

Augmented Antiproliferative Effects of Interferons at Elevated Temperatures against Human Bladder Carcinoma Cell Lines¹

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ABSTRACT

The *in vitro* antiproliferative effects of interferons (IFN) against the human bladder carcinoma cell lines T24, RT4, HT1197, and 647V were evaluated at temperatures ranging from 37–41°. At 37°, the antiproliferative activities of IFN, either naturally produced or produced by recombinant DNA technology, were different against different cell lines. An increase in temperature markedly enhanced the antimitotic effect of IFN for all cells. For example, T24 cells grown at 37° and treated with 200 units naturally produced IFN- α or IFN- β per ml for 7 days were inhibited 50 to 60%. No change in cell proliferation occurred in untreated T24 cells grown at 39.5°. Treatment with 200 units IFN- α or IFN- β per ml at 39.5° inhibited these cells 80 to 90%. Similar results were obtained with IFN produced by recombinant DNA technology and purified to homogeneity. Colony formation by the RT4 cell line, at 37°, was decreased less than 10% with 200 units IFN- α per ml and 63% by 200 units IFN- β per ml. At 39.5°, colony formation by untreated RT4 cells was inhibited 48%. Treatment with IFN- β at 39.5° did not result in an enhancement of the antiproliferative effect; however, treatment with IFN- α enhanced the inhibition from less than 10% to 98%. These results suggest that a supraadditive relationship exists between antiproliferative effects of IFN and temperature elevation. The differences seen between IFN- α and IFN- β may be due to the different stabilities of these two molecules. In order to probe the mechanism of the enhanced antiproliferative effect, activity of an IFN-induced enzyme, 2'-5'-oligoadenylate synthetase, was measured. IFN- α treatment resulted in significantly greater 2'-5'-oligoadenylate synthetase induction at 39.5° than at 37°. Thus, two cellular effects resulting from IFN were augmented by increased temperature.

INTRODUCTION

IFN,⁴ proteins produced in response to viral infections or various nonviral inducers, are potent modulators of cell responses. IFN alter cellular enzymatic activities, inhibit viral replication, inhibit cellular proliferation, influence immunological responses, and suppress tumor growth (3). A family of IFN proteins has been defined physicochemically, antigenically, and by nucleotide sequencing. A comparison of the sequences of human IFN- α and IFN- β revealed 45% homology of nucleotides and 29% homology of amino acids (17). Multiple IFN- α , each of which differs by approximately 10% in nucleotide sequence and 15 to

25% in amino acid sequence, have been identified (6, 15).

Although initial reports suggest the actions of IFN may be enhanced *in vitro* by temperature elevation (5, 8), the effects of increased temperature on IFN action have not been fully evaluated. Hyperthermia results in decreased cell survival. There is a limited literature which suggests transformed cells may be more sensitive to heat than their normal counterparts (11, 14). Heat potentiates the effects of radiation (16) and some cytotoxic drugs (7, 12), probably on the basis of intracellular metabolic changes which may prevent repair of sublethal damage. Various membrane-active and sulfhydryl-rich compounds have additive or synergistic effects when cells are treated in conjunction with increased temperature (13).

The objective of this study was to assess the effects of increased temperature on the antiproliferative actions of IFN. The growth rates and plating efficiencies of human transitional carcinoma cell lines were evaluated *in vitro* at various temperatures. We also determined the effect of temperature on 2-5A synthetase, an enzyme the activity of which is increased in response to IFN, in order to correlate antiproliferative effects with enzyme activity.

MATERIALS AND METHODS

Cells. The T24, 647V, and RT4 cell lines were kindly provided by Jorgen Fogh, Memorial Sloan Kettering Institute, Rye, NY. HT1197 cells were obtained from the American Type Culture Collection, Rockville, MD. All cells were grown as monolayers in 75-sq cm plastic flasks (Corning Glass Works, Corning, NY) at 37° in a 5% CO₂, humidified atmosphere. Growth medium used was Eagle's MEM (Grand Island Biological Co., Grand Island, NY) plus 10% Hyclone fetal calf serum (Sterile Systems, Logan, UT), 2 mM glutamine, sodium bicarbonate (2 μ g/ml), and gentamicin (50 μ g/ml). Medium for the growth of HT1197 cells was supplemented with 0.1 mM nonessential amino acids (Grand Island Biological Co.). Cells were subcultured 1 to 2 times weekly, utilizing a 0.05% trypsin:0.02% EDTA solution (Grand Island Biological Co.). All cell lines were *Mycoplasma free*.

Antiproliferative Assays. In order to evaluate the effects of varied temperatures on IFN activity, 5 \times 10⁴ cells were added to 25-sq cm plastic flasks (Costar, Cambridge, MA). MEM or IFN was added to duplicate flasks, flasks were incubated for 4 hr in a 37°, 5% CO₂ atmosphere, sealed, and incubated at 37°, 38°, 39.5°, or 41° for the remainder of the experiment. At appropriate times, cells were trypsinized and counted in a hemacytometer. Viability was determined using 0.1% eosin exclusion.

In other experiments, cell proliferation was evaluated by adding 1 to 5 \times 10⁴ cells to 35- x 10-mm plastic Petri dishes (Costar). Duplicate plates were treated with MEM or IFN and grown at 37° or 39.5° in a humidified, 5% CO₂ atmosphere. At desired times, cells were trypsinized and counted.

Colony formation was determined by preparing a uniform single cell suspension and adding 100 to 500 cells to each 60- x 15-mm plastic Petri dish (Costar). Medium used was a mixture of 45% α -MEM (without nucleosides; Flow Laboratories, McLean, VA), 45% Ham's F-12 (Flow,

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⁴ The abbreviations used are: IFN, interferon(s); 2-5A synthetase, 2'-5'-oligoadenylate synthetase; MEM, minimal essential medium.

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10% Hyclone fetal calf serum, glucose (3 mg/ml), 2 mM glutamine, and gentamicin (50 μ g/ml). Triplicate plates were treated with IFN or MEM and incubated 10 to 21 days at either 37° or 39.5° in a 5% CO₂, humidified atmosphere. At appropriate times, plates were fixed with 95% ethanol and stained with 0.2% methylene blue for 30 to 60 min. Colony numbers were quantitated using a plaque viewer (Bellco Glass Co., Vineland, NJ). Only colonies with greater than 50 cells were counted.

The pH of the medium used remained 7.2 at all temperatures in these experiments.

Results have been expressed as cell number, colony number, percentage of control

$$\frac{\text{IFN-treated count}}{\text{MEM-treated count}} \times 100$$

or growth index

$$\frac{\text{Final cell no.}}{\text{Starting cell no.}}$$

2-5A Synthetase. This enzyme activity was measured in cells grown in 75-sq cm flasks and treated with IFN or MEM for 24 hr. Thereafter, cells were trypsinized, and pellets of 10⁶ cells were frozen (-20°). Frozen cells were lysed by thawing in a buffer containing Nonidet P-40 detergent (BDH Chemicals, Poole, England). Enzyme in cell-free extracts was bound to and activated by polyinosinic acid: polycytidylic acid copolymer agarose beads (PL Biochemicals, Milwaukee, WI). Reaction mixtures (80 μ l) contained 30 μ l of enzyme-loaded agarose beads, 90 mM KCl, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 6.9), 5 mM magnesium acetate, 10% glycerol, 1 mM dithiothreitol, and 3 mM [³H]ATP (10 Ci/mol) and were incubated for 20 hr at 37°. Aliquots of 20 μ l were digested with bacterial alkaline phosphatase (type III R; Sigma) for 90 min, and then, 6- μ l aliquots were spotted onto 1-sq cm squares of DEAE-cellulose paper (Whatman DE81; Whatman, Inc., Ann Arbor, MI), dried, and washed with distilled water. The incorporated and bound label was eluted from the filters with 0.3 M KCl and counted in Aquasol (New England Nuclear, Boston, MA). Enzyme activity was expressed as 10⁻¹² mol ATP incorporated per hr per 10⁵ cells. The reaction rate was linear and, within limits, the amount of product was proportional to the amount of enzyme assayed.

IFN. Partially purified, buffy coat-produced IFN- α was a gift from K. Cantell, Helsinki, Finland (4). Naturally produced IFN- β was provided by Roswell Park Memorial Institute, Buffalo, NY (9). IFN- α 54 and IFN- β , produced by recombinant DNA technology and purified to a specific activity of >10⁸ units/mg protein, were provided by the Cetus-Shell Interferon Program, Berkeley, CA.⁵ IFN- β was a synthetic mutant protein with a substitution of serine for cysteine at amino acid position 17.⁶ IFN were titered using A549 cells with encephalomyocarditis virus as a virus challenge (10). The titer of each preparation was directly compared to the appropriate international reference standard. International standards used were G023-901-527 and G023-902-527 of the National Institute of Allergy and Infectious Diseases. All IFN units were expressed in relation to standard preparations.

RESULTS

Effects on Different Cell Lines. The effect of IFN- α at elevated temperatures was initially evaluated using the T24 transitional cell carcinoma cell line. The growth rate of T24 cells was similar at 37°, 38°, and 39.5° (Chart 1). A temperature of 41° for 5

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⁶ D. V. Mark, S. D. Lu, A. Creasey, R. Yamamoto, and L. Lin. Site-specific mutagenesis of the human fibroblast interferon gene: a structure-function analysis of the protein, submitted for publication.

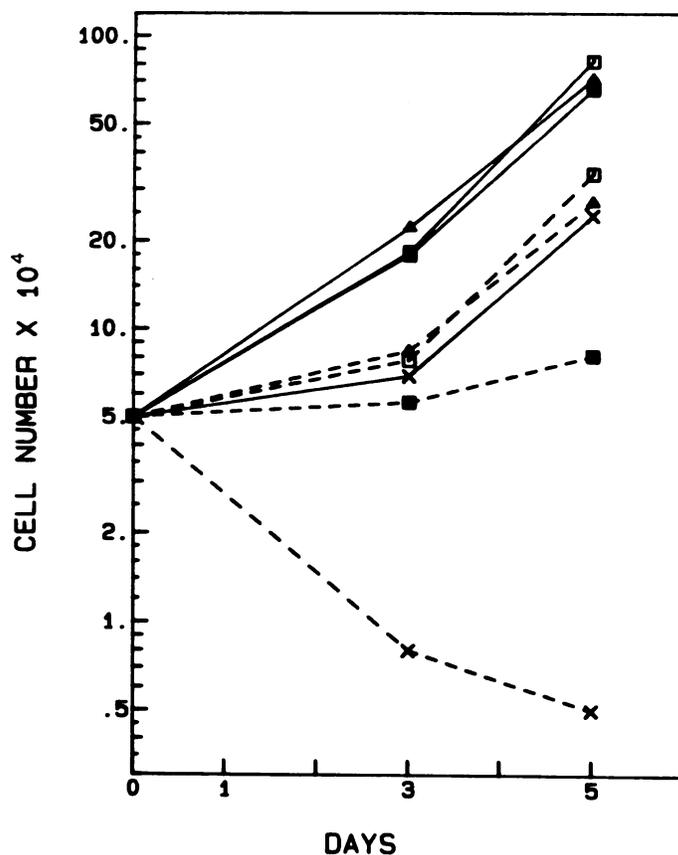


Chart 1. Antiproliferative effects of IFN- α at varying elevated temperatures against T24 cells. T24 cells were left untreated (—) or treated with IFN- α (200 units/ml; ---) and grown at 37° (□), 38° (△), 39.5° (■), or 41° (×). Results are from one representative experiment.

days resulted in a 60 to 70% decrease in cell number at 3 and 5 days. This decrease in cell number reflected an initial inhibition of cell proliferation; the growth rate between 3 and 5 days was equivalent at 37° and 41°. T24 cells grown at 39.5° in the presence of IFN- α (200 units/ml) were inhibited to a greater extent (70 to 90% of 39.5° control). IFN- α temperature at 41° resulted in a loss of viable cells over time (Chart 1). These results suggested that increased temperature enhanced the antiproliferative effects of IFN- α .

To extend these studies, an additional IFN, IFN- β , and a different assay method, colony formation, were assessed. Colony formation of T24 cells was not significantly inhibited after treatment with IFN- α (200 units/ml) at 37° (Chart 2A). The same concentration of IFN- β inhibited colony formation by 61%. Colony formation by untreated T24 cells, grown at 39.5°, was inhibited 27%. T24 cells treated with IFN- α (200 units/ml) and grown at 39.5° were inhibited more markedly than the expected additive effects of IFN plus heat treatment. Although even greater inhibition of cell growth occurred, the combination of IFN- β and 39.5° resulted in a less marked potentiation than occurred with IFN- α at the increased temperature.

Colony formation was also evaluated in the RT4 cell line. IFN- α treatment at 37° did not have a significant effect on colony formation of these cells (Chart 2B). IFN- β inhibited formation of colonies by 63%. The plating efficiency of untreated RT4 cells was reduced by 48% when cultures were grown at 39.5°.

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Chart 2. Effects of IFN on colony formation of T24 (A) and RT4 (B) cells. Cells were left untreated or treated with IFN- α or IFN- β (200 units/ml) and grown at 37° (■) or 39.5° (▨). Colony number was quantitated on Day 10 for T24 cells (A) or Day 21 for RT4 cells (B). Columns, mean colony number of triplicate plates; data are from one representative experiment. Bars, S.E.

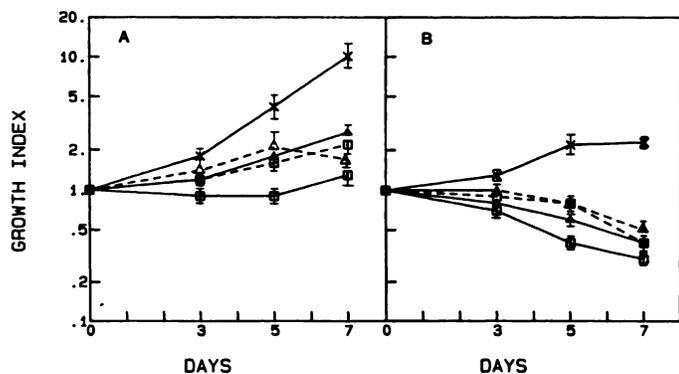
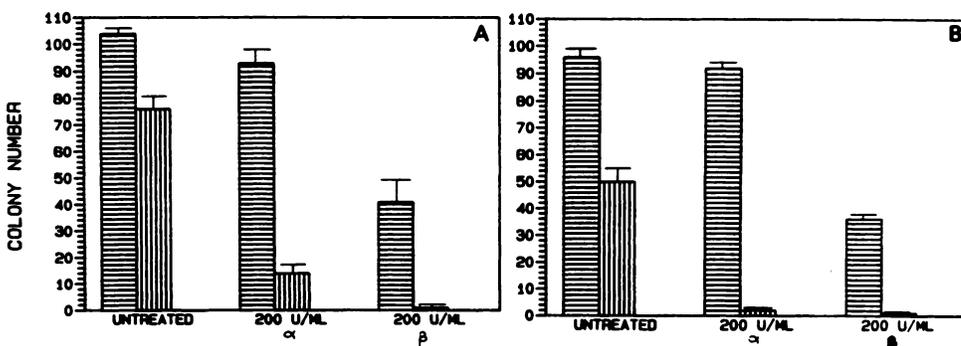


Chart 3. Antiproliferative effects of IFN against HT1197 cells. Cells were left untreated (x) or treated with 200 units naturally produced IFN- α (Δ — Δ), recombinantly produced IFN- α 54 (Δ — Δ), naturally produced IFN- β (\square — \square), or recombinantly produced IFN- β (\square — \square) per ml and grown at 37° (A) or 39.5° (B). Points, mean growth index versus time; bars, S.E. Data are from 2 representative experiments.

Treatment with IFN- β at 39.5° increased inhibition from 63% (IFN- β alone) to 99% inhibition. This increased inhibition of IFN- β at 39.5° was additive. Treatment of RT4 cells with IFN- α increased markedly the inhibition of colony formation from <10% in the 37° cultures treated with IFN to 98% in the 39.5° IFN-treated plates. In contrast to the additive antiproliferative effects of IFN- β and increased temperature, the antiproliferative effect of IFN- α for these cells appeared potentiated in a supraadditive manner by increased temperature.

HT1197, a slowly growing, poorly differentiated transitional cell carcinoma cell line, was extremely sensitive to the antiproliferative effects of IFN (Chart 3A). IFN- β at a concentration of 200 units/ml inhibited cell number up to 80% after 7 days of treatment. Following 7 days of treatment with IFN- α (200 units/ml), a 70% reduction in cell number occurred. Although growth of the T24 cell line was essentially unaltered at 39°, proliferation of HT1197 cells was significantly ($p < 0.001$ at all time points using Student's t test) inhibited by increasing the temperature (Chart 3B). Up to an 80% decrease in cell number was observed when cultures grown at 39.5° without IFN were compared at 7 days to 37° cell cultures without IFN. IFN treatment at 39.5° resulted in an even greater inhibition of cell number (a further 85% decrease in cell counts compared to 39.5° controls) and an actual cell loss over time.

A fourth transitional cell carcinoma cell line, 647V, was less sensitive to the antiproliferative effects of both IFN- α and IFN- β . IFN resulted in less than a 30% decrease in cell number over 7 days at 37° (Chart 4A). Following 7 days at 39.5°, the growth of the untreated 647V cell line in the absence of IFN was also

decreased (approximately a 30% decrease in cell number at 39.5° compared to 37° (Chart 4B). Growth at 39.5° enhanced the antiproliferative effects of IFN- α and IFN- β to inhibition of 65 to 80% compared to untreated 37° cultures.

One explanation for the differences seen between IFN- α and IFN- β was instability of IFN- β at an elevated temperature. IFN- α retained its antiviral activity at both 37° and 39.5° over 7 days. In contrast, IFN- β lost up to 30% of its antiviral activity after 5 days at 37, and up to 65% activity was lost after 3 days at 39.5° (Table 1). Antiviral activity has routinely been used to define a unit of activity for IFN (10). It, however, should be noted that there is no evidence for a common mechanism for IFN antiviral and antiproliferative activities.

Effects of Pure IFN and Influence of IFN Dose. Naturally produced IFN, although partially purified, contains non-IFN impurities. The HT1197 cell line was used to assess whether IFN- α and IFN- β , produced by recombinant DNA technology and purified to homogeneity, would have the same antiproliferative activity as naturally produced IFN. No significant difference existed between naturally produced and recombinant DNA-produced IFN (Chart 3).

The dose response to IFN- α at varying elevated temperatures was evaluated. Untreated cells grown at 37° were the controls to which all other treatments were compared (Chart 5). The number of T24 cells following treatment with IFN (20 units/ml) at 37°, for 96 hr, was decreased 43%. This inhibition was increased to 60% by 200 units/ml and to 76% by 1000 units/ml. A 6% decrease in cell number was seen when untreated cells were grown at 39.5°. Treatment with 20, 200, and 1000 units IFN per ml at 39.5° inhibited cell numbers 70, 94, and 99%, respectively. A temperature of 41° with 20 units/ml resulted in greater than 99% inhibition of cell number.

2-5A Synthetase. 2-5A synthetase activity was measured in T24 cells untreated or treated with IFN- α and grown at 37° or 39.5° (Table 2). The enzyme activity of untreated cells remained the same at both temperatures. After a 24-hr incubation with IFN- α (200 units/ml), a 40-fold increase in enzyme activity occurred. 2-5A synthetase activity increased an additional 2-fold (80-fold over base line) by IFN- α treatment at 39.5°. This increase in an IFN-induced enzyme suggests the effects of IFN- α were increased by elevated temperatures.

DISCUSSION

IFN, *in vivo* and *in vitro*, inhibit the proliferation of many but not all tumor cells. The optimal conditions for this antiproliferative effect have not yet been defined. An increase of temperature

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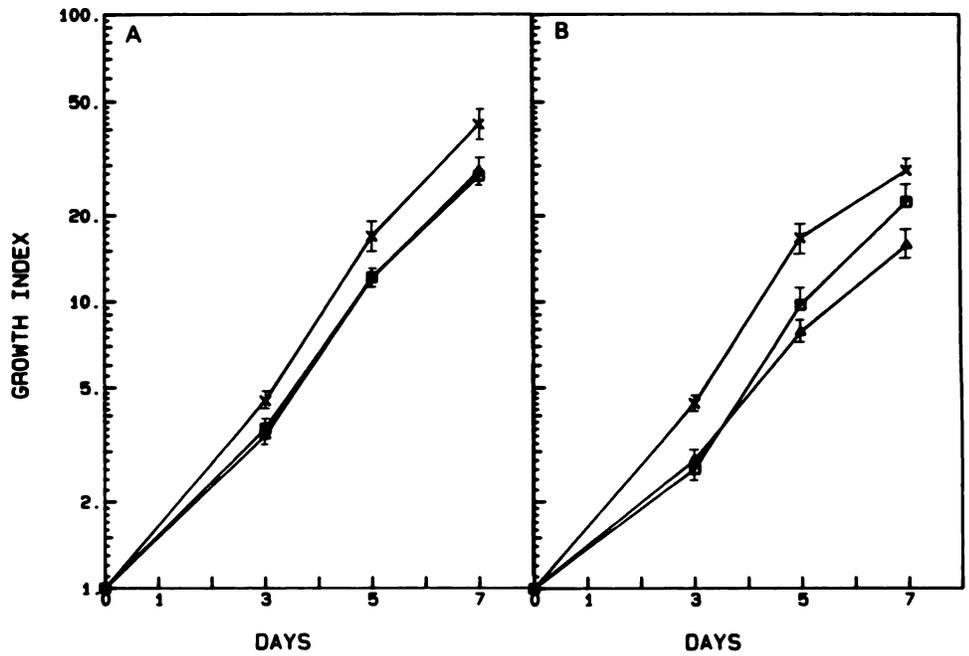


Chart 4. Antiproliferative effects of IFN against 647V cells. Untreated cells (x) or cells treated with 200 units IFN- α (Δ) or 200 units IFN- β (\square) per ml were grown at 37° (A) or 39.5° (B). Data are from 5 experiments. Points, mean growth index versus time; bars, S.E.

Table 1
Stability of IFN at 37° and 39.5°

IFN was measured by 50% reduction of EMC virus yield in A549 cells.

IFN	Temperature	IFN (units/ml) after			
		Day 1	Day 3	Day 5	Day 7
α	37°	270	250	230	230
	39.5	270	230	230	230
β	37	370	340	210	120
	39.5	370	120	110	70

Table 2
2-5A synthetase activity in T24 cells

	Enzyme activity (pmol/hr/10 ⁶ cells)			
	37°		39.5°	
	Untreated	IFN- α (200 units/ml)	Untreated	IFN- α (200 units/ml)
Experiment 1	1.11 \pm 0.35 ^a	46.67 \pm 3.11	0.98 \pm 0.43	74.20 \pm 3.25
Experiment 2	1.35 \pm 0.33	52.82 \pm 3.10	1.01 \pm 0.45	83.68 \pm 8.78

^a Mean \pm S.D.; n = 2.

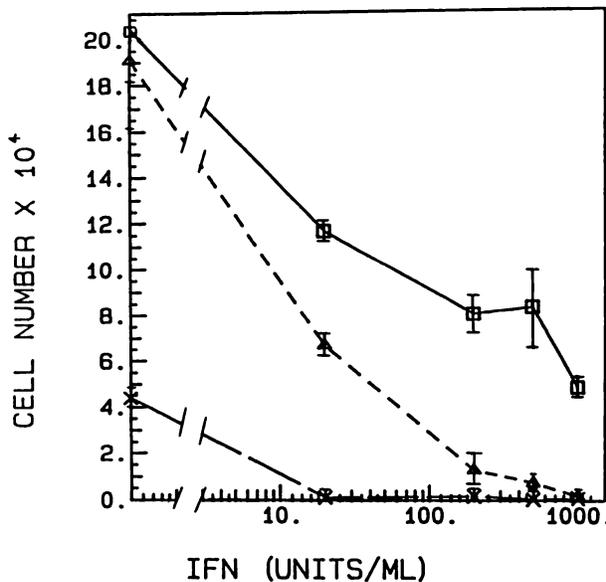


Chart 5. Dose response to IFN- α at varying elevated temperatures. T24 cells were untreated or treated with 20, 200, 500, or 1000 units IFN- α per ml and grown at 37° (\square), 39.5° (Δ), or 41° (x). Cell counts were evaluated at 96 hr. Data are from one representative experiment. Points, mean cell number of duplicate plates versus IFN- α concentration; bars, S.E.

augmented the antiproliferative activity of IFN against transitional cell carcinoma cell lines. Enhanced sensitivity of Daudi cells and rat osteosarcoma cells to the antiproliferative effects of IFN has also been demonstrated at increased temperatures (5, 8). This enhancement occurred with partially purified, naturally produced IFN, as well as IFN produced by recombinant DNA technology and purified to homogeneity. We examined cell lines with a range of sensitivities to IFN and, in each case, the antiproliferative activity was increased at the elevated temperature.

A variety of different criteria have been utilized to define the type of interaction which may exist between biologically active agents (2). Two types of interactions exist: positive or synergistic and negative or antagonistic. Construction of a model which demonstrates no interaction is required in order to detect either departure from a zero interaction. In one such model, an isobole, the interaction is defined using the doses of 2 agents either alone or in combination which result in equieffective responses (2). An examination of the dose response of T24 cells to IFN- α at elevated temperatures suggested a synergistic relationship existed between these 2 agents (Chart 5).

Treatment of cells with IFN results in the induction of several proteins, one of which is an oligonucleotide synthetase (3). The measurement of 2-5A synthetase activity serves as a useful marker for the biological activity of IFN. Induction of 2-5A synthetase in a given cell is a function of IFN dose (1). In the T24 cell line, an increase in temperature during IFN treatment en-

hanced the levels of enzyme activity at a fixed concentration of IFN. The increased temperature may thus have sensitized cells to effects of IFN.

The mechanisms responsible for antiproliferative effects of IFN are unknown. An elevation of temperature may have a direct effect on the cell membrane influencing receptor expression or affinity for IFN. Alternatively, a more complex biochemical event may occur when IFN is introduced to a cell above physiological temperature. Augmented IFN activity at elevated temperatures may have potential for dissecting *in vitro* mechanisms underlying antiproliferative effects of IFN.

IFN has antitumor activity against a variety of neoplastic diseases. Increased temperature sensitizes cells to cytotoxic effects of chemotherapeutic agents (7, 12) and radiation (16). Various technologies for regional and whole-body hyperthermia are thus currently undergoing clinical evaluation. *In vivo*, the combination of IFN and local hyperthermia had greater antitumor effect than either agent alone when tested against transplanted Lewis lung carcinoma in mice (18). Particularly if a selective effect for malignant cells is demonstrated, the combination of IFN and other modalities may prove more effective than IFN alone.

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