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Chemosensitivity and cytokine sensitivity of malignant mesothelioma

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Summary. Malignant mesothelioma arises in serosal tissues, is locally invasive, and is usually resistant to chemotherapeutic agents used clinically. To determine whether resistance to cytotoxic drugs was an inherent characteristic of mesothelioma cells, we performed in vitro chemosensitivity testing on five fully characterised human malignant mesothelioma cell lines and, for comparison, on three lines representative of clinically drug-resistant solid-tissue carcinomas using the MTT (tetrazolium bromide) assay system. Mesothelioma cell lines were intrinsically resistant to eight common antineoplastic drugs, with concentrations that produced a 50% reduction in optical density (IC_{50} values) for all drugs being equivalent, if not higher, for mesothelioma cell lines as compared with lung and colon carcinoma cell lines. We then investigated the direct anti-mesothelioma activity of recombinant human cytokines with their antineoplastic properties. All five mesothelioma cell lines were resistant to tumour necrosis factor, but they displayed varying degrees of sensitivity to interferons (IFNs). IFNγ directly inhibited the growth of two of five mesothelioma lines. IFNα displayed little activity against four of five mesothelioma lines. The mesothelioma cells that were sensitive to IFNα were resistant to IFNγ, indicating that sensitivity to IFNs is not a genetic characteristic of malignant mesothelioma cells. Significant interactions between cytokines in combination were not observed.

Introduction

Malignant mesothelioma is a clinically aggressive tumour that arises in serosal mesothelium and is associated with exposure to asbestos [12]. There is a high incidence of mesothelioma in Western Australia, with most cases occurring in former miners and millers of Wittenoom Gorge crocidolite asbestos [4]. Although the Wittenoom asbestos mining operation closed in 1966, the long latency period between asbestos exposure and tumour development will result in increasing local incidence rates during the next decade [5]. Malignant mesothelioma is clinically resistant to both chemotherapy and radiotherapy and is usually inoperable at presentation [1, 2, 13, 16, 21, 22].

The search for effective therapy for malignant mesothelioma has been difficult because although potentially active chemotherapeutic agents and recombinant anti-cancer cytokines are becoming increasingly available, the number of patients suitable for clinical evaluation of these agents are limited at individual centres. A method of selecting agents for promotion to clinical trial that is based on preclinical biological evaluation would conserve time, expense, and clinical resources. In vitro chemosensitivity testing has the potential of forming the basis of such an approach.

To evaluate in vitro chemosensitivity characteristics of human mesothelioma, we studied five well-established and fully characterised human malignant mesothelioma cell lines [19]. We initially determined optimal assay conditions for these cell lines in short-term culture and then tested a panel of established anti-cancer drugs and cytokines for their anti-mesothelioma activity under standardised conditions.

Materials and methods

Cell lines. Five malignant mesothelioma cell lines established in our own laboratory from pleural fluid cells of patients presenting with pleural mesothelioma were used in the present study. These cell lines, designated NO-36, JU-77, ONE-58, DeH(m), and LO-68, have previously been fully characterised [19]. All cell lines grow adherently in culture and exhibit cytological and histochemical characteristics that are typical of malignant mesothelioma [19, 30]. For purposes of comparison, three non-mesothelioma cell lines representative of tumour types known to be generally drug-resistant were studied in parallel: HT29 – human colon adenocarcinoma (ATCC HTB38) – and A549 – human lung carcinoma (ATCC CCL185) – both of which were purchased from ATCC (USA),

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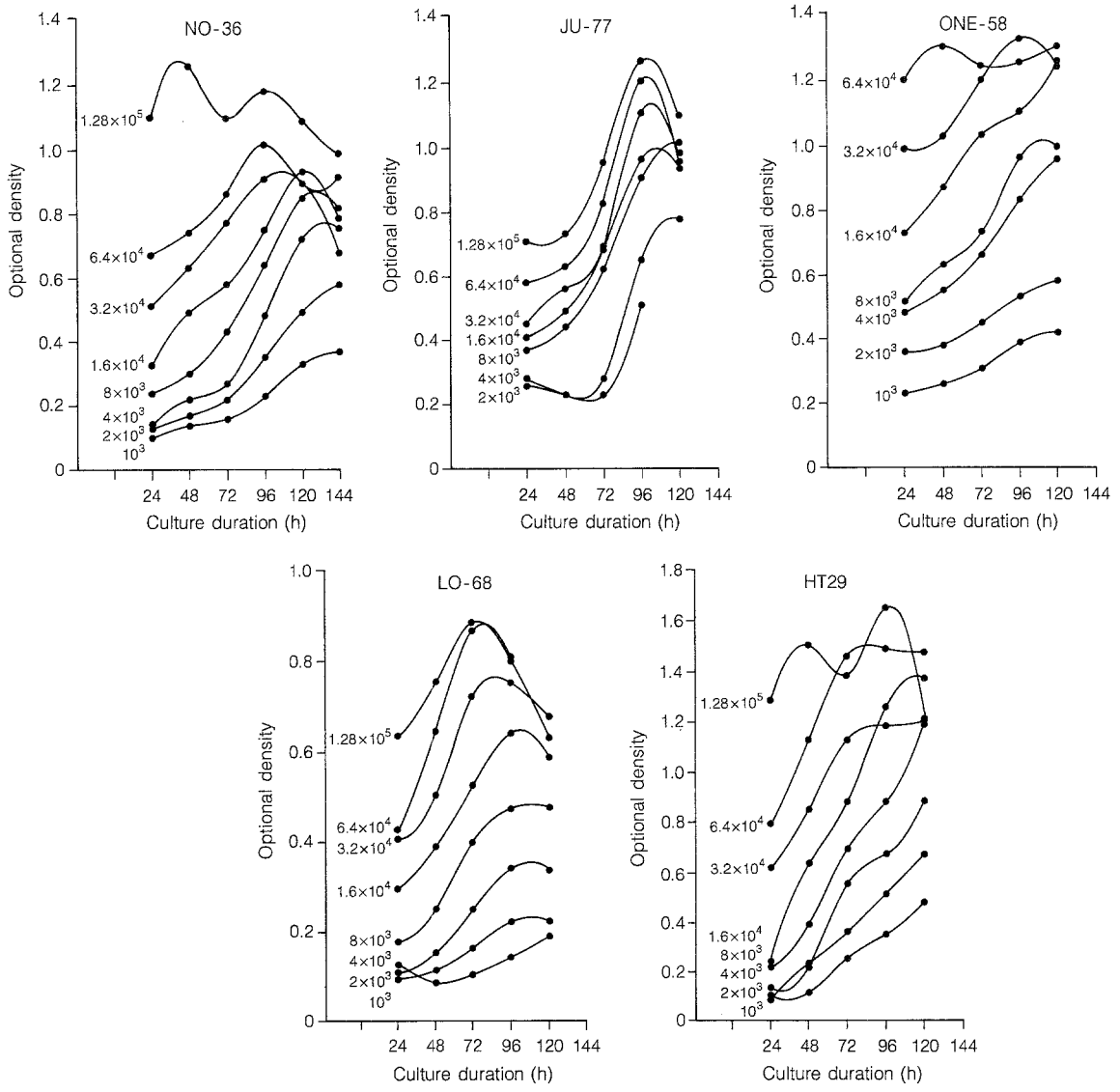

Fig. 1. Growth of cell lines (optical density at 590 nm – *y* axis) depends on seeding density (cells/well, indicated to the *left of curves*) and culture duration prior to MTT substrate handling (*x* axis). Points represent means of quadruplicate measurements. Standard deviations were always <10% of the means. The steepest linearity between the duration of culture and the optical density was obtained when cells were seeded at 4000–8000/well

and NCI-H157 – human large-cell lung carcinoma (kindly provided by Dr. A. Gazdar, National Cancer Institute, Bethesda, Md).

All cell lines were maintained and passaged in RPMI-1640 medium containing 5% foetal calf serum, 2 mM L-glutamine, 1 μM sodium pyruvate, 2.5 mg amphotericin B/ml, 0.1 mM non-essential amino acids (all from Flow Laboratories, Sydney), 5 ng epidermal growth factor/ml (Boehringer Mannheim, North Ryde, Sydney), 100 IU benzyl penicillin/ml (Glaxo Australia, Melbourne), and 50 μg gentamicin/ml (David Bull Laboratories, Melbourne); this medium is hereafter designated R-5. All cell lines tested mycoplasma-free by culture under microaerophilic conditions on mycoplasma agar (State Health Laboratories of Western Australia).

Antineoplastic drugs. Antineoplastic drugs were prepared by the staff of the Sir Charles Gairdner Hospital Pharmacy, including actinomycin D (Cosmegen; Merck, Sharpe and Dohme), mitomycin C (Sigma), cisplatin, doxorubicin, fluorouracil, methotrexate, etoposide, and vinblastine sulphate (all obtained from David Bull Laboratories, Sydney). Drugs were diluted with sterile water for injection, stored light-protected at 4° C, and used within 1 week of preparation.

Recombinant human cytokines. Recombinant human tumour necrosis factor alpha (rHuTNF, TNF) was kindly provided by Cetus Corporation (Emeryville, Calif.; bioactivity, 24×10^6 units/mg). Recombinant human interferon alpha 2a (IFNα) was obtained from Roche (Roferon A; sp. act., 2×10^8IU/mg protein). Recombinant human IFNγ was kindly provided by Boehringer Ingelheim (bioactivity, 2×10^7 units/mg). All were stored at –70° C and according to manufacturers' instructions.

MTT assay. Both drug and cytokine sensitivities were determined using the MTT assay as modified from the method of Mosmann [20]. The assay is based on spectrophotometrical detection of the conversion of MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] to its blue reduction product formazan by mitochondrial enzymes of metabolically active cells. Tumour cells in R-5 were seeded at appropriate densities into 96-well flat-bottom tissue-culture plates. To apply the MTT assay to these cell lines, we first established the optimal cell seeding density and culture duration for the detection of maximal antineoplastic effects. Cells were seeded at densities of 1,000–128,000/well and were cultured for 24–144 h. Antineoplastic drugs were prepared from stock solutions by serial dilution in R-5 to molar concentrations 10 times the final concentration and were added to wells within 1 h of cell

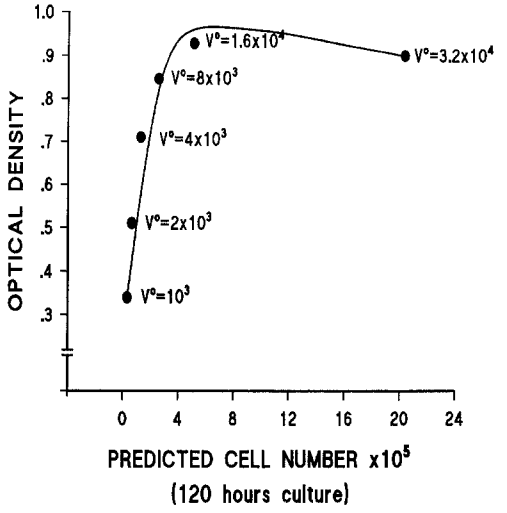

Fig. 2. Measured optical density (y axis) as a function of the predicted number of NO-36 cells in culture after 120 h (x axis) at the indicated seeding densities (cells inoculated/well, N_0). The predicted cell number for logarithmic growth was calculated from the formula $N = N_0 \exp(0.693t/T_D)$, where N = the predicted cell number at the end of culture, N_0 = the seeding density, t = the culture duration (120 h) and T_D = the doubling time (31 h). Points represent means of quadruplicate determinations. Standard deviations were <10% of the means. One representative experiment is shown. At seeding densities of <8000 cells/well the linearity indicated logarithmic growth, whereas at high seeding densities the cultures were supersaturated

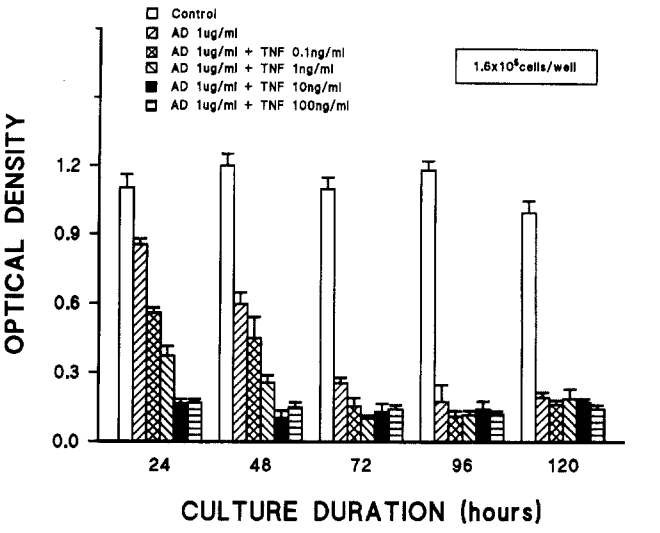

Fig. 3. Measured optical density of supersaturated NO-36 cells (y axis) treated with TNF + actinomycin D as a function of the duration of exposure in culture (x axis). NO-36 cells, resistant to TNF alone (illustrated in Fig. 6A), exhibited maximal sensitivity to actinomycin D plus TNF only after 48 h culture. The dose-response effect of TNF in the presence of actinomycin D seen at 24 and 48 h was no longer apparent after growth inhibition due to actinomycin D alone had become maximal. Bars represent means of quadruplicate determinations +1 SD. One representative experiment is shown

seeding at a 1 : 10 dilution so as to achieve 10^{-9} – 10^{-5} M final concentrations. Initial control experiments indicated that in the absence of cells, none of the eight antineoplastic drugs reduced MTT to formazan (data not shown).

Recombinant human cytokines were prepared by serial dilution in R-5 on each day of assay and were used similarly. Control cell cultures were made up to equivalent volumes (200 µl/well) by the addition of R-5. Plates were incubated at 37° C in 5% CO_2 for 96 h, then 50 µl MTT solution (2 mg/ml, 0.1 mg/well) was added to each well and plates were incubated at 37° C for a further 4 h. Medium was then aspirated and replaced with 100 µl mineral oil to solubilise the formazan over 48 h at 37° C. Optical densities were determined using a Titertek multiscan photometer (Flow Laboratories) equipped with a 590-nm filter. Plates were blanked on cell-free medium-containing wells that had been treated with MTT in a like manner. All experiments were performed in quadruplicate. Data was transferred directly into a computerised data base and statistical system.

Statistical analysis. Data was analysed by Statistical Analysis Systems (SAS) software (SAS Institute, Carey, N. C., USA). Tukey's studentised range test for multiple comparisons (GLM procedure) was used for the comparison of differences between control and treated wells and between $\log_{10}$-differing concentrations of antineoplastic drugs and cytokines. For each agent, the concentration producing a 50% reduction in optical density (IC_{50}) was computed.

Results

Optimal chemosensitivity assay conditions for mesothelioma cell lines

Figures 1–3 demonstrate optimal cell densities and kinetics for testing. Figure 1 shows the relationships between the duration of culture, the cell seeding density, and the measured optical density for four mesothelioma cell lines and for HT 29. The growth and metabolic activity of mesothelioma lines was reflected by optical density plateaus after 120 h and showed the steepest linearity at densities of 8×10^3 and 1.6×10^4 cells/well.

Figure 2 demonstrates the relationship between the optical density and the number of cells predicted to be present after 120 h for various seeding densities of NO-36 cells when cells were growing logarithmically according to the formula $N = N_0 \exp(0.693t/T_D)$, where N represents the number of cells in culture, N_0 stands for the number of cells seeded, t represents the culture duration, and T_D indicates the doubling time (31 h for NO-36). A logarithmic growth phase persisted to 120 h of culture when cells were seeded at $\leqslant 8000$ cells/well, whereas at higher seeding densities growth had reached a plateau by this time.

Figure 3 demonstrates the temporal dependence of the activity of one combination of agents tested (TNF in the presence of actinomycin D) on NO-36 cells. Heavily seeded NO-36 cells demonstrated maximal sensitivity to actinomycin D and to the combination of actinomycin D and TNF only after 48 h of continuous exposure. Based on such analyses, MTT assay conditions involving 96-h culture duration and cell seeding densities of 4,000 or 8,000 cells/well were selected for all mesothelioma cell lines studied. These conditions ensured that untreated control cultures were in logarithmic growth at the time of MTT substrate handling, that sufficient time had elapsed for metabolic inhibition to be maximally reflected by the reduction in measured optical densities, and that control cultures produced optical densities in the range of 0.8–1.2, from which level a reduction caused by antineoplastic agents could be reliably detected.

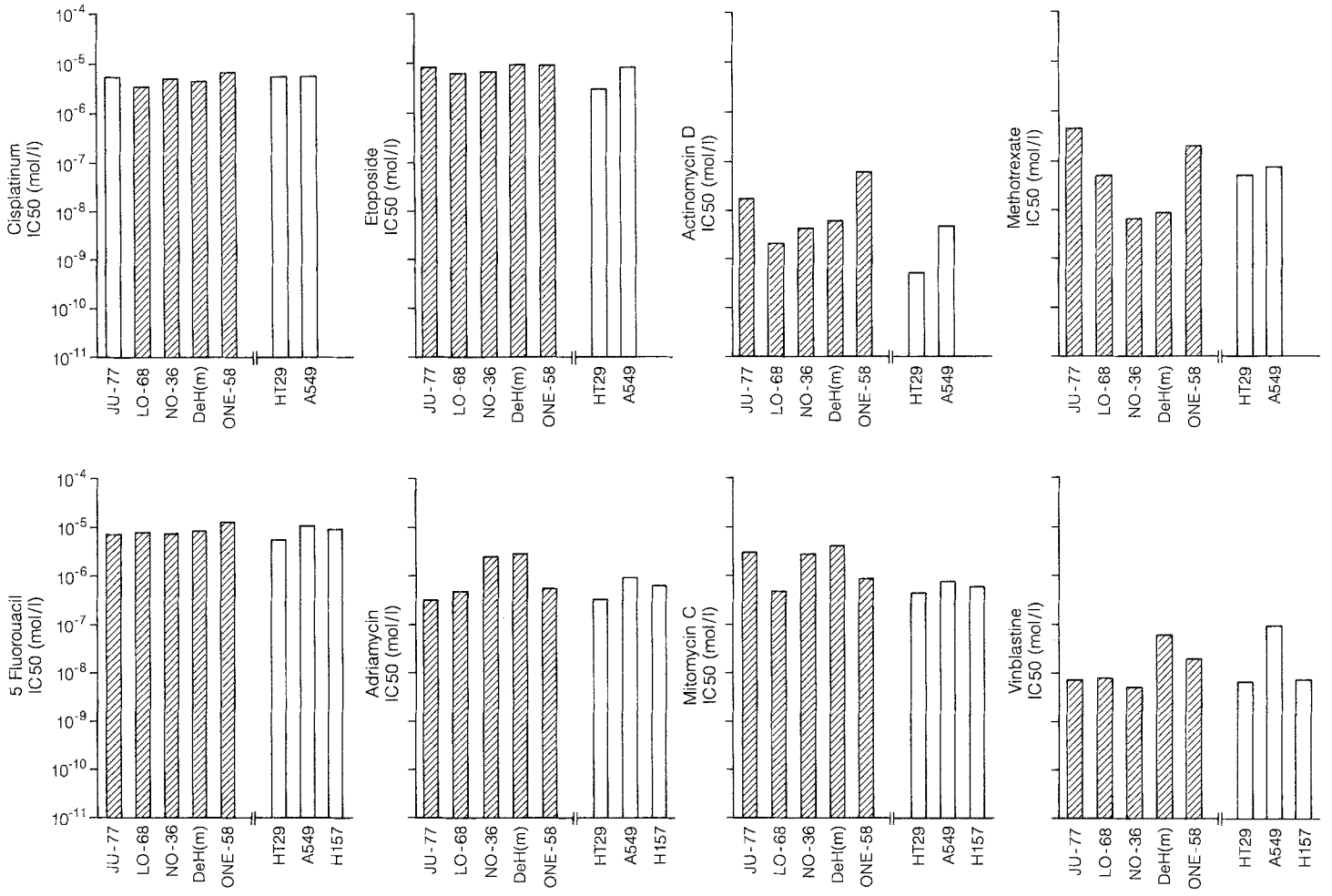

Fig. 4. MTT reactions for mesothelioma (*hatched bars*) and 3 other tumour cell lines (*open bars*; see Materials and methods for a description of tumour cell lines). Optical densities were measured after 96 h culture; cells were exposed to $\log_{10}$ molar concentrations of antineoplastic drugs ranging from $10^{-10}-10^{-5}$ M and were seeded at 8000 cells/well. Dose response curves were plotted for each experiment, and the dose of drug producing a 50% reduction in control (untreated) optical density was calculated (IC_{50}). IC_{50} (bars' length) is plotted on a $\log_{10}$ scale of drug concentration: the shorter the bar, the more potent the agent. For most cytotoxic drugs, IC_{50}s were equivalent or greater for mesothelioma as compared with non-mesothelioma lines. Actinomycin D was the most potent of the drugs tested

Chemosensitivity of malignant mesothelioma cells

The IC_{50}s of fluorouracil ($\sim 10^{-5}$ M), cisplatin ($\sim 5 \times 10^{-6}$ M), and etoposide ($\sim 10^{-5}$ M) were similar on all five mesothelioma and three non-mesothelioma cell lines (Fig. 4). However, the values for doxorubicin, methotrexate, vinblastine, mitomycin C, and actinomycin D showed greater variation among mesothelioma lines. Actinomycin D was the most potent anti-mesothelioma drug in vitro. In general, malignant mesothelioma cell lines showed equivalent or greater drug resistance than did the lung or colon carcinoma lines. The chemosensitivity profile of one of the earliest generated mesothelioma cell lines (JU-77) was reassessed after 2 years, with similar results being obtained (Fig. 5), indicating that the characteristics of these cells remained stable despite repeated passaging of cells in culture over this period.

Sensitivity of mesothelioma cell lines to recombinant cytokines

The direct effects of recombinant human cytokines on malignant mesothelioma and non-mesothelioma cell lines are shown in Fig. 6. None of the cell lines was sensitive to the direct effects of TNF alone (Fig. 6A), although an interactive effect of combined exposure to the protein synthesis inhibitor actinomycin D and TNF could be demonstrated (illustrated for NO-36 cell in Fig. 3). IFNα caused minor growth-inhibitory effects in all mesothelioma cell lines at high concentrations, although the growth of one line, LO-68, was particularly inhibited by IFNα at concentrations of >100 units/ml ($P < 0.05$, 300 units/ml vs control values; Fig. 6B). IFNγ produced direct inhibition of two of five mesothelioma lines, JU-77 and ONE-58 ($P<0.05$, 300 units/ml vs control values), comparable quantitatively with the effect on the colon carcinoma line HT29. This agent also profoundly inhibited the growth of A549 cells at low concentrations ($P < 0.05$, 3 units/ml vs control values; Fig. 6C).

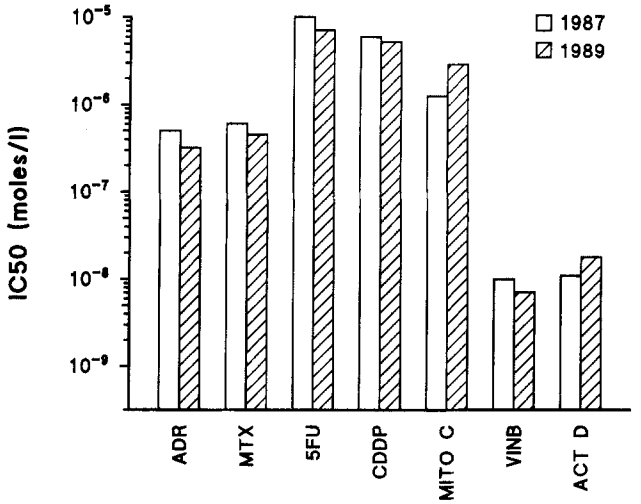

Fig. 5. IC_{50}s derived from MTT chemosensitivity experiments repeated on JU-77 mesothelioma cells at an interval of 2 years have been closely reproduced for all 7 antineoplastic drugs tested, indicating the temporal stability of the chemosensitivity characteristics of this cell line

The capacity of cytokines to produce synergistic or additive antineoplastic effects against tumour cells that were simultaneously exposed to combinations is shown in Fig. 7. Within the indicated dose range, additive or synergistic activities against mesothelioma cell lines were uncommon, exceptions being ONE-58 (IFNα plus TNF) and JU-77 (IFNα plus IFNγ). In contrast, non-mesothelioma cell lines showed varying degrees of sensitivity to all three combinations, the most powerful interaction occurring between IFNα and TNF against the NCI-H157 line.

Discussion

The escalating incidence of malignant mesothelioma in certain parts of the world and the failure of regional therapies, including surgery and radiotherapy, to control the disease clinically have made new approaches to treatment mandatory. These include renewed interest in a systemic treatment approach using chemotherapeutic drugs and recombinant cytokines. We reasoned that systematic testing of the in vitro chemosensitivity of this tumour may provide a basis by which new drugs can be selected for clinical evaluation. We also considered that these studies would provide some insight into mechanisms of the clinical drug resistance of mesothelioma, with the demonstration of in vitro resistance implying an intrinsic cellular resistance mechanism rather than an extrinsic mechanism such as tissue distribution, drug metabolism, or clinical therapeutic index.

The MTT assay method is rapid and reproducible and has been shown to exhibit sensitivity and specificity characteristics for some tumours that suggest that it may be useful in predicting patients' clinical responses to therapy [3]. As a measure of metabolic substrate conversion by cells in culture, it does not directly evaluate a drug's effect on cell division, nor does it enable the assessment of agents such as cyclophosphamide, which require in vivo metabolism to an active agent; however, it is capable of simply

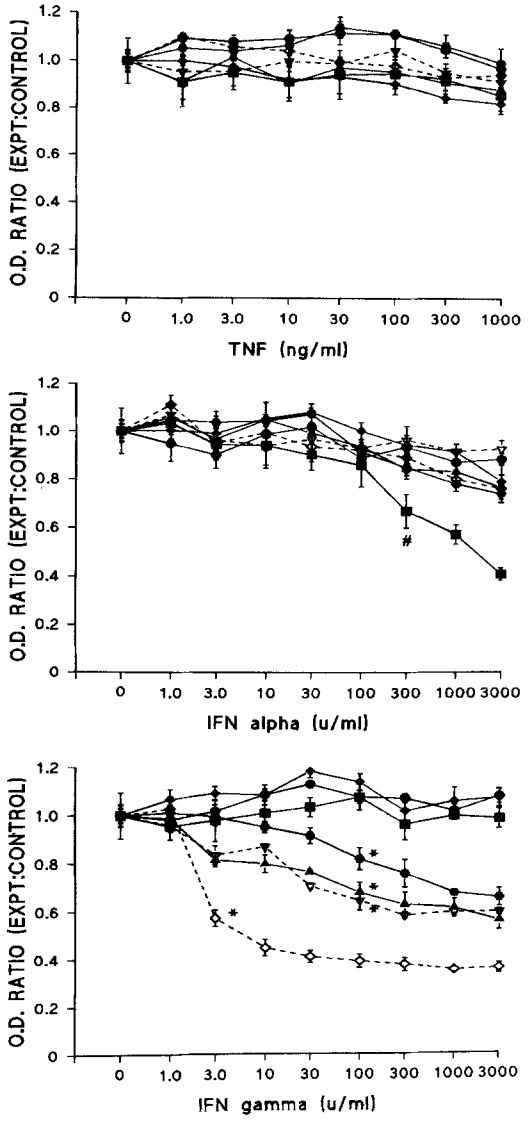

Fig. 6. Sensitivity of 5 malignant mesothelioma and 2 other tumour lines to rHuTNFα (*top*), rHuIFNα (*center*), and rHuIFNγ (*bottom*). The ratio of optical density measured on cells exposed to recombinant cytokines to that measured on control (untreated) cells, representing the mean ±1 SD, as a function of cytokine concentration (note the *x*-axis log scale). All cell lines were resistant to TNF alone (*top*). The growth of all mesothelioma lines was retarded by IFNα (*center*) at high concentrations (3000 units/ml vs control; $P < 0.05$ as computed from raw data). LO-68 was 1 log more sensitive to IFNα than were the other mesothelioma lines (300 units/ml vs control, $^*P < 0.05$). IFNγ (*bottom*) was growth inhibitory to JU-77, ONE-58, and HT 29 (*100 units/ml vs control, $^*P < 0.05$) and to A549 (3 units/ml vs control, $^*P < 0.05$)

and accurately distinguishing varying degrees of drug resistance in neoplastic cells [29]. Because the assay requires standardisation according to tumour-cell doubling time and is affected by the duration of culture prior to and during the MTT-to-formazan reaction [9, 23, 24], it was necessary that optimal conditions be determined for its application to mesothelioma cell lines. We found that the growth characteristics of our established mesothelioma lines were sufficiently similar to enable all of them to be tested under uniform conditions of seeding density and

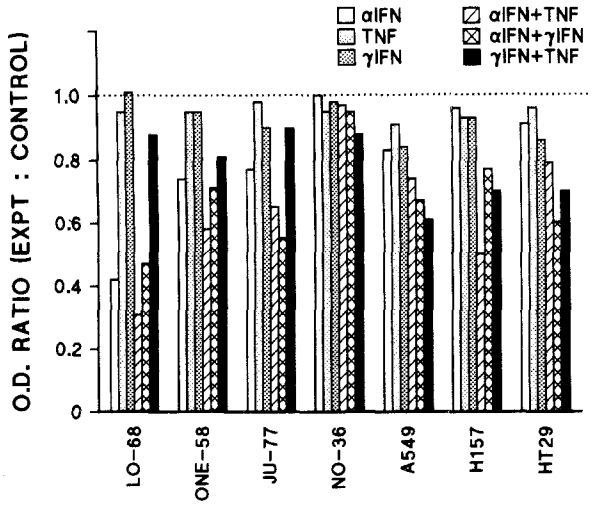

Fig. 7. Effect of recombinant cytokines rHuTNFα (10 ng/ml), rHuIFNα (1000 units/ml), and rHuIFNγ (1 unit/ml) alone and in combination on 4 mesothelioma (LO-68, ONE-58, JU-77, NO-36) and 3 other solid-tissue tumour cell lines (A549, H157, HT29). Data represent mean ratios of treated vs untreated cells (*expt: control*). Standard deviations of the raw data were always <10% of the means. One representative experiment of three is shown

culture duration and to enable the performance of comparative experiments under the same conditions on several other human solid-tumour cell lines representing cancers that are known to be clinically resistant to chemotherapy. Our demonstration that the drug susceptibility of the JU-77 line remained unaltered over 2 years indicates both the temporal stability of the chemosensitivity characteristics of this cell line and the robustness of the in vitro assay system.

None of the eight commonly used anti-cancer drugs tested showed more than 0.5-log greater potency (IC_{50}) for mesothelioma as compared with their activity against non-mesothelioma lines. We concluded that mesothelioma cells were intrinsically resistant to the drugs in the test panel, and this finding was consistent with the clinical behaviour of the tumour. This in vitro resistance is relative rather than absolute, since at high concentrations all of the anti-cancer drugs investigated except fluorouracil produced some metabolic inhibition of mesothelioma cells in culture. The drug resistance of mesothelioma cells may be confined only to the drugs tested, with these cell lines being susceptible to other agents not yet assessed.

However, an alternative possibility would be that the cells express a primary genetic resistance mechanism. A number of such mechanisms have been well characterised, including the drug export system operated by P-glycoprotein under the control of the MDR1 gene [28]. The significance of P-glycoprotein as a clinically important drug-resistance mechanism is uncertain. The MDR1 gene is highly inducible in tumour cell lines by a number of antineoplastic agents [8], implying a role in secondary drug resistance. However, primary expression of P-glycoprotein by tumours has also been described [15]. The MDR1 gene was not constitutively expressed by any of 20 mesothelioma lines investigated by Gerwin et al. (personal communication) or by any of the 5 lines tested in the present study (data not shown) and is therefore not likely to be responsible for the primary drug resistance of mesothelioma.

We also showed that these mesothelioma cell lines are generally resistant to the direct effects of recombinant cytokines. The extent to which this resistance bears upon the clinical efficacy of these agents is unknown. There is considerable evidence that the in vivo activity of cytokines is mediated by indirect means mainly through boosting of immune cell function. Interferons have been shown to boost natural killer cell function [14] and to increase the expression of major histocompatibility complex (MHC) molecules on tumour cells [6], thereby possibly enhancing their recognition by immune effector cells, with both of these functions being of potential importance in immune surveillance and tumour-cell immunocytolysis.

Recent studies investigating the susceptibility of human malignant mesothelioma to immunological mechanisms have demonstrated that mesothelioma cells are susceptible to lysis by lymphokine-activated killer (LAK) cells but are resistant to lysis by natural killer (NK) cells [17], that NK cell susceptibility is increased in the presence of interleukin-2 [17], and that some patients presenting with malignant mesothelioma display diminished LAK cell activity against allogeneic mesothelioma cell targets that is restorable by indomethacin [18]. Direct in vitro inactivity of recombinant cytokines may not predict their in vivo or clinical inactivity against mesothelioma, because the indirect actions of cytokines as regulators of immunocytolytic processes may be of considerable importance in vivo.

Although rHuTNF has been shown to be ineffective even when used together with actinomycin D against a human mesothelioma cell line transplanted into nude mice [7], it has not been evaluated clinically or in an immunocompetent animal model of this disease. On the other hand, recombinant IFNα exhibits some clinical activity in a small proportion of patients presenting with malignant mesothelioma [10]. Possible mechanisms of resistance to the direct effects of recombinant cytokines include the absence of specific receptors on mesothelioma cells, defective signal transduction, or the synthesis of inhibitors by mesothelioma cells. Although specific receptor binding studies have not been performed, it is not likely that mesothelioma cells lack receptors for IFNα, since this substance up-regulates MHC class I mRNA in all of these cell lines [11]. Likewise, IFNγ up-regulates MHC class I mRNA in all lines except DeH(m) and increases the expression of MHC class II (DR) mRNA and surface antigen in three of five lines [11].

The anti-proliferative activity of IFNs against tumour cells has previously been shown to be markedly increased by simultaneous exposure to other cytokines, including combinations of IFNα and IFNγ and of either IFN species with TNF [25–27]. We observed antineoplastic interactions between these recombinant cytokines against some but not all cell lines. On mesothelioma cell lines, the direct activity of cytokines does not correlate with MHC antigen expression or regulation or with susceptibility to immune effector cells. The heterogeneity displayed by individual cell lines may be attributable to quantitative or qualitative intertumour variability in cytokine receptor expression or regulation, or in post-receptor signal-transduction path-

ways. These determinants of the end results of cytokine exposure require further study in mesothelioma.

Overall, these data suggest that immunotherapeutic approaches to mesothelioma may be more effective if they do not rely solely on direct anti-tumour activities. The results of this study, including the relative ease and efficiency with which malignant mesothelioma cell lines can be established from readily available clinical material, the general suitability of such lines to testing by the MTT assay, and their demonstrated intrinsic resistance to standard antineoplastic drugs and cytokines, indicate the potential of in vitro chemosensitivity testing to determine susceptibilities to novel anti-cancer agents, thereby providing a selection mechanism for promotion of the most active agents to clinical trial. The validity and usefulness of such application in mesothelioma requires more extensive laboratory and clinical evaluation. Its direct clinical application to individual tailoring of patient therapy is likely to be limited not only by difficulties involved in establishing cell lines within a useful time-frame but also by the intrinsic resistance of this tumour to existing drugs, such that the frequency of identification of an active agent is likely to be too low to justify the endeavour. Further investigation of the unique biological characteristics of malignant mesothelioma is required to maximise the likelihood of establishing effective therapeutic strategies.

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