon after the initiation of rIL2 treatment. Furthermore, *in vitro* cytotoxicity tests revealed cytotoxic activity of regional lymph node lymphocytes in the IL2-inoculated groups. These effects of IL2 were partially blocked by the administration of anti-IL2 receptor antibody. Taking all these results into consideration, it is suggested that IL2 induces regression of M-MSV-induced sarcoma mainly through activation of IL2 receptor-positive helper T cells in tumor tissues and induction of killer cells in the draining lymph nodes. It is not clear at present what mechanisms are involved in the regression of M-MSV-induced sarcoma, but participation of killer T cells in this system seems to be inevitable.

There are numerous reports concerning the *in vivo* effects of IL2 on tumor cells.[14, 15] In most cases, however, transplanted tumors

were used to examine inhibition of tumor metastasis[7, 14, 16] or reduction in tumor growth,[5] and there have been few studies on the effect of IL2 on primary tumors. In addition, studies reporting complete regression of already developed tumors are rate. Our system, using primary M-MSV-induced tumors which regress completely after rIL2 treatment seems to be an ideal system for studying the effects of IL2 on the primary tumors. It has already been reported that M-MSV-induced tumors have strong antigenicity and induce specific killer T cells in hosts,[17–20] which has not been demonstrated in human tumor systems. Therefore we cannot immediately adapt our results to clinical therapy unless we find similar conditions in human tumor systems.

Although the mechanisms of *in vivo* eradication of primary tumors by $Lyt1^+2^-$ T cells are not clear at present, the importance of $Lyt1^+2^-$ T cells in *in vivo* anti-tumor activity has been reported by others.[21, 22] Fujiwara *et al.*,[23, 24] using transplanted X5563 plasmacytoma, demonstrated that the adoptive transfer of $Lyt1^+2^-$ anti-X5563 immune T cells into B cell mice produced complete protection against subsequent tumor cell challenge without inducing CTL or antibody responses. They interpreted these data in terms of delayed-type hypersensitivity responses.

Greenberg *et al.*[25] reported that mice bearing Friend virus-induced leukemia were successfully treated by a combination of cyclophosphamide and adoptive transfer of syngeneic immune $Lyt1^+2^-$ lymphocytes. The results were interpreted to mean that immune $Lyt1^+2^-$ lymphocytes had an amplifier function in the host to generate tumor-specific CTL. In our system, participation of cytotoxic lymphocytes was suggested by the appearance of cytotoxic activity in regional lymph node lymphocytes of mice with tumor regression, but no direct correlation of $Lyt-1^+2^-$ lymphocytes in tumor tissues and cytotoxic cells in regional lymph nodes has yet been recognized. An adoptive transfer study with distinct subpopulations of lymphocytes may be necessary for the analysis of effector cells in this system.

The time course experiments disclosed that treatment with rIL2 starting from day 8 induced tumor regression while all other schedules were non-effective. This fact indicated the importance of the timing of rIL2 administration.[5] A simillar time-dependent difference was shown by Maekawa *et al.*[22] who reported that administration of rIL2 starting 1 day after X5563 inoculation had a marginal effect on the growth of tumors, whereas rIL2 administration starting 7 days after the tumor inoculation was highly effective and significantly lengthened the survival time compared with the control mice. The reason for this time dependence of IL2 action is not clear at present, but it is suggested that nonspecific expansion of T lymphocytes might lead to inefficient induction of killer T cells.

Although IL2 was originally reported as a factor necessary for the continual proliferation of activated T lymphocytes,[26] various other biological activities have been reported, such as activation of natural killer (NK) cells,[1] or activation of lymphokine activated killer (LAK) cells,[3, 27, 28] as well as induction of other lymphokines. The *in vivo* effects of IL2 have been attributed to the production of these anti-tumor effector cells.[29] In the M-MSV system, the participation of these cells is possible on the basis of the time course experiment, but may not be the main mechanism since the production of these nonspecific killer cells is thought to be most prominent in the early stages of tumor growth.

The results of administration of anti-IL2 receptor antibody suggested the involvement of other mechanisms in the IL2-induced tumor regression, since anti-IL2 receptor antibody could not completely inhibit the effect of rIL2. Furthermore, the high mortality at around 54 days in the rIL2 group requires evaluation of associated mechanisms and possible side effects.

Acknowledgments

This work was supported in part by a Grant-in-Aid (No. 62440053) from the Ministry of Education, Science and Culture, Japan. We are grateful to Mr. Umemiya and Mr. Azuma, First Department of Pathology, Chiba University, for helpful discussions concerning the immunohistological study. We are also grateful to Associate Professer J. Patrick Barron of St. Marianna University School of Medicine for his help in the preparation of the manuscript.

(Received March 15, 1988/Accepted July 2, 1988)

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