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ANTITUMOR ACTIVITY OF RECOMBINANT INTERLEUKIN 6 IN MICE

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IL-6 has been recently cloned and expressed by recombinant DNA techniques in *Escherichia coli* (1–3). It is a 19–30-kD phosphoglycoprotein that can elicit a broad range of immune and acute phase responses. IL-6 can act as a cytotoxic T cell differentiation factor as well as a stimulatory factor for T cells and B cells (4–6). Stimulation of hepatocytes by IL-6 results in the synthesis of major acute-phase proteins associated with inflammation (7). We (8) and others (9) have shown that the systemic administration of human rTNF-α to patients with advanced cancer induced circulating, biologically active IL-6. In addition, IL-2 administration could induce circulating TNF and IL-6 in treated patients (8). In mice, tumor bearers consistently produced more IL-6 in response to rTNF than did nontumor bearers. Moreover, low levels of endogenous circulating IL-6 could be detected as a consequence of the tumor-bearing state, since the sera of mice not receiving recombinant cytokines contained levels of IL-6 that paralleled the extent of tumor burden (10). Based on these studies, it became apparent that IL-6 may be an important cytokine in the host's immune and metabolic responses to cancer. The availability of human rIL-6 prompted us to undertake in vivo studies to examine the therapeutic efficacy of this cytokine in models of established tumors in mice.

Materials and Methods

Mice. Female C57BL/6 mice, 12–16 wk old, were obtained from The Jackson Laboratory (Bar Harbor, ME) and from the Small Animal Section, Veterinary Resources Branch, National Cancer Institute.

Recombinant Cytokines. Human rIL-2 was kindly supplied by the Cetus Corp. (Emeryville, CA) and had a specific activity of 3×10^6 U/mg. The biologic and biochemical activities of rIL-2 have been described elsewhere (11, 12).

Human rIL-6 (Genetics Institute, Cambridge, MA) was purified to homogeneity from *E. coli* supernatants and had an approximate activity of $1–4 \times 10^6$ U/mg, as determined by the plasmacytoma proliferation assay (13, 14); the protein concentration of the stock material was 276–300 μg/ml of buffer (50 mM sodium acetate, 200 mM sodium chloride pH 5–5.5) with an endotoxin level of 2–4 EU/ml.

Human rTNF-α (Cetus Corp.) had a specific activity of 2.2×10^7 U/mg as determined by the L929 cytolytic bioassay (15).

B9 Bioassay. Serum rIL-6 levels were detected by a [^{3}H]TdR uptake assay using the IL-6-dependent murine hybridoma subclone B9 as described previously (13, 14). The specificity

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of proliferation of the B9 hybridoma cells to IL-6 and not to a variety of other known cytokines has been reported elsewhere (8, 10). Known preparations of rIL-6 were diluted in heat-inactivated FCS and tested concurrently as a standard. SD of replicate samples were within 10%.

Tumors. The syngeneic sarcomas (MCA-105, -106, and -203) were induced in our laboratory by 0.1 ml of 1% 3-methylcholanthrene (MCA) in sesame oil injected intramuscularly into C57BL/6 mice (16). The syngeneic colon adenocarcinoma, MC-38, was induced by dimethylhydrazine. Single cell suspensions were prepared for injection by excising growing, fresh tumor transplanted subcutaneously in syngeneic mice (second to ninth passage in vivo), mincing the tissue in HBSS (Biofluids, Rockville, MD), and stirring it in a triple-enzyme mixture (5 ml deoxyribonuclease type IV [0.01-0.02 mg/ml], 5 ml collagenase type IV [1 mg/ml], and 5 ml hyaluronidase type V [0.1 mg/ml; Sigma Chemical Co., St. Louis, MO] in HBSS [35 ml]) for 3 h at room temperature (17). The dispersed tumor cells were collected, passed through 100-guage nylon mesh (Nitex; Lawshe, Rockville, MD), and washed three times in HBSS. The cells were then adjusted to the appropriate cell concentration for injection into mice.

Tumor Therapy Models. C57BL/6 mice were injected intravenously (to generate pulmonary metastases) or intrasplenically (to generate hepatic metastases) with tumor cells suspended in HBSS ($5–8 \times 10^5$ cells/ml) as described previously (17, 18). In some experiments, mice were irradiated by exposure to a cesium-137 source for 4.8 min, which yielded a total body radiation dose of 500 rad. The irradiation was performed 6 h before the injection of tumor cells. Starting on day 3 after tumor induction, mice were given intraperitoneal injections (1 ml thrice daily) of either HBSS, rIL-6, or rIL-2 for 4 to 6 consecutive days. Between days 12 and 19 after tumor induction, the mice were ear tagged, randomized, and killed for enumeration of metastatic lung or liver nodules as described (17, 18). Complete enumeration (blind fashion) of metastases was possible because of the distinct white nodules formed on the blackened surface of the lungs or liver (intratracheal injection [for lungs] or intravenous injection via the lateral tail vein [for liver] with a 15 percent solution of India Ink) when bleached by Fekette's solution (17, 18). Lungs or livers with confluent metastases (or too numerous to count) were assigned an arbitrary value of 250 because in these instances we were able to count reliably only numbers of metastases approaching 250 (with the exception of the MCA-106 sarcoma in which we could count distinct pulmonary metastases approaching 400). Statistical analyses were performed by the Wilcoxon rank sum test. Two-sided p values are presented in all experiments.

In other experiments, C57BL/6 mice were injected subcutaneously with 5×10^5 MCA-106 sarcoma cells suspended in 0.05 ml HBSS as described previously (19). 10 d later, when the tumors had achieved a size of 6–7 mm in diameter, the mice each received a single intravenous injection of 1 ml excipient or rTNF in HBSS containing 0.1% pooled heat-inactivated normal C57BL/6 mouse serum. These mice were then further subdivided into two groups, and received intraperitoneal injections of either 0.5 ml HBSS alone or rIL-6 in HBSS twice a day for 5 consecutive days. Mice were then ear tagged, randomized, and followed for tumor progression/regression and for survival. Statistical significance was determined by χ^2 analysis; two-sided p values are presented.

Results and Discussion

Using the B9 hybridoma cell bioassay (13, 14), we determined that the serum half-life of IL-6 in C57BL/6 mice was ~7 min after an intravenous injection of 1 μg, with 240 hybridoma growth factor (HGF) units detected at 1 h, and disappearance by 2 h (Fig. 1). By the intraperitoneal route, sustained levels of biologically active IL-6 could be detected out to 6 h (240 HGF units) following the administration of 10 μg, with a serum half-life of ~3 h (Fig. 1). Because prolonged, circulating levels of IL-6 could be achieved in sera after an intraperitoneal injection, we examined the effect of rIL-6 on growing syngeneic murine tumors using this route of administration.

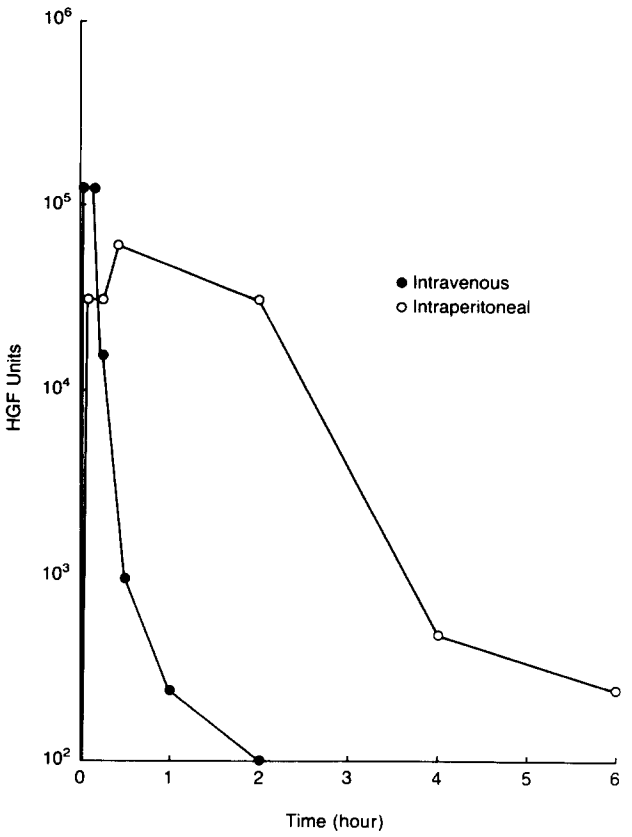

FIGURE 1. Serum half-life of rIL-6 in mice. Normal C57BL/6 mice received a single i.v. or i.p. injection of 1 μg or 10 μg rIL-6, respectively, diluted in 0.5 ml HBSS. Mice were then bled at specified time points after injection; the sera from two mice per time point were pooled. IL-6 levels were determined as described in Materials and Methods.

We (12) and others (20) have shown that the systemic administration of high-dose rIL-2 alone could mediate the regression of a variety of murine tumors established at subcutaneous as well as visceral sites. To examine whether or not rIL-6 could also mediate antitumor effects when used alone at high doses, we directly compared the administration of this cytokine in amounts comparable to known therapeutic doses of rIL-2. C57BL/6 mice were injected intravenously with MCA-105, -106, or -203 sarcoma or MC-38 colon adenocarcinoma cells or intrasplenically with MCA-203 sarcoma cells to induce pulmonary and hepatic metastases, respectively. On day 3 after tumor injection, when multiple foci of micrometastases were established (as assessed by histologic examination of lungs or liver), these mice then received intraperitoneal injections of rIL-6, rIL-2, or HBSS for 4–6 consecutive days. We assayed the effect of these treatments on metastasis development between days 12 and 19. The results of eight separate experiments (Table I) showed that metastases from all four tumors were significantly reduced by the administration of rIL-6 alone. As reported in earlier studies (12), rIL-2 alone at high dose also resulted in marked tumor regression, with the exception of the MC-38 adenocarcinoma. In several experiments the injections of rIL-2 (33.3 or 50.0 μg per injection) were stopped after 12 or 13 doses because mice were showing visible signs of toxicity; manifested as

TABLE I

Comparison between the Antitumor Efficacy of the Systemic Administration of rIL-6 and rIL-2 Against Established Pulmonary or Hepatic Metastases from Four Distinct, Weakly Immunogenic Syngeneic Tumors

Exp.	Tumor	Site	Treatment: μg rIL-2 (per injection)	Treatment: μg rIL-6 (per injection)	Number of treatment-related deaths/ total treated	Mean number of metastases
A	MCA-203	Lungs	–	–	0/6	205
			33.3 (13)*	–	0/6	181
			–	33.3 (15)	0/6	23‡
B	MCA-203	Lungs	–	–	0/12	>250
			33.3 (12)	–	0/6	67‡
			–	33.3 (15)	0/6	46‡
C	MCA-106	Lungs	–	–	0/12	385
			33.3 (12)	–	1/6	137‡
			–	33.3 (15)	0/6	158‡
D	MCA-203	Liver	–	–	0/6	>250
			33.3 (15)	–	0/6	11‡
			–	6.6 (15)	0/5	101
			–	33.3 (15)	0/6	11‡
E	MC-38	Lungs	–	–	0/12	207
			10.0 (18)	–	0/6	237
			50.0 (12)	–	1/6	221
			50.0 (18)	–	4/6	>250
			–	10.0 (18)	0/6	98‡
			–	50.0 (18)	0/6	2‡
F	MCA-105	Lungs	–	–	0/10	225
			10.0 (18)	–	0/6	63‡
			50.0 (12)	–	2/6	14‡
			50.0 (18)	–	3/6	25‡
			–	10.0 (18)	0/5	19‡
			–	50.0 (18)	0/6	5‡
G	MCA-105	Lungs	–	–	0/12	>250
			50.0 (18)	–	7/12	2
			–	50.0 (18)	0/6	27‡
H	MC-38	Lungs	–	–	0/12	>250
			10.0 (15)	–	0/6	243
			50.0 (12)	–	0/6	>250
			–	10.0 (15)	0/6	4‡
			–	50.0 (15)	0/6	0‡

* Number in parentheses; total number of cytokine injections administered.

‡ $p < 0.01$ compared with the group receiving no treatment (HBSS).

tachypnea, edema, lethargy, and piloerection (21). In contrast, mice treated with rIL-6 at similar doses did not show outward signs of toxicity. Moreover, the administration of high dose rIL-6 did not cause substantial increases in wet weights of the lungs compared with HBSS control mice (data not shown); thus, unlike rIL-2, rIL-6 did not appear to elicit the vascular leak syndrome (21). In three combined experiments (E, F, G), when rIL-2 was continued for a full 6-d course of 18 total injections

(50.0 μg per injection), 14 of 24 mice died from treatment compared with 0 of 18 mice receiving rIL-6 ($p^2 < 0.005$). Thus, rIL-6 at relatively high doses could mediate the regression of established, 3-d metastases from four distinct syngeneic tumors located in liver or lungs without apparent toxicity.

As an approach to study the mechanism of the rIL-6 effect we examined this treatment simultaneously in normal and in immunosuppressed mice. Either normal C57BL/6 mice or those that had received 500 rad of total body irradiation were injected intravenously with MCA-203 sarcoma cells and then treated beginning on day 3 with rIL-6 alone; comparisons were made with rIL-2 at comparable doses. As shown in Fig. 2, host immunosuppression by irradiation eliminated the rIL-6- (and rIL-2, as reported elsewhere [12]) mediated tumor regression in the lungs. A similar finding was obtained in two additional experiments, using the MCA-105 and -106 sarcomas (not shown). Therefore, the marked reduction of established micrometastatic disease mediated by rIL-6 was not a direct effect on the tumor per se, but rather was through a relatively radiosensitive host component.

Since rTNF injections in tumor bearing mice were accompanied by the production of detectable, albeit short-term, levels of circulating IL-6 (10), we next studied what effect, if any, prolonged administration of relatively low doses of rIL-6 (5 μg per injection) might have on the antitumor therapeutic efficacy of rTNF in vivo. The doses of rTNF chosen were based on our earlier in vivo studies of the synergistic antitumor effects obtained when this cytokine was combined with rIL-2 (19). The long term survival of C57BL/6 mice bearing a subcutaneous, 10-d weakly immunogenic MCA-106 sarcoma after combination cytokine immunotherapy is shown in the representative experiment of Fig. 3 (graph), in which repetitive injections of rIL-6 substantially augmented the small prolongation in survival achieved with 2 or 5 μg of rTNF alone. The combined cure rates of three separate experiments are shown in the inset of Fig. 3. Treatment of the tumor-bearing mice with the combination of a subtherapeutic dose of rTNF plus rIL-6 resulted in eradication of the tumor in a significant proportion of animals, which was not observed with treatment by either cytokine alone.

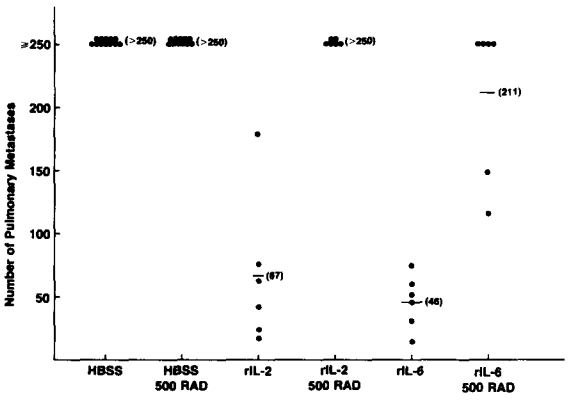

FIGURE 2. Effect of prior host immunosuppression by sublethal, total body irradiation on the antitumor effect of rIL-6 in C57BL/6 mice bearing 3-d MCA-203 pulmonary metastases. The nonirradiated arm of this experiment is presented as experiment B in Table I. Mice were injected i.p. with either HBSS, rIL-2 (33.3 μg/injection), or rIL-6 (33.3 μg/injection), thrice daily for 4 (rIL-2) or 5 (HBSS, rIL-6) consecutive days, beginning 3 d after tumor injection. The number of pulmonary metastases was counted on day 13. Each dot in the figure represents the number of lung nodules from an individual animal, with the mean value for each treatment group given in parentheses.

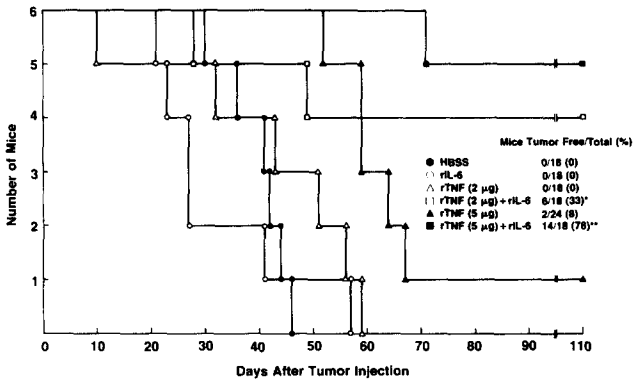

FIGURE 3. Therapeutic efficacy of the combination of rIL-6 and TNF. Treatment with rTNF (2 or 5 μg) alone, rIL-6 (5 μg per injection) alone, or the two cytokines combined was initiated 10 d after the s.c. injection of MCA-106 sarcoma cells as described in Materials and Methods. Mice without detectable tumor at 110 d had been rendered completely free of tumor for at least 80 d. The data in this figure are representative of three individual studies, with the combined cure rates from all experiments presented in the insert. *, $p = 0.05$; **, $p = 0.001$; compared with group receiving no treatment.

Collectively, these data show that the systemic administration of rIL-6 alone at high dose or at lower doses in combination with rTNF can mediate the regression of murine syngeneic tumors in vivo. The mechanism(s) by which this antitumor effect occurs does not appear to be through a direct action of rIL-6 on the tumor, since host immunosuppression eliminates successful therapy. This latter finding differs, therefore, from that reported by others in which IL-6 had a direct inhibitory (22) or stimulatory (23) effect on the growth of certain tumor cells in vitro. It is conceivable that secondary cytokines may be released or that cellular immune mechanisms may be activated following rIL-6 administration that directly or indirectly result in antitumor effects. Moreover, since IL-6 can have a variety of growth and differentiation effects on T cells, B cells, hepatocytes, and hematopoietic stem cells, the cellular mechanism(s) operational in vivo during rIL-6-mediated tumor regression may involve a complex interaction of several cell types. Preliminary histologic analysis of the lungs of mice receiving high dose rIL-6 has revealed infiltration with morphologically activated lymphoid cells during the regression of pulmonary metastases (not shown). The mechanism(s) of IL-6 action in vivo as well as whether rIL-6 alone, or in combination with other recombinant cytokines, will impact on more advanced metastatic disease remain to be examined.

Summary

IL-6 possesses multiple biologic activities that affect a broad range of cells including those directly involved in immune responses as well as cells important in the systemic response to infection or trauma. We now show that purified human rIL-6, when administered alone at relatively high doses that are comparable to therapeutic levels of IL-2, mediated substantial reductions in the number of pulmonary and hepatic micrometastases from four distinct syngeneic tumors. Unlike IL-2, IL-6 injections resulted in neither observable toxicity nor death of the treated mice at the dose regimens used. Host immunosuppression by sublethal total-body irradiation before the initiation of therapy prevented the IL-6 antitumor effect, thus suggesting that IL-6 acted through a radiosensitive host component rather than directly on the

tumor itself. Moreover, the systemic administration of relatively low doses of IL-6 in combination with subtherapeutic doses of TNF to mice bearing an established weakly immunogenic, syngeneic tumor at a subcutaneous site resulted in marked tumor regression and cure rates. These studies represent the first demonstration of tumor regression mediated by recombinant IL-6 in vivo.

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