Prevention of Death from Metastases by Immune RNA Therapy

Abstract. *The effect of immune RNA treatment on the incidence of death from pulmonary metastases was studied in C57BL/6J mice after excision of a B16 murine melanoma. Immune RNA was extracted from the lymphoid tissues of guinea pigs immunized with B16 tumor and then incubated in vitro with normal C57BL/6J mouse splenocytes. Mice receiving intraperitoneal injections of these RNA-treated syngeneic splenocytes after the primary B16 isograft was resectioned showed significantly improved long-term survival (42 to 67 percent in three successive experiments) as compared to control mice (0 to 20 percent survival) receiving untreated splenocytes. The effect of RNA treatment was tumor-specific and ribonuclease sensitive. The results suggest that immunotherapy with immune RNA may be of benefit to certain patients after surgery for cancer.*

Immune RNA (I-RNA) extracted from the lymphoid tissues of sensitized animals has been reported to transfer specific immune responsiveness both in vitro and in vivo (*1*). Its ability to transfer tumor-specific immunity has potential clinical application. Despite a number of reports of successful immunoprophylaxis against experimental animal tumors with I-RNA (*2*), its effects on established tumors have been disappointing; temporary interference with tumor growth has been the maximum result achieved in most experiments (*2*).

Treatment with I-RNA shares with most other forms of immunotherapy a lack of effectiveness in animals with a sizable tumor burden. A more logical setting for potential application of I-RNA would be the circumstance in which all gross tumor has been excised, and in which the goal of I-RNA immunotherapy is the prevention of metastatic tumor recurrence. However, the data in which I-RNA has been successful in preventing death from metastases have been insufficient (*3*). We now report repeated and consistent reduction in death from pulmonary metastases in C57BL/6J mice by I-RNA treatment after excision of a primary B16 melanoma isograft.

The B16 melanoma and Lewis lung carcinoma (3LL) were originally obtained from the Jackson Laboratory, Bar Harbor, Maine, and from Dr. A. E. Bogden, Mason Research Institute, Worcester, Mass., respectively. They were maintained by serial transplantation in adult male C57BL/6J mice. Both tumors cause death from pulmonary metastases in a high percentage of mice after excision of primary tumor isografts. The tumors were antigenically distinct from each other in a standard in vitro [^{125}I]deoxyuridine release microcytotoxicity assay (*4*).

Immune RNA was prepared (*5*) by injecting into the foot pads of Hartley guinea pigs a mixture of tumor cells in complete Freund's adjuvant (CFA). Two weeks later, spleens and lymph nodes of immunized animals were harvested, and I-RNA was extracted by the hot phenol method and stored in ethanol at −20°C until use. Only nondegraded I-RNA as determined by ultracentrifugation in sucrose density gradients was used. After the ethanol was completely removed, I-RNA was dissolved in RPMI 1640 medium and added to a tris-ammonium chloride–treated splenocyte suspension prepared from normal C57BL/6J mice, at a concentration of 750 to 1000 μg for every 5×10^6 splenocytes in 1 ml of RPMI 1640 medium. After incubation at 37°C in a 10 percent CO_2 atmosphere for 30 minutes, the cells were collected by centrifugation, and RPMI 1640 medium with 1 percent penicillin and streptomycin was added until 1 ml of cell suspension contained approximately 75×10^6 RNA-incubated splenocytes.

The effectiveness of I-RNA in preventing metastases was tested by injecting 50 to 60 C57BL/6J mice with 2×10^3 B16 tumor cells into the left hind limb. Two to three weeks later, after isografts became palpable, the limbs were amputated. Beginning 2 days later, each animal received five intraperitoneal injections, administered at 2-day intervals, of 75×10^6 syngeneic normal splenocytes that had been treated in several ways in vitro. The survival rate of each group of 10 to 13 mice was recorded until 100 days after the primary isograft excision. Selected survivors were killed and autopsied to prove absence of metastases. The significance of the difference in survival between groups was analyzed by Fisher exact probability test.

In the first experiment, all animals that received untreated splenocytes or splenocytes that had been incubated in vitro with nonspecific I-RNA extracted from the lymphoid tissues of 3LL tumor–immunized guinea pigs died of proven pulmonary metastases by 46 days after resection of the primary B16 isografts (Fig. 1). However, treatment with splenocytes incubated with specific B16 I-RNA significantly increased long-term survival to 42 percent (P = .013). Treatment of B16 I-RNA with pancreatic ribonuclease (50 μg/ml) for 30 minutes before incubation with splenocytes destroyed its therapeutic capability (survival rate = 8 percent). Furthermore, administration of splenocytes that had been incubated with B16 I-RNA had no effect on animals that had had 3LL iso-

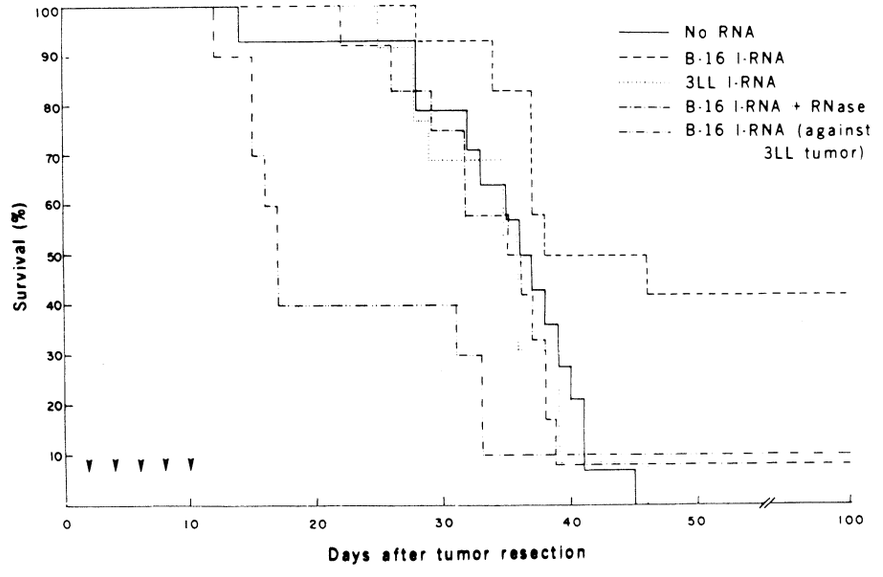

Fig. 1. C57BL/6J mice were injected with 2×10^3 B16 tumor cells into the left hind limb. The limbs were amputated after tumor isografts became palpable. Beginning 2 days later, all animals received five injections (indicated by arrows) of syngeneic splenocytes. Thirteen animals received normal untreated splenocytes (____), 12 animals received splenocytes that had been treated in vitro with B16 I-RNA (-----), 13 animals received splenocytes treated with nonspecific 3LL I-RNA (·····), and 12 animals received splenocytes incubated with ribonuclease-treated B16 I-RNA (—·—·—·). Ten animals that had had 3LL tumors amputated and were then treated with B16 I-RNA–incubated splenocytes were also tested (—··—··—). The survival rate was recorded until 100 days after the primary isograft excision.

0036-8075/78/1006-0059$00.50/0

grafts amputated (survival rate = 10 percent), indicating the specificity of the tumor immunity transferred by I-RNA. A similar therapeutic effect of I-RNA–incubated splenocytes was shown in the second experiment (Fig. 2). The survival rate of B16 I-RNA–treated animals was 67 percent compared to 20 percent for control animals that received untreated splenocytes (P = .038). RNA extracted from guinea pigs that had been immunized with CFA without tumor was ineffective (survival rate = 14 percent). The treatment of B16 I-RNA with ribonuclease again abolished its effectiveness (survival rate = 20 percent). The effect of varying the frequency of treatment with I-RNA–incubated splenocytes was tested in a third experiment (Fig. 3). Both three and nine injections of I-RNA–incubated splenocytes increased survival rates to 57 percent (P = .06) and 44 percent (P = .11), respectively, compared to 10 percent survival for control animals. Because of the smaller number of animals being tested in this experiment, the statistical difference in survival between I-RNA–treated and control animals was not significant. However, when all three experiments are considered together, the survival of I-RNA–treated animals was significantly improved compared to that of control animals (P = .0001).

No animal in any experiment manifested evidence of toxicity after administration of I-RNA–treated splenocytes. No animal surviving 100 days after amputation of the primary tumor had gross or microscopic evidence of pulmonary metastases. As previous studies have shown (*6*), I-RNA appeared to transfer significant and specific immune reactivity to previously unsensitized lymphocytes. The effectiveness of I-RNA was abolished by treatment with ribonuclease, suggesting the necessity for an intact RNA molecule for the production of the biological effect.

Although we did not achieve 100 percent prevention of death from metastases, a 34 to 47 percent improvement in survival rate was consistently seen. The long-lasting effect (more than 100 days) of I-RNA immunotherapy is encouraging for its future clinical application since this experimental model resembles the clinical situation in which I-RNA immunotherapy might be employed—that is, the patient who has had all gross tumor removed by surgery and who then receives therapy with I-RNA in an attempt to prevent the future development of distant metastases. Deckers and Pilch (*7*) have proposed a similar clinical immunotherapy regimen in which animals are immunized with the human tumor removed from patients at surgery. The I-RNA extracted from these immunized animals is incubated in vitro with autologous peripheral blood lymphocytes and then reinfused into the same patient. Our results support the clinical trial of this kind of therapy.

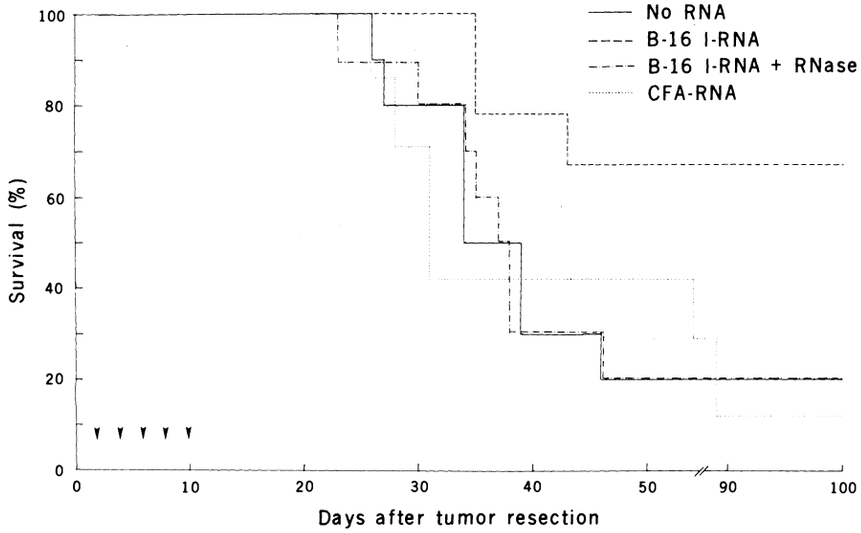

Fig. 2. Ten C57BL/6J animals received untreated splenocytes (____), nine animals received splenocytes that had been incubated with B16 I-RNA (-----), ten animals received splenocytes incubated with ribonuclease-treated B16 I-RNA (—·—·—), and seven animals received splenocytes treated with RNA extracted from guinea pigs immunized with CFA without tumor (·····). The survival rate was recorded until 100 days after the primary isograft excision.

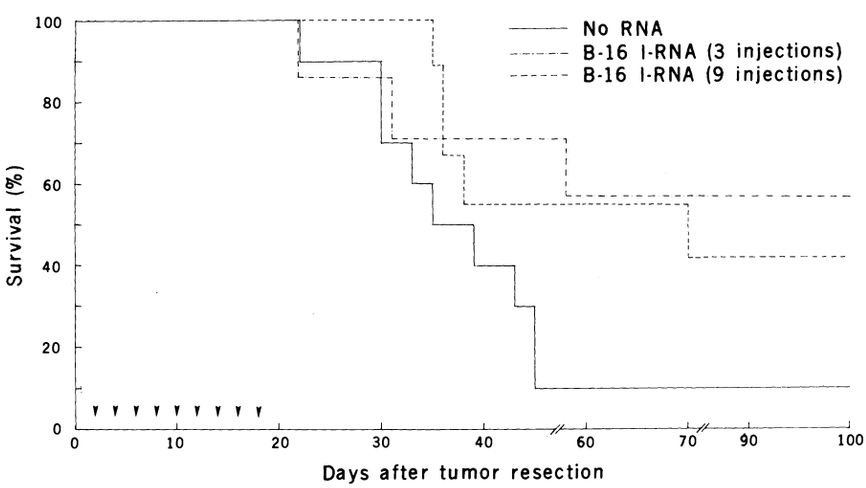

Fig. 3. Ten animals received nine injections (indicated by arrows) of untreated splenocytes (____), nine animals received nine injections of splenocytes previously incubated with B16 I-RNA (------), and seven animals received three injections of I-RNA–incubated splenocytes (—·—·—). The survival rate was recorded until 100 days after the primary isograft excision.

BOSCO SHANG WANG
SUZANNE R. ONIKUL
JOHN A. MANNICK
Departments of Surgery and *Pathology,*
Peter Bent Brigham Hospital,
Harvard Medical School,
Boston, Massachusetts 02115

References and Notes

1. J. A. Mannick and R. H. Egdahl, *Science* **137**, 976 (1962); M. Fishman and F. L. Adler, *J. Exp. Med.* **117**, 595 (1963); J. A. Mannick and R. H. Egdahl, *J. Clin. Invest.* **43**, 2166 (1964); D. E. Thor, *Science* **157**, 1567 (1967); E. Sabbadini and A. H. Sehon, *Int. Arch. Allergy* **32**, 55 (1967); B. S. Wang, P. A. Stuart, J. A. Mannick, *Cell. Immunol.* **12**, 114 (1974); B. S. Wang, P. J. Deckers, J. A. Mannick, *Clin. Immunol. Immunopathol.* **9**, 218 (1978).
2. K. P. Ramming and Y. H. Pilch, *J. Natl. Cancer Inst.* **46**, 735 (1971); S. I. Schlager, R. E. Paque, S. Dray, *Cancer Res.* **35**, 1907 (1975); P. J. Deckers, B. S. Wang, J. A. Mannick, *Ann. N.Y. Acad. Sci.* **277**, 575 (1976).
3. Y. H. Pilch, D. Fritze, J. B. deKernion, K. P. Ramming, D. H. Kern, *Ann. N.Y. Acad. Sci.* **277**, 592 (1976).
4. A. M. Cohen, J. F. Burdick, A. S. Ketcham, *J. Immunol.* **107**, 895 (1971).
5. B. S. Wang and J. A. Mannick, *Cell. Immunol.* **37**, 358 (1978).
6. D. H. Kern and Y. H. Pilch, *Int. J. Cancer* **13**, 679 (1974); B. S. Wang, S. R. Onikul, J. A. Mannick, *Cell. Immunol.*, in press.
7. P. J. Deckers and Y. H. Pilch, *Cancer* **28**, 1219 (1971).

8. Supported by NIH grant CA 21644. We thank I. Saporoschetz, K. H. Collins, P. F. Henry, and S. Quimby for technical assistance and Drs. M. Zelen and G. Flowerdew for statistical help.

17 May 1978; revised 6 July 1978