# The Role of Interferon-α in a Successful Murine Tumor Therapy

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Combination therapy using reovirus type 3 and the chemotherapeutic agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) is sufficient to cure approximately 80% of EL-4 lymphoma tumor–bearing BD2F1 male mice. Cured animals can be challenged with the EL-4 tumor, in the absence of the therapy, to yield 100% survival, whereas those challenged with heterologous tumor produce 0% survival. These results strongly suggest that a host-immune response is responsible for the observed therapeutic effect. Reovirus, a double-stranded RNA virus, is an efficient inducer of type I interferon. In an effort to determine the role of virus in this therapy, we substituted interferon-α (IFN-α) for reovirus in the therapy. Doses of IFN-α from 1000–10,000 U were capable of replacing reovirus to produce cure rates similar to reovirus. Spleen cells isolated from therapy-treated animals demonstrated high levels of cytotoxicity against the natural killer cell–sensitive cell line YAC-1, but not against EL-4 tumor. *In vitro* stimulation of isolated spleen cells by IFN-α resulted in a high level of natural killer cell activity, but no cytotoxicity against the EL-4 tumor. A significant antiproliferative effect against the EL-4 tumor in cell culture was demonstrated by IFN-α. Finally, therapy-treated, tumor-bearing mice that were injected with anti–IFN-α + -β antibodies had similar survival levels as control mice, indicating that other cytokines might also play a role in promoting tumor killing. These investigations suggest that IFN-α may be a mediator of antitumor activity in the reovirus therapy system. Exp Biol Med 230:487–493, 2005

Key words: reovirus; interferon-α; immunotherapy; 1,3-bis(2-chloroethyl)-1-nitrosourea; EL-4 lymphoma

## Introduction

Cancer immunotherapy is being extensively investigated to determine how to induce effective host antitumor responses to eradicate tumor burden. Many different approaches exist, each having its selective advantages and disadvantages. Some viruses are known to selectively kill tumor cells, and using viruses as oncolytic agents is gaining widespread support (1, 2). Reovirus is composed of a segmented, double-stranded RNA genome contained within a nonenveloped icosahedral capsid. Although the virus can be isolated from the respiratory and gastrointestinal tracts of humans, no disease has been definitively assigned to reovirus; therefore, it is felt that the virus is nonpathogenic in immunocompetent hosts (3). Recently, reovirus has been shown to infect and kill many types of human tumor cells including ovarian and colon cancer (4), breast cancer (5), pancreatic cancer (6), lymphoid malignancies (7), malignant gliomas (8), and meduloblastoma tumor cells (9).

Our laboratory has demonstrated that reovirus type 3, in combination with the chemotherapeutic agent BCNU, can cure a high percentage of mice that bear a lethal dose of the EL-4 lymphoma tumor (10). Treatment with reovirus or BCNU alone produces little or no survival in tumor-bearing animal groups. Cured animals can be rechallenged with homologous EL-4 tumor in the absence of the reovirus therapy to yield 100% survival. However, challenge with heterologous tumor results in 100% mortality, suggesting that a specific antitumor immune response was generated. Additional support for the role of the immune system in tumor eradication is that cyclosporine, an immunosuppressive drug, abrogates the effects of the reovirus therapy (10). A major advantage of the reovirus therapy over other viral approaches is that tumor cells need not be pretreated with virus for a therapeutic effect to occur.

Because reovirus is known to be a potent inducer of interferon (11), it was of interest to us to determine whether IFN-α could play a role in the eradication of tumor in our reovirus therapy system. Several effects that might play a role in tumor eradication in our reovirus therapy system are mediated by IFN-α. First, IFN-α is known to powerfully enhance the cytolytic activity of natural killer cells (12),

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which can lyse tumor cells and virus-infected cells. Second, IFN-α can inhibit tumor-cell proliferation (13) which, in our system, could slow down tumor growth to allow a potent antitumor immune response to be elicited. Third, class I major histocompatibility protein expression is enhanced (14), potentially allowing a better cytotoxic T lymphocyte response. Cytotoxic T lymphocytes are dependent on class I major histocompatibility proteins to lyse tumor cells and virus-infected cells. Although IFN-α is currently being tested for activity against a variety of tumors, the effectiveness of IFN-α as an antitumor agent has been clearly demonstrated in the treatment of hairy cell leukemia (15–17). Our investigation demonstrated that IFN-α could substitute for reovirus to produce a survival level comparable to that of the reovirus-containing therapy. These results suggest that IFN-α may be a mediator of antitumor activity in the reovirus therapy system.

## Materials and Methods

**Mice.** Male BD2F1 mice (C57/BL × DBA/2; Harlan Sprague Dawley, Indianapolis, IN) were housed and fed under standard laboratory conditions in cages provided with adequate ventilation and light (approximately 10 hrs daily) and with appropriate constant temperature and humidity. The mice ranged from 4 to 6 weeks in age and weighed 18–24 g. The animals were housed at the University of Wisconsin-Milwaukee, Department of Clinical Laboratory Sciences, according to the university's Research Animal Resources guidelines. Use of animals in this research project was approved by the Institutional Animal Care and Use Committee.

**Propagation and Purification of Reovirus.** Reovirus was grown in L-929 cells (American Type Culture Collection, Rockville, MD) cultured in Joklik-modified minimal essential medium (MEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% bovine calf serum (Life Technologies, Inc., Gaithersburg, MD) and 0.5% penicillin-streptomycin solution (Sigma Chemical). The L-929 cells were brought to a concentration of $1 \times 10^7$ cells/ml by growing them in suspension culture at 37°C. Cell viability was determined by trypan blue exclusion. Reovirus type 3 (American Type Culture Collection) from a third-passage lysate was sonicated to disrupt aggregation and added to the cells. Cell viability was monitored until 80% viability of the L-929 cells was obtained. The cells were pelleted and used for virus purification.

The L-929 cells were homogenized with homogenizing solution (i.e., Trizma base, NaCl in distilled water, pH 8) and with freon. The homogenate was centrifuged, and the whole process was repeated three times to collect a pellet. The aqueous phase was homogenized with freon only and centrifuged. The aqueous phase from both extractions were pooled and extracted three times with freon.

The final aqueous phase was carefully layered onto a preformed, 16-ml cesium chloride gradient and ultracentrifuged at 21,000 *g* for 3 hrs at 4°C using a SW 27.1 rotor. The viral bands were harvested, and viral particles were enumerated with a UV/VIS spectrophotometer (Lambda 3; Perkin Elmer, Wellesley, MA) where 1 $ODU_{260nm} = 2.1 \times 10^{12}$/ml reovirus particles. This sample was then diluted to the proper concentration for therapy usage and stored at 4°C.

**Plaque Assay.** Six-well tissue culture plates of L-929 cells were prepared by adding 2 ml of MEM (Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum (Sigma Chemical) and 0.5% penicillin-streptomycin solution (Sigma Chemical). The plates were incubated at 37°C in 5% $CO_2$ until they were a confluent monolayer. Dilutions of $1 \times 10^{-5}$, $1 \times 10^{-6}$, $1 \times 10^{-7}$, $1 \times 10^{-8}$, and $1 \times 10^{-9}$ reovirus were prepared, and 0.2 ml of each dilution were added to each of two wells of the six-well plates. The virus was allowed to absorb to the cells by incubating the plates at 37°C in 5% $CO_2$ for 60 mins. Overlay media was prepared as follows: 2X Noble agar (Difco Laboratories, Detroit, MI) at 2 g/100 ml $dH_2O$ was autoclaved for 15 mins and allowed to cool to 45°C. Then, an equal volume of warmed 2X-supplemented MEM was added to the agar. Two ml of this mixture was added to the plates, and they were incubated at 37°C and 5% $CO_2$. Another layer was added after 48 hrs. The third layer, which contained 0.2 ml neutral red (Sigma Chemical) was added after 48 hrs in reduced light. The plaques were counted after 24 hrs and plaque-forming units (PFUs)/ml were calculated. In our therapy, $1 \times 10^9$ PFUs were found to be therapeutically equivalent to $1 \times 10^{12}$ viral particles.

**Therapy Protocol.** The therapy protocol used in our laboratory has been used successfully in the treatment of EL-4 and other murine tumors such as L1210 and A10 adenocarcinoma. Male BD2F1 mice that were 4–6 weeks of age received an intraperitoneal (ip) injection of $1 \times 10^5$ (0.2 ml) of EL-4 tumor cells. Four days later, they received 9 mg/kg BCNU (Bristol Laboratories, Princeton, NJ) ip, followed 2 days later by an injection of $1 \times 10^{12}$ particles of reovirus type 3 (0.2 mL). The IFN-α (Sigma Chemical) groups received EL-4 tumor followed by the injection of BCNU. The reovirus step, however, was replaced by 3 consecutive days (i.e., Days 6, 7, and 8 after tumor injection) of 10,000, 5000, 1000, or 500 U of IFN-α in a volume of 0.2 ml. All of the control mice received the tumor, but were lacking one component of the therapy. These groups consisted of BCNU alone, reovirus alone, 5000 U IFN-α alone for 3 consecutive days, and BCNU + 1000 U inactivated IFN-α (boiled for 30 mins) for 3 consecutive days. All groups contained 10 mice, and survival was monitored for 60 days. The cured therapy and IFN-α–treated mice were then challenged with a lethal dose ($1 \times 10^5$) of EL-4 tumor. Survival of the challenged groups was monitored for 30 days.

For the anti–IFN-α experiments, mice received an ip injection of $1 \times 10^5$ (0.2 ml) of EL-4 tumor cells. Four days later, they received 9 mg/kg BCNU ip, followed 2 days later by an injection of $1 \times 10^{12}$ particles of reovirus type 3 (0.2

mL). All of the control mice received the tumor, but were lacking one component of the therapy. These groups consisted of BCNU or reovirus alone. Additional therapy-treated groups received either 5000 IU of mouse anti–IFN-α + -β serum (0.2 ml; Lee Biomolecular Research Laboratories, San Diego, CA) or preimmune control serum (0.2 ml). All groups contained 10 mice, and survival was monitored for 60 days.

**Tumor Cell Harvest Protocol.** Mice were sacrificed by cervical dislocation. The EL-4 cells in the ascites were harvested by injecting RPMI-1640 (Mediatech) into the peritoneal cavity and then aspirating the ascites fluid through a small surgical opening. The fluid was pelleted at room temperature and then washed twice with phosphate-buffered saline (PBS). Viability was checked with trypan blue, and these cells were then brought to the desired cell concentration.

**Spleen Cell Harvest Protocol.** Spleens were harvested from sacrificed mice under aseptic conditions. The organs were disrupted in RPMI-1640 using two frosted microscope slides. These cells were pelleted and resuspended in 8 ml RPMI-1640. Red blood cells were removed from the spleen-effector cells using ficoll-hypaque solution (Sigma Chemical) and then pelleted at room temperature. Spleen cells were washed twice with PBS, which was followed by dilution to the desired cell concentration.

**Target Cell Labeling.** The EL-4 and YAC-1 cells were labeled for use in the cytotoxicity assay using chromium-51 ($^{51}Cr$; 1 mCi/ml; New England Nuclear/DuPont, Boston, MA). The target cells were washed three times, and then 0.2 ml $^{51}Cr$ (0.2 mCi) was added to the cell pellet with 0.5-ml supplemented RPMI-1640. The cells were incubated for 3 hrs at 37°C and 5% $CO_2$. Following incubation, the cells were washed four times.

**Chromium Release Assay.** Spleen effector cells (0.1 ml) were dispensed into wells of 96-well, round-bottom, microtiter plates in quadruplicate. Labeled target cells (0.1 ml; $1 \times 10^5$/ml) were then added to each appropriate well. Plates were incubated for 4 hrs at 37°C in a humidified 5% $CO_2$ incubator. Following incubation, the plates were centrifuged for 5 mins at 25 *g*. Using a Supernatant Collection System (Skatron Instruments Inc., Sterling, VA), the supernatants were removed without disturbing the cell pellet. The radioactivity in the supernatants was measured in a gamma counter. Maximal release (MAX) of $^{51}Cr$ was estimated using 0.1 ml of the radiolabeled target cells absorbed into harvesting filters.

Measurement of the spontaneous release (SPON) of $^{51}Cr$ was performed using 0.1 ml of radiolabeled target cells mixed with 0.1 ml of serum-supplemented RPMI-1640 and dispensed into the microtiter plates. The levels of specific lysis were quantitated using the following formula:

$$\% \text{ cytotoxicity} = \text{test samples (cpm)} - \text{SPON (cpm)} \div \text{MAX (cpm)} - \text{SPON (cpm)} \times 100$$

**Culture Conditions for EL-4 Growth Studies.** The EL-4 tumor cells ($2 \times 10^5$) were added to each of five cell-culture flasks. Four of the flasks received murine IFN-α so that final concentrations reached 1000, 500, 100, or 50 U/ml. The fifth flask received no IFN-α. Supplemented RPMI-1640 was added to all flasks to reach a final volume of 5 ml. The cells were then measured for viable cell concentration *via* trypan blue exclusion and incubated at 37°C in 5% $CO_2$. The viable cell numbers were measured every 24 hrs for a total of an additional 4 days.

***In Vitro* IFN-α Effector Cell Stimulation.** Spleen effector cells were harvested, as previously described. The cells were incubated for 3 hrs and 40 mins in supplemented RPMI-1640 containing 1500 U/ml IFN-α at 37°C and 5% $CO_2$. Control cells without IFN-α were incubated under the same conditions. These cells were then used in the $^{51}Cr$-release assay at a 200:1 effector-to-target ratio. All measurements were performed in quadruplicate.

**Statistical Analysis.** The Student's *t* test was used to determine significant differences among treatment groups.

## Results

**Reovirus Therapy.** To demonstrate that our therapy regimen could cure animals of EL-4 tumor burden, we performed the therapy as described in the Materials and Methods section. Figure 1 illustrates that tumor-bearing control animals treated with either BCNU or reovirus gained little or no survival benefit. However, the therapy group demonstrated a high percentage of survivors at 60 days. It was determined that 60-day survivors very rarely died at a later date (data not shown); therefore, the 60-day time limit proved to be an accurate assessment of long-term survival.

**Replacement of Reovirus With IFN-α.** Because reovirus is an efficient inducer of type 1 IFN in mice, we investigated whether IFN-α could effectively replace reovirus in our therapy system. Reovirus was replaced in the therapy by an injection of IFN-α on Days 6, 7, and 8 following tumor injection. The dose of IFN-α was varied in each experimental group and consisted of 10,000, 5,000, 1,000, or 500 U for each injection. The reovirus therapy group became a control to determine whether IFN-α could provide comparable survival benefit. Table 1 demonstrates that with IFN-α doses of 1000 U or greater, there was no significant difference in survival values when compared to the reovirus therapy group. In contrast, 500 U of IFN-α provided a relatively high level of survival, but varied significantly from the reovirus therapy control group ($P < 0.05$). Tumor-bearing animals given only IFN-α did not survive, signifying that it was important to first reduce tumor burden using BCNU. Tumor-bearing animals given BNCU and heat-inactivated IFN-α survived to the level observed with BCNU alone, as would be expected if the heat treatment destroyed the biologic activity of IFN-α. Therefore, IFN-α could effectively replace reovirus, suggesting a potential role for IFN in our therapy system.

**Tumor Challenge of Cured Animals.** To determine whether IFN-α therapy-treated animals, cured of

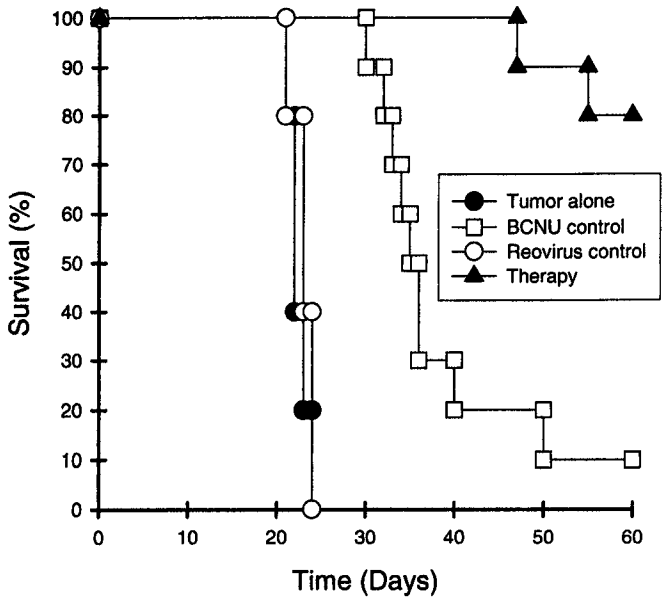


**Figure 1.** Reovirus therapy. All animals were injected on Day 0 with $1 \times 10^5$ EL-4 tumor cells. Tumor alone, tumor-bearing animals received no treatment. BCNU, animals received only chemotherapy. Reovirus, animals received only reovirus. Therapy, animals received both BCNU and reovirus. The results shown are representative of three experiments.

tumor, could successfully eliminate a challenge dose of tumor we injected the cured animals with $1 \times 10^5$ EL-4 tumor cells in the absence of any other treatment. Survival was monitored for 30 days. This time period was chosen because it has been demonstrated that mice that survive tumor challenge for 30 days will remain tumor free for the remainder of their natural lives (unpublished observations). All of the animals survived for the 30-day observation period, with no signs of tumor development (data not shown). Previous studies have shown that animals cured of the EL-4 tumor cannot survive a challenge of an equivalent dose of heterologous L1210 tumor cells (10). These studies indicate that a specific acquired immunity was likely induced by the reovirus therapy in response to EL-4 tumor cells.

***In Vivo* Cytotoxicity Generated by the Reovirus Therapy.** Because the reovirus therapy can eliminate tumors in a manner consistent with the production of an immune response, we examined the production of cytotoxicity against EL-4 tumors. At 5, 10, and 20 days posttherapy, we isolated spleen cells from therapy-treated animals and assayed for cytotoxicity against EL-4 and YAC-1 tumor lines. The YAC-1 tumor cell line is sensitive to lysis by murine natural killer cells. Table 2 demonstrates that a cytotoxic response was generated against YAC-1 tumor cells; however, no cytotoxicity was observed against EL-4 tumor cells. The generation of cytotoxicity against YAC-1 tumor cells is consistent with the production of reovirus-stimulated IFN, known to be a potent activator of natural killer cell activity.

**Table 1.** Therapy Using IFN-α Replacement of Reovirus

| EL-4 | BCNU | Reovirus | Interferon[a] | % survival[b] |
|---|---|---|---|---|
| + | | | | 0[c] |
| + | + | | | 20 ± 10[c] |
| + | | + | | 0[c] |
| + | + | + | | 90 ± 6 |
| + | + | | 10,000 U | 95 ± 5 |
| + | + | | 5,000 U | 73 ± 9 |
| + | + | | 1,000 U | 73 ± 15 |
| + | + | | 500 U | 63 ± 7[c] |
| + | | | 5,000 U | 0[c] |
| + | + | | 1,000 U[d] | 20 ± 0[c] |

[a] Recombinant murine IFN-α.
[b] Values represent the means ± SEM from three separate experiments.
[c] Statistically significantly different ($P < 0.05$) from therapy control (EL-4 + BCNU + reovirus).
[d] The IFN-α was heat activated by boiling for 30 mins.

***In Vitro* Stimulation of Cytotoxicity Using IFN-α.** In an effort to get optimal stimulation of the spleen cells, we exposed the cells to 1500 U/ml IFN-α and tested for cytotoxic activity against EL-4 and YAC-1 tumor cells lines. As shown in Figure 2, we again observed a high degree of cytotoxicity against the natural killer cell–sensitive YAC-1 tumor cells with the IFN-α–stimulated spleen cells, but no cytotoxicity was observed against the EL-4 tumor cells. Untreated spleen cells exhibited little, although statistically significant, cytotoxic activity against the YAC-1 cell line. These results, combined with the previous results, demonstrate that natural killer cells cannot lyse EL-4 tumor cells and, therefore, natural killer cells are unlikely to be the effector cells that are responsible for eliminating tumors in our reovirus therapy.

**Antiproliferative Effect of IFN-α.** Other investigators have described the ability of the interferon to inhibit tumor-cell proliferation (13). In an effort to determine whether IFN-α could be exerting antiproliferative effects on EL-4 tumor growth in our reovirus therapy system, we investigated whether IFN-α could inhibit EL-4 tumor growth *in vitro*. The EL-4 tumor was cultured in varying concentrations of IFN-α that were added at the initiation of the culture period. The cells were allowed to grow for a total of 4 days, and cell counts were performed on each day. Concentrations of IFN-α greater than or equal to 100 U/ml were found to inhibit the growth of EL-4 tumor cells relative to control cells in cultures devoid of IFN-α (Fig. 3). The antiproliferative effect observed suggests that this mechanism may play a role in tumor elimination *in vivo*.

**Effect of Anti-Interferon Antibodies on the Therapy.** Because IFN-α could effectively replace reovirus in our therapy system, we investigated whether anti–IFN-α + -β antibodies could abrogate the effects of the reovirus therapy. Figure 4 shows that ip injection of anti–IFN-α + -β antibodies or preimmune control serum into

**Table 2.** *In Vivo* Cytotoxicity of Reovirus Therapy–Treated Animals

| Day | EL-4 | | YAC-1 | |
|---|---|---|---|---|
| | Control | Therapy | Control | Therapy |
| 5 | 0 | 2.1 ± 1.4[a] | 6.8 ± 0.4 | 34.7 ± 5.4[b] |
| 10 | 0.5 ± 0.3 | 0.6 ± 0.3 | 9.4 ± 7.8 | 13.1 ± 5.3 |
| 20 | 1.6 ± 1.5 | 1.9 ± 0.7 | 6.8 ± 1.1 | 7.9 ± 1.7 |

[a] Values represent the percent cytotoxicity of the mean ± SEM from three separate experiments.
[b] Significantly different ($P < 0.05$) from therapy control.

therapy-treated mice could not reverse the effects of the therapy. Survival kinetics and 60-day survival levels were unaffected by the presence of the anti-IFN antibodies. These results suggest that, in addition to IFN-α, other cytokines may play an important and potentially redundant antitumor role.

## Discussion

In light of the success of reovirus therapy in curing animals with EL-4 tumor burden, we investigated one potential role of reovirus, namely the induction of IFN-α. We demonstrated that IFN-α, when used at doses from 1,000–10,000 U, could yield cures of EL-4–bearing animals (Table 1). In addition, cured animals could be rechallenged with a lethal dose of the EL-4 tumor to yield 100% survival and no apparent morbidity, all in the absence of virus or chemotherapy (data not shown). These results strongly suggest a role for the immune system in reovirus therapy to result in tumor elimination and are consistent with the antitumor effects of IFN-α observed in a number of experimental systems.

Consistent with our results, IFN-α has been demonstrated to have significant antitumor effects on a number of human and murine tumors. Utilizing a murine intracranial glioma model, Tsugawa *et al.* (18) demonstrated that transduction of an adenoviral vector containing an IFN-α gene along with the transfer of bone marrow–derived dendritic cells prolonged survival of about half of the tumor-bearing animals. Tumor-specific cytotoxic T lymphocyte activity was generated, and protection against rechallenge with glioma tumors was demonstrated. *In vitro,* IFN-α caused growth suppression *via* cell cycle arrest in some human gastric cancer cell lines (19). In addition, IFN-α augmented the ability of tumor necrosis factor-α to induce apoptosis in a human cervical cancer cell line (20). Treatment of human neuroendocrine tumors in nude mice with IFN-α resulted in antiproliferative effects on the tumor and suppression of angiogenesis (21). In a phase II clinical trial, chronic lymphocytic leukemia and low-grade non-

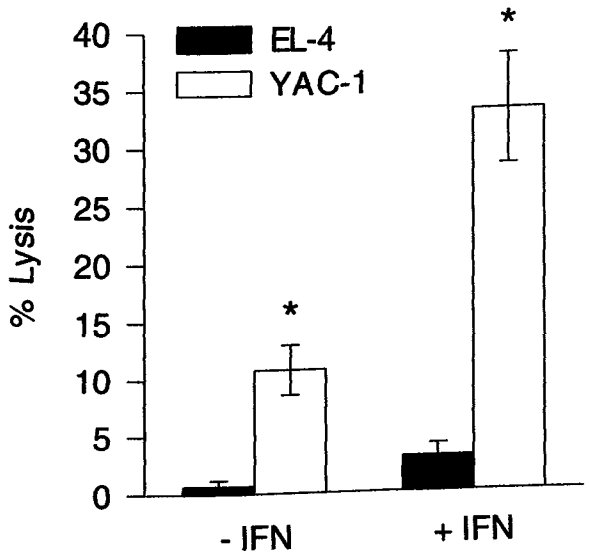


**Figure 2.** *In vitro* stimulation of cytotoxicity using IFN-α. Spleen cells were isolated from animals, incubated with 1500 U/ml IFN-α, and tested for cytotoxicity against the EL-4 and YAC-1 cell lines. The values represent the means ± SEM of three experiments. *, significantly different from untreated control values at $P < 0.05$.

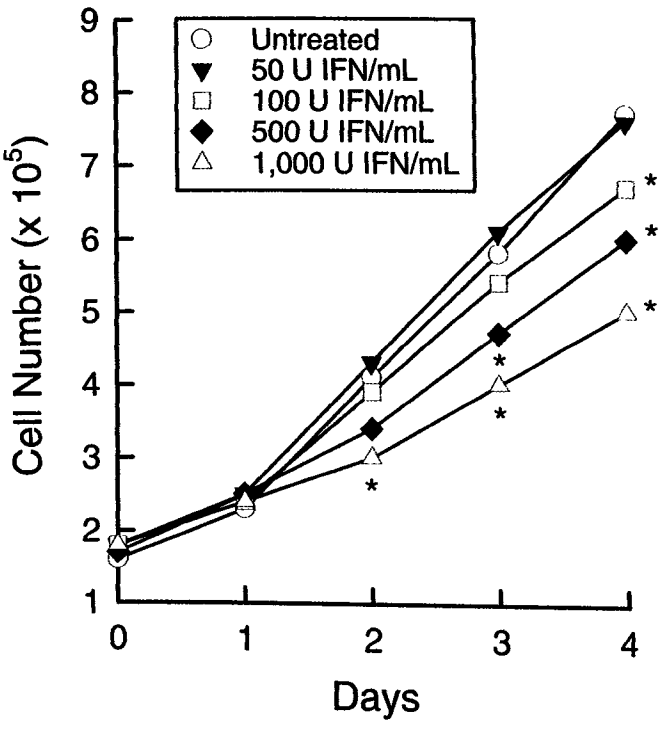


**Figure 3.** Antiproliferative effect of IFN-α. The EL-4 tumor cells were cultured in the presence of IFN-α, and viable cell counts were determined daily for 4 days. The values represent the means ± SEM of four experiments. *, significantly different from untreated control values at $P < 0.05$.

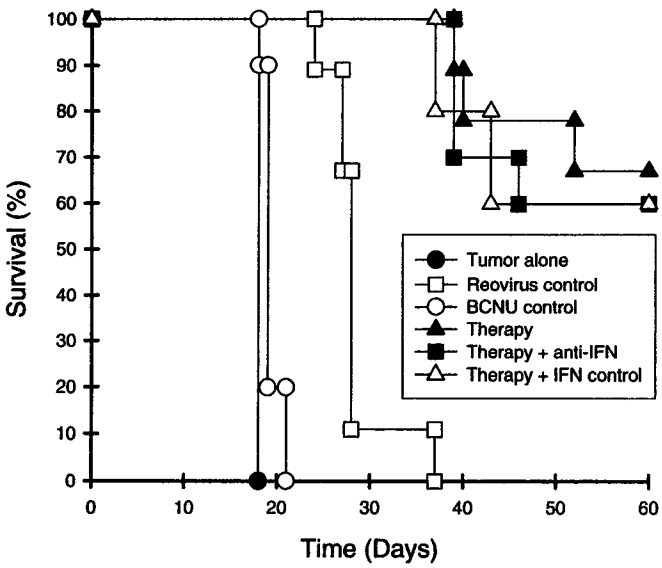


**Figure 4.** Anti–IFN-α + -β antibodies do not abrogate the effect of the reovirus therapy. Tumor-bearing, therapy-treated mice received either 5000 IU of mouse anti-IFN-α + -β sera (0.2 ml) or preimmune control serum (0.2 ml). Control groups consisted of therapy, BCNU only, or reovirus-only groups. All groups contained 10 mice, and survival was monitored for 60 days.

Hodgkin's lymphoma patients were treated with IFN-α as a maintenance drug following fludarabine therapy (22). In those patients that responded favorably to fludarabine, IFN-α significantly prolonged the duration of remission from disease. Finally, some data suggest that IFN-α may reverse tumor-induced immunosuppression, as evidenced by the enhanced production of interleukin-2 (IL-2) by multiple myeloma patients treated with IFN-α-2a (23).

Recent investigations have demonstrated that IFN-α can be used in combination with BCNU clinically to induce antitumor outcomes. A combination of chemotherapeutics, including BCNU, IFN-α-2b, and tamoxifen was shown to induce durable remissions in patients with metastatic melanoma (24). To improve the response rates, BCNU was added to IL-2 and IFN treatment of patients with advanced melanoma (25). These studies are among others that have demonstrated that cytotoxic chemotherapy can be effectively administered concurrently with biologic therapy.

The reovirus therapy was capable of producing a significant increase in natural killer cell activity (Table 2), which can most likely be attributed to reovirus-induced interferons. Natural killer cells are potently activated by the interferons (12). However, because EL-4 tumor cells are not susceptible to natural killer cell–mediated lysis *in vitro* (data not shown), it is unlikely that these cells play a direct cytotoxic role in tumor elimination. No significant cytotoxic T lymphocyte activity was demonstrable in the *in vitro* cytotoxicity assay from the isolated spleen cells. However, T lymphocytes have been implicated as being important in the reovirus therapy as cyclosporine, an immunosuppressive drug that inhibits mainly T lymphocytes, completely abrogates the therapy (10). It is conceivable that our cytotoxicity assay was not sensitive enough to detect cytotoxic T lymphocyte activity against the EL-4 tumor cells at the tested time points.

The important role of T lymphocytes, however, might lie with the regulator T helper lymphocytes activating other effector cells to lyse the tumor cells. Or, its important role may be in inducing the production of antibody to facilitate antibody-dependent, cell-mediated cytotoxicity or complement-dependent mechanisms of tumor killing. Interestingly, Kollmorgen *et al.* (26) showed that spleen and lymph node lymphocytes derived from reovirus therapy–treated animals that had rejected EL-4 tumors demonstrated cytotoxic T lymphocyte activity against EL-4 tumor cells, but not EG2 lymphoma cells. Lymphocytes from untreated tumor controls and non–tumor-bearing untreated mice exhibited no cytotoxic activity against either EL-4 or EG2 tumor cells. This investigation demonstrated that the reovirus therapy can generate cytotoxic activity against tumors.

Direct antiproliferative effects of IFN-α on tumor growth were observed *in vitro* (Fig. 3). Interferons have been shown to inhibit the growth of primary and transplanted tumor cells, as well as prevent tumor metastasis (13). Caraglia *et al.* (27, 28) described how IFN-α can upregulate proteins important to the apoptotic process, potentially accounting for the antiproliferative effects of the cytokine on certain tumor cells. Although we have not investigated possible apoptotic effects of IFN-α in our tumor cell lines, the inhibition of cell growth that we observed is consistent with this mechanism. In the reovirus therapy, the induction of IFN-α might serve to inhibit tumor cell growth long enough for an immune response to be generated. As an antiproliferative agent and an immunostimulant, IFN-α has emerged as an important biologic response modifier.

We were surprised to demonstrate that, although IFN-α was capable of replacing reovirus in therapy to produce significant survival of tumor-bearing mice, inhibition of IFN-α by neutralizing antibodies failed to abrogate the therapy (Fig. 4). According to the manufacturer of the anti–IFN-α + -β antibodies, each neutralizing unit will result in complete neutralization of 3–10 U of interferon activity. Therefore, the 5000 IU of anti–IFN-α + -β antibodies had the potential to neutralize 15,000–50,000 U of interferon. Direct measurement of peritoneal cavity levels of interferon would be difficult to interpret because cellular receptors would quickly bind free interferon. However, sufficient neutralizing antibody would be present to significantly reduce, if not completely remove, available interferon, yet we observed no difference in the survival values or kinetics. It is possible that other noninterferon cytokines may play a role in reovirus therapy.

In summary, the present study demonstrated that IFN-α may play an important role in tumor eradication in our reovirus therapy. In addition, our investigation confirmed that a biologic response modifier, in combination with chemotherapy, can synergize to eliminate tumor burden. Studies are continuing to characterize the immune response

generated by the reovirus therapy to better understand how biologic therapy might be used to combat cancer.


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