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Effects of Human Recombinant Interferons-α_2, -β and -γ on Growth and Survival of Human Cancer Nodules Maintained in Continuous Organotypic Culture

R. BEAUPAIN,* C. BILLARD† and E. FALCOFF†

**Institut Curie — Section de Biologie, 26 rue d'Ulm, 75231 Paris Cedex 05, and †Institut Curie — Section de Biologie and INSERM U196, 26 rue d'Ulm, 75231 Paris Cedex 05, France*

Abstract—*Alveolar II pulmonary tumor cells (A549 cells) maintained in continuous tridimensional organotypic culture were used to test the effects of recombinant human interferons -α_2, -β and -γ on growth inhibition and survival of the tumor nodules. The organotypic culture method has several advantages: the three-dimensional structures of the cells as well as some cell differentiation are maintained and the extremely low traumatizing culture conditions offer injured cells the maximum chance of survival. A continuous treatment lasting* 65 days *(three weekly interferon changes) with* 10, 10^2, 10^3 *and* 10^4 U/ml *doses of the three interferons led to growth inhibition and necrosis only in the presence of the two highest doses* (10^3 *and* 10^4 U/ml) *of IFN-α_2 and -γ. IFN-β had no inhibitory effect. Some nodules, especially at the lower dose levels* (10^2 U/ml), *showed enhanced growth in presence of the three types of interferons. After stopping the treatments, all the necrotic and disintegrating nodules resumed growth. Growth of the recovered nodules was followed in the absence of interferon for another period of* 70 days. *The growth rate of IFN-β and -γ-treated nodules was similar to that of the controls, but was slowed down for the regenerated IFN-α_2-treated nodules. Hence, in A549 organotypic cancer nodules and under our experimental conditions, only high doses of IFN-α_2 and -γ appeared to have a partial cytolytic, but finally no tumoridical action; IFN-β was inactive. At the lower doses growth stimulation was found during the treatments with the three interferons.*

INTRODUCTION

INTERFERONS (IFNs) have an antitumor activity [1–4] but their antiproliferative effects may differ according to the type of cells treated and a great heterogeneity in the sensitivities of tumor cells has been reported in a number of *in vitro* and *in vivo* studies [5, 6]. In addition, IFNs were found to differ not only by their structure, physico-chemical and antigenic properties, but also in their various biological activities [2]. Therefore the direct comparison of the now-available highly purified human interferons of the three major types may contribute to a better understanding of their action in view of cancer therapy. In the present study we compared the effects of three recombinant human IFNs, IFN-α_2, -β and -γ, on A549 human lung carcinoma cells [7] maintained in organotypic culture [8]. The organotypic culture method, which derived originally from Wolff and Wolff's method [9], is a continuous three-dimensional culture system where tumor nodules are grown on top of a semi-solid medium and where no enzymatic dissociation procedures are used for subculturing. Moreover, growth evolution can be followed for long periods of time (2–3 months and even longer) without handling the nodules. Hence this culture method makes it possible to maintain injured cells *in situ* for several months after the different antitumoral treatments without touching them, giving them a maximal survival chance, which may mimic the *in vivo* situation. Another similarity with the *in vivo* situation is the presence of some cell differentiation in the nodules. The A549 nodules develop alveolar structures which are filled with mucus. This mucus appears to be the pulmonary surfactant [10]. It has been shown previously [8] that the organotypic nodules are more resistant to drug (*cis*-platinum) and X-ray treatments than the same cells maintained in monolayer or suspension culture, as in the case of the multicellular spheroids, indicating that the differences in response of the cells depend on their spacial configuration and on cell-to-cell contact [11]. Stimulation of organotypic nodule growth has been observed after treatment with misonidazole [10] and likewise it has been

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observed that besides their antitumoral activities, IFNs stimulate growth of malignant cells [12]. These later results were obtained with the tumor clonogenic assay [12].

Taking all these points together, it was interesting to compare the effects of three types of bacteria-produced human IFNs-α_2, -β and -γ on the growth of three-dimensional organized organotypic cancer nodules and to see whether both antitumoral and stimulating effects could be found in A549 nodules. Above all, comparison was made of the long-term effects of the IFNs over a time span of about 6 months.

MATERIALS AND METHODS

Organotypic culture

The method for nodule formation has been described elsewhere [8]. The A549 nodules, which originated from monolayer cells, derived from an alveolar II pulmonary adenocarcinoma [7]. The organotypic nodules were obtained by scraping cultured cells from the culture surface of plastic flasks (Falcon) followed by centrifugation at 2000 rpm (540 ***g***) during 20 min. The pellet was placed on top of a semi-solid culture medium in a Petri dish (Falcon 1006) which can be closed with a tight-fitting lid. The semi-solid medium was obtained by mixing 1% bacto agar (Difco) in distilled water in a 1:1 ratio with two fold-concentrated RPMI 1640 culture medium containing 20% heat-inactivated fetal bovine serum. The nodules were subcultured every 10 days by dividing them into two (or more) pieces with microchirurgical scissors and placing them on fresh medium previously prepared in the Petri dishes.

Interferons

Three highly purified *Escherichia coli*-derived human interferons were studied: IFN-α_2 obtained from Shering-Plough (1×10^8 U/mg protein), IFN-β, from Cetus (1.3×10^8 U/mg protein), and IFN-γ, from Biogen (2×10^7 U/mg). The three IFNs were found to be electrophoretically pure.

The doses of interferons in cell cultures were based on the antiviral titers, as determined by the cytopathic effect inhibition assay on human Wish cells, challenged with Vesicular stomatitis virus, as already described [13]. All three IFNs were assayed simultaneously, and the titers were standardized to the NIH human leukocyte interferon No GA 902 530.

IFN treatment of the nodules

In order to perform the IFN treatments, we modified the original organotypic culture method. At subculture, the nodules were transferred on Millipore filters (HABPO 2500, pores size 0.45 μm) which had previously been placed on the semi-solid agar medium. Five days later, when wound healing of the nodules was finished, the filters containing the nodules (having a healthy alveolar aspect, eight nodules per filter) were transferred to new Petri dishes without the semi-solid medium. Liquid medium (1.3 ml) was added with or without IFN and the media were changed three times per week. The IFN doses used were 10, 10^2, 10^3 and 10^4 U/ml. The nodules on the filter were individualized to enable the following of the growth or degradation of each individual nodule. Evolution of the nodules was observed for 65 days, without subculturing and without handling the nodules. For sterility purposes, the filters were placed in new Petri dishes every 14 days. Two series of experiments were done. Curves of one experiment are presented. In Tables 1 and 2 are presented the data of all the treated nodules of the two series.

Measurements of nodule growth

Two-dimensional nodule measurements (the greatest diameter and a second measurement at right angles to the first) were taken every 10–14 days. The 'size' (S) of the nodules was expressed in mm^2, S_0 being the nodule size before treatment and S_t the size at time t after the treatment. The nodule growth is given as a percentage S_t / S_0 of each individual nodule at the different times of measurement. Two filters each containing eight nodules were used for each dose of IFN. The mean growth percentage of the 16 nodules was calculated and regression lines were established. In order to establish whether the differences in slopes (growth %/day) of the regression lines of the treated nodules and those of the controls were significant or not, Student's t test was done for all the treated nodules (Tables 1 and 2). Individual nodules which apparently grew faster than the fastest growing control nodule were scored. The slope of their regression line was calculated and the χ^2 test was done with the data of the fastest growing control.

Culturing of the regeneration nodules

The healthy and outgrowing zones of regenerating nodules (presenting the same morphological characteristics as the original nodules) were excised and put on Millipore filters which had previously been placed on the semi-solid agar medium. This was done to reduce the destructive effects of the wavelets of the liquid medium especially on the very necrotic and brittle IFN-γ (10^4 U/ml dose)-treated nodules. The filters were transferred to a fresh medium every 10 days for 70 days. Nodule growth measurements were made as described above.

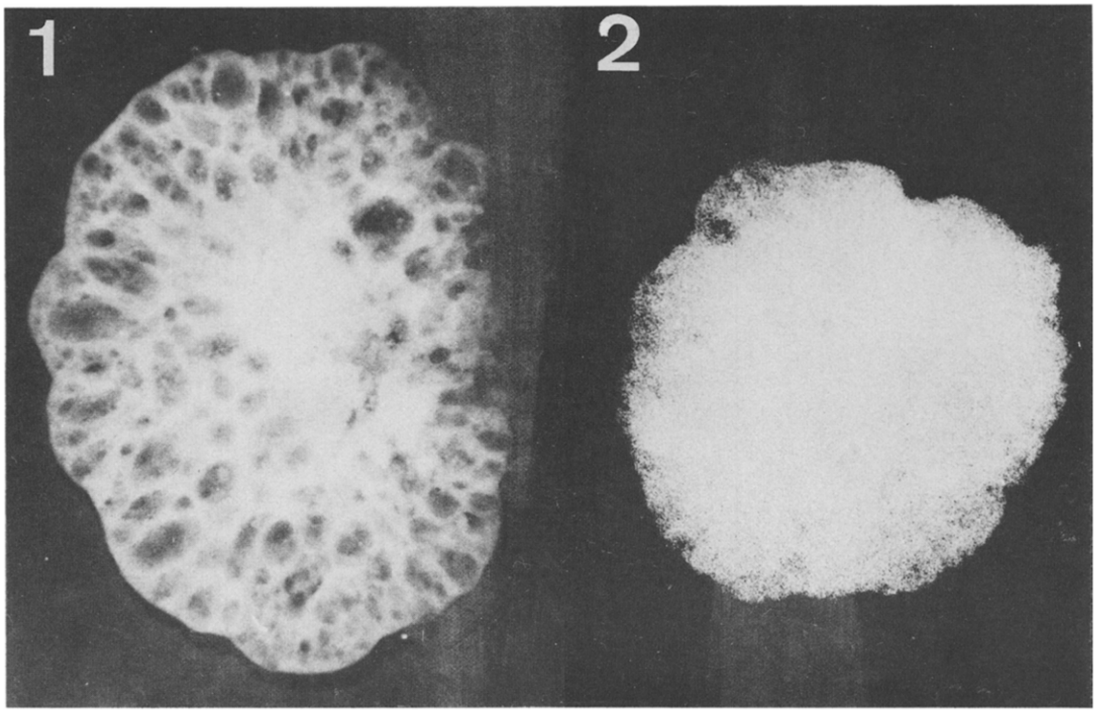

Fig. 1. *A typical healthy A549 nodule growing on a Millipore filter in RPMI* 1640 *medium supplemented with* 10% *fetal bovine serum, 'size'* 7.1 mm^2 (1 cm = 0.38 mm). *Note the translucent areas which are the alveoli* (× 26).

Fig. 2. *A549 nodule at the end of* 65 *days of IFN-*α_2 *treatment* (10^4 U/ml). *The alveoli have disappeared and the nodule has become necrotic. Nodule size* 4.4 mm^2 (1 cm = 0.38 mm) (× 26).

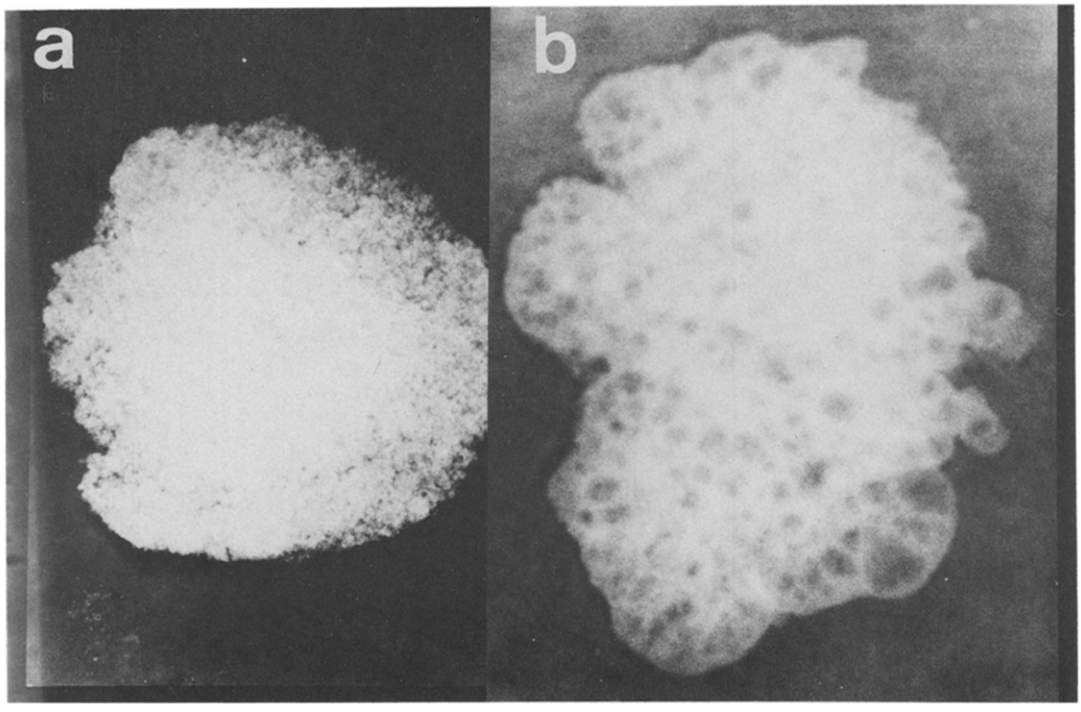

Fig. 5. *In A is shown a disintegrating A549 nodule after* 65 days *of treatment with* 10^4 U/ml *IFN-*γ. *Note the crumbling border of the nodule. The same nodule* 35 days *after stopping the treatment is shown in B. Healthy alveolar structures are present in outgrowing protrusions.*

Table 1. *Comparison of growth percentage of A549 nodules during the different IFN treatments*

IFN (U/ml)	Time (days) after treatment	Growth (%/day)	*P*
Control	0–65	5.62 ± 0.48	
α_2			
10	0–65	5.60 ± 0.48	N.S.
10^2	0–65	5.35 ± 0.35	N.S.
10^3	0–41	6.20 ± 0.41	N.S.
	41–65	0.66 ± 0.17	<0.001
10^4	0–55	2.98 ± 0.31	<0.001
	55–65	regression	
β			
10	0–65	5.21 ± 0.31	N.S.
10^2	0–65	5.57 ± 0.32	N.S.
10^3	0–65	5.09 ± 0.47	N.S.
10^4	0–65	4.95 ± 0.41	N.S.
γ			
10	0–65	5.61 ± 0.63	N.S.
10^2	0–65	5.92 ± 0.41	N.S.
10^3	0–65	3.73 ± 0.51	<0.02
10^4	0–55	2.88 ± 0.27	<0.001
	55–65	regression	

Values are mean ± S.D. of all treated nodules. NS, not significant. *P*, difference in growth of IFN treated nodules vs control.

Table 2. *Comparison of growth percentage of A549 nodules in the absence of IFN after the different IFN treatments*

IFN (U/ml/	Growth (%/day)	*P*
Control	6.06 ± 0.56	
α_2 10^2	5.06 ± 0.34	N.S.
α_2 10^4	4.16 ± 0.25	<0.05
β 10^2	6.31 ± 0.79	N.S.
β 10^4	5.40 ± 0.32	N.S.
γ 10^2	5.78 ± 0.33	N.S.
γ 10^4	5.15 ± 0.31	N.S.

Values are mean ± S.D. of all treated nodules. NS, not significant. *P*, difference in growth of IFN treated nodules vs control.

RESULTS

Figure 1 shows an untreated control A549 nodule of size 7.13 mm^2 (magnification × 26) and Fig. 2 a necrotic 10^4 U/ml IFN-γ-treated nodule 65 days after the beginning of the treatment. Nodule size in this case is 4.4 mm^2 (magnification × 26). Growth evolution of IFN-α_2-treated nodules is represented in Fig. 3. The controls had a growth rate of 5.62 ± 0.48% per day (Fig. 3A). During 10 and 10^2 U/ml IFN-α_2 treatment the growth rate was respectively 5.35 ± 0.35 and 5.52 ± 0.49% per day. Treatment with 10^3 U/ml IFN-α_2 had no effect up to the 41st day: the nodules grew 6.20 ± 0.41% per day. Afterwards growth was inhibited 8.5-fold and nodules grew only 0.66 ± 0.17% per day. Afterwards growth was inhibited 8.5-fold and nodules grew only 0.66 ± 0.17% per day. The nodules, however, remained healthy. A 10^4 U/ml dose of IFN-α_2 occasioned a 1.89-fold growth inhibition which was present from the beginning of the treatment onwards; nodules grew 2.98 ± 0.31% per day (Fig. 3A, Table 1). By the 55th day necrosis appeared in the nodules, and healthy alveolar structures remained present in only three of the 16 treated nodules.

IFN-γ had a similar effect on nodule growth as IFN-α_2 (Fig. 3B, Table 1). No growth inhibition

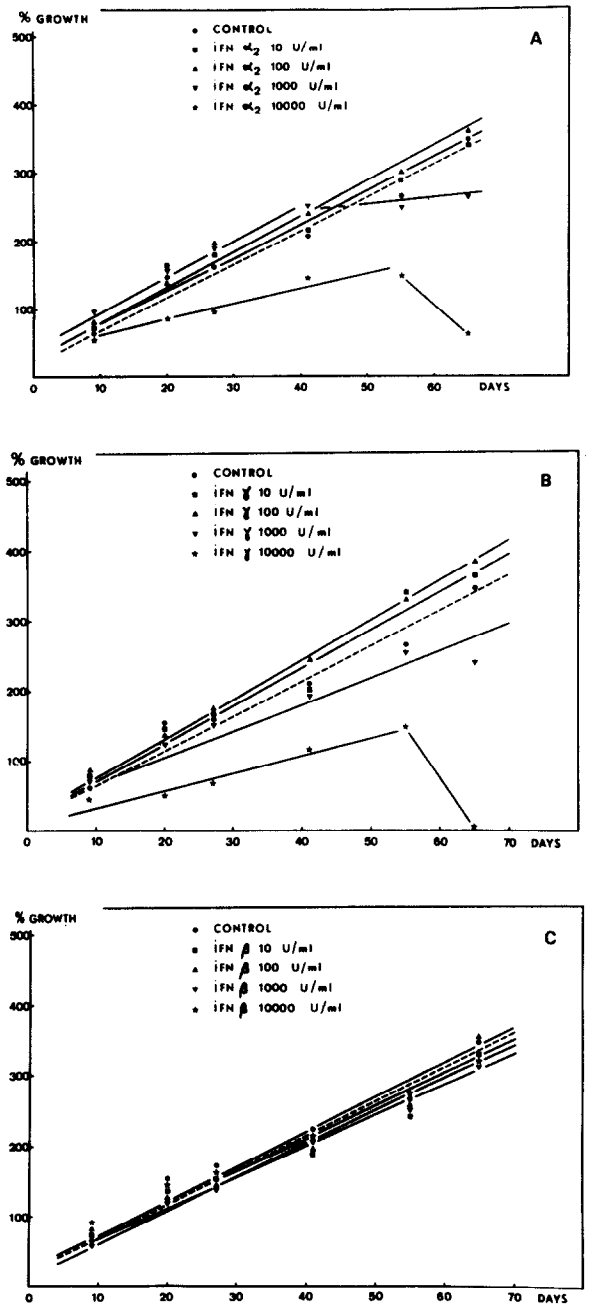

Fig. 3. *Growth curves of A549 nodules treated for* 65 days *with different doses of IFN-α_2 (A), IFN-γ (B) and IFN-β (C). Untreated controls (●···●), 10 (■), 10^2 (▲), 10^3 (▼) and 10^4 (★) U/ml.*

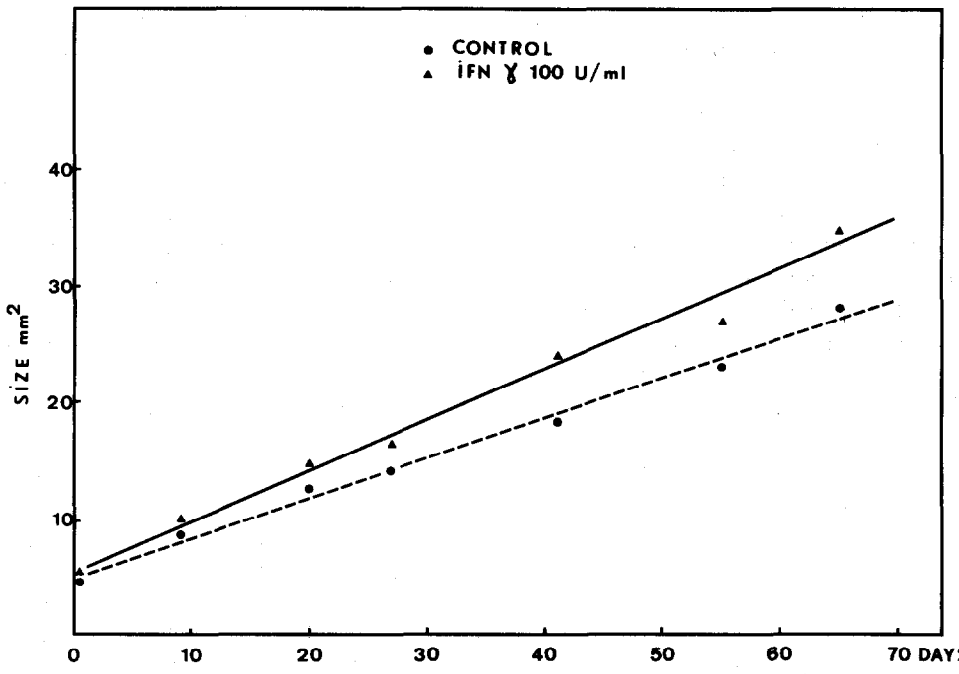

Fig. 4. Comparison of growth of the fastest IFN-γ (100 U/ml) treated nodule (▲) and the fastest growing control (●). Difference in growth rate is significant for $P < 0.01$ *from the 41st day on.*

was observed during treatment with low doses. Growth rates of the 10 and 10^2 U/ml doses were respectively 5.61 ± 0.63 and 5.92 ± 0.41% per day. Growth was slowed down 1.51 times when the nodules were maintained in the presence of the 10^3 U/ml dose and growth was 3.73 ± 0.51% per day. A greater effect was obtained with the 10^4 U/ml dose. A 2.30-fold growth inhibition was observed and all the nodules became highly necrotic from the 55th day of treatment onwards. The nodules tended then to disintegrate.

No effect was observed during a treatment with IFN-β (Fig. 3C, Table 1). The daily growth rates for the 10, 10^2, 10^3 and 10^4 U/ml doses were respectively 5.21 ± 0.31, 5.57 ± 0.32, 5.09 ± 0.47 and 4.95 ± 0.41% (Fig. 3C). The differences are not statistically significant for $P = 0.01$ as compared to untreated controls.

When studying the growth rates of the individual nodules, it became clear that some nodules grew faster than the fastest growing nodule of the untreated controls, and this occurred for the three IFNs used. An example of this phenomenon is shown in Fig. 4, where growth of an IFN-γ-treated nodule (10^2 U/ml) is compared with the fastest growing control. Taking together all the treated nodules, 16/192 (8%) showed enhanced growth (from 1 to 29% stimulation), but a statistically significant difference with the fastest growing untreated control nodule was found in two nodules treated with 10^2 U/ml IFN/α_2, one nodule treated with 10^2 U/ml and one with 10^3 U/ml IFN-β, and in three nodules grown in the presence of 10^2 U/ml IFN-γ.

The IFN treatments were stopped once the nodule necrosis was developing. In order to verify whether all the disintegrating nodules were dying, the filters containing the nodules were placed on top of a semi-solid medium to reduce the disintegrating effect of the wavelets of the liquid medium. Under these culture conditions all the necrotic nodules regenerated forming several extrusions of healthy tissue showing alveoli, as exemplified in Fig. 5, which shows the same nodule immediately after stopping the treatment (Fig. 5A) and 35 days later (Fig. 5B). These healthy regeneration zones of the IFN-α_2-, -β- and -γ-treated nodules at the 10^2 and 10^4 U/ml doses were excised, placed on new filters and their growth measured for another period of 70 days (Fig. 6). The controls, which were parts cut off from the original untreated control nodules of the experimental series, grew 6.13 ± 0.56% per day. The 10^2 and 10^4 U/ml IFN-α_2-treated nodules showed respectively a 1.23- and 1.43-fold growth inhibition, with growth percentages respectively of 4.97 ± 0.34 and 4.28 ± 0.25 per day (Fig. 6A, Table 2). Compared with the controls, growth rates of the IFN-β and -γ showed no significant difference (Fig. 6B and C, Table 2). The growth percentages for the 10^2 and 10^4 U/ml doses of IFN-β were respectively 6.31 ± 0.79 and 5.40 ± 0.32, and for IFN-γ the values were 5.78 ± 0.32 and 5.15 ± 0.31.

DISCUSSION

The opposite effects of IFNs, their antiproliferative and stimulatory actions, which have been reported in human tumor cells [1, 2, 12] were also

observed during treatment of cancer nodules maintained in organotypic culture. However, the three IFNs tested did not act in the same way. The cytostatic effect was observed using IFN-α_2 and -γ at relatively high doses (10^3–10^4 U/ml) compared with the serum peak levels of IFN found in IFN-treated patients [14], and growth inhibition of nodules during treatment with IFN in this low dose range (100 U/ml) did not occur even after long-term treatment. IFN-β had no inhibitory effect on A549 nodules after a 2-month treatment with doses as high as 10^4 U/ml. There was some central necrosis in IFN-β-treated nodules but only at the end of the IFN treatment. Central necrosis appeared also in the controls at this stage, nodule size having increased notably and exceeding 25 mm^2. Growth measurements were stopped at this point. The absence of activity was not the result of the inactivation of IFN-β during the time of contact with the nodules, as was the case elsewhere [6], since the biological activities of the three IFNs as measured by the persistence of antiviral activities remained unchanged between the successive medium renewals, and the presence of the Millipore filters did not cause a detectable loss of IFN activity either (data not shown). The lack of activity of IFN-β may be occasioned by the low level of IFN-β receptor found in A549 cells, which appeared to be lower than IFN-α_2 receptors [15], despite the close relationship of IFN-α_2 and -β binding sites [16, 17]. However, a difference in affinity cannot be excluded. The difference between the action of IFN-α_2 and -β may be situated at another level, for instance, at the induction of 2′-5′-oligo A synthetase activity, which has been suggested to be a biological marker of IFN action on proliferation [18], or at another subsequent step. Such possibilities have been investigated and the differences in effect on cell growth (both *in vitro* and *in vivo*) of these two IFNs have been reported [19–22]. Likewise, our data clearly indicate that human tumor cells which are resistant to IFN-β can be more sensitive to IFN-α_2 or IFN-γ. The fact that the effects of IFNs were observed only after long-term treatment (65 days) may exclude the possibility of a non-specific cytotoxic effect. The antitumor capacities of interferons can result from two possible mechanisms: (i) a direct cytostatic effect on tumor cells; and (ii) an indirect stimulation of the immune system [1]. Our biological system does not allow us to decide which of the two mechanisms is involved. The data, however, indicate that there is a direct effect of IFN on growth, although in the case of A549 nodules high doses are necessary.

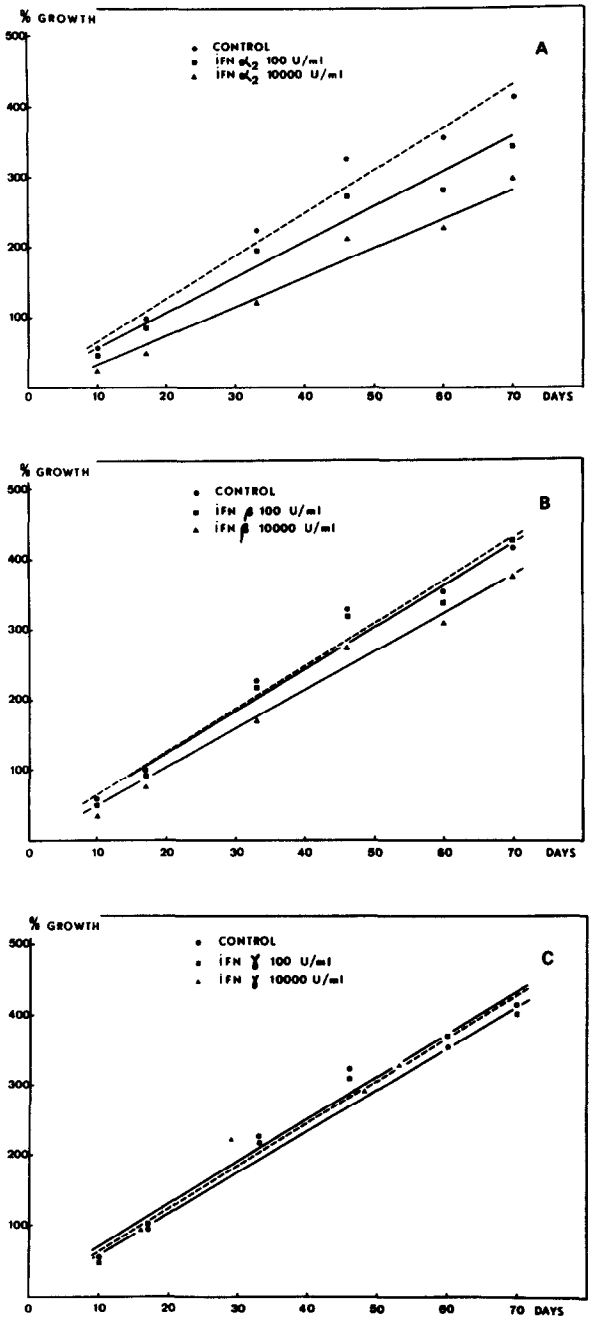

Fig. 6. Growth curves of regenerated A549 nodules after stopping the 65-day treatment with IFN-α_2 (A), IFN-β (B) and IFN-γ (C). Untreated controls (●_ _ _●), 10^2 U/ml (■) and 10^4 U/ml (▲).

Resumption of growth occurred even in the totally necrotic nodules which developed at the end of the treatment with the highest doses of IFN-α_2 and -γ. Contrary to regeneration in the *cis*-platinum-treated A549 nodules [8], where regrowth appeared as healthy clusters in the necrotic tissue, the regrowth after IFN treatment occurred all over the necrotic nodules, as can be seen in Fig. 5. This indicates that a network of living cells persists and that these cells develop rapidly when freed from IFN. This rapid development indicates that the surviving cells were not in the tumor dormant state [23], as was probably the case with the X-ray- or *cis*-platinum-treated nodules, where regrowth occurred 90 and 30 days respectively after the treatment [8]. In this respect, the most

active IFN tested had a different action on the A549 nodules than X-rays or *cis*-platinum.

Lung cancers are known to be resistant to *in vivo* IFN treatments [24, 25], and likewise in our tumor model system, A549 alveolar II lung cancer nodules actually resist. This resistance may be due to the three-dimensional organization of the A549 cells, as the A549 monolayer cells were about 20 times more sensitive to *cis*-platinum treatment [26] than the A549 nodules. Cell necrosis appeared not only at the border but also in the center of the nodules (Figs 2 and 5A), and this suggests that the IFNs penetrated all over the nodules, at least at the highest doses. For this well-characterized type of cancer the organotypic culture model may be a useful tool to attempt to ameliorate efficacy of IFN treatments in resistant cancers by combining IFN-α_2 with IFN-γ or IFNs with drugs.

Stimulation of nodule growth occurred during the treatment with the three IFNs. The dose levels which induced the stimulation were situated in the range of the IFN serum levels in patients treated with IFN [14]. Our observations are in agreement with data obtained with a human myeloma cell line using the human tumor clonogenic assays [12]. In accord with a remark made by these authors, it is clear that the possibility of such an undesirable effect of IFN-α_2, -β and -γ when using them clinically should be kept in mind even if it is difficult to extrapolate results obtained with cultured cells to the *in vivo* situation. There were, of course, differences in the growth rates of the controls of the two experimental series. However, the means of the growth percentages per day of the two series (respectively 5.7 ± 0.88 and 5.61 ± 0.48%) are not significantly different. The mechanism whereby this growth stimulation occurred is unknown. On the other hand, the antitumor effect of IFN-α_2 and -γ seems to result from the tumoristatic rather than the tumoricidal capacities of these interferons. Actually, a complete regeneration with reappearance of alveoli, mucus secretion and regrowth occurred 18 and 55 days after the end of the treatments with the IFN-α_2 and -γ respectively. It has to be noted, however, that once the IFN treatment stopped, the growth stimulation disappeared and the nodules which had been in contact with IFN-β and -γ showed a growth rate identical to that of the controls. Growth of the IFN-α_2 regenerated nodules was slowed down and the reduced growth rate continued during the 70 days of the test period. All the treated nodules, i.e. those which had been stimulated as well as those which were necrotic and which regenerated afterwards, are now routinely maintained in continuous organotypic culture and we are testing them for their sensitivity to renewed treatments with IFNs.

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