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Potentiation of Antiproliferative Activity by Mixtures of Human Recombinant IFN-α2 and -γ on Growth of Human Cancer Nodules Maintained in Continuous Organotypic Culture

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Abstract—*Alveolar II pulmonary tumor cells (A549 cells) maintained in continuous tridimensional organotypic culture were used to evaluate the eventual potentiation effect of mixtures of recombinant human interferon-α2 and -γ on growth inhibition of the tumor nodules. A continuous 45 day treatment (interferon renewed three times a week) with 10, 10^2 or 10^3 U/ml of IFN-α2 or -γ combined with a fixed high dose (10^3 U/ml) of either IFN-α2 or -γ resulted in an additive or synergistic growth inhibition according to the doses used. There was a close dose–effect relation, the percentage of inhibition increasing proportionally to the variable IFN doses added to the fixed high dose; moreover, the growth inhibition effect occurred earlier with the mixtures than with IFNs used separately. Furthermore, the growth inhibition observed with 2000 U/ml of the mixture (1000 U/ml of each IFN) was greater than that induced by 2000 U/ml of IFN-α2 or -γ used alone. A 35-day treatment with IFN-α2 1000 U/ml plus IFN-γ 1000 U/ml led to a complete growth inhibition and necrosis of the nodules. These data demonstrate that IFN-α2 and -γ cooperate to potentiate the IFN antiproliferative activity.*

INTRODUCTION

THE ANTIVIRAL, antiproliferative and immunomodulatory activities of IFNs have been extensively described [1–3]. Concerning their antiproliferative activity on tumor growth, the effects observed, either *in vitro* on murine or human tumor cell lines or *in vivo* in animals and in man, are variable and sometimes disappointing; particularly if one keeps in mind their enhancing effect on tumor growth [4–6], especially when used at low doses, and their toxicity at high doses [7].

Although the mechanisms involved in IFN antitumor activity are still poorly understood, it can be assumed that this activity could result from a direct cytostatic effect and/or from an indirect effect involving different limbs of the immune system. Combination of IFN-α/β and IFN-γ, all produced as highly purified recombinant IFNs, could result in association of complementary effects at the tumor target cell level. Such an association could provide a more efficient use of IFNs in human therapy in abolishing the enhancement or the toxicity effects.

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In a previous work [8], we studied the antiproliferative activity of the three recombinant human IFN-α2, -β and -γ on A549 human lung carcinoma cells maintained in organotypic culture [9] derived from the method of Wolff and Wolff [10]; this model mimics the *in vivo* situation with respect to the stage of cell differentiation of the nodules which develop an alveolar structure with secretion of pulmonary surfactant [11]. A continuous 65 day treatment with each of the three IFNs (10, 10^2, 10^3, 10^4 U/ml) resulted in a growth inhibition and necrosis only with the highest doses (10^3 and 10^4 U/ml) of IFN-α2 and -γ; we observed no inhibitory effect with IFN-β whereas all three IFNs when used at low doses (10, 10^2 U/ml) induced an enhanced growth, at least in some percentage of nodules; regeneration and regrowth of all the necrotic nodules appeared afterwards when the IFN treatment was discontinued.

The aim of this study is to evaluate the effect of the combinations of IFN-α2 and -γ on tumor growth using the same *in vitro* model, i.e. A549 cells in organotypic culture, to see whether additive or synergistic effect could be obtained. Our results

clearly demonstrate that such potentiation can be expected.

MATERIALS AND METHODS

Organotypic culture

The methods to obtain and culture the organotypic nodules from A549 monolayer cells have been previously described [8]. A549 cells were derived from an alveolar II pulmonary adenocarcinoma. The nodules were subcultured every 10 days and maintained in Petri dishes (Falcon 1006) on a 0.5% agar (Difco) semi-solid culture medium with RPMI 1640 supplemented with 10% fetal serum.

Interferons

Two highly purified *E. coli*-derived human IFNs were used: IFN-α2 kindly provided by Shering-Plough (specific activity: 1×10^8 U/ml protein) and IFN-γ from Biogen (specific activity: 2×10^7 U/ml protein). These IFNs were electrophoretically pure.

Interferon titers were determined by cytopathic effect inhibition assay with Vesicular Stomatitis Virus (VSV) on human Wish cells [12]. The IFN-α2 samples were also calibrated against the N.I.H. international human leukocyte interferon standard No. GA 902.530 and the IFN-γ samples were calibrated against the N.I.H. human IFN-γ No. Gg 23.901.530. Interferons were stored at −80°C.

IFN treatment of the nodules

In order to perform the IFN treatment, the nodules cultured on the semi-solid agar medium were transferred on Millipore filters (AABPO 2500, pore size 0.8 μm) as described [8]. Filters with eight nodules each were put in Petri dishes containing RPMI 1640 plus 10% FCS with or without IFN; the medium was renewed three times per week. IFN-α2 and -γ were used alone or in mixtures at the following concentrations: -α2 1000 and 2000 U/ml; -γ 1000 and 2000 U/ml; -α2 1000 U/ml plus -γ 10, 100 or 1000 U/ml; -γ 1000 U/ml plus -α2 10 or 100 U/ml. In order to complete the experiment and to allow us to calculate the eventual additive or synergistic effects, low doses (10 and 100 U/ml) already studied in our previous work, were tested again for both IFNs. The evolution and growth of the nodules were observed for 45 days without subculturing and handling. Two series of experiments were done. Curves of one experiment are shown, but the growth index and the combination effect were evaluated from the results of the two series of experiments.

Measurement of the nodule growth

Every 8 days, a two dimensional measurement of each nodule was done. The size(s) was expressed in mm^2 and the nodule growth was expressed as percentage S_t/S_0 where S_0 is the size before treatment and S_t is the size at time t during the treatment. The two experiments were done in duplicate. The mean growth percentage was calculated and the regression lines were determined and gave the growth rate (percentage per day). A Student's test, done for all the treated nodules, was used to compare the growth percentage of the treated and control nodules, all P values below 0.05 were considered to be significant (Table 1). The growth index was the ratio $\frac{\text{growth \% of IFN treated cells}}{\text{growth \% of control cells}}$ (Table 2). The analysis of the combined effects of the two IFNs was performed according to the definitions of Denz *et al.* [13].

RESULTS

To determine whether IFN-α2 and -γ could interact in an additive or synergistic way in our tumor model, the IFNs were applied separately and in combinations of A549 cells maintained in organotypic culture.

Figure 1(A) shows the effect of IFN-α2 used at a concentration of 1000 U/ml, alone or added with various concentrations of IFN-γ: 10, 100 and 1000 U/ml. The controls had a growth rate of 6.8 ± 0.5% per day; a treatment with 1000 U/ml of IFN-α2 had no effect up to the 28th day with a growth of 6.6 ± 0.8%, afterwards the nodules grew only 0.34 ± 0.22% per day, whereas treatment with the same dose of IFN-α2 plus 10, 100 and 1000 U/ml IFN-γ resulted respectively in growth rates of 6.42 ± 0.69, 2.95 ± 0.25 and 1.85 ± 0.6% per day, thus demonstrating a dose–effect phenomenon as growth inhibition was well correlated with the IFN-γ concentrations; on the contrary, when used alone at doses of 10 and 100 U/ml, IFN-γ gave a growth rate of 9.92 ± 0.97 and 7.26 ± 0.67% per day, this indicates a stimulation growth effect of the low doses, an effect specially marked with 10 U/ml (Table 1).

Moreover, the dramatic growth inhibition observed on the 35th day in the nodules treated with IFN-α2 1000 U/ml plus -γ 1000 U/ml was accompanied by the complete disappearance of the healthy alveolar structure leading to a total necrosis.

Figure 1(B) shows the results obtained with IFN-α2 or -γ used at a dose of 2000 U/ml and compared with the combination using 1000 U/ml of each IFN; at day 21, the growth rate per day was respectively 6.70 ± 0.89 and 5.38 ± 0.94 for IFN-α2 and -γ vs. 1.96 ± 0.33 for the nodules treated by the mixture, thus indicating an earlier effect of the combination; between the 21st and the 45th day, the growth rate was no more than 0.54 ± 0.24% for the IFN-α2 and 1.03 ± 0.24% for the

Table 1. Comparison of growth percentage of A549 nodules treated with IFN-α2 and/or IFN-γ

IFN (U/ml)	Time after treatment (days)	Growth (%/day)	*P*
1. *Fixed dose of IFN-α2, variable doses of IFN-γ*			
Control	0–45	6.8 ± 0.50	
α2 (1000)	0–28	6.6 ± 0.80	N.S.
	28–45	0.34 ± 0.22	< 0.001
α2 (2000)	0–21	6.70 ± 0.89	N.S.
	21–45	0.54 ± 0.15	< 0.001
γ (10)	0–45	9.92 ± 0.97	< 0.01
γ (100)	0–45	7.26 ± 0.67	N.S.
γ (1000)	0–45	2.54 ± 0.49	< 0.001
γ (2000)	0–21	5.38 ± 0.94	N.S.
	21–45	1.03 ± 0.24	< 0.001
α2 (1000) + γ (10)	0–45	6.42 ± 0.69	N.S.
α2 (1000) + γ (100)	0.45	2.95 ± 0.25	< 0.001
α2 (1000) + γ (1000)	0–28	2.47 ± 0.39	< 0.001
	28–45	Regression	
2. *Fixed dose of IFN-γ, variable doses of IFN-α2*			
Control	0–45	10.06 ± 1.23	
γ (1000)	0–45	5.55 ± 0.73	< 0.01
α2 (10)	0–45	8.49 ± 1.01	N.S.
α2 (100)	0–45	8.63 ± 0.77	N.S.
γ (1000) + α2 (10)	0–45	5.23 ± 0.44	< 0.001
γ (1000) + α2 (100)	0–45	2.17 ± 0.28	< 0.001
γ (1000) + α2 (1000)	0–28	2.45 ± 0.33	< 0.001
	28–45	Regression	

Table 2. Potentiation of IFN antiproliferative activity by mixing IFN-α2 and IFN-γ

IFN (U/ml)	Growth index*	Combination effect†
No IFN	1	
α2 (10)	0.84	
α2 (100)	0.85	
α2 (1000)	0.75	
α2 (2000)	0.75	
γ (10)	1.33	
γ (100)	1.01	
γ (1000)	0.55	
γ (2000)	0.58	
α2 (1000) + γ (10)	0.86	Additive
α2 (1000) + γ (100)	0.53	Synergistic
α2 (1000) + γ (1000)	0.15	Synergistic
γ (1000) + α2 (10)	0.48	Additive
γ (1000) + α2 (100)	0.15	Synergistic

*Ratio $\frac{\text{growth \% of IFN-treated cells}}{\text{growth \% of control cells}}$.

†Additive and synergistic effects of the combinations were evaluated according to the definitions of Denz *et al.* [13].

IFN-γ with complete regression of the nodules treated by the mixture.

Finally, the effects obtained using 1000 U/ml IFN-γ associated with 10 or 100 U/ml IFN-α2 are reported in Fig. 1(C), compared to those induced when IFNs were used separately at the same doses. In order to present a complete evaluation, 1000 U/ml of IFN-α2 were tested again when combined with 1000 U/ml of IFN-γ. In this experiment, the growth rate of the controls was of 10.06 ± 1.23 per day; no significant growth inhibition was observed in the nodules treated with 10 and 100 U/ml IFN-α2 with respectively a growth rate of 8.49 ± 1.01 and 8.63 ± 0.77% per day; the percentage growth of IFN-γ-treated nodules (1000 U/ml) was 5.55 ± 0.73% per day; this percentage decreased to 5.23 ± 0.44 and 2.17 ± 0.28 when IFN-γ was combined with 10 and 100 U/ml IFN-α2. In the group treated by 1000 U/ml IFN-γ plus 1000 U/ml IFN-α2, the growth rate was 2.45 ± 0.33% per day up to the 28th day, with regression between the 28th and the 45th day (Table 1).

The growth index was calculated for all series and the effects of the combinations were evaluated according to the definitions listed by Denz *et al.* [13]. These evaluations were made from the data obtained on day 35, considering the state of necrosis of the nodules treated with 1000 U/ml IFN-α2 plus 1000 U/ml IFN-γ. Additive or synergistic effects resulting from the use of mixtures of IFN-α2 and -γ are reported in Table 2.

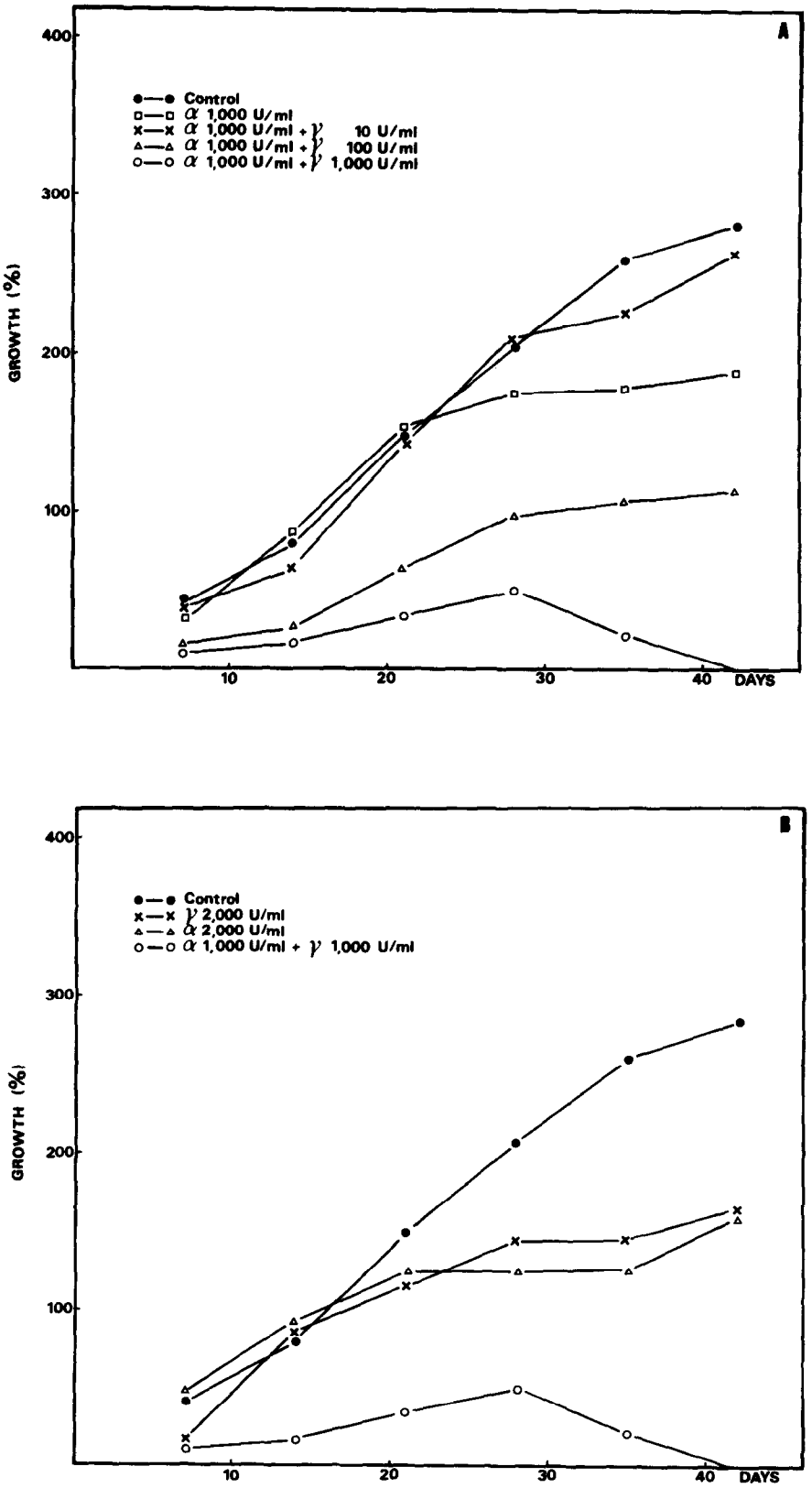

Fig. 1. Growth curves of A549 nodules treated for 45 days with IFN-α2 and -γ used at different doses, separately or in combination.

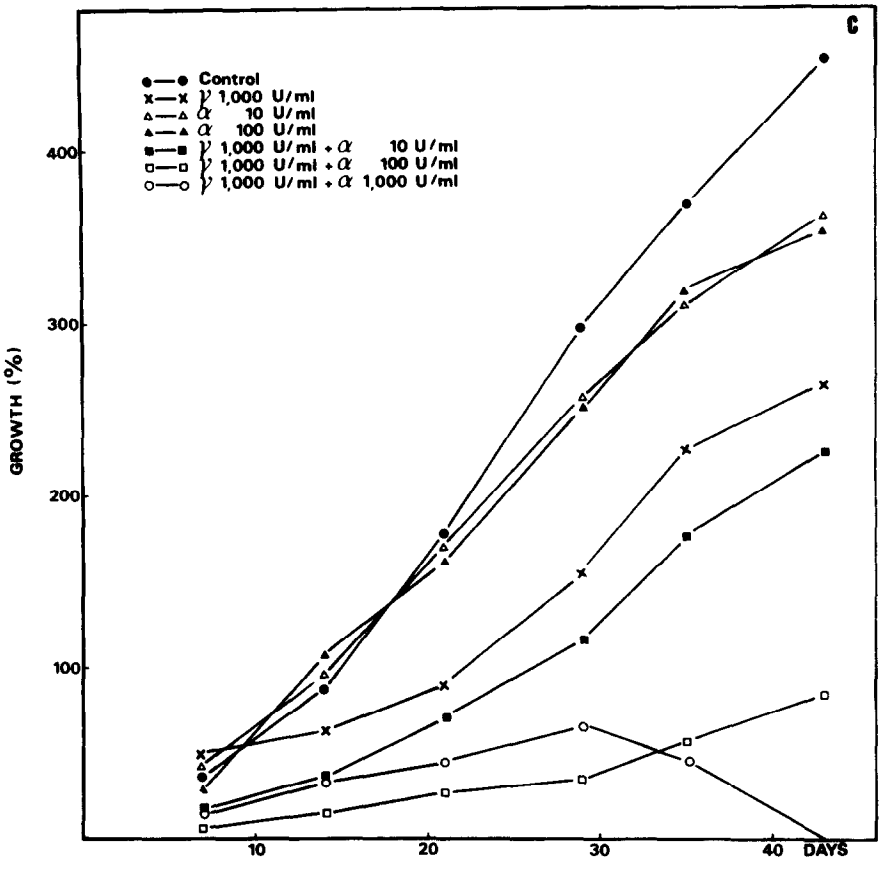

Fig. 1. (*cont.*)

DISCUSSION

The purpose of this study was to investigate whether combination of IFN-α2 and -γ resulted in the potentiation of the growth inhibition of A549 human lung carcinoma cells maintained in organotypic culture, an *in vitro* model which mimics the *in vivo* situation. Our results showed that such an association acts additively or synergistically on tumor growth inhibition, according to the doses used. In fact, the percentage of inhibition increases proportionally to the doses of IFN (-α2 or -γ) added to a fixed high dose of either IFN-α2 or γ (1000 U/ml); this was observed in the two combination schedules, thus indicating a close dose–effect correlation. Nevertheless, it should be emphasized that using 1000 U/ml IFN-γ plus 100 U/ml IFN-α2 resulted in a greater growth inhibition than the combination of 1000 U/ml IFN-α2 plus 100 U/ml IFN-γ; even the growth inhibition was equivalent to that observed using 1000 U/ml IFN-α2 plus 1000 U/ml IFN-γ; however, it should be noted that, in the latter case, a 35-day treatment resulted in complete growth inhibition and necrosis which led to the disaggregation of the nodules on the 45th day.

It should also be pointed out that the inhibition of tumor growth observed with 2000 U/ml of the mixture (1000 U/ml of each IFN) is considerably greater than that obtained with 2000 U/ml of IFN-α2 or -γ used separately, which indicates once again that the activities of these two types of IFN are complementary.

Lastly, the growth inhibition occurred much earlier when the IFNs were used in association than when used separately; this was observed in all cases except one, interestingly when 1000 U/ml IFN-α2 were combined with 10 U/ml IFN-γ which, when used alone, induced a stimulation of nodule growth.

Such a potentiation of the anticellular activity by mixtures of IFN-α2 or -β and -γ have been already described, in particular by Fleischmann *et al.* [14, 15], efficient doses varying according to the assay system and to the cell type [13, 16, 17]. However, until now, the methods for studying the antiproliferative effects of IFNs, used separately or in association, on cells in culture, were commonly monolayer, suspension culture or a soft agar cloning assay. The three-dimensional organotypic culture is, therefore, of interest for this kind of trials since this type of culture more accurately reflects cells *in vivo* and allows one to perform long-term treatment aproaching the clinical ones. This model was helpful to observe synergistic antiproliferative effect of mixtures of IFN-α2 and -γ on the growth of A549 cells

(a human lung carcinoma), an effect which was not detectable in a clonogenic assay or in monolayer culture [13].

Although experimental data dealing with the various effects that IFNs can have on different cell types are exhaustive, it is not yet possible to discern a basis for a common mode of action [18, 19]. It is now well admitted that IFN-α and -γ interact at the cell surface with distinct receptors; moreover it has been shown that they potentiate each other with respect to the induction of the 2′,5′-oligo(A) synthetase, a marker for IFN activity on proliferation [20]. Our data strengthen the idea according to which within the complex framework of mechanisms involved in the IFN-α2 and -γ antiproliferative and antitumoral activities, at least any are different but presumably somehow complementary. This is also suggested by the *in vivo* production of more than one kind of IFN (mixtures of the HuIFN-α with either HuIFN-γ or -β in different proportions) that has been shown to occur simultaneously [19].

The regeneration of tumor nodules after treatment with IFNs and the possibility of observing this clinically relevant phenomenon in the organotypic culture model [8] emphasize the interest of the nodule model in the approach of human malignant pathology; it provides a possibility for the setting up of the most efficient not only palliative but also curative therapeutic modalities.

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