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IN VITRO EFFECTS OF RECOMBINANT HUMAN INTERFERON GAMMA ON HUMAN MESOTHELIOMA CELL LINES

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Malignant mesothelioma is a tumor arising from serous surfaces and often related to asbestos exposure. Malignant mesothelioma is resistant to various forms of therapy. Radiotherapy, surgery or chemotherapy only slightly improve prognosis. IFN-γ produces complete or partial responses in stage-I patients with malignant mesothelioma. The *in vitro* biological effect of IFN-γ on malignant mesothelioma cells remains poorly elucidated. In the present study, 32 well-characterized human mesothelioma cell lines (HMCL) were treated with r-hu IFN-γ at 4 doses and cell growth was determined by a colorimetric method (MTT assay). Among the 32 HMCLs tested, 11 exhibited significant cell-growth inhibition; 16 were insensitive to r-hu IFN-γ, and 5 were slightly inhibited. The sensitive cell lines were strongly inhibited by r-hu IFN-γ. Our results show that HMCL exhibit a large range of responses to r-hu IFN-γ, some of which can be compared with those obtained *in vivo* in humans.

Malignant mesothelioma is a tumor arising from serous surfaces. Pleural malignant mesothelioma is often related to asbestos exposure, but other etiological factors might also play a role in the development of mesothelioma (Peterson *et al.*, 1984). The incidence of pleural malignant mesothelioma is increasing by 13% per year in American men (Pisani *et al.*, 1988). Increased rates are also reported in Australia (Musk *et al.*, 1989). Five hundred to six hundred cases of mesothelioma were diagnosed from death certificates between 1979 and 1982 in France (Bignon *et al.*, 1990) and 200 to 300 cases are diagnosed yearly in the Netherlands (Van Gelder *et al.*, 1989). Two major problems are associated with mesothelioma: first, the diagnosis is difficult; second, the tumor is very resistant to chemotherapy and radiotherapy. Mesothelial differentiation of a pleural tumor is usually identified by standard histology and by immunohistochemistry on the basis of the co-expression of cytokeratin and vimentin, and the absence of reactivity with carcinoembryonic antigen (CEA) or Leu-M1 (Sheibani *et al.*, 1992). Ultrastructural characteristics are also useful to identify mesothelial tumor cells; intermediate filaments and cell junctions are found in mesothelial cells as well as in adenocarcinoma cells, but perinuclear microfilaments and long and flexible microvilli are specific for mesothelial cells (Wang, 1985).

So far, mesothelioma has responded poorly to various sorts of therapy. Radiotherapy, surgery or chemotherapy have only slightly improved the prognosis (Pisani *et al.*, 1988). Cytokines such as IFN-β have been used without improvement of the outlook (Von Hoff *et al.*, 1990). However, a combination of IFN-α and chemical drugs seems to exert inhibition of tumor growth when using in *in vivo* models of transplantation into nude mice (Sklarin *et al.*, 1988); moreover, malignant mesothelioma cells resistant to natural-killer (NK) cells could be killed by the lymphokine-activated killer (LAK) cells *in vitro* (Manning *et al.*, 1989). IFN-γ has been found to produce complete or partial responses in stage-I patients with malignant mesothelioma (Boutin *et al.*, 1991). These results suggest that cytokine treatment could be useful to treat mesothelioma and that further studies should be conducted in order to more clearly determine the effect of cytokines on mesothelial cells and on mesothelioma.

For several years our laboratory has developed cultures of rat pleural mesothelial cells (Jaurand *et al.*, 1981). We have collected human mesothelioma cases to characterize mesothelioma cells; so far, we have obtained 32 mesothelioma cell lines from 22 mesothelioma cases. It was therefore of great interest to use these cell lines to study the *in vitro* effects of molecules used as anti-tumor agents in order to investigate their antiproliferative action.

The aim of the study was to investigate the *in vitro* effect of r-hu IFN-γ on the proliferation of well-characterized mesothelioma cell lines. Characterization was performed by cytology and immunocytochemistry. The tumorigenesis of the cell lines was also investigated after s.c. inoculation in nude mice. The sensitivity of r-hu IFN-γ was determined by measurement of cell proliferation by a colorimetric assay using MTT [3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide], a tetrazolium salt which is reduced to a colored formazan product by reducing enzymes present only in metabolically active cells (Alley *et al.*, 1988).

MATERIAL AND METHODS

Source of tumors

Specimens were obtained from 22 patients with pleural malignant mesothelioma, from thoracoscopic or surgical biopsy material and/or from pleural effusions. Two mesotheliomas with a peritoneal location were studied; samples were peritoneal (case 1) and pleural and peritoneal fluids (case 8). All cases were diagnosed as malignant mesothelioma, according to histopathological characterizations, including biphasic differentiation, formation of tubular and papillary structures, and sarcomatoid changes. Complementary immunohistochemistry was carried out using commercially available monoclonal antibodies (MAbs) raised against cytokeratin, carcinoembryonic antigen and vimentin. Some specimens were examined by electron-microscopy analysis for detection of characteristic microvilli, intermediate filaments, and cell junctions. All cases were confirmed by the French mesothelioma panel of pathologists.

Development and culture of mesothelioma cell lines

Cell cultures were performed according to standard methods. Briefly, mesothelioma samples were minced with a scalpel into pieces of less than 0.5 mm^3, then transferred to a tissue-culture dish for adhesion. The medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 mM HEPES buffer, 50 U/ml penicillin and 50 μg/ml streptomycin. Pleural effusions were spun at 300 *g* for 10 min and medium was added to the cell pellet, which was then transferred to a 25-cm^2 tissue-culture flask at a concentration of about 10^6 cells/ml. If necessary, 0.87% of ammonium

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chloride was used to lyse the erythrocytes prior to centrifugation. Cultures were examined at regular intervals with a phase-contrast microscope to detect cell clusters or monolayer growth. In some cases, cells were plated at low density to allow colony formation. When colonies exhibited different morphological features, they were sub-cultured independently after selection by trypsination using hollow cylinders. When the cells were confluent, a trypsin-EDTA mixture (0.25% trypsin, 0.02% EDTA in PBS) was used to detach the cells. Cells then were seeded at the concentration of 5×10^5 cells per 25-cm^2 flask, and maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells were established by sub-culturing approximately every 2 weeks, depending on the cell line. The cultures were used between passages 1 and 15.

TABLE I – CLINICAL AND CELL-LINE DATA OF 22 PATIENTS

Case	Sex	Age	Asbestos exposure	Histological type[1]	Cell line	Cell-line morphology
1	M	65	Possible	E	7	E
2	F	87	Yes	E	17	E
3	M	59	No[2]	E	23	E
4	M	71	Yes	E	21	E
5	M	79	Yes	E	22	E
6	M	65	Unknown	E	29	E
					30	E
7	M	78	Yes	E	6	M
8	M	47	No	M	8	S
					5	M
					16	E
					9	E
9	M	68	No	E	31	E
10	M	66	Yes	E	2	E
11	M	68	No	E	32	E
12	F	65	Yes	E	25	E
13	M	60	Unknown	E	13	E
14	M	65	Yes	E	4	M
15	M	70	No	M	12	S
16	M	71	Yes	E	15	E
					20	E
					19	E
17	M	66	Yes	E	18	E
18	F	64	No	E	11	S
19	M	49	No	E	24	E
					26	E
					27	E
20	M	70	Yes	E	28	E
21	M	69	Yes	E	3	E
					1	E
					14	E
22	M	65	Possible	E	10	E

[1]E = epithelial; S = sarcomatous; M = mixed.–[2]No exposure detected.

Immunocytochemistry

Commercially available primary MAbs raised against different antigens were used. Anti-cytokeratin (56 kDa) was purchased from Immunotech (Marseille, France), carcinoembryonic antigen (CEA) and vimentin from Dako (Copenhagen, Denmark). To perform immunocytochemistry, cells were cultured in plastic tissue-culture chamber slides (Labtek, Corsico Milano, Italy, 8 chambers). Sub-confluent cells were washed with PBS, air dried for 6 hr at room temperature, and then fixed in cold acetone (4°C) for 10 min. Subsequently, an alkaline-phosphatase anti-alkaline-phosphatase (APAAP) technique was used to identify the antigens. Tris buffer was substituted for the primary antibodies in the negative control.

Test substances

Recombinant human interferon-gamma (r-hu IFN-γ) was obtained from Roussel UCLAF (Romainville, France), and had a specific activity 2×10^7 units/mg protein and a purity of at least 95% based on analysis by SDS-PAGE. MTT [3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] was purchased from Sigma (St. Louis, MO, M2128). It was dissolved at a concentration of 2 mg/ml in sterile PBS, and stored at 4°C for less than one week.

Cell-growth-inhibition assay

Mesothelioma cells were counted (Coulter Counter ZM, Margency, France) and dispensed into 96-well tissue-culture plates (Costar, Cambridge, MA 81451) at the concentration of 2,000–4,000 cells/well in 100 μl of culture medium (RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 10 mM HEPES buffer, 50 U/ml penicillin and 50 μg/ml streptomycin). Following a 24-hr incubation at 37°C, 5% CO_2, 100% relative humidity, 100 μl of culture medium containing r-hu IFN-γ at the concentrations of 0, 10, 10^2, 10^3 and 10^4 units/ml were dispensed into 8 wells for each concentration group. Culture plates were then incubated for different times until 120 hr and cell proliferation was measured every 24 hr. Medium was renewed after 72 hr of incubation with the same concentration of r-hu IFN-γ. The formazan reduced by living cells was measured every 24 hr to assess the cell growth, according to the method described by Alley *et al.* (1988); 25 μl of MTT solution were added to 5 of the 8 culture wells for each concentration and were referred to as "wells with MTT"; the remaining 3 culture wells were referred to as "wells without MTT". The cultures were incubated at 37°C for 3 hr. Following

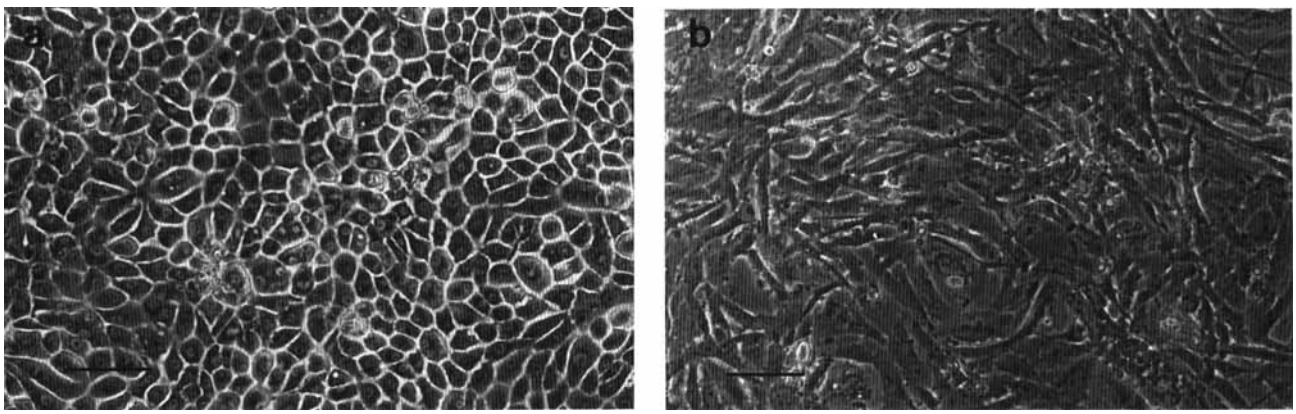

FIGURE 1 – Micrographs of HMCL in phase-contrast microscopy. Epithelial cells *(a)*, spindle cells *(b)*; bar = 100 μm.

TABLE II – KINETICS OF OPTICAL DENSITY AND GROWTH INHIBITION (GI) OF 32 MESOTHELIOMA CELL LINES IN THE PRESENCE OF SEVERAL CONCENTRATIONS OF r-hu IFN-γ

Dose (U/ml)		Incubation time with r-hu IFN-γ (hr)				
		24	48	72	96	120
0	O.D.[1]	0.34 ± 0.12	0.45 ± 0.16	0.56 ± 0.18	0.65 ± 0.24	0.71 ± 0.25
	GI[2]	0	0	0	0	0
10	O.D.	0.31 ± 0.12	0.40 ± 0.15	0.46 ± 0.16	0.54 ± 0.25	0.55 ± 0.23
	GI	8 ± 8	11 ± 9	16 ± 11	17 ± 13	23 ± 13
10^2	O.D.	0.30 ± 0.11	0.38 ± 0.15	0.39 ± 0.16	0.44 ± 0.20	0.41 ± 0.19
	GI	12 ± 10	16 ± 13	27 ± 18	30 ± 21	39 ± 22
10^3	O.D.	0.30 ± 0.11	0.35 ± 0.14	0.36 ± 0.16	0.40 ± 0.20	0.37 ± 0.20
	GI	11 ± 9	20 ± 15	32 ± 20	35 ± 24	44 ± 26
10^4	O.D.	0.29 ± 0.11	0.35 ± 0.14	0.35 ± 0.16	0.37 ± 0.19	0.34 ± 0.21
	GI	14 ± 11	21 ± 16	34 ± 22	40 ± 23	50 ± 27

[1]Optical density: mean value of optical density ± standard deviation on 32 cell lines.–[2]GI(%): 100-percentage of optical density in the treated culture in comparison with untreated culture (mean ± SD on 32 cell lines).

incubation, the culture medium was removed by careful aspiration through a Pasteur pipette and replaced with 200 μl of DMSO to solubilize the formazan. Formazan solubilization was achieved by using a plate shaker for 10 min, and the absorption of each well was measured using a spectrophotometer (Multiskan, Flow, McLean, VA MCC/340) at 540 nm. Subsequently, data were stored and analyzed using Cricket Graph Software. Cell growth (G) was expressed in terms of mean optical density ± SD by the difference between optical density of wells with MTT and the mean optical density of wells without MTT.

Effect of r-hu IFN-γ on cell viability

The effect of r-hu IFN-γ on cell viability was assessed by determining the reversibility of its anti-proliferative action and by measurement of cell viability on non-proliferating cells. Cells were incubated with r-hu IFN-γ at the same doses as described above for 72 hr, then medium with r-hu IFN-γ was removed and replaced with r-hu IFN-γ-free medium; and cell incubation was continued until 216 hr. Cell proliferation was measured every 24 hr by the method described above.

Mesothelioma cells sensitive to r-hu IFN-γ were cultured as described above until confluence, when the medium was replaced by medium containing r-hu IFN-γ at the concentrations of 0, 10, 10^2, 10^3 and 10^4 unit/ml. Viability was measured after 48, 72 and 96 hr of incubation using the MTT assay.

Tumorigenicity

Some cell lines were inoculated into nude mice to assess the tumorigenic potency. Three million mesothelioma cells in 0.2 ml of PBS were inoculated s.c. into athymic mice (IFFA, CREDO, L'Arbresle, France) to observe tumor formation. Ten mice were inoculated per cell line. Mice were observed weekly until the appearance of tumors. Tumors were measured twice weekly and their volume was calculated. All mice were killed at the 20th week.

RESULTS

Morphological features of mesothelioma cell lines

Data on mesothelioma cases are reported in Table I. A total of 32 HMCL has been studied. Microscopically, 26 cell lines showed an epithelial appearance (Fig. 1*a*); 98% of the cells presented abundant cytoplasm, central nuclei, some with numerous nucleoli; 3 lines presented the spindle-shaped pattern (Fig. 1*b*), and 3 lines had a mixed appearance, with mostly epithelial cells and a few spindle cells. Multinucleate cells and mitotic figures frequently occurred in all mesothelioma cell lines. No differential characteristics could be discerned between peritoneal and pleural origin of the cells.

TABLE III – GROWTH INHIBITION OF 32 MESOTHELIOMA CELL LINES IN THE PRESENCE OF SEVERAL CONCENTRATIONS OF r-hu IFN-γ FOR 96 HR INCUBATION (PERCENTAGE ± SD)

Cell line	Response[1]	Concentration of r-hu IFN-γ		
		10 U/ml	10^2 U/ml	10^3 U/ml
1	S	49 ± 11	70 ± 10	79 ± 8
2	R	4 ± 6	9 ± 2	12 ± 6
3	S	6 ± 7	55 ± 5	68 ± 5
4	S	20 ± 10	47 ± 8	58 ± 10
5	S	8 ± 5	39 ± 6	50 ± 2
6	I	12 ± 9	35 ± 3	38 ± 9
7	R	15 ± 5	13 ± 7	23 ± 8
8	R	12 ± 5	13 ± 2	18 ± 1
9	I	22 ± 5	28 ± 5	37 ± 5
10	I	30 ± 5	37 ± 8	38 ± 5
11	R	2 ± 7	25 ± 6	25 ± 5
12	R	12 ± 3	24 ± 3	24 ± 2
13	R	10 ± 7	13 ± 5	15 ± 5
14	S	56 ± 7	85 ± 5	91 ± 2
15	S	29 ± 1	47 ± 1	57 ± 2
16	R	14 ± 4	27 ± 5	27 ± 8
17	S	20 ± 6	41 ± 7	46 ± 4
18	R	21 ± 4	22 ± 7	27 ± 4
19	I	28 ± 5	35 ± 3	38 ± 3
20	S	27 ± 9	72 ± 2	81 ± 2
21	R	20 ± 13	22 ± 8	28 ± 12
22	R	5 ± 3	0 ± 9	2 ± 8
23	R	15 ± 5	15 ± 6	23 ± 9
24	I	32 ± 3	32 ± 1	35 ± 3
25	R	16 ± 4	14 ± 1	16 ± 2
26	R	0 ± 6	19 ± 6	24 ± 5
27	S	34 ± 3	35 ± 2	40 ± 3
28	S	18 ± 5	49 ± 3	59 ± 1
29	R	13 ± 6	11 ± 2	11 ± 4
30	R	6 ± 11	7 ± 7	4 ± 4
31	R	12 ± 9	19 ± 6	22 ± 6
32	S	23 ± 15	40 ± 17	58 ± 12

[1]S, sensitive cell lines; I, intermediate; R, insensitive.

Characterization of mesothelioma cell lines

All 32 cell lines used in the present study had the immunocytological characteristics of mesothelial cells, *i.e.,* co-expression of cytokeratin and vimentin and negative reactivity with antibodies to CEA. Only 15 cell lines were inoculated into nude mice; 10 (60%) formed tumors. The interval between inoculation and tumor formation ranged between 1 and 14 weeks. The tumors grew progressively.

Inhibitory effect of r-hu IFN- γ on growth of mesothelioma cell lines

The mean value of optical density representing the cell growth of all 32 cell lines used in the present experiment is reported in Table II. Mesothelioma cell lines exhibited a large

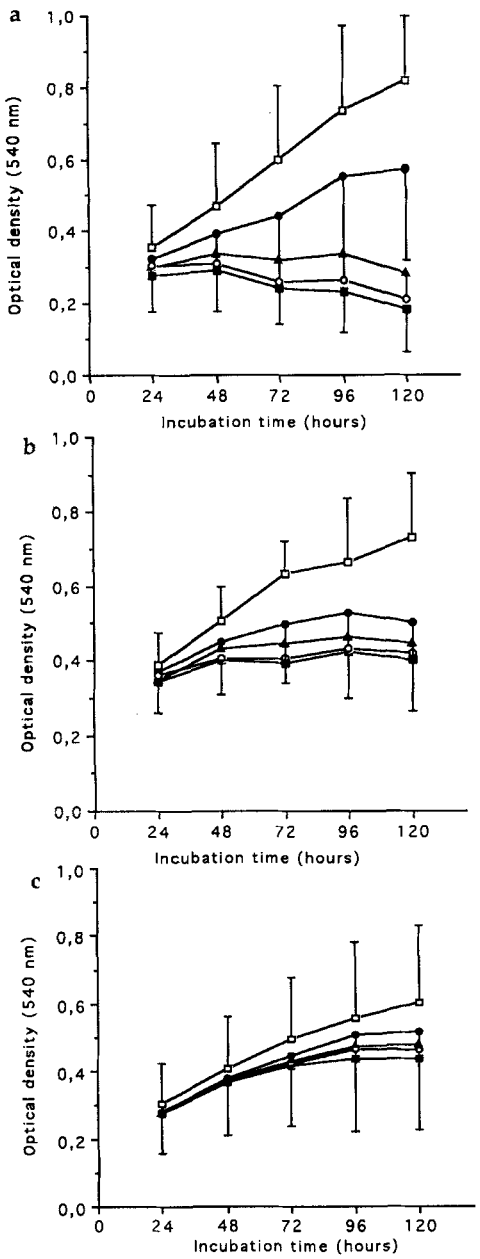

FIGURE 2 – Effect of r-hu IFN-γ on cell growth of mesothelioma cell lines, assayed by the induction of MTT in formazan as described in "Material and methods". Mean optical density ± standard deviation in the presence of different concentrations of r-hu IFN-γ. *(a)* 11 HMCL classified as sensitive to r-hu IFN-γ; *(b)* 5 intermediate-sensitive HMCL; *(c)* 16 insensitive HMCL. □ control (not treated by r-hu IFN-γ); ● 10 U/ml; ▲ 10^2 U/ml; o 10^3 U/ml; ■ 10^4 U/ml.

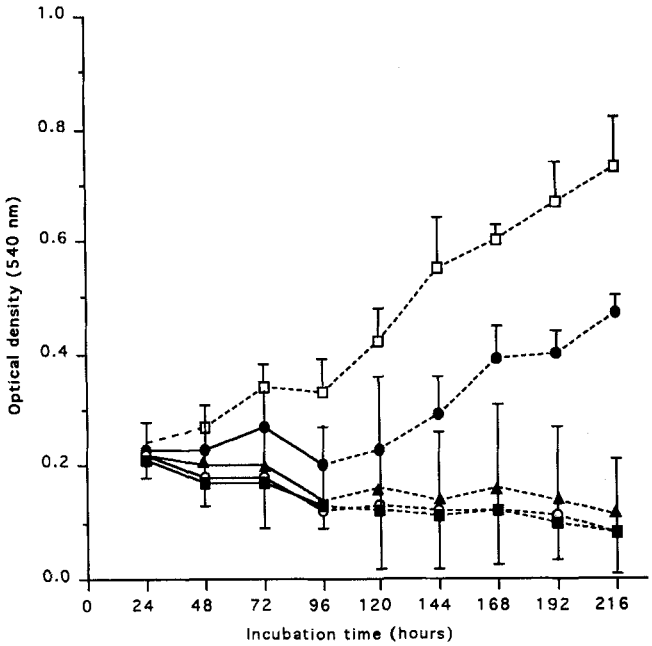

FIGURE 3 – Effect of removing r-hu IFN-γ on proliferation of mesothelioma cell lines sensitive to r-hu IFN-γ. Each point represents mean ± SD of 3 HMCL. Cell proliferation re-started after removal of r-hu IFN-γ at the concentration 10 U/ml, while cell growth was inhibited at the other concentrations, suggesting a certain cytotoxicity of r-hu IFN-γ. □ control (not treated by r-hu IFN-γ); ● 10 U/ml; ▲ 10^2 U/ml; o 10^3 U/ml; ■ 10^4 U/ml; -------- without r-hu IFN-γ.

range of sensitivity to r-hu IFN-γ. To compare the effect of r-hu IFN-γ between the different cell lines, the growth inhibition of each cell line by r-hu IFN-γ at 96 hr is reported in Table III, which represents the beginning of the plateau phase of cell growth in untreated cells. The extent of growth inhibition depends on the cell line. With 10^2 U/ml, values between 85% and 0% were obtained. In order to classify our results, we considered that our data could correspond to 3 classes of sensitivity to r-hu IFN-γ: sensitive, intermediate and insensitive. If we define "sensitive" as a reduction of cell growth compared with the control value of more than 30% with 10^2 U/ml associated with 40% with 10^3 U/ml after 96 hr of incubation, 11 out of 32 cell lines (34.4%) were found to be sensitive, 16 cell lines (50%) were insensitive (no cell-growth inhibition more than 30% of the control value at any concentration) and 5 cell lines were intermediate, exhibiting an optical density reduced by 20% to 30% and 30% to 40% with 10^2 U/ml and 10^3 U/ml, respectively. Figure 2*a* shows the growth pattern of the 11 mesothelioma cell lines sensitive to r-hu IFN-γ. After 48 hr of incubation with r-hu IFN-γ, cell growth was reduced, in comparison with untreated cells, in a dose-dependent manner and the rate of inhibition increased with the incubation time. Figure 2*b,c* expresses the results obtained with 5 intermediate and 16 insensitive cell lines. Other experiments were performed to determine whether the effect of r-hu IFN-γ is related to cell death and/or to arrest of cell growth. Figure 3 shows that cell proliferation restarts after removing r-hu IFN-γ only when low doses of r-hu IFN-γ have been applied to the cells, as higher doses seemed to exert a certain cytotoxicity.

Effect of r-hu IFN-γ on cell viability

The effect was determined on mesothelioma cell lines sensitive to r-hu IFN-γ. With growing cells, the inhibitory effect of low doses of r-hu IFN-γ on cell proliferation was reversible and could be suppressed by the removal of r-hu IFN-γ (Fig. 3). With non-proliferative cells, r-hu IFN-γ did not reduce cell viability at doses of 10 U/ml and 10^2 U/ml after 96 and 72 hr of incubation respectively. However, with the highest doses, r-hu IFN-γ exerted some cytotoxicity (Table IV).

Tumorigenicity in nude mice

The comparison between sensitivity to r-hu IFN-γ and tumorigenicity is reported in Table V. No correlation was

TABLE IV – VIABILITY[1] OF NON-PROLIFERATING MESOTHELIOMA CELL LINE TREATED WITH r-hu IFN-γ

Incubation time (hr)	Interferon-γ concentration (U/ml)				
	0	10	100	1000	10000
48	100 ± 0	100 ± 6.2	100 ± 9.0	100 ± 5.2	93.3 ± 6.0
72	100 ± 0	100 ± 3.0	100 ± 3.3	93 ± 6.5	91.5 ± 16.0
96	100 ± 0	100 ± 3.6	92.4 ± 3.8	74.7 ± 2.2	64.5 ± 1.5

[1](Optical density in r-hu-IFN-γ-treated cells/optical density in r-hu-IFN-γ-untreated cells) × 100 mean ± SD of 5 wells for each dose.

TABLE V – COMPARISON BETWEEN SENSITIVITY TO r-hu IFN-γ AND TUMORIGENICITY IN NUDE MICE

Tumorigenicity	Sensitivity to r-hu IFN-γ[1]			
	Sensitive	Intermediate	Insensitive	Total
+	4 (40)	2 (20)	4 (40)	10
–	2 (40)	1 (20)	2 (40)	5

[1]Number of cell lines, and, in parentheses, percentage.

found between r-hu IFN-γ sensitivity and ability to grow in nude mice.

DISCUSSION

We present evidence that r-hu IFN-γ can affect the proliferation of human mesothelioma cells *in vitro*. IFN-γ has been shown to have a potent direct anti-tumor activity, and has been used clinically to treat patients with certain types of tumors including pleural mesothelioma. Several *in vitro* experiments have demonstrated that r-hu IFN-γ exerts a direct effect on different tumor cell lines, for example, human ovarian carcinoma, melanoma, cervical carcinoma, breast carcinoma, renal-cell carcinoma, endometrial carcinoma and colon carcinoma (Pfizenmaier *et al.*, 1985; Saito *et al.*, 1986). Mesothelioma cells obtained from different donors are characterized by a great variability in several aspects: morphology (Jaurand *et al.*, 1993), karyotype (Hagemeijer *et al.*, 1990) and production of growth factors (Gerwin *et al.*, 1987; Demetri *et al.*, 1989). Moreover, mesothelial cells respond to a broad range of growth factors and individual variations have been observed (Lechner *et al.*, 1989). Therefore, a great number of well-characterized mesothelioma cell lines should be utilized to determine the effect of a given agent. So far, the *in vitro* effects of IFN-γ on 3 and 5 human mesothelioma cell lines have been reported (Hand *et al.*, 1991; Bowman *et al.*, 1991). Hand *et al.* (1991) found one mesothelioma cell line established from a metastatic tumor to be sensitive to IFN-γ. Bowman *et al.* (1991) found that all 5 human mesothelioma cell lines studied displayed varying degrees of sensitivity to interferons, including IFN-γ.

In the present experiment, we investigated 32 human mesothelioma cell lines originated from 22 patients with malignant mesothelioma. We found a reduction of cell growth in 16 cell lines (50%) in the cell lines studied, but 11 cell lines (34.4%) showed high sensitivity to r-hu IFN-γ, with obvious growth inhibition (Fig. 2*a*). In contrast, some other cell lines did not demonstrate strong (Fig. 2*c*) or intermediate sensitivity to r-hu IFN-γ (Fig. 2*b*).

The reduction of cell growth with low doses might be due to impairment of cell proliferation and not to cytotoxicity; first, the inhibitory effect of r-hu IFN-γ was reversible; second, no decrease of cell viability was detected with r-hu IFN-γ concentrations of up to 10^2 U/ml.

Our *in vitro* data can be compared with the results obtained in human therapy based on use of cytokines. Boutin *et al.* (1991) observed that 6 (32%) of 19 patients with malignant pleural mesothelioma demonstrated total or partial response to intrapleural administration of r-hu IFN-γ. In a phase-II study of r-hu IFN-α therapy, only 15% of patients showed partial response and 30% had stable disease (Christmas *et al.*, 1990). Other cytokines have been tested; recombinant human α-interferon-2a (Roferon-A) produced partial reduction in tumor bulk in 3 out of 25 patients with malignant mesothelioma, and stable disease in 12 patients (Robinson *et al.*, 1993). A phase I–II study of intrapleural IL-2 in 10 patients with malignant pleural mesothelioma showed partial response in 3 cases (33%) (Stoter *et al.*, 1990). Compared with *in vivo* observations, these results agree with the response to r-hu IFN-γ in patients with malignant pleural mesothelioma, with only a few total or partial responses; however, *in vivo,* other events related to the immunological response may account for the effect of r-hu IFN-γ.

It is interesting to note that different cell lines obtained from one patient exhibited different sensitivities to r-hu IFN-γ. However, these cell lines were obtained from the same material (except lines 9 and 16, which were ascites, while 5 and 8 came from the pleura). This may be explained by the peculiarities of mesothelioma cells, *i.e.*, polyclonal constitution of the tumor resulting from the diffuse pattern of the tumor and from the polyclonal progression of the tumor cells.

The reasons for different responses of the different cell lines to r-hu IFN-γ are not clear, and further research is necessary to investigate the mechanisms of action of r-hu IFN-γ. In humans, only Stage-IA mesothelioma cases responded to r-hu IFN-γ (Boutin *et al.*, 1991), while later stages did not. Mesothelioma cells from later stages may be in a more advanced phase of neoplastic transformation than early stages and may be more tumorigenic in nude mice. The inoculation into nude mice of several cell lines of different sensitivities did not result in a correlation between IFN-γ sensitivity and tumorigenicity, suggesting that poor *in vitro* response to r-hu IFN-γ is not a specific feature of tumorigenic cells.

The precise mechanisms of the anti-neoplastic action of IFN-γ are not fully elucidated. At the cellular level, IFN-γ exerts its action through a membrane receptor different from that used by other interferons (Branca, 1988). Whether a relationship exists between the sensitivity of mesothelioma cells to IFN-γ and IFN-γ-receptor expression should be further investigated. Recently, we demonstrated, by an immunocytochemical method, that all 25 human mesothelioma cell lines in culture so far tested possess the interferon-gamma receptor (data not shown here).

In summary, r-hu IFN-γ has inhibitory effects *in vitro* on the proliferation of human malignant mesothelioma cells, but there are some differences in the sensitivity to IFN-γ for different cell lines, in agreement with observations made in human therapy. Our results favor the search for improvement of the effect of r-hu IFN-γ, especially, in association with other drugs on tumor cells, and the use of an *in vitro* system to assess the effect of cytokines.

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