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Local and Systemic Effects during Interleukin-2 Therapy of Mouse Mammary Tumors[1]

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ABSTRACT

The therapeutic effects of 12 daily peritumor injections of from 100 to 300,000 units of recombinant human interleukin-2 were tested against the syngeneic, immunogenic mammary carcinoma MC2 implanted s.c. into C3H/He mice. Local therapeutic effect on injected tumors was observed down to 300 units of interleukin-2 per injection. Cures of injected tumors were obtained with 1,000 units and more per injection. Systemic therapeutic effect on contralateral, uninjected tumors in treated mice was discernible at 5,000 units and more per injection. Hepatic periportal cellular swelling with mononuclear infiltration, and renal tubular edema were observed at 7,000 units or more per injection. Hepatic and renal repairs were rapid and complete with 50,000 units and less per injection. Hepatic necrosis developed above 50,000 units per injection. Deaths resulted from 100,000 units and more per injection. It is concluded that interleukin-2 can be a safe and effective therapeutic agent at a wide range of doses well below those that may be expected to have serious negative side effects.

INTRODUCTION

T-cell growth factor (1), now referred to as IL-2,[3] is of primary practical importance in the long-term culture of T-lymphocytes, and is of central biological interest in the study of cellular interaction mechanisms.

The potential that IL-2 may have in cancer therapy has been indicated by animal studies where local, peritumor injections have sometimes resulted in cures (2–6). Most investigators have, however, found that not IL-2 injections alone, but only IL-2 in combined treatment with adoptive transfer of activated splenocytes had therapeutic effect (7–10). The administration of very high doses (200,000 units) of IL-2 i.p. three times daily has been reported to produce toxic side effects in mice (11). The clinical usefulness of IL-2 is now a prominent question in practical tumor immunology.

Animal tumor models such as mouse mammary tumors, can be used to provide basic and preliminary information that may suggest, or warn against, approaches to clinical testing of IL-2. The present investigation therefore undertook to study the therapeutic effects of peritumor injections of highly purified human recombinant IL-2 given repeatedly in daily doses from 100 units to 300,000 units. Could a range of effective doses from minimal to maximal be determined? At what dose-level could toxic side effects be expected? Would local and systemic therapeutic effects be determined by the dose? Local therapeutic effects would be indicated by inhibition of the injected (left) tumors, systemic therapeutic effects by inhibition of the uninjected (right) tumors in treated mice.

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[3] The abbreviations used are: IL-2, interleukin 2; NK cell, natural killer cell.

MATERIALS AND METHODS

Mice. All animals used in this study were 8- to 10-week old female mice of the inbred C3H/He strain, raised and kept in an infection-controlled environment.

Tumor. The mammary carcinoma MC2 had developed spontaneously in a multiparous C3H/He mouse, and has been transplanted in syngeneic female mice. The second transplant generation is stored in liquid N_2, and the tumor was used in the third to seventh transplant generations. The tumor is immunogenic, and the primary, local immune reaction displays a substantial accumulation of T-cells, B-cells, and macrophages (12). The evidence for the immunological specificity of the anti-MC2 systemic resistance as well as the local stromal reaction was presented in a previous publication (13).

Tumor tissue was removed from normal donor mice under the short-acting methoxyfluorane anesthetic Penthrane (Abbott Laboratories, North Chicago, IL). The soft, translucent, viable tumor tissue was separated from the necrotic tumor and the surrounding stroma by careful microdissection. Two 1-mm^3 pieces of tumor were placed s.c. by trocar in the right and left flanks of experimental mice. Of the two implants, only the left implant in each mouse received local IL-2 therapy.

Recombinant IL-2. The IL-2 used in these studies was an oxidized, highly purified (>99% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) human recombinant IL-2 produced in *Escherichia coli* (14). Specific activity was 3×10^6 Cetus units/mg (8×10^6 Biological Response Modifiers Program units/mg). The IL-2 preparation (lots LP315 and LP338A) contained less than 0.01 ng of endotoxin per mg IL-2. This material was provided by Dr. Kirston Koths, Cetus Corporation, Emeryville, CA. Stock concentrates of 1.0×10^6 IL-2 units per 1.7 ml were prepared from lyophilized IL-2 using a Cetus diluent containing sodium dodecyl sulfate. Further dilutions to provide doses from 100 to 300,000 units IL-2 per 50 μl were made in phosphate buffered saline containing 10% normal C3H/He serum. Aliquots of 1.2 ml were stored at −70°C for up to 3 weeks.

IL-2 at sublethal doses was given as 12 daily (five times per week) peritumor injections. From the day after the test implantation of MC2, the IL-2 was injected close to, but not into, the left implants. Control groups of mice received similar injections of the IL-2 free liquid vehicle (Cetus Corp.) diluted with phosphate buffered saline with 10% normal C3H/He serum.

Statistical Analysis. The incidence and the growth of tumors were recorded twice weekly. Two bisecting diameters of each tumor were measured with calipers, and the volume calculated by the formula 0.4 (ab^2) where a is the larger and b the lesser diameter.

Differences in tumor incidence were evaluated with the χ^2 test. Differences in mean tumor volume at different treatment schedules were evaluated by Student's t test in the following comparisons: (*a*) Local effects of treatment by comparing the volumes of IL-2 treated (left) tumors *versus* placebo treated (left) tumors; (*b*) systemic effects of treatment by comparing the volumes of uninjected (right) tumors in the IL-2 treated mice *versus* uninjected (right) tumors in placebo-treated mice. Differences between local and systemic therapeutic effects were determined by the paired t test comparing the volumes of right and left tumors in individual mice. Differences between groups were considered significant when the p value of comparison was 0.05 or less.

RESULTS

Therapeutic Effects and Side Effects at Increasing Daily Doses. The results shown in Fig. 1 represent the summaries of

tests repeated from three to seven times at each indicated dose level. Local therapeutic effects were not observed at 100 and 200 units IL-2. Consistent local therapeutic effects were not observed below 500 units per dose. Complete local cures were obtained most consistently with 1,500 to 10,000 units per dose.

Not shown in Fig. 1 (for the sake of graphic neatness) are the following observations: (*a*) 1,000 unit doses gave complete cures only when treatments were started no later than 1 day after tumor implantation; 2,000 unit doses only when started no later than the 5th day (histologically detectable tumor growth); 3,000 and 5,000 unit doses only when started no later than the 9th day (palpable tumor growth). (*b*) Below 5,000 units per dose, only the injected (left) tumors were affected by the treatment. At 5,000 units and more per dose, both the injected and the uninjected (right) tumors were affected. (See Table 1.)

Complete cures were obtained less frequently with daily doses above 10,000 units ($P > 0.05$). This coincided with dose levels at which systemic toxic side effects were observed. After 12 injections of 7,000 units IL-2 (on day 16), histological examination of all visceral organs revealed liver and kidney damage. The liver showed periportal mononuclear infiltration, hydropic cells, and pyknotic nuclei; the kidneys showed cellular edema (cloudy swelling) of the tubular epithelium in the cortex. Recovery was nearly complete by day 30 in the liver, and by day 40 in the kidneys.

At high doses, the pathological signs became more prominent. After 12 injections of 50,000 units, the liver showed periportal necrosis and the kidneys showed tubular cloudy swelling and protein casts. Signs of recovery were seen by day 38, and only minor changes remained evident in the liver by day 47 and in the kidneys by day 70.

Death occurred in four of 36 mice after seven to 11 injections (days 9 to 15) of 100,000 units, in six of 10 mice after nine to 10 injections (day 11 to 14) of 200,000 units, and in 10 of 10 mice after three to five injections (days 3 to 5) of 300,000 units. In all of the dead mice periportal liver necrosis was extensive. The mice that survived 12 injections of 100,000 units and 200,000 units showed signs of hepatic repair by days 42 to 47, which was incomplete on day 70. The pathological signs in the kidneys were undiminished on day 70. Because of the high toxicity of 200,000 and 300,000 unit doses, their therapeutic effects could not be determined.

Control mice received 12 injections of 200,000 unit-equivalents of the liquid vehicle (Cetus Corp.) containing everything except IL-2. These mice showed no outward signs of adverse reaction and no histological signs of damage in their viscera.

Local and Systemic Therapeutic Effects of Peritumor IL-2 Injections. The results shown in Table 1 represent the combined data from four to five similar, repeated tests. The tests compared the local and systemic effects of 12 peritumor injections of IL-2 at four dose levels: 1,000 units, 5,000 units, 50,000 units, and 100,000 units. In IL-2-treated mice the left tumor implant was injected, the right tumor implant was not injected.

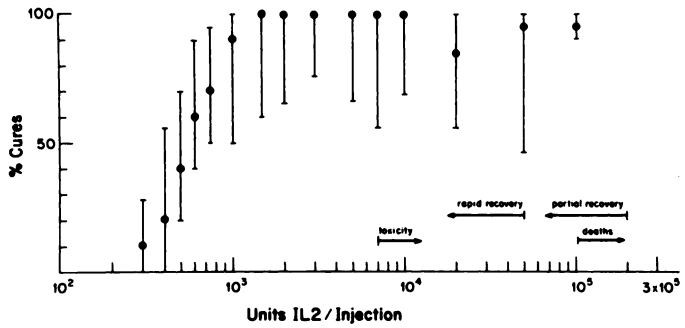

Fig. 1. Local therapeutic effect and toxic side effects of peritumor injections of graded doses of IL-2. *Points* and *bars*, modes and ranges of complete cures in repeated tests recorded from 10 to 14 weeks after the start of peritumor IL-2 injections. The IL-2 was given in 12 daily doses of from 300 units to 100,000 units each.

Table 1 *Local and systemic therapeutic effects of IL-2 injections on the growth of MC2*

Group	Treatment[a]	Incidence and mean volume (± SD) day 24[b]	Incidence day 71[c]
1	1,000 U (lt)	17/30 (57%) 260 ± 52[d, e]	5/30 (17%)[d, e]
	None (rt)	30/30 (100) 609 ± 89	27/30 (90)
2	5,000 U (lt)	11/30 (37) 179 ± 31[d, e]	2/30 (7)[d, e]
	None (rt)	28/30 (93) 424 ± 40[d]	20/30 (63)[d]
3	50,000 U (lt)	5/20 (25) 137 ± 26[d, e]	1/20 (5)[d, e]
	None (rt)	13/20 (65) 292 ± 48[d]	11/20 (55)[d]
4	100,000 U (lt)	4/20 (20) 151 ± 29[d]	1/20 (5)[d]
	None (rt)	11/20 (55) 142 ± 21[d]	3/20 (15)[d]
5	Placebo (lt)	50/50 (100) 840 ± 110	46/50 (92)
	None (rt)	49/50 (98) 834 ± 129	47/50 (94)

[a] Each mouse carried two MC2 pieces implanted s.c. on day 0. The implant on the left side (lt) was treated, the implant on the right side (rt) was not treated. Each mouse received 12 injections of 1,000 to 100,000 units of IL-2 from Days 1 to 16 after tumor inplantation.

[b] Includes palpable but unmeasurably small tumors (2 mm) recorded 1 week before the mice with the largest tumors had to be killed.

[c] By day 71, tumors had either regressed, or grown to a size beyond likelihood of regression, or grown to a size necessitating euthanasia.

[d] Significantly different from group 5.

[e] Significant difference between the left (treated) and right (untreated) tumors in individual mice.

The results show that there was a significant reduction in the growth of injected (left) tumors at all four dose levels. At 5,000 and 50,000 units per dose, there was a significant, but lesser therapeutic effect also on the uninjected (right) tumors. At 100,000 units per dose, there was a strong and nearly equal suppression of the growth of both injected and uninjected tumors. These results agree with a related, earlier study which found only a transient systemic tumor inhibition with 12 low-dose (1,500 units) peritumor injections of IL-2 (6).

DISCUSSION

Several investigators have reported that injections of IL-2 alone, at the tumor (10), i.v. (10), or i.p. (7–9, 15) had no significant effect on tumor growth. Other investigators have reported that peritumor injections of natural IL-2 (2, 4, 16) or recombinant IL-2 (3, 6) as sole therapeutic agent, inhibited the growth of tumors. Several investigators have reported that only the combined treatment with adoptive transfer of activated lymphocytes plus IL-2 injections had therapeutic effect (7–10). These divergent observations in comparable experimental systems could depend on differences in tumor characteristics as well as on varying methods of IL-2 administration. The mammary carcinoma used in this investigation is known to cause a substantial and early peritumor accumulation of T-cells and macrophages, and later, accumulation of B-cells (12). The conditions for direct action of IL-2 on responsive effector cells were therefore present at the IL-2 injection site, and presenting, naturally, the condition that Forni needed to create by implanting tumor cells mixed with immune lymphocytes before peritumor IL-2 injections would have therapeutic effect (10). Steller and coworkers observed a similar phenomenon of increased IL-2 therapeutic effect when a local inflammatory response had been created by mixing allogeneic tumor cells with the syngeneic tumor target (17). Cheever and coworkers reasoned that the

same T-cells that responded to IL-2 by proliferating *in vitro*, responded similarly to injected IL-2 by proliferating *in vivo* (18). Forni and coworkers, on the other hand, concluded that local IL-2-dependent T-cell expansion was not important, because irradiated immune lymphocytes mixed with the tumor cell implant, were just as effective as unirradiated cells in causing the rejection of IL-2 treated tumor implants (10). IL-2 has also been shown to induce proliferation in NK cells (19–21). Several types of lymphocytes can therefore be expected to respond to local IL-2 treatment by lymphokine production (21, 22) as well as by proliferation. The simultaneous IL-2 stimulation of different classes of cells could significantly enhance the systemic results of cellular cooperation at the tumor. By inducing T-cells and NK-cells to release γ-interferon at the tumor, the attraction of macrophages (23) and NK cells (24) to the tumor could be enhanced. Preliminary studies[4] found that pretreatment of the implantation site with daily doses of 500 to 1,500 units of IL-2 for 1 week before tumor implantation had no effect. This is probably because IL-2 will not by itself attract effector cells to prepare an unfavorable implantation site, but needs to act on the effector cells that are attracted to the MC2 implant. Earlier studies found that i.p. or s.c. injections 2 cm from a tumor implant, at doses from 1,000 to 10,000 units, had no antitumor effect (6). The importance of the recruitment of systemic effector cells at the tumor target was demonstrated by Forni when tumor destruction by peritumor IL-2 treatment of s.c. implants of tumor cells mixed with immune lymphocytes was prevented by prior sublethal whole-body irradiation (10).

Both the systemic and the local benefits of peritumor IL-2 treatments were, in the present study, dose dependent (Table 1). The short *in vivo* half-life of IL-2 (25, 26) and normal serum inhibitors (27) makes it likely that the systemic effect was not by disseminated IL-2, but by action on systemic effector cells by lymphokines disseminated from the host cells at the treated tumors.

The more frequent local cures observed with daily doses from 1,500 to 10,000 units given 12 times, and the slightly reduced local benefit at 20,000 to 100,000 units given at the same schedule (Fig. 1), may be related to the toxic side effects of IL-2 treatments seen at the higher doses. The toxicity and the slightly reduced local benefit at high doses (Fig. 1) must however, be measured against the systemic therapeutic benefits achieved at high doses (Table 1).

The present observations of 60% deaths after nine to 10 daily s.c. injections of 200,000 units of IL-2 is not in conformity with a report that s.c. tumor growth was controlled by giving mice 39 i.p doses of 200,000 units each of recombinant IL-2 over a period of 13 days (15). Preliminary studies in this laboratory found that i.p. administration of IL-2 at toxic doses produced more severe side effects than s.c. administration (data not shown).

The observations that good therapeutic effects were obtained with daily doses (1,500 to 10,000 units) well below the highest dose with completely repaired toxic side effects (50,000 units), indicates that judicious use of recombinant IL-2 may be beneficial and safe.

REFERENCES

1. Gillis, S., Ferm, M. M., Ou, W., and Smith, K. A. T cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol., *120:* 2027–2032, 1978.
2. Bubenik, J., Indrova, M., Perlman, P., Berzins, K., Mach, O., Kraml, J., and Toulcova, A. Tumor inhibitory effects of TCGF/IL-2/containing preparations. Cancer Immunol. Immunother., *19:* 57–61, 1985.
3. Bubenik, J., Kieler, J., and Indrova, M. Local treatment with human recombinant interleukin 2 inhibits growth of MC-induced sarcomas in syngeneic mice. Folia Biol. (Praque), *32:* 209–211, 1986.
4. Bubenik, J., Pearlman, P., Indrova, M., Simova, J., Jandlova, T., and Neuwirth, J. Growth inhibition of an MC-induced mouse sarcoma by TCGF (IL-2)-containing preparations. Cancer Immunol. Immunother., *14:* 205–206, 1983.
5. Pactkau, V., Mills, G. B., and Bleackley, R. C. Enhancement of antitumor immune responses with interleukin 2. Prog. Cancer Res. and Ther., *22:* 147–159, 1982.
6. Vaage, J., Pauly, J. L., and Harlos, J. P. Influence of the administration schedule on the therapeutic effect of interleukin. Int. J. Cancer, *39:* 530–533, 1987.
7. Cheever, M. A., Greenberg, P. D., Fefer, A., and Gillis, S. Augmentation of the anti-tumor therapeutic efficacy of long-term cultured T lymphocytes by *in vivo* administration of purified interleukin-2. J. Exp. Med., *155:* 968–980, 1982.
8. Donohue, J. H., Rosenstein, M., Chang, A. E., Lotze, M. T., Robb, R. J., and Rosenberg, S. A. The systemic administration of purified interleukin 2 enhances the ability of sensitized murine lymphocytes to cure a disseminated syngeneic lymphoma. J. Immunol., *132:* 2123–2128, 1984.
9. Mulé, J. J., Shu, S., Schwarz, S. L., and Rosenberg, S. A. Adoptive immunotherapy of established pulmonary metastases with LAK cells and recombinant Interleukin-2. Science (Wash. DC), *225:* 1487–1489, 1984.
10. Forni, G., Giovarelli, M., and Santoni, A. Lymphokine-activated tumor inhibition *in vivo*. I. The local administration of Interleukin-2 triggers nonreactive lymphocytes from tumor-bearing mice to inhibit tumor growth. J. Immunol., *134:* 1305–1311, 1985.
11. Rosenstein, M., Ettinghausen, S. E., and Rosenberg, S. A. Extravasation of intravascular fluid mediated by the systemic administration of recombinant interleukin 2. J. Immunol., *137:* 1735–1742, 1986.
12. Vaage, J., and Pepin, K. Morphological observations during developing concomitant immunity against a C3H/He mammary tumor. Cancer Res., *45:* 659–666, 1985.
13. Vaage, J., and Pepin, K. Plasma cell infiltrate of the primary tumor as a source of early systemic protection against metastases in mice. J. Natl. Cancer Inst., *71:* 147–155, 1983.
14. Wang, A., Lu, S. D., and Mark, D. F. Site-specific mutagenesis of the human interleukin-2 gene: structure-function analysis of the cystine residues. Science (Wash. DC), *224:* 1431–1433, 1984.
15. Rosenberg, S. A., Mulé, J. J., Spiess, P. J., Reichert, C. M., and Schwarz, S. L. Regression of established pulmonary metastasis and subcutaneous tumor mediated by the systemic administration of high-dose recombinant Interleukin-2. J. Exp. Med., *161:* 1169–1188, 1985.
16. Pizza, G., Severin, G., Menniti, D., DeVinci, C., and Corrado, F. Tumor regression after intralesional injection of interleukin 2 (IL-2) in bladder cancer. Preliminary report. Int. J. Cancer, *34:* 359–367, 1984.
17. Steller, E. P., Eggermont, A. M. M., Mathews, W., and Sugarbaker, P. H. Recruitment of inflammatory cells to a tumor deposit protentiates the immunotherapeutic effects of Interleukin-2. Cancer Immunol. Immunother., *23:* 165–168, 1986.
18. Cheever, M. A., Greenberg, P. D., Irle, C., Thompson, J. A., Urdal, D. L., Mochizuki, D. Y., Henney, C. S., and Gillis, S. Interleukin 2 administered *in vivo* induces the growth of cultured T cells *in vivo*. J. Immunol., *132:* 2259–2265, 1984.
19. Dennert, G., Yogeeswaran, G., and Yamagata, S. Cloned lines with natural killer activity. J. Exp. Med., *153:* 545–556, 1981.
20. Hefeneider, S. H., Conlon, P. J., Henney, C. S., and Gillis, S. *In vivo* Interleukin-2 administration augments the generation of alloreactive cytolytic T lymphocytes and resident natural killer cells. J. Immunol., *130:* 222–227, 1983.
21. Handa K., Suzuki, R., Marsui, H., Shinizu, Y., and Kumagai, K. Natural killer (NK) cells as responder to interleukin 2 (IL-2). II. IL-2 induced interferon-α production. J. Immunol., *130:* 988–992, 1983.
22. Kasahara, T., Djeu, Y. J., Dougherty, S. F., and Oppenheim, J. J. Capacity of human large granular lymphocytes to produce multiple lymphokines: interleukin 2, interferon, and colony-stimulating factor. J. Immunol., *131:* 2379–2385, 1983.
23. Ruco, L. P., and Meltzer, S. M. Macrophage activation for tumor cytotoxicity. Development of macrophage cytotoxic activity requires completion of a sequence of short-lived intermediary reactions. J. Immunol., *121:* 2035–2042, 1978.
24. Henney, C. S., Kuribayashi, K., Kern, D. E., and Gillis, S. Interleukin-2 augments natural killer activity. Nature (Lond.), *291:* 335–338, 1981.
25. Muhlradt, P. F., and Opitz, H. G. Clearance of interleukin 2 from the blood of normal and T-cell-depleted mice. Eur. J. Immunol., *12:* 983–985, 1982.
26. Donohue, J. H., and Rosenberg, S. A. The fate of interleukin 2 after *in vivo* administration. J. Immunol., *130:* 2203–2208, 1983.
27. Wagner, H., Hardt, C., Heeg, K., Pfizenmaier, K., Stotter, H., and Rollinghoff, M. The *in vivo* effects of interleukin 2 (TCGF). Immunobiol., *161:* 139–156, 1982.

[4] J. Vaage, unpublished data.

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