Ad-IFNγ Induces Antiproliferative and Antitumoral Responses in Malignant Mesothelioma[1]

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ABSTRACT

Purpose: **The aim of the work was to investigate the effects of adenovirus (Ad)-mediated *IFN*γ gene transfer on human mesothelioma (HM) cell proliferation *in vitro* and growth in nude mice.**

Experimental Design: **We constructed an E1E3-deleted replication-defective recombinant Ad carrying the human *IFN*γ gene (Ad-IFNγ) and tested its activity *in vitro* on HM cell lines established in our laboratory and in a nude mice model.**

***Results: In vitro*, infection of HM cells with Ad-IFNγ led to a prolonged production of an active cytokine in the 10 HM cell lines tested and also led to an antiproliferative effect on the HM cells previously demonstrated as responsive to exogenous recombinant human IFNγ. In nude mice, s.c. inoculation of HM cells from one responsive HM cell line previously infected with Ad-IFNγ resulted in a delay in tumor development, and injection of Ad-IFNγ in preestablished tumors restrained tumor development.**

Conclusions: **These results indicate for the first time that HM cells are efficiently transduced by Ad-IFNγ and produce an active cytokine for several days. IFNγ produced by gene transfer is shown to have both an antiproliferative effect *in vitro* and an antitumoral effect *in vivo* in a nude mice model.**

INTRODUCTION

MM[4] is a fatal disease consistently related to past asbestos exposure (1). Its incidence is still increasing, and deaths would reach a peak or would stay at a saturation level by 2020/2040 (2, 3). Approximately 3,000 patients die of MM each year in the United States alone (4), and 250,000 deaths due to MM are expected in Western Europe in the next 35 years (4). Classical treatments, such as chemotherapy, radiotherapy, and surgery, currently fail to improve the prognosis of this disease (5). In consequence, multimodality treatments have been developed (1), and the most favorable outcomes have been reported with a combination of surgery, chemotherapy, and radiation therapy in highly selected groups of patients. However, mortality and morbidity are still high, and this strategy is not curative. It is therefore necessary to develop innovative therapies that may offer hope for improved palliation, prolonged survival, and even potential cure for certain mesotheliomas (5).

Several authors have developed new strategies using antitumoral cytokines in MM (5, 6), and interest in IFNγ for adjuvant or single therapy of various types of cancer, such as renal cell cancer, malignant melanoma, ovarian cancer, and non-small cell lung cancer, has been reported (7–10). With regard to mesothelioma, the most impressive clinical results of cytokine therapy were obtained with intrapleural delivery of r-hu-IFNγ (5). However, r-hu-IFNγ efficiency was demonstrated mostly in early stages of MM and was limited by the short half-life of the recombinant cytokine (6). Thus, further investigations for developing methods allowing continual treatment of MM are required.

Among potential candidates, gene therapy is likely to be useful in MM, and a surgical debulking procedure to remove gross disease, followed by gene therapy to remove residual disease, would be technically feasible (5). Thus far, several recombinant Ads have been constructed carrying various genes of interest, such as *interleukin-12* (11), *P16/INK4a* (12), *P14(ARF)* (13), *BAK* (14), or *P53* (15). Moreover, a suicide gene strategy using an Ad-TK has given encouraging results, showing minimal side effects (16). Viral constructs carrying the *IFN*γ gene showed efficiency in animal models of diverse tumors (17, 18) and in patients with metastatic melanoma (19), but to date, gene transfer of IFNγ has never been applied to MM.

Based on these data and on previous reports from our laboratory showing an IFNγ-induced inhibition of cell prolifer-

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[4] The abbreviations used are: MM, malignant mesothelioma; Ad, adenovirus; HM, human mesothelioma; r-hu-IFNγ, recombinant human IFNγ; TK, thymidine kinase; IRF, interferon regulatory factor; CMV, cytomegalovirus; $TCID_{50}$, 50% tissue culture infective dose; GFP, green fluorescence protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; βGal, β-galactosidase.

ation in HM cells *in vitro* (20, 21), we developed a recombinant Ad carrying the *IFN*γ gene (Ad-IFNγ). The mechanisms whereby IFNs exert their growth-inhibitory effect are not well understood. Earlier findings suggested that IFNγ may act by controlling metabolic pathways involving activation of indoleamine-2,3-dioxygenase and NO synthase (22). However, these pathways did not appear to be involved in r-hu-IFNγ-induced HM cell cytostasis (23). Recently, several pathways have been suggested, involving IRF-1-regulated genes, antagonism with the action of growth factors, regulation of proteins that regulate cell cycle progression (up-regulation of cyclin-dependent kinase inhibitors and down-regulation of regulatory cyclin subunits; Ref. 24), and up-regulation of proapoptotic genes (25). In HM cell lines, IFNγ produces an arrest in G_1 and G_2-M phases of the cell cycle, and the down-regulation of cyclin A has been suggested to play a role (26). No apoptosis was detected. Moreover, from the study of resistant cell lines, it seems that IRF-1 activation is important to account for the antiproliferative action of IFNγ in HM cells (21). Thus, IFNγ could possibly produce an *in vivo* antitumor response through a direct antitumor effect and/or through an indirect mechanism involving immunomodulation.

The present work had two objectives: first, to determine whether Ad-mediated IFNγ transfection resulted in an antiproliferative effect on HM cell lines; and second, to evaluate the effect of transfection on the growth of these human cells in a nude mice model. Our results demonstrate a strong infectability and transduction of mesothelioma cells and, more importantly, a direct antiproliferative effect of the transduction product both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Generation of an E1E3-deleted Recombinant Ad Encoding Human *IFN*γ Gene. Ad-IFNγ was constructed as described previously (27). The human IFNγ cDNA PTG13 was inserted into the *Sma*I-*Nhe*I sites of a pCI plasmid (Promega, Charbonnières, France) under the control of the CMV immediate early promoter/enhancer and containing a chimeric intron and a downstream polyadenylation signal from SV40. Ad-TK and Ad-βGal were prepared in parallel. The titer of recombinant virus stock was determined by plaque assay on 293 cells and expressed as the $TCID_{50}$ per ml. In some experiments, Ad encoding GFP (Ad-CMV-GFP) was also used.

Cell Lines. HM cell lines were cultured according to standard methods used in the laboratory, as described previously (21), in RPMI 1640 supplemented with 25 mM HEPES, 8% fetal bovine serum, 80 μg/ml streptomycin, and 80 IU/ml penicillin (all from Life Technologies, Inc., Cergy-Pontoise, France). Ten HM cell lines were selected for the present study, according to previous results on signal transduction and growth response to r-hu-IFNγ (20, 21): eight cell lines (BT, DV, HB, MR, QR, BL, RV, and BN) were growth-inhibited after treatment with r-hu-IFNγ (Imukin; Boehringer Ingelheim, Paris, France), and two cell lines (CR and FR) did not respond to r-hu-IFNγ. Henceforth, they will be referred to as "responsive" and "unresponsive," respectively.

Cell line 293 expressing adenoviral genes was obtained from the American Type Culture Collection. WISH cells were provided by J. Wietzerbin. Both cell lines were routinely cultured in DMEM (Life Technologies, Inc.), supplemented as described for RPMI 1640.

Cell Infection and Collection of Conditioned Medium. HM cells were dispensed in 24-well tissue culture plates (Costar; Dutscher, Brumath, France; 140,000 cells/ml/well). The desired amount of recombinant Ad was added to the cultures 24 h after plating, on a per cell basis. The plates were incubated at 37°C in 95% air, 5% CO_2 for 1.5 h. Thereafter, the culture medium was removed and replaced with fresh medium, and plates were incubated for the indicated time. Then the conditioned medium was removed, centrifuged at $300 \times g$ to get rid of cell debris, and stored at −20°C until further use.

IFNγ Production by Ad-IFNγ-infected HM Cell Lines. IFNγ concentration was measured in duplicate with an ELISA (Quantikine TM; R&D Systems, Abingdon, United Kingdom) in the conditioned media from Ad-IFNγ-treated HM cells, according to the manufacturer's recommendations. The activity of IFNγ produced by gene transfer was assayed by inhibition of the cytopathic effect of vesicular stomatitis virus on human WISH cells (28).

***In Vitro* Investigation of the Antiproliferative Effect of IFNγ Produced by Gene Transfer.** The effect of Ad-IFNγ infection on HM cell growth was determined in the 24-well plates used to collect conditioned medium for ELISA measurement of IFNγ concentration, as described above. The effect of conditioned medium from Ad-IFNγ-treated cells on HM cell growth was evaluated according to the following procedures: HM cells were dispensed into 24-well tissue culture plates at a concentration of $1.5–4 \times 10^4$ cells/ml/well, depending on the cell line. After incubation for 24 h at 37°C in 5% CO_2, the cells were either exposed to conditioned medium from cells previously infected with 100 $TCID_{50}$ Ad-IFNγ/cell or cocultured with infected HM cells from the same or a different HM cell line. In coculture assays, HM cells were first plated on a Transwell membrane (Dutscher, Brumath, France) in a 24-well multiwell plate and then infected with 50 $TCID_{50}$ Ad-IFNγ/cell and incubated under standard conditions. After 24 h, the membranes were washed with complete culture medium and transferred into 24-well tissue culture plates containing the untreated growing HM cells.

Cell proliferation was investigated using a MTT assay (20). The absorbance was measured at 540 nm using an automated microplate reader (EL 800; BIO-TEK Instruments, Fischer Scientific).

Effect of Ad-IFNγ Infection on the Tumorigenic Potency of HM Cell Lines. Female nude mice (Swiss $^{nu}/_{nu}$; 7 weeks old) were obtained from Iffa Credo (L'Arbresle, France) and housed according to European Union Guidelines (29). After 1 week of adaptation, groups of six or seven mice were inoculated s.c. in the scapular region with 3×10^6 HM cells, which were either untreated or previously infected with 50 $TCID_{50}$ Ad-IFNγ/cell. Two HM cell lines were tested: one responsive cell line (BT) and one nonresponsive cell line (FR). Tumor volume (V) was measured twice a week, according to the formula: $V = L \times W^2/2$ (L, length; W, width). After sacrifice, tumors were either fixed in 10% formalin or cultured to control their responsiveness to r-hu-IFNγ using the MTT assay.

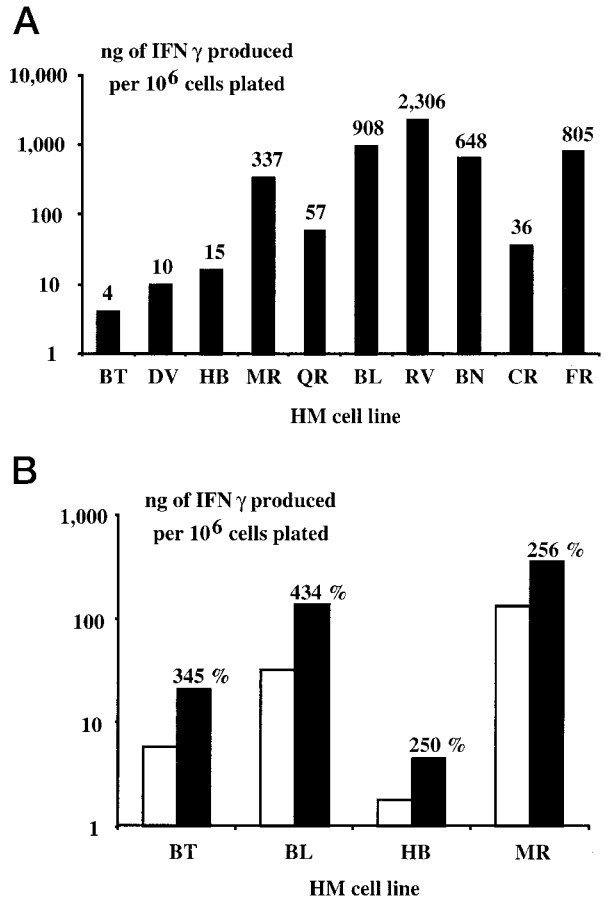

Fig. 1 IFNγ production was monitored after infection of HM cell lines with Ad-IFNγ. IFNγ concentration was measured by ELISA and expressed as ng IFNγ/10^6 cells plated. No IFNγ was found in the culture medium from uninfected cells. *A*, cumulative amount over a period of 6 days. Ten HM cell lines were infected for 1.5 h with 100 $TCID_{50}$ Ad-IFNγ/cell 24 h after plating. Exact values are displayed *above* the bars. *B*, production over a period of 24 h at different Ad-IFNγ concentrations. Four cell lines (BT, BL, HB, and MR) were exposed to 50 $TCID_{50}$ (□) and 100 $TCID_{50}$ (■) Ad-IFNγ/cell. *Numbers* above the bars represent the percentage of IFNγ production at 100 $TCID_{50}$/cell, relative to the production at 50 $TCID_{50}$/cell.

Effect of Ad-IFNγ Treatment on Growth of Preestablished Tumors. Two groups of 24 female nude mice (Swiss $^{nu}/_{nu}$; 7 weeks old) were inoculated s.c. in the scapular region with 3×10^6 cells from BT and FR cell lines. Six mice inoculated with PBS served as control. Tumor nodules were visible after less than 1 week. Three weeks after inoculation, all tumors were >40 mm^3. Each treatment was randomly attributed to six mice in each group: 2×10^8 $TCID_{50}$ of recombinant Ad (Ad-IFNγ, Ad-TK, or Ad-βGal) or PBS alone was injected intratumorally in 50 μl of PBS. Tumor volumes were measured twice a week.

Statistical Analyses. Results were evaluated using Dunnett's, log-rank tests and linear regression with Graph Pad Prism Software V2.0 for Macintosh. The difference was considered significant when $P \leq 0.05$.

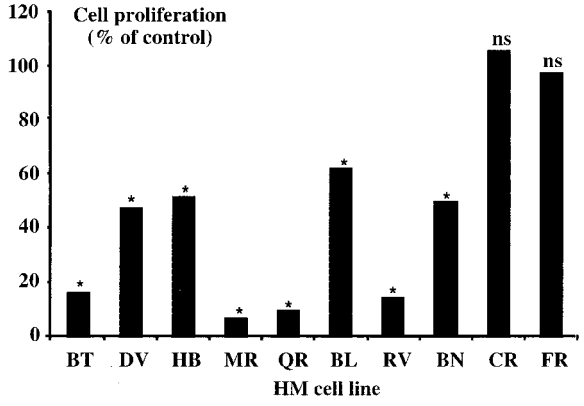

Fig. 2 Proliferation of 10 HM cell lines 7 days after infection with 100 $TCID_{50}$/cell, as described in "Materials and Methods." Cell growth was determined with the MTT assay on the plates used to investigate IFNγ production. Ad-βGal was used as control vector. The results are expressed as a percentage of absorbance value in the treated culture, in comparison with untreated cells. Difference was assessed by Dunnett's test (treated *versus* untreated): ∗, statistically significant; *ns*, not significant.

RESULTS

IFNγ Production by Ad-IFNγ-infected HM Cell Lines. To evaluate the efficiency of gene transfer and the capability of transduced cells to produce the transgene, we investigated IFNγ cumulative production over the course of 6 days. All cell lines produced IFNγ after infection (Fig. 1*A*). All cell lines previously found to be responsive to r-hu-IFNγ produced amounts of IFNγ greater than their IC_{30} (concentration of r-hu-IFNγ reducing growth by 30%; Ref. 20).[5] No IFNγ was found in the culture medium from uninfected cells.

The transgene production was evaluated daily, 1, 3, 7, and 14 days after infection. All cell lines still produced IFNγ at day 7, but the production was lower than that seen 3 days after infection. At day 14, IFNγ was still detected in the conditioned medium from four HM cell lines (DV, RV, BN, and FR). Furthermore, the FR cell line still produced IFNγ 28 days after infection (data not shown).

Four cell lines, BT, BL, HB and MR, were treated with two different doses of Ad-IFNγ, and subsequent production of IFNγ was compared. Increasing the Ad-IFNγ concentration from 50 to 100 $TCID_{50}$/cell resulted in more than a doubling of the IFNγ production (Fig. 1*B*).

Activity of IFNγ Produced by Gene Transfer. To compare the effect of IFNγ produced by gene transfer with that of r-hu-IFNγ produced by *Escherichia coli*, the specific activity of the IFNγ produced by gene transfer was determined in the conditioned medium from three HM cell lines (BT, BL, and FR) collected 24 h after infection with 100 $TCID_{50}$ Ad-IFNγ/cell. The r-hu-IFNγ (20 IU/ng) from Boehringer Ingelheim was used as standard. The activity of IFNγ produced by gene transfer was

[5] L. Zeng and M. C. Jaurand, unpublished data.

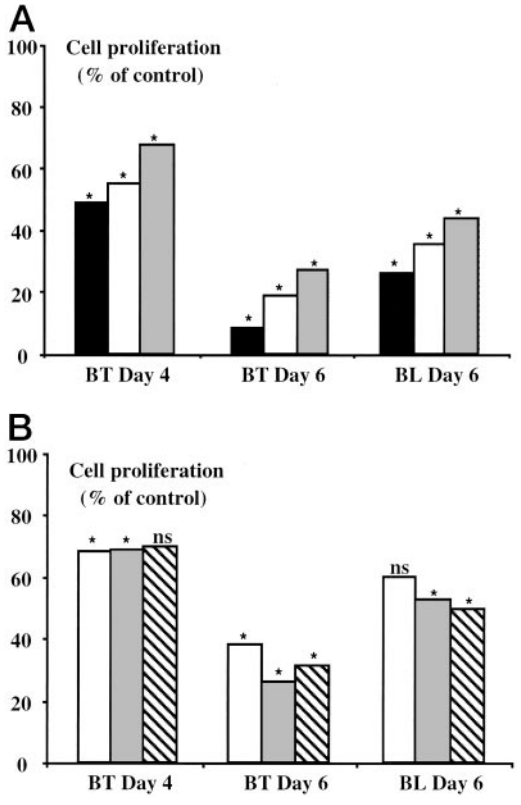

Fig. 3 Proliferation of two HM cell lines 4 days (BT) and 6 days (BT and BL) after infection with 100 $TCID_{50}$ Ad-IFNγ/cell in coculture conditions or after exposure to conditioned medium as described in "Materials and Methods." *A,* cells were exposed to Ad-IFNγ (■) or cocultured with the same cell line (□) or with the cell line FR (▨). *B,* cells were exposed to the conditioned medium from the same cell line (□) or from another cell line (FR, ▩; CR, ▧). The results are expressed as the percentage of absorbance value in the treated culture, in comparison with untreated culture. Difference was assessed by Dunnett's test (treated *versus* untreated): ∗, statistically significant; *ns,* not significant.

found to be 11, 24, and 61 IU/ng in the HM cell lines FR, BL, and BT, respectively.

Antiproliferative Action of IFNγ Produced by Gene Transfer. Mesothelioma cell growth was significantly reduced in the cultures treated with Ad-IFNγ (Fig. 2), except in two HM cell lines (CR and FR) previously demonstrated to be unresponsive to r-hu-IFNγ (20, 21). No antiproliferative effect was observed after infection with Ad-βGal (data not shown). Two cell lines (BT and BL) were also cultured in conditioned medium from the same or a different HM cell line or cocultured with cells of the same or a different HM cell line previously infected with 100 $TCID_{50}$ Ad-IFNγ/cell. Cell proliferation was inhibited in comparison with the control cultures (Fig. 3).

Influence of Ad-IFNγ Concentration on the Proliferation of HM Cell Lines. These experiments were carried out in two responsive cell lines (BT and BL) and one unresponsive cell line (FR). Fig. 4 shows that the cell growth was significantly reduced in the responsive cell line in a dose-dependent manner, but not in FR.

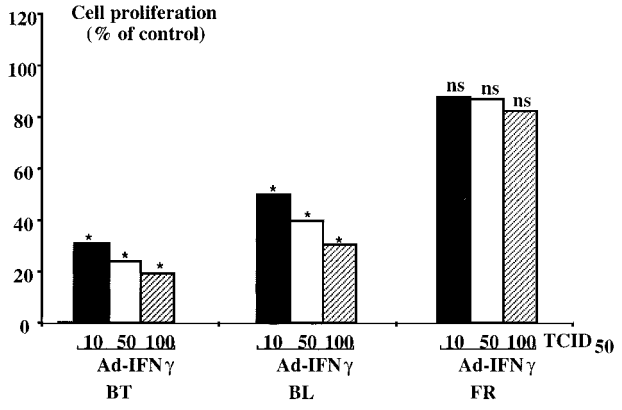

Fig. 4 Proliferation of three HM cell lines (BT, BL, and FR) in the presence of 10 $TCID_{50}$ Ad-IFNγ/cell (■), 50 $TCID_{50}$ Ad-IFNγ/cell (□), and 100 $TCID_{50}$ Ad-IFNγ/cell (▨). Cell growth was determined with the MTT assay. Results are expressed as the percentage of absorbance value obtained in the MTT assay in comparison with untreated cells. Difference was assessed by Dunnett's test (treated *versus* untreated): ∗, statistically significant; *ns,* not significant.

Tumorigenic Potency of Ad-IFNγ-treated HM Cell Lines. Fig. 5 shows the time course of the percentage of tumor-free mice after treatment with BT and FR cells. All animals inoculated with uninfected cells from the BT cell line exhibited nodules 12 days after inoculation, whereas mice inoculated with Ad-IFNγ-infected BT cells did not develop nodules earlier than 58 days after inoculation. Time between inoculation and tumor appearance was found to be statistically different between treated and untreated cells (log-rank test). In contrast, both infected and noninfected FR cells produced nodules without significant difference in the time of tumor appearance. To determine whether the delayed growth of the Ad-IFNγ-treated BT cells could be due to a phenotypic reversion (resistance to IFNγ), we verified that the *in vitro* growth of cells cultured from the tumors was still inhibited by r-hu-IFNγ (data not shown).

Effect of Ad-IFNγ Treatment on Growth of Preestablished Tumors. Fig. 6*A* shows the time-dependent evolution of tumor volumes in mice inoculated with the BT cell line: tumors treated with Ad-IFNγ grew significantly slower than those treated with control Ads (linear regression, $P < 0.01$). Tumors treated with PBS grew significantly faster than all of the others ($P = 0.011$). For mice inoculated with FR cells, tumor development was also significantly reduced after all treatments, when compared with PBS-treated tumors (linear regression, $P < 0.001$; Fig. 6*B*). However, no significant difference among the three treatments (*i.e.,* Ad-βGal, Ad-TK, or Ad-IFNγ) was observed ($P > 0.08$).

DISCUSSION

In the present study, we demonstrate IFNγ production and inhibition of cell proliferation and tumor growth in different HM cell lines after infection with an E1E3-deleted recombinant Ad encoding human IFNγ. We observed that HM cell lines were all able to produce levels of IFNγ that were previously shown to have an antiproliferative action in responsive cells (20). How-

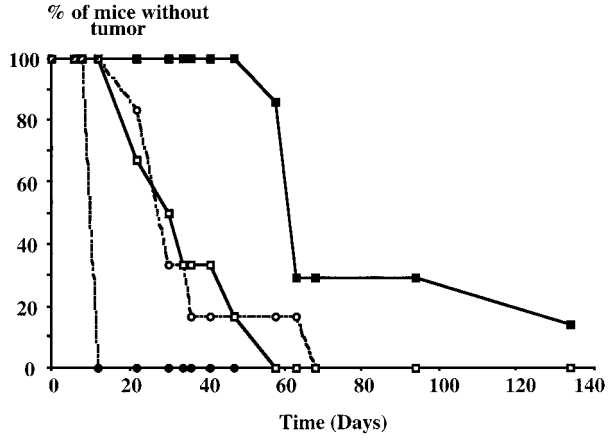

Fig. 5 Tumorigenic potency of HM cell lines BT (■ and ●) and FR (□ and ○) either infected (*squares*) or not infected (*circles*) with 50 $TCID_{50}$ Ad-IFNγ/cell. HM cell lines were infected *in vitro*, as described in "Materials and Methods." The graph represents the percentage of tumor-free mice. Nodules did not appear before 12 days.

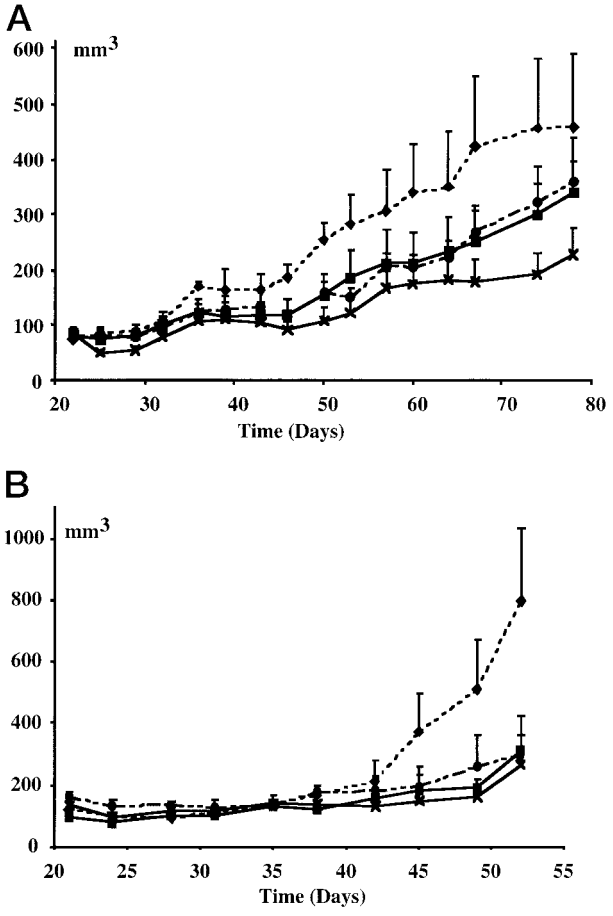

Fig. 6 Effect of treatment with PBS (♦), Ad-βGal (●), Ad-TK (■), or Ad-IFNγ (X) on growth of tumors preestablished by s.c. inoculation of BT (*A*) or FR (*B*) cells. The graph represents the time-dependent evolution of tumor volume (mean + SE) in five to six mice. Data were treated statistically using linear regression.

ever, IFNγ production differed between HM cell lines (4–2306 ng produced over a 6-day period, for instance). The differences in IFNγ production could not be attributed to different infectabilities because the percentage of cells transduced after exposure to 100 $TCID_{50}$/cell, on the basis of the results obtained with Ad-GFP, was similar between the cell lines (data not shown). However, the degree of GFP expression did not correlate with the degree of IFNγ production. Therefore, differences in the mechanisms leading to gene expression in the different cell lines could account for these results.

In vitro, all of the HM cell lines tested were growth-inhibited after infection with Ad-IFNγ, with the exception of CR and FR, which were previously found to be unresponsive to r-hu-IFNγ because they have an impaired Janus-activated kinase/signal transducers and activators of transcription signal transduction pathway, *i.e.*, FR was shown to lack Janus-activated kinase 2 expression, whereas a poor activation of IRF-1 was demonstrated in CR (21). Interestingly, all sensitive cell lines produced amounts of IFNγ higher than their IC_{30} and were, accordingly, more than 30% growth-inhibited. Cell growth inhibition observed *in vitro* likely results from a specific effect of IFNγ, as suggested by different findings. Firstly, no growth inhibition was observed after treatment with Ad-βGal. Secondly, growth inhibition in the presence of conditioned medium from cells infected with Ad-IFNγ and coculture assays demonstrated that the medium from transfected cells exerted a cytostatic effect that was not observed with conditioned medium from uninfected cells.

Intracavitary therapy has become accepted for treatment of MM because it permits a greater concentration of drug within the cavity compared with systemic administration. However, the half-life of IFNγ appears limited, likely because of its degradation (30). Thus, local production of IFNγ at the tumor site deserves to be investigated. From the time course of IFNγ production, we concluded that transfection of HM cells permits a longer exposure to IFNγ because all HM cell lines still produced a detectable amount of the transgene 7 days after infection. Furthermore, IFNγ was still detected in four HM cell lines 14 days after infection and was detected in FR even 28 days after infection. However, a progressive time-dependent decrease in IFNγ production was observed. A time-dependent decrease in the amount of IFNγ transcript was also found, except in the FR cell line, as demonstrated by quantitative real-time PCR analysis (data not shown). This could be due to a slowdown of the cell machinery induced by IFNγ and perhaps to the lack of fresh media because the medium was not changed through a 6-day period, or it could be explained by the CMV promoter being down-regulated by IFNγ (31). This decrease may also be related to a dilution effect of the vector because the cells did not stop growing immediately after infection but stopped growing at least 2 days later (data not shown). Such a delay was also observed with direct addition of r-hu-IFNγ (20, 21). A time-dependent decrease in IFNγ expression was also found in fibroblasts infected with Ad-IFNγ (32).

In immunocompetent animals, Ad-IFNγ can stimulate the host immune response against the tumor cells (17) and then have both a direct antiproliferative effect and an indirect immune

effect toward the tumor cells. Alternatively, Ad-IFNγ has been demonstrated to repress growth of established brain tumors by antiangiogenesis (18). Thus, given the pleiotropic effects of Ad-IFNγ, some response could be also obtained *in vivo* with cells unresponsive to the antiproliferative effect of IFNγ.

Activity of IFNγ produced by gene transfer ranged from 11 to 61 IU/ng in three HM cell lines studied. This value was of the same order as that of r-hu-IFNγ (20 IU/ng) used in clinical trials (6). The differences in IFNγ activity between cell lines could be related to different proteolytic activities in the culture medium between the cell lines, possibly leading to differences in the activation or inactivation of the cytokine (30).

Because immunocompetent animals cannot be used to investigate the effect of drugs on human cells, the nude mice model appears useful for assessing the effect of drugs (33). Injection of *in vitro*-transfected BT cells resulted in a significant delay in tumor development when compared with untreated BT cells, in agreement with the antiproliferative action of IFNγ on this cell line. In contrast, no change in tumor incidence and delay of tumor formation was observed in the cell line FR that did not display an antiproliferative response to IFNγ. This result suggests that the antiproliferative action demonstrated *in vitro* also occurred *in vivo* and produced a delay in tumor growth. We found that the lack of duration in the inhibition of tumor development in mice inoculated with Ad-IFNγ-infected BT cells was not caused by a selection of resistant cells. This effect was more likely due to a dilution of the transgene along with cell division and/or to a down-regulation of the gene promoter, as discussed above. Alternatively, the persistence of the expression seems to be dependent on the vector backbone and host background in a tissue-specific manner (34).

When Ad-IFNγ was inoculated in preestablished tumors, a significant decrease in tumor growth was observed with both cell lines tested when compared with PBS. However, with the cell line unresponsive to IFNγ, the inhibitory effect observed with Ad-IFNγ was not significantly different from that of control Ads. In contrast, in the responsive cell line, Ad-IFNγ was significantly more efficient in reducing tumor growth than control Ads. An inhibitory effect of nonrelevant Ads has been described elsewhere in immunocompetent animals and could be explained in our system by an activation of the residual immune system of nude mice, including natural killer cells (35) and macrophages.

Additional studies are still needed to understand the mechanisms involved in the antiproliferative and antitumoral effects of IFNγ produced by gene transfer and to determine the best conditions for a possible treatment of human MM by *IFN*γ gene therapy. Moreover, experimental trials are necessary before testing the promising new therapy on human patients to be as efficient and safe as possible (36).

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