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Interferon (IFN)-α and IFN-γ in combination with methotrexate: *in vitro* sensitivity studies in four human mesothelioma cell lines

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Mesothelioma is a malignant tumor of the serous surfaces in the thorax and abdomen, which has proved exceptionally resistant to treatment. A recent phase II trial of a high-dose methotrexate regime on 63 Norwegian patients has, however, achieved a response rate of 37%. Some responses have also been achieved using interferon (IFN)-γ administered intrapleurally or recombinant (r) IFN-α administered subcutaneously. Our earlier *in vitro* sensitivity studies of mesothelioma cell lines showed that IFN augments the response to chemotherapeutic agents in mesothelioma. The aim of this study was to assess the response of four mesothelioma cell lines, derived from diffuse asbestos-related pleural malignant mesothelioma, to methotrexate alone and in combination with recombinant IFN-α and IFN-γ. Anti-proliferative effects were assayed by vital dye exclusion. A combination of IFN-α and IFN-γ consistently augmented the response of the cell lines to methotrexate, by as much as 75% for one cell line, although the response to the individual IFNs was variable. We were also able to compare the effects of natural IFN-β with those of IFN-α and IFN-γ. The IFN-β sensitivity profile for each of the four cell lines was similar to that of IFN-α. In two cell lines, the combination of IFN-β and IFN-γ produced a similar effect to the IFN-α and IFN-γ combination.

Key words: Human malignant mesothelioma, interferon-α, interferon-β, interferon-γ, *in vitro* testing, methotrexate.

Introduction

Mesothelioma is a malignant tumor of the serous surfaces of the thorax and abdomen,[1,2] the development of which is almost always associated with a history of occupational exposure to asbestos.[1] There is no standard treatment for this disease and median survival is typically less than 1 year from diagnosis.[3–5]

Diagnostic techniques have improved so that mesothelioma can now be identified earlier and be reliably distinguished from adenocarcinoma, which is important for the selection of appropriate therapies. Various chemotherapeutic regimes have been tested, but assessment has been hampered by the low overall numbers of patients.[6] Doxorubicin and cisplatin have usually been among the most consistently effective, achieving response rates of 15–18%.[3,7,8] However, an overall response rate of 37% has recently been achieved using high-dose methotrexate in a Norwegian study.[9]

Interferon (IFN) has been of interest to cancer researchers since it was first discovered in 1959.[10] IFNs are involved directly and indirectly in the host response to malignancy.[11–13] Much interest has been created recently by their ability as biological response agents to augment or maintain the effects of chemotherapeutic agents in both experimental and clinical situations.[14–18] In experimental studies, Sklarin showed that recombinant (r) IFN-α augmented the activity of cisplatin in mesothelioma xenografts[14] and Ohnuma *et al.* have shown that natural IFN-α alone also has an inhibitory effect in these circumstances.[19] Intrapleural IFN-γ is active against early-stage malignant mesothelioma[20,21] and intramuscular rIFN-α2a has also produced some partial responses in an Australian study of 25 patients with malignant mesothelioma.[22] A trial of combined systemic cisplatin and IFN-α initiated in an attempt to improve on the minor responses achieved with the individual agents, recorded partial responses in seven out of 19 evaluable patients.[23]

In vitro screening of new agents and new combinations of agents is essential if effective therapies are to be found when the low overall number of patients prevents systematic clinical testing of possible agents. We have established a number of continuously-growing human mesothelioma cell lines from fresh tumor tissue,[24] and have already reported on the sensitivity of three of these cell lines to recombinant human cytokine combinations;[25] and of four cell lines to various chemotherapeutic

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agents, with and without IFN.[26] In this paper we report on additional experiments to assess the sensitivity of our cell lines to methotrexate in combination with rhIFN-α and IFN-γ, and to natural human IFN-β.

Materials and methods

Four human mesothelioma cell lines were used in these experiments.[24] Three of the cell lines (M9K, M33K and M38K) were from tumors with mixed histology and one (MI4K) from an epithelial-type tumor. Three cell lines (M14K, M33K and M38K) were established from the primary tumors of previously untreated patients. Cell line M9K was established from the metastatic tumor of a patient who had previously been treated with mitoxantrone and radiotherapy, although the tumor sample was taken from an area outside the radiation field.

From continuously growing monolayer cultures, 1.0–1.5 × 10^5 cells were plated into 6-well plates (10 cm^2, Nunc Denmark) in a 3 ml medium. We used RPMI 1640 medium supplemented with 10% fetal calf serum, 0.03% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRLU) for cell lines M9K, M14K and M38K; and RPMI 1640 supplemented with 3% fetal calf serum, 0.03% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco) and 2 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml selenite (all from Sigma, St Louis, MO) for cell line M33K. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 40–50 h to establish exponentially growing cultures.[24] The medium was then replaced by fresh medium supplemented with methotrexate and/or IFN and incubated for a further 48 h before harvesting. The drugs and IFN were diluted in medium to give final concentrations of 10^{-4} to 100 μg/ml per well. The cells were detached with 0.05% trypsin–EDTA, centrifuged (1000 r.p.m. for 5 min) and stained with Trypan blue for 5 min. Finally, the viable cells were counted.

For each cell line we assessed sensitivity to methotrexate (Methotrexate; Lederle Parenterals, Carolina, Puerto Rico), rIFN-α (Berofer®; Boehringer, Ingelheim, Germany; specific activity 32 × 10^7 U/mg), rIFN-γ (Imukin®; Boehringer; specific activity 2 × 10^7 U/mg) and natural human IFN-β (Pantaferon®; Asta Medica ; Germany). We also tested sensitivity to rIFN-α combined with rIFN-γ, and methotrexate in combination with rIFN-α, rIFN-γ and rIFN-α plus rIFN-γ. In all our experiments, methotrexate and IFNs were tested individually over a range of concentrations from 10^{-4} to 1 μg/ml. IFN-β was tested over the same range of biological activity as rIFN-α. IFN was added at a fixed concentration (0.01 μg/ml) over the range of methotrexate concentrations. This dose was established from the experiments testing IFNs alone and in combination. In the combined IFN experiments, equal amounts of rIFN-α and rIFN-γ were tested over the whole range of concentrations. The duration of treatment was 48 h.

The results are presented as follows: the number of viable test cells as a percentage of the number of viable control cells (% viable cells) against the concentration of drug(s) used. Each point on the figures represents the mean result of at least two independent experiments of duplicate cultures. The reduction in growth which would be expected from the fixed amount of IFN-α and IFN-γ used in the methotrexate and mitoxantrone experiments is represented by the horizontal lines on each graph in Figures 1 and 3.

Results

The results of the experiments using methotrexate, and methotrexate with a fixed concentration (0.01 μg/ml) of rIFN-α, rIFN-γ or of both IFN-α and rIFN-γ, are shown in Figure 1. The sensitivities of the different cell lines to methotrexate were very similar. They were all most sensitive to methotrexate plus the IFN combination, although their sensitivity to methotrexate with the individual IFNs was variable: M9K responded to methotrexate with either IFN; M14K responded to neither individual IFN with methotrexate; and M33K responded to methotrexate and rIFN-γ but not to methotrexate and rIFN-α. For M33K this is clearly consistent with the pattern of response to the IFNs alone (Figure 2c), but for the other cell lines there is no obvious correspondence.

Figure 2 shows the results of the experiments using the individual IFNs and their combinations. In addition to rIFN-α, rIFN-γ and their combination, M9K and M38K were tested with nIFN-β and nIFN-β and rIFN-γ, and M14K and M33K with nIFN-β. In all four cell lines the response to nIFN-β was similar to the response to rIFN-α. The response to nIFN-β plus rIFN-γ was very similar to that to rIFN-α plus rIFN-γ.

In Figure 3 we compare the sensitivity of two cell lines to methotrexate and mitoxantrone, and to both drugs combined with IFN-α and IFN-γ. Both cell lines were marginally more sensitive to mitoxantrone than to methotrexate, both with and without the IFN combination.

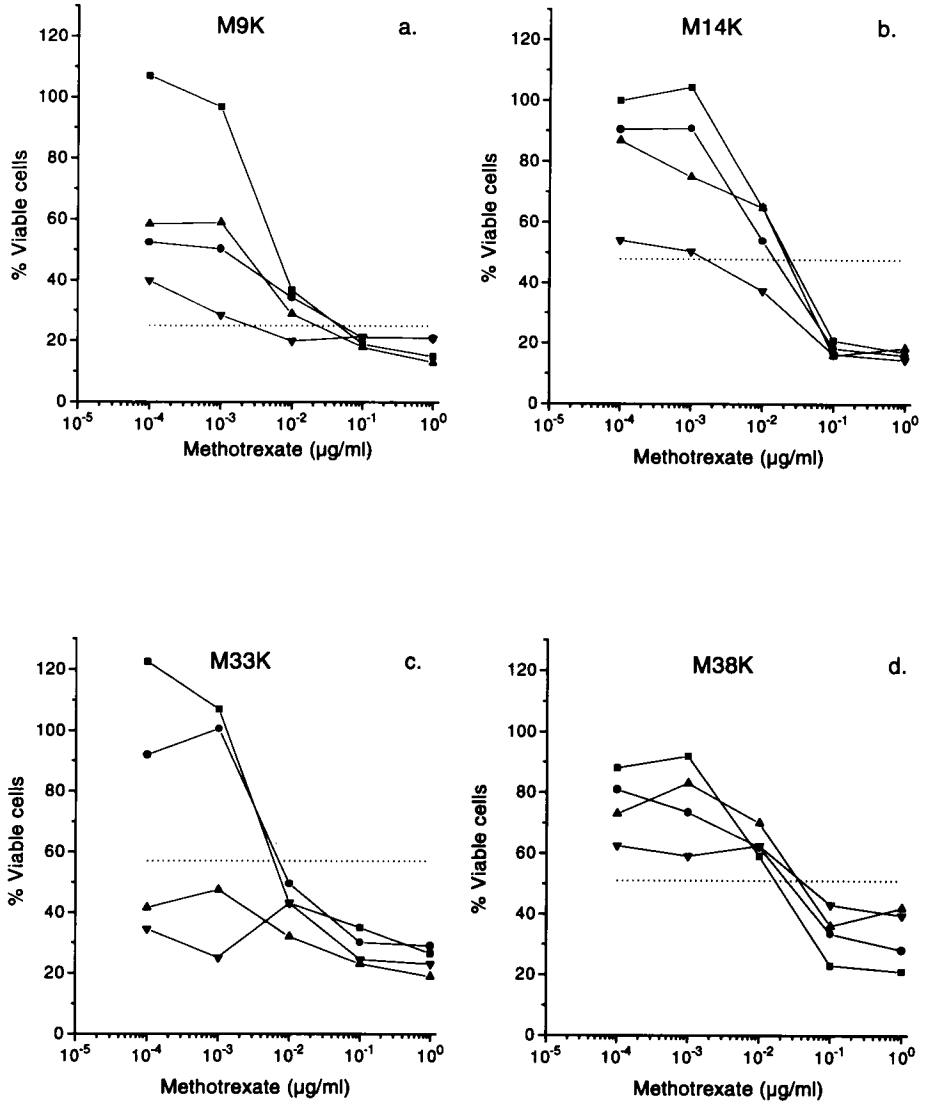

Figure 1. The effects of methotrexate and rIFN on four mesothelioma cell lines: ■, methotrexate alone; ●, methotrexate plus 0.01 μg/ml rIFN-α2c; ▲, methotrexate plus 0.01 μg/ml rIFN-γ1b; ▼, methotrexate plus 0.01 μg/ml rIFN-α2c and rIFN-γ1b.

Discussion

The results of our earlier sensitivity studies suggested that mesothelioma cells are more sensitive to the DNA intercalating anthracyclines, especially mitoxantrone, than to either etoposide or cisplatin—which interact with DNA in other ways;[27] but this activity has not been seen in clinical studies.[28] It may be significant that only a very low dose of mitoxantrone (14 mg/m^2) was used in Van Breukelen's study.[28] However, high-dose methotrexate, an anti-foliate, has shown activity against mesothelioma, albeit in small studies,[9,29] and this result prompted our *in vitro* investigation. Our earlier study[26] showed that mesothelioma cell lines respond more consistently to a combination of rIFN-α and rIFN-γ than to the individual IFNs. Zeng and coworkers have also observed the variable sensitivity of mesothelioma cell lines to rIFN-γ.[30] They found that although 50% of their 32 cell lines responded to rIFN-γ, only 34% were actually sensitive (>30% growth inhibition). This corresponds well with the 32% of patients who responded to intrapleural rIFN-γ therapy.[20] Interestingly, they found that sensitivity to rIFN-γ did not depend on the level of expression of IFN-γ receptors and that different cell lines from the same patient often showed differing sensitivities to rIFN-γ. Christmas *et al.* also found that there was a variable response to IFN-α and IFN-γ in terms of changes in HLA antigen expression in mesothelioma cell lines.[31]

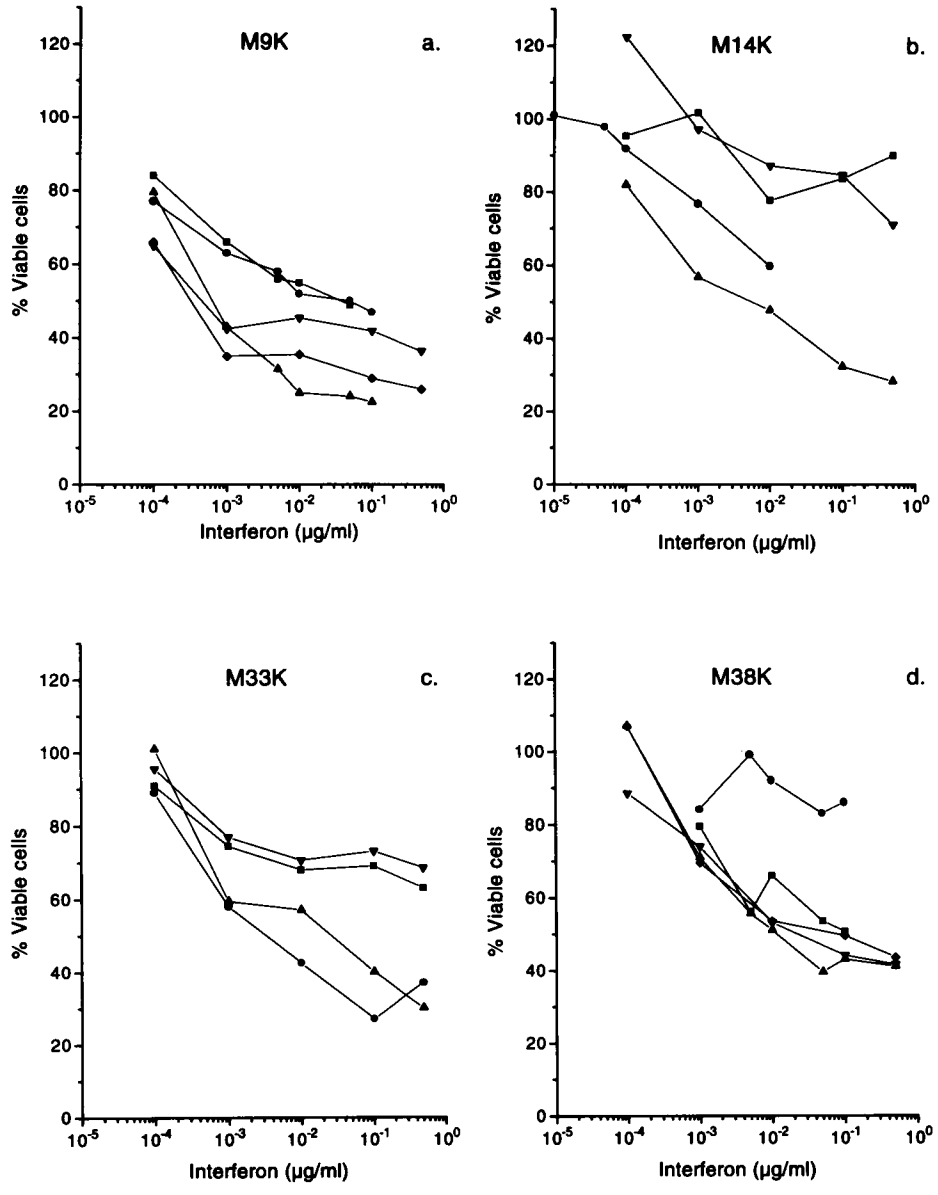

Figure 2. The effects of IFNs and their combination on four mesothelioma cell lines: ■, rIFN-α2c; ●, rIFN-γ1b; ▲, IFN-α2c plus rIFN-γ1b; ▼, nIFN-β; ◆, nIFN-β plus rIFN-γ1b.

It can be seen from Figure 1, where the reduction in growth to be expected from 0.01 μg/ml of both rIFN-α and rIFN-γ is shown by a horizontal line on each graph, that there is a noticeable interaction in only one cell line (M33K) between the methotrexate and the IFN combination. In M9K and M14K there is barely any reduction in the numbers of viable cells over that to be expected from the IFN combination alone. In M38K, no combination of methotrexate with IFN appears to reduce growth as much as the IFN combination alone. Cell line M33K was established from the tumor of a patient with stage IV disease, whereas the other cell lines were established from less advanceed tumors.[24] This cell line required a different growth medium to the others, and was the only one of the four to lack expression of the gap junction channel protein, connexin 43, and the cell–cell adhesion protein A-CAM (Pelin *et al.*[32]). However these findings do not explain why M33K was more sensitive to methotrexate plus IFN.

In Figure 3 we compare the effects of methotrexate and mitoxantrone (the most effective drug in our earlier studies) on two cell lines. The drugs have very similar effects on cell line M9K (Figure 3A), but cell line M38K appears to be slightly more sensitive to mitoxantrone and this effect is augmented by the addition of rIFN-α and rIFN-γ (Figure 3B).

Our results indicate that mesothelioma cells are also sensitive to IFN-β and as sensitive to IFN-β plus IFN-γ as to IFN-α plus IFN-γ. This might be expected

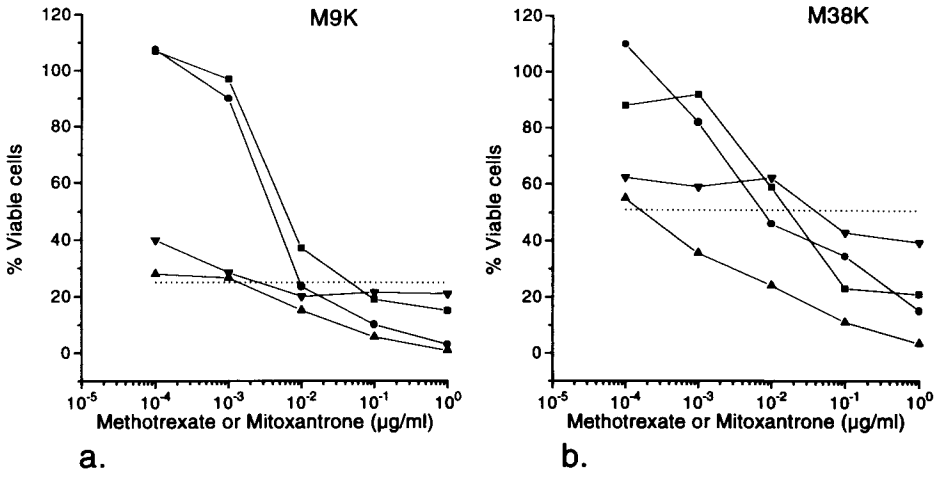

Figure 3. A comparison of the effects of methotrexate and mitoxantrone with and without IFN in two mesothelioma cell lines; ■, methotrexate alone; ●, mitoxantrone alone; ▼, methotrexate plus rIFN-α2c and rIFN-γ1b; ▲, mitoxantrone plus rIFN-α2c and rIFN-γ1b.

given that IFN-α and IFN-β share a cell surface receptor.[13] One group has already investigated rIFN-β in the treatment of diffuse mesothelioma.[33] No responses were obtained in this study, but the authors suggested that the serum level of IFN-β may have been too low to be effective. A very encouraging result has, however, been obtained in non-small cell lung cancer using rIFN-β in conjunction with radiotherapy.[34] Radiotherapy usually forms part of multimodality therapy for mesothelioma.[5]

Our results appear to show that methotrexate activity can be potentiated by IFN *in vitro*, although they should be interpreted with caution because methotrexate acts indirectly on DNA, via the cellular folate pool, and the dynamics of this interaction are not yet understood. A new Scandinavian mesothelioma trial, inspired by these results, has recently started using high-dose methotrexate with Leucovorin administered with rIFN-α and rIFN-γ, followed by IFN-α and IFN-γ maintenance therapy.

Conclusion

We conclude that our results support the continued search for a role for IFN in the treatment of malignant mesothelioma. Cell line studies should be encouraged to screen new agents and new combinations of agents, as they will increase the chances of finding an active treatment for this distressing and fatal condition.[6,35]

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