

Combined Interleukin 1/Interleukin 2 Therapy of Mice Injected with Highly Metastatic Friend Leukemia Cells: Host Antitumor Mechanisms and Marked Effects on Established Metastases

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Summary

Peritumoral injection of recombinant human interleukin 1 β (IL-1 β) in mice transplanted subcutaneously with Friend erythroleukemia cells (FLC) resulted in a marked increase in survival time and inhibition of metastatic tumor growth in liver and spleen. In contrast, IL-2 treatment alone did not significantly inhibit the development of FLC metastases. A synergistic antitumor effect was observed after combined IL-1/IL-2 therapy of these mice. The antitumor action of IL-1/IL-2 treatment was abolished or markedly reduced in mice treated with antibodies to CD4 or CD8 antigens, whereas antibodies to asialo-GM₁ were ineffective. A clear-cut increase in the percentage of CD4⁺ cells was observed in the spleens of cytokine-treated mice on days 17 and 23. On day 23 of cytokine therapy, CD8⁺ cells were increased in both spleens and lymph nodes. On day 17, infiltrates of host-reactive cells (i.e., lymphocytes, granulocytes, and monocytes) were observed in both spleen and liver from FLC-injected mice treated with IL-1/IL-2, in association with tumor cells. On days 17 and 23, spleen cells and cells recovered from mesenteric lymph nodes of IL-1/IL-2-treated mice exerted a potent antitumor effect as determined by Winn assay experiments. This antitumor activity was abolished by preincubation of spleen cells with anti-CD8 antibody, but not by treatment with antibodies to asialo-GM₁; antibodies to CD4 exerted only a slight effect. Combined IL-1/IL-2 therapy was more effective on established (i.e., 6–7-d) FLC tumors than on early (i.e., 1-d) tumor-transplanted mice. IL-1/IL-2 treatments were also highly effective in increasing survival time of mice from which the subcutaneous primary tumors were excised 7 d after FLC injection. These data indicate that in mice injected with FLC, the antitumor effects of IL-1/IL-2 are mediated by CD4⁺ and CD8⁺ cells (but not NK cells), and suggest that this combined cytokine treatment may be effective against established metastatic tumors.

Recent data indicate that the combined treatment of tumor-bearing animals with multiple cytokines results in a more potent antitumor effect than single cytokine therapy (1–7), thus suggesting that combined cytokine therapy represents an interesting approach to the treatment of some human cancers. In the present study, we have investigated the effects and antitumor mechanisms of combined treatment with IL-1 and IL-2 in mice transplanted with highly malignant Friend erythroleukemia cells (FLC),¹ which metastasize to the liver and the spleen (8).

IL-1, a cytokine mainly produced by stimulated monocytes/macrophages, promotes T and B lymphocyte activation and induces a wide range of biological effects in vitro and in vivo (9–11). However, little data are available on the antitumor effects of IL-1 in experimental tumors (12–17). In contrast, a number of studies have reported that IL-2 exerts marked antitumor effects in both experimental animals and cancer patients (18–20). It has been suggested that the antitumor effects of IL-2 are mediated by the in vivo generation of lymphokine-activated killer (LAK) cells (21, 22), although a direct correlation has not been shown between the in vivo antitumor efficacy of IL-2 and the in vitro cytolytic activity of LAK cells against a variety of mouse tumors (17, 23).

¹ Abbreviations used in this paper: FLC, Friend erythroleukemia cells; LAK, lymphokine-activated killer.

In past studies, we have used mice transplanted with highly metastatic FLC to investigate the antitumor effects of IFN- α/β (24–28), TNF- α (29, 30), IL-1 (16, 17), and IL-2 (17). Peritumoral injections of IL-2, alone or in combination with LAK cells, did not inhibit the development of spleen or liver metastases, although some inhibition of the primary subcutaneous FLC tumor was observed (17). In contrast, injection of IL-1 β resulted in a marked reduction of tumor growth and in an increased survival time, as compared with control mice (16, 17). In preliminary experiments, we observed that the combined treatment with IL-1 and IL-2 of mice transplanted with FLC resulted in an antitumor effect greater than that observed with either cytokine alone (17). In the present study, we have described the synergistic antitumor effects of the combined IL-1/IL-2 treatment in mice injected with FLC and we have characterized the host mechanisms involved in this antitumor response. Furthermore, we provide evidence that this combined cytokine treatment is markedly effective in inducing the regression of established liver and spleen metastases.

Materials and Methods

Mice. 6–8-wk-old male DBA/2 mice were obtained from Charles River Breeding Laboratories (Italia Calco, Italy). Nude mice were kept under specific germ-free conditions using sterile filters.

Tumor Cells. IFN- α/β -sensitive 745 and IFN- α/β -resistant 3Cl-8 FLC (31) were serially passaged intraperitoneally in syngeneic DBA/2 mice (in vivo passaged FLC). These FLC are highly metastatic to the spleen and to the liver (8) and exhibited a specific membrane glycoprotein pattern as compared with that of the non-metastatic in vitro passaged FLC (32).

IL-2, IL-1 β , and Control Preparations. Human rIL-2 (sp act, 3×10^6 U/mg of protein) (Hoffmann-La Roche, Inc., Nutley, NJ) was obtained through the National Cancer Institute (Dr. Michael J. Hawkins, Chief of the Investigational Drug Branch). Electrophoretically pure human rIL-1 β (mature fragment 117–269, expressed in *Escherichia coli*) had a specific activity of $\sim 5 \times 10^8$ half-maximal U/mg of protein in the D10.G4.1 proliferation assay (33). IL-1 β was provided by Scavo (Siena and Cassina de Pecchi, Italy), through the courtesy of Dr. D. Boraschi (Centro Ricerche Scavo, Siena). Cytokines were diluted in physiologic solution (153 mM NaCl); endotoxin contamination was <0.1 ng/mg as determined by limulus amoebocyte lysate assay. Control preparations consisted of physiologic solution unless otherwise stated.

Antibodies to Asialo-GM₁, CD4, and CD8. Anti-asialo-GM₁ rabbit antibody (10 mg/ml) (34, 35) was purchased from Wako Chemicals (Neuss 1, FRG). DBA/2 spleen cells taken from mice injected three times intravenously with 50 μ g of antibody to asialo-GM₁ (days 0, +7, +11) showed no significant NK cell cytotoxicity for mouse YAC cells in vitro.

The rat hybridoma cell mAb GK1-5 (36, 37) producing mAb to CD4 (L3T4) was provided by Prof. G. Forni (Institute of Microbiology, University of Turin, Turin, Italy). Hybridoma cells were passaged intraperitoneally in BALB/c nude mice in our laboratory. The Ig fraction of ascitic fluids was separated by ammonium sulfate precipitation. The protein concentration was ~ 10 mg/ml. Intravenous injection of DBA/2 mice with 500 μ g of the anti-CD4 Ig resulted in complete depletion of CD4⁺ spleen cells, 6–9 d after inoculation of antibody, as determined by flow cytometric analysis

using a FITC-labeled anti-CD4 mAb (Becton Dickinson & Co., Mountain View, CA).

The Ig fraction of ascitic fluids from the rat hybridoma cell mAb 53-6.7 (38) producing mAb to CD8 (Lyt-2) was separated by ammonium sulfate precipitation.

Intraperitoneal injection of DBA/2 mice with 1 mg of anti-CD8 Ig resulted in complete depletion of CD8⁺ spleen and lymph nodes cells when tested 15 d thereafter by flow cytometric analysis using a FITC-labeled anti-CD8 mAb.

Silica. Silica particles (average size $<5 \mu$ m) were provided through the generosity of Dr. K. Röbock (Steinkohlenbergbauverein, Essen-Krey, FRG). Mice were injected intravenously with 5 mg of silica particles. This dose had been shown to be effective in destroying peritoneal macrophages after intraperitoneal injection.

Flow Cytometric Analysis of Host Cells from FLC-injected Mice. The phenotypes of lymphoid cells present in lymph nodes and spleens of animals injected with FLC were evaluated by direct immunofluorescence using specific mAbs.

Spleen and mesenteric lymph nodes were removed aseptically and washed with the hub of a syringe in RPMI 1640. The cell suspension and spleen or lymph node fragments were then passed through a single layer of 100-gauge nylon mesh, and the cells were centrifuged on a Ficoll-Hypaque gradient. After three washes in RPMI 1640, light density cells were resuspended in RPMI 1640 and aliquots of 10^6 cells were incubated for 60 min at 4°C in the presence of a 1:20 dilution of one of the following mAbs conjugated with either FITC or PE (Becton Dickinson & Co.): control rat IgG1 or IgG2; anti-Lyt-2 (anti-CD8); anti-L3T4 (anti-CD4); anti-Thy-1.2. After three washes in cold Hank's saline solution, the cells were resuspended in Hank's formalin and analyzed with a FAC-Scan (Becton Dickinson & Co.).

Winn Assay. Peritoneal cells and cells recovered from the spleens or mesenteric lymph nodes were washed with saline, counted, and mixed with 5×10^4 in vivo passaged 3Cl-8 FLC (E/T cell ratios were 20:1). Cells were subsequently injected subcutaneously in 6–8-wk-old DBA/2 mice. The percentage of mice that developed subcutaneous tumors was determined 30–90 d after the subcutaneous injection.

Statistical Analyses. Data were analyzed by student's *t* test.

Light Microscopy Studies. Mice were killed by ether. Primary subcutaneous tumors, spleens, and livers were removed, cut into two pieces of ~ 0.5 cm³, and fixed in 10% formalin for several days. Fixed tissues were subsequently processed for paraffin embedding, cut into 2–3- μ m-thick slices, and stained with hematoxylin and eosin.

Results

Synergistic Antitumor Effect of Combined IL-1 and IL-2 Treatment. Table 1 summarizes the results of five experiments in which DBA/2 mice were injected subcutaneously with different FLC types and subsequently treated with IL-2, IL-1, or a combination of both cytokines. In all cases, combined IL-1/IL-2 treatment resulted in an antitumor effect greater than that observed with either cytokine alone, even though some variability of the antitumor activity was observed.

Fig. 1 (A) shows a representative experiment on the synergistic antitumor effect of combined IL-1/IL-2 treatment. Thus, DBA/2 mice were injected subcutaneously with highly metastatic 3Cl-8 FLC and, after 1 d, the mice were treated

Table 1. Synergistic Antitumor Effects of Combined IL-1/IL-2 Treatment in Mice Injected Subcutaneously with Different FLC Types

Exp.	FLC type	No. of FLC injected	Treatment	No. of mice dead at day 60/ total No. of mice	No. of tumor-free mice/ total No. of mice
1	in vivo passaged 745	5×10^6	Saline	6/6	0/6
			IL-2	6/6	0/6
			IL-1	4/6	1/6
			IL-1/IL-2	1/6	3/6
2	in vivo passaged 745	2×10^6	Saline	10/10	0/10
			IL-2	9/9	0/9
			IL-1	8/10	0/10
			IL-1/IL-2	3/10	2/10
3	in vitro passaged 3Cl-8	5×10^6	Saline	6/6	0/6
			IL-2	5/6	0/6
			IL-1	4/6	1/6
			IL-1/IL-2	1/6	3/6
4	in vivo passaged 3Cl-8	2×10^6	Saline	10/10	0/10
			IL-2	9/9	0/9
			IL-1	6/9	1/9
			IL-1/IL-2	1/9	4/9
5	in vivo passaged 3Cl-8	2×10^6	Saline	10/10	0/10
			IL-2	10/10	0/10
			IL-1	5/10	3/10
			IL-1/IL-2	2/10	6/10
Total values			Saline	42/42	0/42
			IL-2	39/40	0/40
			IL-1	27/41	6/41
			IL-1/IL-2	8/41	18/41

6–8-wk-old male DBA/2 mice were injected with different FLC types. 24 h after tumor injection, mice were treated peritumorally (0.2 ml per mouse, twice a day: approximately 10 am and 6 pm) on days 2, 3, 4, 7, 8, 9, 14, 15, 16, 20, 21, and 22. IL-1 and IL-2 were injected together in a total 0.2 ml volume. IL-1, 250 ng/treatment; IL-2, 20,000 U/treatment. Survived mice were killed 100–135 d after FLC injection and were found to be tumor free.

subcutaneously (i.e., peritumorally) with IL-1 β , IL-2, or a combination of both cytokines. Subcutaneous injection of IL-1 β resulted in marked inhibition of tumor growth (data not shown) and a clear-cut increase in survival time, as compared with control FLC-injected mice (Fig. 1 *A*). In agreement with previous findings (17), subcutaneous treatment with IL-2 alone resulted in some inhibition of tumor growth, but only a slight increase in survival time as compared with control mice was observed. As shown in Fig. 1 (*A*), the concomitant treatment with both IL-1 and IL-2 resulted in a synergistic antitumor effect. On days 8–10, all treated mice in this group developed small FLC tumors, comparable with those of control mice. On day 10, the mean surface area of these tumors was 60.8 ± 5.0 . These IL-1/IL-2-treated tumors regressed 3–4 wk after tumor cell injection and a few days after treat-

ment had been discontinued. All control and IL-2-treated mice died with extensive liver and spleen metastases. In contrast, 30% of the IL-1-treated mice were tumor free 140 d after tumor cell injection. The combined IL-1/IL-2 treatment resulted in complete regression of the tumor and recovery in 60% of the FLC-injected mice. At death, the IL-1/IL-2-treated mice injected with the in vivo passaged FLC exhibited liver and spleen metastases, indicating that this combined cytokine treatment did not modify the in vivo behavior of FLC. As shown in Fig. 1 (*B*), combined IL-1/IL-2 peritumoral treatment resulted in a slight decrease in body weight as compared with control animals, within 1–2 wk of cytokine administration. However, no toxicity was observed at any time during IL-1/IL-2 treatment.

In vitro treatment of FLC with IL-1 (50 ng/ml), IL-2

(2,000–20,000 U/ml), or a combination of both cytokines did not result in significant inhibition of cell proliferation 1–3 d after cell seeding (data not shown).

Efficacy of IL-1/IL-2 Treatment in Mice Injected Subcutaneously with FLC and Treated with Antibodies to CD4, CD8, or Asialo-GM₁. It was of interest to determine the antitumor effects of IL-1/IL-2 treatment in mice whose immune response was defective because of the injection of antibodies directed against specific cell types. As shown in Table 2, the antitumor effect of IL-1/IL-2 therapy was completely abrogated in mice treated with anti-CD4 antibody. Injection of anti-CD8 antibody also resulted in a marked reduction of the IL-1/IL-2 antitumor effects. In contrast, treatment with anti-asialo-GM₁ antibody, which completely inhibited specific NK cell activity, did not significantly affect the antitumor activity of IL-1/IL-2 (Table 2). Injection of mice with silica, which has been shown to be toxic for macrophages, partially inhibited the antitumor effects of the IL-1/IL-2 treatment (data not shown).

Effects of IL-1/IL-2 Treatment on Lymphocyte Subsets in the Spleen and Lymph Nodes of FLC-injected DBA/2 Mice. IL-1/IL-2 treatment of mice injected subcutaneously with FLC resulted in a marked increase in the percentage of CD4⁺ spleen cells on days 17 and 23 after tumor cell injection (i.e., a twofold and a fivefold increase, respectively). On these days, a slight increase in the percentage of CD4⁺ cells was also observed in the mesenteric lymph nodes of IL-1/IL-2-treated mice (data not shown). Moreover, the flow cytometric analyses of host cells recovered on day 23 from FLC-injected mice revealed a clear-cut increase in the percentage of CD8⁺ cells in the spleens and lymph nodes of IL-1/IL-2-treated mice (i.e., a 12-fold and a twofold increase, respectively) (data not shown).

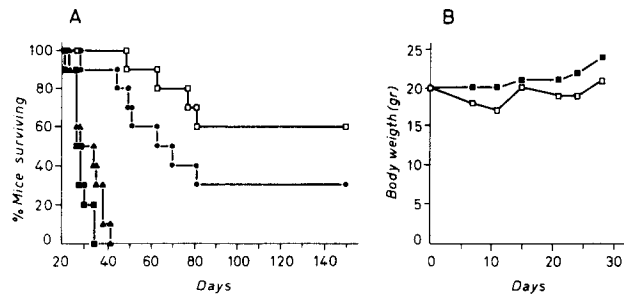


Figure 1. Antitumor effects of peritumoral injection of IL-1 β , alone or in combination with IL-2, in mice transplanted subcutaneously with 3Cl-8 FLC. 7-wk-old male DBA/2 were injected subcutaneously with 2×10^6 in vivo passaged 3Cl-8 FLC. On day 1, mice were divided into the following groups: (a) saline; (b) IL-1 β ; (c) IL-2; (d) IL-1 β + IL-2. Treatments were performed as described in detail in the footnote to Table 1. There were 10 mice in each experimental group. On day 140, surviving mice were killed and found to be tumor free. (A) The percent of surviving mice at different days after tumor cell injection. (■) Saline-treated mice; (▲) IL-2-treated mice; (●) IL-1-treated mice; (□) IL-1/IL-2-treated mice. (B) The mean body weight of mice treated with saline (■) or IL-1 + IL-2 (□). The SEs are within the symbols.

Generation of Effector Cells Endowed with Potent Antitumor Activity during IL-1/IL-2 Treatment of FLC-injected Mice. The livers and spleens of FLC-injected mice were examined by light microscopy at different times after IL-1/IL-2 treatments. On days 10, 14, 17, and 20, the histologic examination of livers and spleens of FLC-injected mice revealed the presence of both tumor cells and infiltrating host cells. Apparent micrometastases were observed in the livers of IL-1/IL-2-treated

Table 2. Effects of Injection of Antibodies to Specific Cell Types on the Antitumor Activity of IL-1/IL-2 Treatment

Exp.	Treatment	Antibody	Mean day of death \pm SE	Tumor-free mice
1	Saline	-	34 \pm 3.2	0/8
	Saline	Anti-CD4	27 \pm 3.0	0/8
	IL-1 β + IL-2	-	57 \pm 10.4	2/7
	IL-1 β + IL-2	Anti-CD4	25 \pm 1.1	0/8
2	Saline	-	28 \pm 1.1	0/8
	Saline	Anti-CD8	27 \pm 1.4	0/10
	IL-1 + IL-2	-	48 \pm 2.6	1/5
	IL-1 + IL-2	Anti-CD8	29 \pm 6.5	1/5
3	Saline	-	25 \pm 1.7	0/8
	Saline	Anti-asialo-GM ₁	25 \pm 3.1	0/8
	IL-1 β + IL-2	-	65 \pm 11.4	1/7
	IL-1 β + IL-2	Anti-asialo-GM ₁	51 \pm 4.4	0/8

6–8-wk-old male DBA/2 mice were injected subcutaneously with 5×10^6 3Cl-8 FLC. Some mice were treated with different antibodies as described in Materials and Methods. 1 d after FLC injection, mice were treated subcutaneously with saline or IL-1 + IL-2 as described in the legend to Fig. 1, on days 1, 2, 3, 7, 8, 9, 13, 14, and 15. Surviving mice were killed 120 d after tumor injection and found to be tumor free.

* $p < 0.001$.

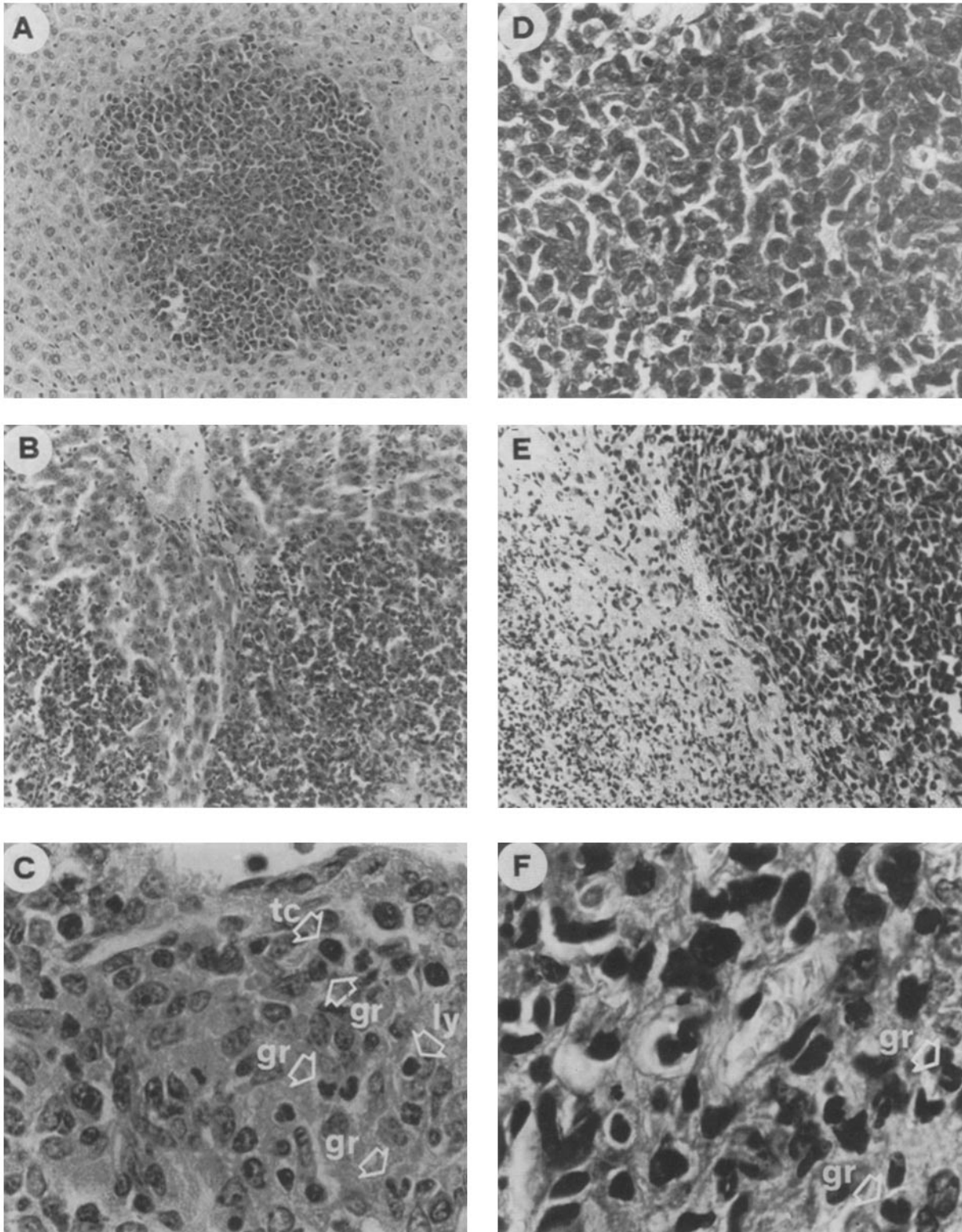


Figure 2. Histologic changes in the liver and primary subcutaneous tumor of IL-1/IL-2-treated mice injected with FLC. (A) Liver of a saline-treated mouse on day 17 after FLC injection. A typical FLC metastasis is observed without evidence of host cell infiltrates ($\times 130$). (B) Liver of a mouse treated with IL-1/IL-2 (day 17). Large areas of tumor cells are observed together with the appearance of host cell infiltrates ($\times 130$). (C) Same liver, as in B, at a higher magnification ($\times 670$). Infiltrating lymphocytes (ly) and granulocytes (gr) as well as tumor cells (tc) are observed. (D) Primary subcutaneous tumor from a saline-treated mouse (day 17) ($\times 330$). (E) Primary subcutaneous tumor from a mouse treated with IL-1/IL-2 (day 17). Note the hemorrhagic tumor necrosis, host cell infiltrates, and degenerating tumor cells ($\times 130$). (F) Same tumor, as in E, at higher magnification ($\times 840$). Lymphocytes, granulocytes (gr), and monocytes are occasionally observed.

mice on days 10 and 17. On these days, aliquots of cells recovered from the spleens of IL-1/IL-2-treated FLC-injected mice were seeded in medium containing agarose to determine the number of FLC colonies (24).

Values ranging from 1.2×10^4 to 2.5×10^5 FLC per spleen were found by counting the FLC colonies grown in agarose, indicating that a consistent number of tumor cells were present in the spleens of IL-1/IL-2-treated mice. On day 17, infiltrates of host-reactive cells (i.e., lymphocytes, granulocytes, and monocytes) were clearly observed in both spleen (data not shown) and liver (Fig. 2, B and C) from FLC-injected mice treated with IL-1/IL-2. At this time, large areas of hemorrhagic tumor necrosis were present within the primary subcutaneous tumors from the treated mice (Fig. 2 E), and similar host cell infiltrates were detected (Fig. 2 F).

Experiments were then performed to determine whether host cells from cytokine-treated mice might exert some antitumor activity in typical Winn assays. No antitumor activity was found using spleen cells, peritoneal cells, or cells from lymph nodes of mice on day 10 of cytokine therapy (data not shown). In contrast, a clear-cut antitumor activity was exhibited by host cells recovered from the spleens and lymph nodes of mice on days 17 and 23 of treatment with IL-1/IL-2 (Table 3). This antitumor activity was abolished by pretreatment of spleen cells with anti-CD8 antibody, but not by preincubation with anti-asialo-GM₁ antibodies (Fig. 3). A slight inhibition of tumor growth was observed by treatment of spleen cells with antibodies to CD4 (Fig. 3).

IL-1/IL-2 Treatment Is More Effective in Mice with Established FLC Tumors Than in Early Transplanted Mice. The data presented above suggested that IL-1/IL-2 treatment was effective

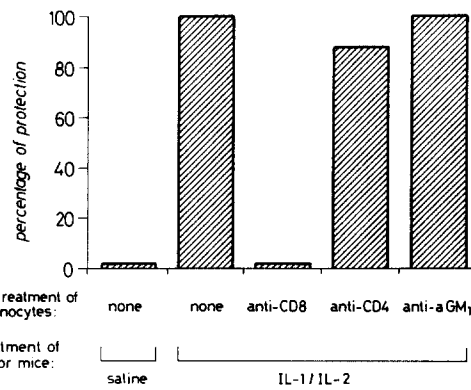


Figure 3. Characterization of cells endowed with antitumor activity in spleens of cytokine-treated mice. 7-wk-old male DBA/2 mice were injected subcutaneously with 2×10^6 in vivo passaged 3Cl-8 FLC. Treatment was as follows: 0.2 ml of either saline or IL-1 β + IL-2 (250 ng and 20,000 U, respectively) injected twice a day (i.e., at approximately 10 am and 6 pm) on days 1, 2, 3, 7, 8, 9, 14, 15, and 16. Cells recovered from the spleens of FLC-injected mice on days 17 and 23 were preincubated for 30 min at 37°C with a suitable dilution of different antibodies. Cells were then mixed with 5×10^4 3Cl-8 FLC and subsequently injected subcutaneously in 6-wk-old male DBA/2 mice. The E/T cell ratio was ~20:1. There were eight mice per each group. The values represent the percentage of protection by: $100 \times (\text{No. of mice without tumor}) / (\text{Total No. of mice})$. All the mice injected only with FLC developed subcutaneous tumors within 15 d, similarly to mice injected subcutaneously with tumor cells plus splenocytes from saline-treated mice. The number of tumor-free mice in each group was determined 30 d after FLC injection.

in curing some FLC-injected mice even though some tumor cells were present in their spleens and livers. It was of interest, therefore, to study the antitumor effects of IL-1/IL-2 treatments in mice with established solid tumors, as com-

Table 3. Antitumor Activity of Host Cells Recovered from Cytokine-treated FLC-injected Mice (Winn test)

Treatment	Day	No. of tumor-free mice (day 90)/total No. of injected mice with effector cells from:		
		Spleen*	Lymph nodes*	Peritoneal cavity†
Saline	17	0/6	0/6	0/6
IL-1 β + IL-2	17	6/6	2/6	0/6
Saline	23	0/6	0/6	ND
IL-1 + IL-2	23	3/6	6/6	ND

7-wk-old male DBA/2 mice were injected subcutaneously with 2×10^6 in vivo passaged 3Cl-8 FLC. Treatment was as follows: 0.2 ml of either saline or IL-1 β + IL-2 (250 ng and 20,000 U, respectively) injected twice (i.e., at approximately 10 am and 6 pm) on days 1, 2, 3, 7, 8, 9, 14, 15, and 16. On day 17 of tumor growth, pooled cells recovered from each experimental group were mixed with 5×10^4 in vivo passaged FLC and injected subcutaneously into DBA/2 mice. There were six mice in each group.

* The E/T cell ratio was ~20:1.

† The E/T cell ratio was ~10:1.

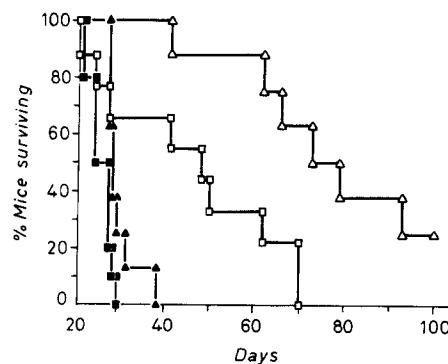


Figure 4. Efficacy of IL-1/IL-2 treatment in mice with 1-d or 6-d established FLC subcutaneous tumors. 6-wk-old male DBA/2 mice were injected subcutaneously with 2×10^6 in vivo passaged 3Cl-8 FLC. Mice were treated with saline or IL-1 + IL-2 on day 1 or 6 after tumor cell implantation. IL-1/IL-2 treatments were performed as described in the legend to Fig. 3. There were 10 mice in each group. 1-d tumors were treated on days 1, 2, 3, 7, 8, 9, 13, 14, and 15. 6-d tumors were treated on days 6, 7, 8, 13, 14, 15, 20, 21, and 23. (■) Saline-treated mice (1 d); (□) IL-1/IL-2-treated mice (1 d); (▲) Saline-treated mice (6 d); (△) IL-1/IL-2-treated mice (6 d).

pared with mice transplanted with FLC 1 d before the start of cytokine treatment.

Fig. 4 shows a representative experiment in which the efficacy of IL-1/IL-2 treatment was compared in mice with 1- or 6-d implanted tumors. IL-1/IL-2 therapy appeared to be more effective on established FLC tumors than on early FLC-transplanted mice. Similar results were obtained comparing the efficacy of IL-1/IL-2 treatments on 1- and 7-d FLC tumors (data not shown).

Fig. 5 shows the effect of the combined cytokine treatment on the growth of established (i.e., 6-d) FLC tumors. IL-1/IL-2 treatment resulted in a complete arrest of tumor growth. In this experiment, 30% of the IL-1/IL-2-treated mice exhibited a complete tumor regression and were considered to be tumor free 120 d after FLC injection.

IL-1/IL-2 Treatment Is Effective as Adjuvant Therapy after Surgery in the Inhibition of Liver and Spleen Metastases. The experiments described above were performed by treating FLC-injected mice peritumorally. Therefore, it was not possible to distinguish between the effects of cytokines on the primary subcutaneous tumor vs. the effects on metastases. On the other hand, intraperitoneal or intravenous treatments with IL-1/IL-2 of mice injected subcutaneously with FLC were less effective in inhibiting tumor growth and exhibited some toxicity, which was not observed after subcutaneous cytokine treatments (data not shown).

It was of interest, therefore, to study the antitumor effect of the subcutaneous IL-1/IL-2 treatment after surgical ablation of the subcutaneous primary tumor. Thus, we excised the established subcutaneous tumors on day 7, when tumor cells had metastasized to the spleen and to the liver of most

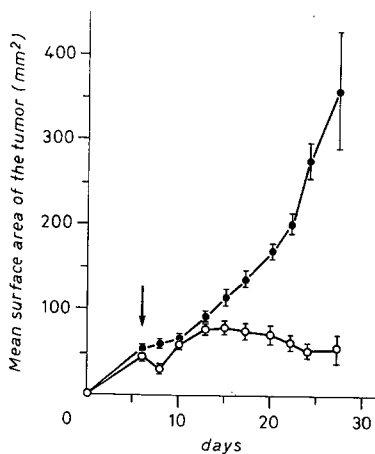


Figure 5. Effect of IL-1/IL-2 treatment on the growth of established FLC subcutaneous tumors. 6-wk-old male DBA/2 mice were injected subcutaneously with 2×10^6 in vivo passaged 3Cl-8 FLC. Mice were treated with saline or IL-1 + IL-2 on day 6 after tumor cell injection. IL-1/IL-2 treatments were performed as described in the legend to Fig. 3, on days 6, 7, 8, 13, 14, 15, 20, 21, and 23. There were 10 mice in each group. 3 of 10 IL-1/IL-2 treated mice exhibited a complete tumor regression 40–52 d after FLC injection and were considered to be tumor free on day 120. The arrow indicates the initiation of tumor therapy. (●) Saline-treated mice; (○) IL-1/IL-2-treated mice.

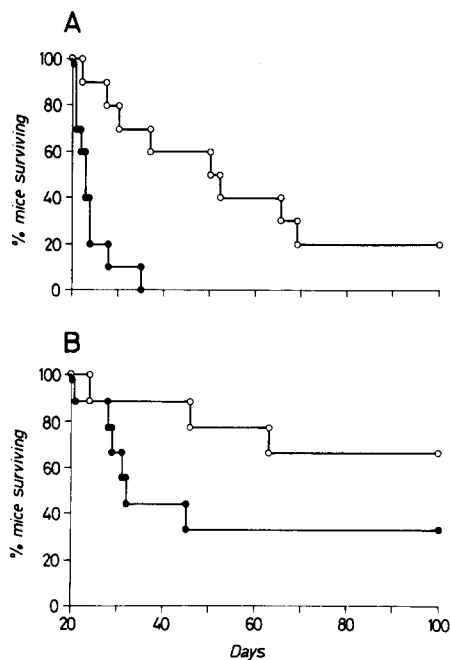


Figure 6. Effect of IL-1/IL-2 treatment as adjuvant therapy after surgery of subcutaneous primary FLC tumors. 6-wk-old male DBA/2 mice were injected subcutaneously with 5×10^6 3Cl-8 FLC in the dorsal flank region. After 7 d, some mice were anesthetized by ether, the tumor nodule and immediate surrounding tissue were excised, and the skin edges were tied with sutures; the remaining mice were left with the primary subcutaneous tumors. On day 9 of tumor growth, untreated mice (A) and surgically treated mice (B) were randomized and treated with IL-1/IL-2 (□) or saline (■). Subcutaneous treatments were performed as described in the legend to Fig. 3, on days 9, 10, 11, 16, 17, 18, 22, 23, and 24. There were 10 mice in each experimental group.

of the injected mice (data not shown). On day 9, we treated the mice subcutaneously with IL-1/IL-2 or a control preparation. As shown in Fig. 6, the combination of higher tumor resection and IL-1/IL-2 therapy resulted in a higher rate of survival as compared with control mice.

Discussion

The present results indicate that combined IL-1/IL-2 treatment of mice injected subcutaneously with FLC results in a synergistic host-dependent antitumor effect as compared with single cytokine treatment (Table 1 and Fig. 1). Synergistic antitumor effects have also been described in a variety of experimental tumors treated with different cytokines (1–7). In the IL-1/IL-2 treatment of FLC tumors, it is reasonable to assume that most of the antitumor effect observed is caused by IL-1 β , as IL-2 alone does not significantly increase the survival time of FLC-injected mice. Little information is available on the antitumor effects of IL-1 in mice (11–17), and to the best of our knowledge, no data have been published on the antitumor effects of IL-1 in mice transplanted with highly metastatic tumor cells. We have observed that small tumors initially developed in IL-1-treated mice and subsequently regressed between the second and third week of treatment.

This tumor regression was more rapid in mice treated with both IL-1 and IL-2, resulting in the cure of 10–60% of tumor-bearing mice. The fact that IL-1 β alone or in association with IL-2 did not exhibit a significant inhibitory effect on the in vitro multiplication of FLC suggests that the IL-1/IL-2-induced antitumor effect was likely to be host mediated and not related to direct cytostatic or cytotoxic effects of these cytokines on the tumor cell themselves. In particular, the finding that the IL-1/IL-2 antitumor effect was completely abrogated or markedly reduced in mice injected with antibodies to CD4 and CD8 (Table 2) suggests that T cells, particularly T helper and cytotoxic lymphocytes, are involved in the generation of this antitumor response. In contrast, NK cells do not appear to play a relevant role in the antitumor effects of IL-1/IL-2, as the injection of antibody to asialo-GM₁ did not significantly affect the antitumor effect of the combined cytokine treatment (Table 2).

It is emphasized that, although tumor cells were detected by morphologic and biologic assays in the organs of cytokine-treated mice, some of these mice were cured after IL-1/IL-2 therapy, thus suggesting the involvement of potent immune mechanisms. In this regard, the following observations are of interest: (a) an increased percentage of CD4⁺ and CD8⁺ cells was detected in the spleens of FLC-injected mice on days 17 and 23; (b) at this time, host cell infiltrates (mostly lymphocytes and granulocytes) were clearly detected in situ (i.e., primary subcutaneous tumor, liver, or spleen metastases), in close association with tumor cells, in the spleen, liver (Fig. 2, B and C), and primary subcutaneous tumor (Fig. 2 E and F); (c) likewise, host cells recovered from the spleens and lymph nodes of these mice exhibited marked antitumor activity as determined by Winn test experiments (Table 3); and (d) the antitumor activity of spleen cells was abolished by preincubation with antibodies to CD8 (but not with antibodies to CD4 or asialo-GM₁) (Fig. 3). The finding that the anti-CD4 mAb is highly effective in vivo in abolishing the antitumor effect of the IL-1/IL-2 therapy, but is not significantly effective in inhibiting the ability of spleen cells to function in a Winn assay, might be due to the fact that CD4⁺ cells play

a crucial role in the generation process of the antitumor immune response, but they are not directly involved in the cytotoxic effector mechanism. The final effector cells responsible for the tumor regression are likely to be CD8⁺ cytotoxic T lymphocytes, as suggested by the results of the Winn assay experiments (Fig. 3).

The finding that the IL-1/IL-2 treatment was more effective on established (6–8-d) than on early (1-d) implanted subcutaneous tumors (Fig. 4) is consistent with the observation that some tumor cells were present in the liver and spleen of cytokine-treated mice, before the in situ generation of host immune cells could occur. In fact, at 6–8 d after subcutaneous FLC injection, when IL-1/IL-2 therapy was started, FLC micrometastases were already detectable in the spleen and liver of the majority of these mice (data not shown). In this regard, North et al. (14) have recently reported that IL-1 β caused regression of the immunogenic SA1 sarcoma, growing subcutaneously in syngeneic mice, when given on days 6–8 of tumor growth, but not when given on days 1–3. It was thus suggested that the antitumor action of IL-1 was based on an underlying host-immune response that required some tumor growth to be fully generated. Although the highly metastatic in vivo passaged FLC appeared to be nonimmunogenic when injected in DBA/2 mice (8, 17), the data presented in this article on IL-1/IL-2-treated mice seem to be in agreement with those obtained by North et al. (14) in IL-1-treated mice. It is possible to envisage that injection of FLC primes the host's immune system, which is then markedly amplified by IL-1/IL-2 treatment.

The antitumor effect of IL-1/IL-2 therapy was also observed when the primary tumors were surgically removed (Fig. 6 B), demonstrating that this combined cytokine treatment can exert antimetastatic effects, which are independent from the effects on the primary tumor. It is reasonable to conclude that knowledge of the host mechanisms operative in the potent antimetastatic effect exerted by combined IL-1/IL-2 treatment of established FLC tumors may provide some insights in defining new strategies for more effective combined cytokine therapies in patients with advanced tumors.

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