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Effects of scheduling and ascites-associated macrophages on combined antiproliferative activity of alpha-2b interferon and gamma-interferon in a clonogenic assay

Junichiro Higashihara, Toshiaki Saito*, Michael E. Berens, and Charles E. Welander**

Section on Gynecologic Oncology, Bowman Gray School of Medicine of Wake Forest University, 300 South Hawthorne Road, Winston-Salem, NC 27103, USA

Summary. Effects of combination treatment with human recombinant alpha-2b interferon (IFN-α2b) and gamma interferon (IFN-γ) and sequencing of the combination on colony formation of human tumor cells were studied in a human tumor clonogenic assay (HTCA) with or without ascites-associated macrophages (AAM). Five different human tumor cell lines were studied. Three of the five cell lines (ovarian cancer cell line BG-1, cervical cancer cell line ME-180, and melanoma cell line SK-MEL 28) were sensitive to both IFNs. Cervical cancer cell line CaSki was sensitive to IFN-α2b but resistant to IFN-γ. Endometrial cancer cell line HEC-1A was resistant to both IFNs. Synergistic interaction was observed in BG-1 and SK-MEL 28 with a combination of the IFNs. ME-180 did not exhibit a positive interaction, in spite of its sensitivity to each IFN. CaSki and HEC-1A also did not exhibit a positive combined interaction at clinically achievable concentrations. One sequential combination method (method 1: IFN-α2b $\rightarrow$ IFN gamma with a 24-h interval) resulted in a similar antitumor effect as the simultaneous combination. A reversed sequential method (method 2: IFN-$\gamma \rightarrow$ IFN-α2b with a 24-h interval) was less effective in three of the five cell lines. In BG-1, AAM enclosed in the lower layer markedly enhanced the antitumor effect of combined IFNs as well as each IFN alone. The antitumor effect with method 1 was significantly greater than that achieved with simultaneous combination or combination according to method 2 in the presence of AAM ($P < 0.01$). These results suggest that (1) a synergistic antitumor effect of IFN-α2b and IFN gamma is demonstrable in selected types of tumors, depending upon the sensitivity of each tumor cell line to both IFNs; (2) optimal scheduling for the direct antitumor effect of combined IFNs seems to be long-term exposure of cells to the IFN, the cells being treated with both IFNs either simultaneously or sequentially (IFN-α2b preceding IFN-γ); and (3) AAM potentiate the antitumor effect of IFNs either alone or in combination. Finally, IFN-α2b may have some priming effects for the indirect effect of IFN gamma mediated through AAM in certain tumor cells.

Introduction

Thirty years have passed since Isaacs and Lindenmann described interferon (IFN) as a naturally occurring antiviral substance [17]. Since then, three antigenically different types of IFN (IFN-α, IFN-β, and IFN-γ) have been identified, and it has been demonstrated that they have not only antiviral, but also antiproliferative and immunomodulatory activities [11, 15, 32]. Recombinant DNA technology now permits the production of large quantities of purified human IFNs, and has provided an opportunity for systematic study of the in vitro and in vivo effects of each type of human recombinant IFN (rIFN) on tumor cells. Each type of IFN has been investigated as a possible anticancer drug, and each has been shown to have some direct antitumor effects [4, 12]. Clinical trials have been performed with each type of IFN. However, the clinically observed antitumor effect of these IFNs as single agents have been reported as minimal in the common solid tumor types [6, 14].

In an effort to improve the usefulness of IFNs in clinical oncology, combinations of IFN plus other agents have been tried. IFN plus chemotherapeutic agents has been tested in preclinical trials, with positive interactions reported [2, 22, 37]. Combinations of two types of IFN have also been tested, both in murine systems with IFN-γ and IFN-α/β [12], and with human rIFNs in human tumor cell lines. Potentiation of antiproliferative effects has been reported for human rIFN-γ and α/β combined [9, 30]. Beyond the simple in vitro combination of IFN with other agents, the impact of host cells on IFN effects is an important phenomenon. Preclinical testing of macrophages as mediators of IFN activity has been done, demonstrating that IFN can mediate indirect effects on tumor cell growth through host effector cells [24].

In this preclinical investigation, the interaction of human rIFN-α2b and rIFN-γ was tested against five different human tumor cell lines and five fresh human ovarian tumor samples in a colony-forming assay. Three specific questions are raised: (1) Is it possible to demonstrate combined antiproliferative activity of rIFN-α and rIFN-γ on these cell lines? (2) If there is combined antiproliferative activity, is it altered by different sequencing? (3) Is this interaction potentiated by the presence of added effector cells?

Materials and methods

Cell lines. Five different human cancer cell lines were used as target cells in a colony-forming assay. They were derived from a human ovarian adenocarcinoma (BG-1) [36], two cervical carcinomas (CaSki [25] and ME-180 [33]), an endometrial carcinoma (HEC-1A) [20], and a malignant melanoma (SK-MEL 28) [8]. All were serially maintained

* *Current address:* Department of Obstetrics & Gynecology, Kyushu University, Faculty of Medicine, Fukuoka, Japan
** *Current address:* Brain Tumor Research Center, University of California at San Francisco, San Francisco, CA, USA
Offprint requests to: C. E. Welander

as monolayer cultures in McCoy's 5A medium (Gibco, Grand Island, NY) enriched with 10% heat-inactivated fetal calf serum (FCS), 0.1% insulin, and 1% penicillin/streptomycin and passaged after mild trypsinization. Cultures were routinely tested for mycoplasma contamination (Hoechst stain) and found to be negative. Cells were plated in soft agarose cultures 72 h after prior reseeding in liquid culture to achieve logarithmic phase cell growth.

Fresh human ovarian tumor cells. Human tumor specimens were obtained from five ovarian cancer patients who had malignant effusions drained. Ficoll-Paque (Pharmacia, Piscataway, NJ) gradient separation of the cell suspension was performed to remove erythrocytes, cellular debris, and non-viable cells from the plating suspension. Cells from the interface were counted and used for drug exposure [13]. These cells taken from the gradient interface included monocytes and lymphocytes as well as tumor cells.

Preparation of ascites-associated macrophages. Macrophages from human malignant effusions were used as effector cells in the group of experiments investigating the indirect effects of IFNs, as previously described in detail [28]. Briefly, malignant effusions were obtained from patients with ovarian cancer. Mononuclear cells were separated by gradient centrifugation on Ficoll-Paque. After washing with phosphate-buffered saline (PBS), the cells were resuspended in RPMI-1640, supplemented with 20% heat-inactivated FCS, and decanted into culture flasks (3375; Costar, Cambridge, Mass) at a concentration of 10^6 cells/ml. The cells were then incubated and allowed to adhere to plastic for 1 h at 37° C in a humidified atmosphere of 7.5% CO_2 in air. After a thorough wash to remove nonadherent cells, the adherent cells were removed with 0.2% EDTA in PBS with 5% FCS for 20 min at 37° C and mixed into the agarose underlayer at a density of 5×10^4 cells per dish. More than 90% of these adherent ascites cells were cytochemically positive macrophages by nonspecific esterase staining [23]. In this colony-forming assay system, physical contact between target cells and effector cells (ascites-associated macrophages; AAM) is impossible.

Soft agarose clonogenic assay and drug exposure method. Tumor cells were cloned in a two-layer agarose matrix modified slightly from the method described by Hamburger and Salmon [16]. Plating densities of the cell line were between 3×10^4 and 1×10^5 cells per 35-mm dish, selected to achieve at least 100 colonies per control dish. For primary human tumors the plating density was 5×10^5 nucleated cells per 35-mm dish. Total volume of agarose matrix per 35-mm dish was 2 ml. IFNs were diluted in PBS. To test the effect of a single IFN, 0.22 ml of 10× concentration of drug was applied to the upper layer as liquid overlay and remained present throughout the incubation of the culture. For in vitro simultaneous combination treatment, 0.11 ml of 20× concentration of each drug was applied at the same time. The ratio of IFN-α2b to IFN-γ in each combination experiment was 1:1, based on international units (IU). Control cultures were incubated with 0.22 ml PBS alone on the upper layer. Each culture was set up in triplicate. Sequential combination treatment of IFN-α2b with IFN-γ was administered in two ways (Table 1). As an additional experiment, the effects of sequence interval variation (0–48 h) on the antitumor activity of 1000 IU/ml of each IFN were examined in the BG-1 cell line, IFN-α2b being added at the time of plating, followed by IFN-γ at varied time intervals up to 48 h after. In all of the combination experiments described above, the treatment effects of IFNs alone on tumor colony growth were investigated to allow calculation of the expected colony growth. *Agarose cultures were incubated at 37° C in a humidified incubator with 7.5% CO_2 for 7 days in cell lines and 14–21 days in fresh tumor cells, depending upon the time needed to form definite colonies.* Colonies were counted with a computerized feature analysis system (Omnicon FAS-II, Baush & Lomb, Rochester, NY) [19]. Colonies were defined as cell aggregates of at least 40 μm in diameter arising from a single-cell suspension. Results were expressed as the percentage colony growth in the treated cultures compared with the untreated controls.

Table 1. Sequential combination methods employed in the HTCA

Method 1:	Continuous exposure of IFN-α2b at time of plating followed 24 h later by IFN-γ
Method 2:	Continuous exposure of IFN-γ at time of plating followed 24 h later by IFN-α2b

Source of interferons. Human recombinant IFN-α2b and human recombinant IFN-γ were provided by Schering (Kenilworth, NJ). They were stored at −20° C until use. These IFNs were free of endotoxin according to Limulus amebocyte lysate assay (Whittaker Bioproducts, Walkersville, Md).

Statistical methods to determine drug combination effects. The interaction between two types of IFNs was interpreted using a multiplicative model developed by Valeriote and Lin [35] with some modifications (Table 2). Statistical significance ($P < 0.05$) between fractional colony growth (FCG) of combination- and single-treatement cultures was determined using Student's *t*-test (two-tailed). Positive drug interactions were further categorized according to the observed FCG of drug combination cultures relative to an "expected" FCG value, which was calculated as the prod-

Table 2. Definitions of drug combination interactions

Negative interaction		FCG [AB] ≥ FCG [B] when FCG [A] ≥ FCG [B]
Positive interaction		FCG [AB] < FCG [B] when FCG [A] ≥ FCG [B]
	Subadditive	FCG [AB] > FCG [A] × FCG [B]
	Additive	FCG [AB] = FCG [A] × FCG [B]
	Synergistic	FCG [AB] < FCG [A] × FCG [B]

A negative and a positive interaction was defined as a case where the FCG of the combination treatment was greater than or equal to that of more effective single drug (negative) and a case where the FCG of the combination treatment was less than that of more effective single drug (positive), respectively ($P < 0.05$). Positive interaction was further categorized using a statistical model that tests for additivity. Statistical significance for synergism or subadditivity was achieved by rejection of the null hypothesis for additivity ($P < 0.05$)

FCG, Fractional colony growth; [AB], drugs used in combination; [A], [B], drugs used as a single agent

Table 3. Interaction of IFN-α2b and IFN-γ (1 : 1 combination)

Cell line	IFN conc. (IU/ml)	Tumor colony growth (% control)				Combined interactiion
		IFN-α2b alone	IFN-γ alone	Expected colony growth	Observed colony growth	
BG-1	10	83.2 ± 2.8[a]	106.2 ± 9.6	88.3	83.5 ± 11.5	Negative
	100	70.3 ± 8.0	102.7 ± 5.9	72.2	72.2 ± 1.7	Negative
	1 000	50.5 ± 4.2	98.5 ± 14.4	49.7	9.0 ± 0.3	Synergistic ($P < 0.02$)
	10 000	7.6 ± 0.8	44.2 ± 1.4	3.4	0.05 ± 0.05	Additive
ME-180	1	46.1 ± 2.7	96.4 ± 8.9	44.4	48.1 ± 2.0	Negative
	10	40.7 ± 2.7	52.2 ± 4.6	21.2	37.9 ± 4.6	Negative
	100	29.8 ± 4.0	24.7 ± 1.0	7.3	20.9 ± 1.6	Negative
CaSki	10	70.6 ± 2.0	110.7 ± 3.8	78.2	91.1 ± 7.1	Negative
	100	38.9 ± 3.6	133.1 ± 6.3	51.8	70.5 ± 1.4	Negative
	1 000	15.8 ± 1.0	137.3 ± 5.8	21.9	19.7 ± 2.3	Negative
	10 000	5.2 ± 0.8	122.9 ± 9.0	6.3	2.9 ± 0.3	Additive
HEC-1A	100	88.5 ± 7.6	89.3 ± 1.2	79.1	84.9 ± 2.9	Negative
	1 000	92.4 ± 1.0	78.9 ± 4.6	72.9	87.4 ± 2.7	Negative
	10 000	98.7 ± 4.6	81.3 ± 2.9	80.1	92.0 ± 5.3	Negative
SK-MEL	10	103.7 ± 0.8	104.3 ± 5.0	108.2	90.9 ± 3.1	Additive
	100	84.3 ± 0.7	92.8 ± 1.5	78.2	68.9 ± 2.3	Additive
	1 000	57.4 ± 2.8	59.1 ± 0.5	33.9	10.0 ± 1.0	Synergistic ($P < 0.01$)
	10 000	19.9 ± 1.8	18.6 ± 1.4	3.7	1.0 ± 0.2	Additive

The control colony numbers (mean ± SD) and plating efficiency (%) in the different cell lines were BG-1: 1791 ± 199 (6.0%), ME-180: 131 ± 2 (0.44%), CaSki: 1614 ± 14 (5.4%), HEC-1A: 427 ± 11 (1.4%), and SK-MEL: 858 ± 58 (2.9%)

[a] Mean ± SE of triplicate cultures

uct of the FCG observed from each of the single-drug cultures [3]. These interactions were termed "subadditive," "additive," and "synergistic" when the observed FCG was greater than, equal to, or less than the calculated FCG, respectively. The data were tested for statistical significance ($P < 0.05$) using a previously described analytical model [37]. This analysis takes into consideration the variance of each series of replicate measurements. These results were also analyzed using the isobole method, whenever possible, because this method can be applied to all cell systems regardless of the dose-response curve [3]. In the isobole method, the equation $A_c/A_e + B_c/B_e = D$ is employed, where A_c and B_c are the concentrations of drugs in the combination treatment and A_e and B_e are the concentrations of the drugs used individually to achieve the same effect as that combination (the equi-effective concentrations). If $D < 1$, the interaction is synergistic; if $D = 1$, the interaction is additive; and if $D > 1$, the interaction is antagonistic. In some cell lines, equi-effective concentrations could not be achieved because of the marked differences in the antiproliferative effect between single and combination treatment. We have therefore presented most of our data using the modified multiplicative model.

Results

Antiproliferative effects of IFN as a single drug

The direct antiproliferative effects of IFN-α2b and IFN-γ were studied in five different human cancer cell lines (Table 3). Both IFN-α2b and IFN-γ demonstrated dose-responsive antiproliferative activities against three of the five cell lines (BG-1, ME-180, and SK-MEL 28). The endometrial cancer cell line HEC-1A was resistant to both IFNs even at the highest concentration tested. The cervical cancer cell line CaSki showed a mixed response. It was sensitive to IFN-α2b but resistant to IFN-γ. In fact, IFN-γ significantly stimulated the colony growth of CaSki at all concentrations tested ($P < 0.05$). The other cervical cancer cell line, ME-180, was the most sensitive cell line to both IFNs.

Antiproliferative effects of IFN combinations

The direct effects of IFN-α2b and IFN-γ were tested in simultaneous combination against the five cell lines, using a concentration ratio of 1:1 (Table 3). In three of five cell lines (BG-1, SK-MEL 28, and CaSki), the combination of IFNs demonstrated additive or synergistic interactions. In cell line BG-1, the combination treatment of IFNs resulted in additive or synergistic interaction only at IFN concentration of 1000 IU/ml or higher. *From the dose-response curve for each IFN,* the concentrations of IFN-α2b and IFN-γ that reduced colony growth by 50% (IC_{50}) were 1000 IU/ml and 7000 IU/ml, respectively. The combination of approximately 150 IU/ml of each IFN would achieve the same effect as that of either IFN alone, according to the combination dose-response curve (Fig. 1; D = 0.15; synergy). In cell line SK-MEL 28, additive or synergistic interaction was observed at all IFN concentrations tested. According to the isobole analysis, the IC_{50} of either IFN-α2b or IFN-γ alone was 1400 IU/ml, as against an IC_{50} of 150 IU/ml for each when the two IFN types were combined (D = 0.21; synergy). In cell line CaSki, additive interaction was noted only at an IFN concentration of 10000 IU/ml. The combined antiproliferative effects were smaller than those of IFN-α2b alone at IFN concentrations lower than and up to 1000 IU/ml. Since CaSki cells showed resistance to IFN-γ at all concentrations tested, this combination was defined as heterergic [3]. Combina-

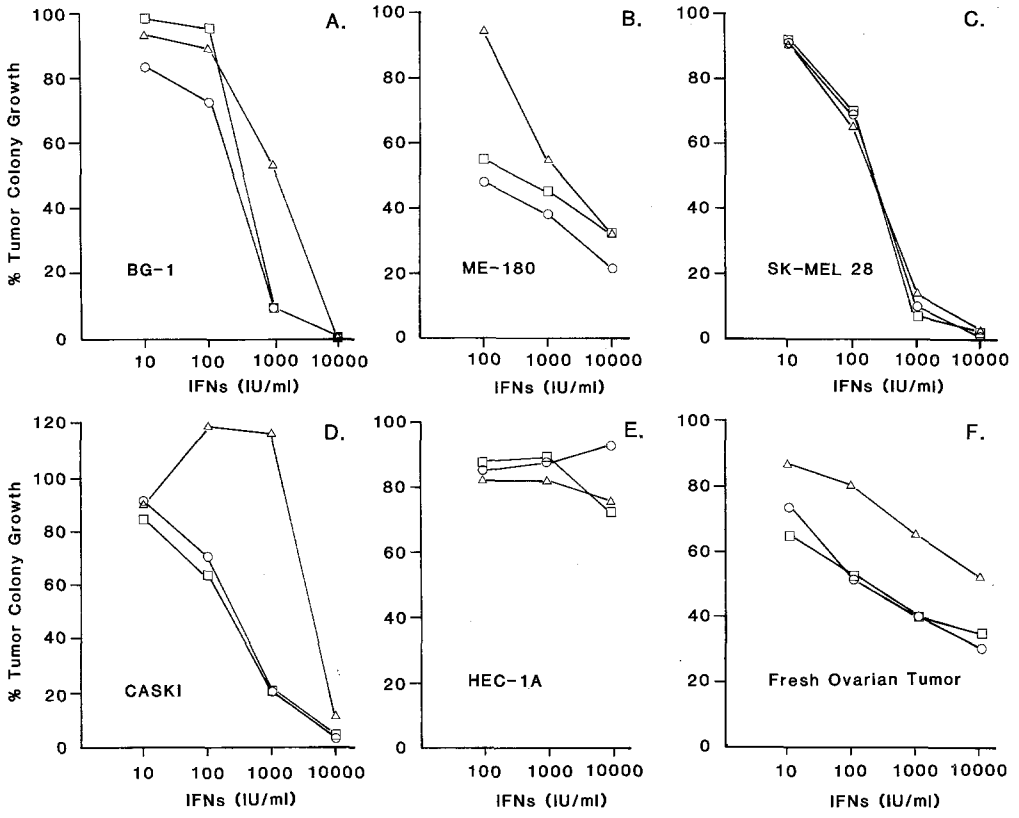

Fig. 1. Comparison of antiproliferative effect between the simultaneous and sequential combination treatment of IFN-α2b with IFN-γ against human tumor cell lines and fresh human ovarian tumor cells. Combination ratio was 1:1 based on antiviral activity. ○, Simultaneous combination; □, method 1 (IFN-$\alpha \rightarrow$ IFN-γ); △, method 2 (IFN-$\gamma \rightarrow$ IFN-α). Each *point* is the mean of triplicate plates, expressed as percentage of control colony number

tion treatment of 10000 IU/ml of IFNs demonstrated 97% colony growth inhibition, whereas when IFN-α2b was applied more than 10000 IU/ml was needed to achieve an equivalent effect. In the isobole analysis, since the equi-effective concentration of IFN-γ was regarded as infinite, where $D < 1$ the interaction was noted as synergy. However, because no significant difference was found between the expected and the observed value by statistical analysis, we have interpreted the interaction as additive. In cell line ME-180, the combination treatment showed dose-responsive antiproliferative effects, although the interaction was negative. Cell line HEC-1A was very resistant even in the combination treatment of both IFNs at all concentrations tested.

Effects of sequencing in combinations of IFNs

The direct antipr[illegible] ffects of [illegible] ial combination [illegible]

simultaneous method [illegible] method 2 at IFN concentration of 10 IU/ml and below, but at 100 IU/ml both sequential methods were less effective than the simultaneous method. Similarly, in cell line CaSki, method 1 was as effective as the simultaneous combination, and more effective than method 2 at IFN concentrations of 100 IU/ml and more. The colony growth of the CaSki was stimulated by method 2 at the IFN concentrations of 100 IU/ml ($P < 0.05$). In cell line SK-MEL 28, no statistically significant difference in the antiproliferative effects was observed between the simultaneous method and either sequential method, but method 1 was more effective than method 2 ($P < 0.02$) at an IFN concentration of 1000 IU/ml. Sequential combination treatment did not alter the resistance of HEC-1A to IFNs.

The same methods for testing IFNs were used in fresh human ovarian cancer cells derived from five patients. In four of the five samples, method 1 was as effective as simultaneous treatment and more effective than method 2 (Fig. 1) ($P < 0.05$). In the one remaining sample, the three combination methods exhibited almost the same antiproliferative effects (data not shown). In the experiments using fresh tumor samples, however, no synergistic interactions were detected in spite of their individual sensitivity [illegible] IFN [illegible] Experiments were done to investigate the optimal [illegible] terval with cell line BG-1. Method 1 with [illegible] after a 6-h interval was significantly more [illegible] was simultaneous treatment ($P < 0.01$) [illegible] 3-h point, the antiproliferative activities [illegible] based sig[illegible] and returned to the same level as simultaneous combination by 12 h. Although the antiproliferative effect of the sequential combination treatment, IFN-$\alpha \rightarrow$ IFN-γ, was similar to the level of simultaneous combi-

Table 4. Simultaneous and sequential combinations of IFN-α2b and IFN-γ (1 : 1 combination)

Cell line	IFN conc. (IU/ml)	Tumor colony growth (% control)					
		Simultaneous combination		Sequential combination (24-h interval)			
				IFN-α → IFN-γ		IFN-γ → IFN-α	
		Expected	Observed	Expected	Observed	Expected	Observed
BG-1	10	88.3	83.5 ±11.5[a]	94.9	98.4±11.3	91.8	93.0±6.8
	100	72.2	72.2 ± 1.7	66.7	95.6±10.7	108.3	89.6±5.4
	1 000	49.7	9.0 ± 0.3[b]	38.6	9.0± 0.5[b]	98.5	53.2±2.9[c]
	10 000	3.4	0.05± 0.05[c]	2.4	0.1± 0.1[c]	15.7	0.3±0.1[b]
ME-180	1	44.4	48.1 ± 2.0	35.5	54.5± 5.1	99.2	94.4±6.7
	10	21.2	37.9 ± 4.6	20.9	45.0± 8.2	34.5	54.5±1.7
	100	7.3	20.9 ± 1.6	11.1	32.3± 2.0	19.0	31.8±0.5
CaSki	10	78.2	91.1 ± 7.1	86.1	84.1± 5.6	98.9	89.2±2.7
	100	51.8	70.5 ± 1.4	48.4	63.8± 2.6	92.1	119.2±6.9
	1 000	21.6	19.7 ± 2.3	23.8	20.1± 0.9	79.7	116.2±7.8
	10 000	6.3	2.9 ± 0.3[c]	7.0	3.6± 0.7[c]	13.9	11.6±1.3
HEC-1A	100	79.1	84.9 ± 2.9	71.6	87.6± 2.0	80.9	82.1±5.5
	1 000	72.9	87.4 ± 2.7	89.1	88.5± 2.3	76.6	82.1±4.1
	10 000	80.1	92.0 ± 5.3	93.5	71.8± 5.7	84.3	75.0±2.5
SK-MEL 28	10	108.2	90.9 ± 3.1[c]	102.9	91.7± 5.0[c]	88.4	90.6±4.8
	100	78.2	68.9 ± 2.3[c]	75.1	69.9± 4.2[c]	65.2	65.2±2.3[c]
	1 000	33.9	10.0 ± 1.0[b]	38.0	7.1± 0.8[b]	30.5	13.7±1.4[c]
	10 000	3.7	1.0 ± 0.2[c]	6.3	1.5± 0.6[c]	3.6	2.9±1.4[c]

[a] Mean ± SE of triplicate cultures
[b] Synergistic interaction ($P < 0.05$)
[c] Additive interaction

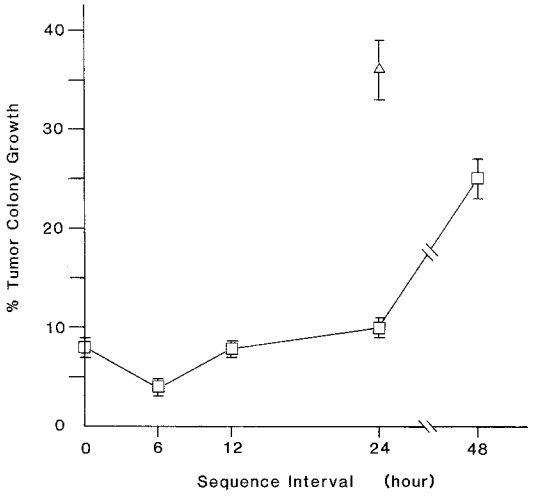

Fig. 2. Effect of the sequence interval variation on the combined antiproliferative activity of IFN-α2b with IFN-γ against BG-1. Concentrations of both IFNs were 1000 IU/ml. □, Simultaneous combination (0 h) and method 1 (6 h to 48 h); △, method 2 (24 h). *The colony formation of control culture was 1239± 55 (SD).* Each *point* is the mean of triplicate plates, expressed as percentage of control colony number. *Bars*, SD

nation between 12 h and 24 h, beyond 24 h it markedly decreased up to 48 h. The sequential combination treatment, IFN-γ → IFN-α, was even less effective beyond a 24-h interval than either simultaneous combination or reversed sequential combination treatment ($P < 0.001$).

Result of the inclusion of ascites macrophages on the effect of IFNs

The antiproliferative effects of both IFN-α2b and IFN-γ as a single agent against BG-1 were significantly enhanced ($P < 0.05$) by the presence of AAM in the underlayer at IFN concentrations over 100 IU/ml and over 10 IU/ml, respectively (Fig. 3). The magnitude of potentiation was greater in IFN-γ than in IFN-α2b. For instance, while IC_{50} of IFN-α2b with AAM was 10-fold less than the IC_{50} without AAM (200 IU/ml and 2000 IU/ml), the IC_{50} of IFN-γ with AAM was over 30-fold less than the IC_{50} without AAM (220 IU/ml and 7000 IU/ml). The effects of AMM on the antiproliferative activity of IFNs against BG-1 in simultaneous and sequential combination treatments were also tested (Fig. 4). The combined antiproliferative effects of IFNs were significantly enhanced ($P < 0.02$) by the presence of AAM in both simultaneous and sequential combination methods at IFN concentrations 100 IU/ml and 1000 IU/ml. In particular, the effect of method 1 was most enhanced by AAM and, at IFN concentrations of 1000 IU/ml and more, method 1 was significantly more effective than either simultaneous combination treatment or method 2 ($P < 0.01$). The antiproliferative effects of the simultaneous combination and method 2 were not significantly different at any IFN concentration tested in the presence of AAM.

Discussion

Results of published clinical trials testing IFN-α demonstrate activity as a single agent in patients with malignancies, including hairy cell leukemia [26], AIDS-related Ka-

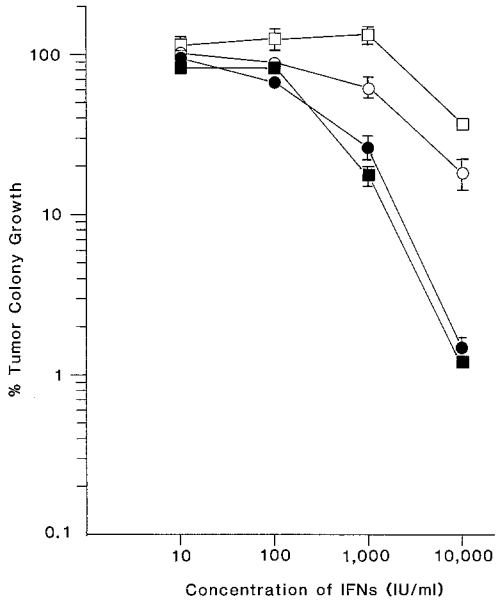

Fig. 3. Effect of AAM on the antiproliferative activity of IFNs as a single agent against BG-1. The ratio of target cells to effector cells was 0.6. ○, IFN-α2b without AAM; □, IFN-γ without AAM; ●, IFN-α2b with AAM; ■, IFN-γ with AAM. *The colony formations of control culture with or without AAM were 2523± 221 (SD) and 1791± 199 (SD), respectively. Each point* is the mean of triplicate plates, expressed as percentage of control colony number. *Bars*, SD

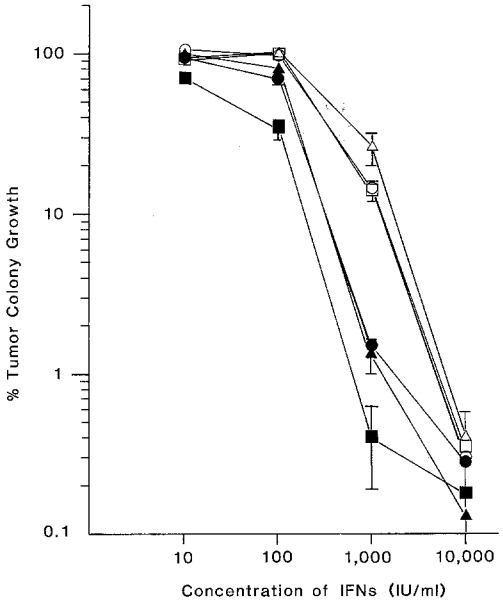

Fig. 4. Effect of AAM on the combined antiproliferative activity of IFN-α2b with IFN-γ against BG-1 in the simultaneous and sequential (24-h interval) combination treatment. The ratio of target cells to effector cells was 0.6 (3×10^4:5×10^4). Combination ratio of both IFNs was 1:1 based on antiviral activity. ○, Simultaneous method without AAM; □, method 1 without AAM; △, method 2 without AAM; ●, simultaneous method with AAM; ■, method 1 with AAM; ▲, method 2 with AAM. The colony formations of control culture with or without AAM were 2523±221 (SD) and 1791±199 (SD), respectively. Each *point* is the mean of triplicate plates, expressed as percentage of control colony number. *Bars*, SD

posi's sarcoma [27], chronic myelogenous leukemia [34], renal cell carcinoma [18], and ovarian carcinoma [36]. There are fewer clinical trials testing IFN gamma, but renal cell carcinoma may be among the solid tumor types for which IFN-γ is effective [6]. To date, studies on cytotoxic chemotherapeutic agents have been concentrated on the design of multiple-agent treatment regimens for treating resistant tumors. By using combinations of agents, each of which has some independent activity, greater antitumor activity might be achieved. This idea has been applied to the use of biological response modifiers (BRMs); combinations of agents with different mechanisms of action have been sought in the hope that there will not be additive toxicity. Although there is not yet a clear outline of the mechanisms of IFN effect on tumor cells, clinical oncologists have been anxious to try combinations of IFN-α and IFN-γ in patient treatment trials, and some clinical protocols have already been implemented [21]. While our current understanding of IFN activity may seem to make clinical treatment trials premature, preclinical models enabling the study of human rIFNs have become a valuable tool. The studies reported here were performed in attempt to find in vitro clues to the optimal conditions for combined treatment with IFN-α and IFN-γ. Empiric observations have been made, using a colony-forming assay as the reproducible endpoint for study. Effector cells (AAM) have been added to some of the cultures to determine whether indirect mechanisms of IFN action can change the growth of tumor cells.

The five cell lines tested in this set of experiments demonstrate that not all tumor types respond to individual IFNs or to combinations in the same way. Since it is not possible to describe common mechanisms of IFN effect on various types of solid tumors, we are left with empiric testing as the only means of learning whether a particular tumor type is sensitive to IFN or not. It appears from this report that cell lines which show individual sensitivity to IFN-α and IFN-γ also show positive interactions when the two IFNs are combined. However, individual sensitivity to both IFNs may not always be a sufficient condition for positive interaction in combination treatment. In the case of cell line ME-180, we observed negative interaction *despite* its sensitivity to both individual types of IFN. When one of the IFNs is inactive as a single agent (e.g., cell line CaSki), there is no positive interaction of the two IFNs except at the highest in vitro concentrations, which cannot be achieved in patients. Cell line HEC-1A is insensitive to both IFNs as single agents and remains resistant to the combination treatment of IFNs. This study has not provided any hopeful empiric evidence to suggest that tumors that are historically resistant to the antiproliferative effect of individual IFNs will respond any differently when combinations are tested.

There is some discrepancy between the results in this report and data published elsewhere on IFN activity. One report has described synergy with IFN-α/β and IFN-γ in nine out of ten cell lines tested, regardless of the individual IFN sensitivities [30]. One explanation may be the differences in target cells used, or in the use of strict criteria to define positive interactions and synergy. In some reports, statistical analysis intended to show positive interaction of two drugs seems to be obscure or inappropriate, i.e., no statistical difference from single IFN treatment was determined. As Berenbaum mentions [3], adequacy of the anal-

ytical model must be confirmed before any conclusions of combined drug activity can be made. On the basis of the stricter endpoint evaluation we applied, our results indicate that positive interactions can be achieved only in selected tumor types.

The second question posed in this report concerns the sequencing of IFN-α and IFN-γ. Is there any change in antiproliferative effects that can be reproducibly measured if tumor cells are exposed to the IFNs sequentially? A clinical corollary to the question of sequencing would be the impact on toxicity seen when IFNs are administered to patients sequentially. Since dose-limiting side-effects are similar for both IFN-α and IFN-γ [6, 31], separation the administration of the two by 24–48 h would be desirable, provided that this timing does not decrease the overall benefit of the IFN combination. When tumor cells are first exposed in vitro to IFN-α2b followed 24 h later by IFN-γ (method 1), the degree of antiproliferative effect is similar to that achieved with simultaneous exposure in most of the cell lines tested and in four of five fresh ovarian tumors tested. Beyond a 24-h interval between the two IFNs, a declining antiproliferative effect was observed.

Proposals for mechanisms to explain the combined effect of IFN-α and IFN-γ are based at best on conjecture. It has been reported that IFN-γ needs longer than IFN-α/β to induce an antiviral state [10] and also to synthesize 2',5'-oligoadenylate synthetase (2'5'A) [1]. There are differing indirect effects on feeder cells in cultures of IFN-γ as opposed to IFN-α/β [5, 24]. The receptors for IFN-γ and IFN-α/β are different [7]. We cannot speculate on the mechanisms of IFNs from these data. We note merely that there is no apparent loss of effect on tumor cell growth when cells are exposed to these IFNs separately 24 h apart.

BRMs are thought to have multiple effects on cell growth, by both direct and indirect means. Previous work has shown that clonogenic assays are able to measure both direct antiproliferative effects of BRMs as well as indirect effects mediated through "feeder cells" [28]. We have also reported that supernatants from IFN-γ-pretreated AAM cultures inhibit the colony growth of various tumor cell lines and that some part (40%) of this antitumor effect is inhibited by neutralization antiserum against tumor necrosis factor (TNF) [29]. In the present study, the antiproliferative effects of both IFN-α2b and IFN-γ were potentiated by AAM. The degree of potentiation was greater with IFN-γ than with IFN-α2b. We confirmed that these IFNs were free of endotoxin by the Limulus amebocyte lysate assay. Since target cells and AAM cannot physically contact each other in our assay system, it is concluded that some soluble substances, that potentiate the antitumor effect are secreted from activated AAM after IFN exposure. Cell line BG-1 is sensitive to TNF (data not shown). It would be reasonable to consider that TNF contributes in part to the potentiation of the combined antitumor effect of both IFNs when AAM are enclosed in the underlayer. The consumption and/or depletion of nutrients by AAM do not appear to affect tumor colony growth, because the colony number in control cultures with AAM was larger than that in control cultures without AAM ($P < 0.02$). We have confirmed that this density of AAM (5×10^4) does not change the dose-response pattern of tumor cells to cytotoxic agents (unpublished data). We observed a greater antiproliferative effect with method 1 than with the simultaneous method or method 2 in the presence of AAM. The mechanism of this interaction is unclear. IFN-α2b may have some priming effect for the potentiation of the indirect effect of IFN gamma mediated through AAM. Consequently, method 1 showed a greater antitumor effect against BG-1 than other methods tested.

In summary, this study provides in vitro data suggesting that selected tumor types are senstive to both IFN-α and IFN-γ. When a tumor shows some sensitivity to each of the IFNs, additive or even synergistic combined antiproliferative effects are frequently observed. Optimal scheduling seems to be long-term exposure of cells to IFN, the two IFNs either simultaneously or sequentially, with IFN-α2b preceding IFN-γ. Indirect mechanisms of action may be a part of the overall antiproliferative effect observed. In the presence of AAM, IFN-α2b may have some priming effect for the potentiation of the indirect effect of IFN-γ against some tumor cells.

Our aim in these studies is not necessarily to provide information that can be applied directly in clinical oncology. However, since clinical trials are already open and patients are being enrolled, any clues that might help in the selection of optimal tumor types for therapy or the determination of optimal drug scheduling and sequencing may be useful.

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