Anti-Cancer Drugs, 4, pp. 265–272

Antiproliferative and chemomodulatory effects of interferon-γ on doxorubicin-sensitive and -resistant tumor cell lines

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Biological agents might offer various therapeutic opportunities in the treatment of cancer, including a direct and/or host-mediated antiproliferative effect and also the possibility to favorably modulate tumor resistance to antineoplastic drugs. We studied the *in vitro* antiproliferative effects of interferon (IFN)-γ on the mouse B16 melanoma and Friend erythroleukemia, and the human K562 erythroleukemia, as doxorubicin (DXR)-sensitive and -resistant (multidrug resistant) variants. These effects were marked in B16 melanoma and rather slight in K562 erythroleukemia, without any difference between the DXR-sensitive and -resistant lines. The chemosensitive variant of Friend erythroleukemia showed an intermediate response, which was greater than that seen in its resistant counterpart. There was no apparent relationship between the antiproliferative activity of IFN-γ and the glutathione content of the cell lines. On the other hand, this activity was enhanced by co-treatment with glutathione-depleting concentrations of buthionine sulfoximine, but only in the cell lines which had responded better to IFN-γ alone. This result probably confirms that a free radical mechanism plays a part in the antitumor effect of the cytokine. Finally, a range of concentrations of IFN-γ, including slightly cytotoxic ones, did not substantially improve the antiproliferative effects of doxorubicin on the various cell lines, except in the DXR-sensitive variant of Friend erythroleukemia where a synergistic effect of the combination was observed. Thus, our results are not very promising with regard to a possible favorable modulatory activity by IFN-γ of DXR (multidrug)-resistance.

Key words: Antiproliferative effects, interferon-γ, multidrug resistance.

Introduction

One of the main reasons for the failure of anticancer chemotherapy is drug resistance, which is often of multiple type (multidrug resistance, MDR) and associated with various mechanisms, especially the over-expression at the tumor cell level of an energy-dependent multidrug efflux pump known as P-glycoprotein (for review, see, e.g. refs 1 and 2). Thus, at the present time considerable research is being made to develop therapeutic modalities which are capable of overcoming this problem, either by producing antiproliferative effects in drug-resistant populations or by conferring or restoring drug-sensitivity.

With regard to this, great interest is being directed towards biological agents, including interferons (IFNs). In theory, such compounds might in fact control neoplastic growth both by stimulating the host's response and by direct antiproliferative effects (for review, see ref. 3). In addition, they may positively interact with the conventional antitumor agents.[4] However, information on their activity on chemoresistant tumors is still scanty.[5–7] On the other hand, recent data have indicated the possibility that the IFNs, in particular type I IFNs (IFN-α and IFN-β), may favorably modulate the level of chemoresistance in certain MDR models.[8,9]

In this paper, we have focused on IFN-γ and studied its *in vitro* effects on the growth of three tumor cell lines, the mouse B16 melanoma and Friend erythroleukemia, and the human K562 erythroleukemia, both as doxorubicin (DXR)-sensitive and -resistant variants. The chemoresistant variants had characteristics of 'typical' MDR,[1,2] including cross-resistance to vincristine (VCR) and over-expression of P-glycoprotein.[10–12] We also examined one of the possible causes which might determine the cell responsiveness to IFN-γ; since in some tumors IFN-γ might act through oxidative stress with greater efficacy on cells depleted of their glutathione content,[13] we studied

This work was partially supported by AIRC and MURST 60%.

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the antiproliferative effects of IFN-γ combined with glutathione-depleting concentrations of buthionine sulfoximine (BSO). Finally, other experiments were performed in order to ascertain whether a co-treatment with IFN-γ could modify the sensitivity to DXR of the drug-sensitive and -resistant cell lines.

Materials and methods

Drugs

Doxorubicin hydrochloride was supplied by Farmitalia Carlo Erba (Milan, Italy). Hi-pur recombinant mouse IFN-γ (CHO derived, specific activity about 10×10^6 U/mg IFN) and recombinant human IFN-γ (*Escherichia coli* derived, specific activity about 20×10^6 U/mg IFN) were supplied by Holland Biotechnology BV (Leiden, The Netherlands). DL-buthionine-[S, R]-sulfoximine was from Sigma (St Louis, MO).

Cell lines and cytotoxicity assays

Mouse B16 melanoma cells as parental (B16) or DXR-resistant variants (B16/DXR) were obtained *in vivo* as previously described.[10] Mouse Friend erythroleukemia cells as parental (FLC) or DXR-resistant (FLC/DXR) variants, and human K562 erythroleukemia cells as parental (K562) or DXR-resistant variants (K562/DXR) were kindly supplied by Dr Haim Tapiero (ICIG, Villejuif, France).[11,12]

All the cell lines were grown in RPMI 1640 (Gibco, Grand Island, NY) containing 10% fetal calf serum (Gibco), and 1% penicillin and streptomycin; in the case of B16 and B16/DXR 1 mM sodium pyruvate was also added to the culture medium and, in order to maintain the level of the resistance, B16/DXR cells were routinely grown in the presence of DXR 15 ng/ml. There was a humidified atmosphere of 5% CO_2 in air at 37°C. B16, B16/DXR, K562 and K562/DXR were subcultured twice a week; FLC and FLC/DXR three times a week.

For the experiments, the cells were generally seeded at 1×10^5/ml in 24-well culture plates (Nunc, Roskilde, Denmark) in the presence of various concentrations of the drugs. At 72 h later the viable cells were counted through the microscope with Trypan blue exclusion. In the case of B16 and B16/DXR the cells were harvested by trypsin-EDTA because they had grown as monolayer cultures.

Glutathione assay

The cells were seeded at 1×10^5/ml in the presence or not of BSO. At 72 h later the cells were washed twice with PBS, counted and pelleted. Their content of total glutathione was determined as previously described[10] according to Griffith.[14]

Results

Drug-sensitivity profiles of the cell lines

The MDR phenotypes of B16/DXR, FLC/DXR and K562/DXR have already been described.[10,12] The drug-sensitivity profiles and some other characteristics of the cell lines are shown in Table 1. When compared with their parental counterparts, B16/DXR, FLC/DXR and K562/DXR proved to be resistant both to DXR and VCR. The MDR

Table 1. Some characteristics of B16 melanoma, Friend erythroleukemia and K562 erythroleukemia as DXR-sensitive or -resistant variants.

	IC_{50} DXR (ng/ml)[a] (RI)	IC_{50} VCR (ng/ml)[a] (RI)	Doubling time[a] (h)	Total glutathione[b] (nmol/mg protein)
B16	5.5	24.0	13.6	35.4 ± 2.6
B16/DXR	65.0 (11.8)	170.0 (7.1)	11.1	47.8 ± 1.1
FLC	5.3	12.0	13.3	8.8 ± 0.5
FLC/DXR	460.2 (86.8)	155.0 (12.9)	13.5	23.6 ± 0.9
K562	10.1	14.0	22.8	22.0 ± 1.4
K562/DXR	280.4 (27.7)	659.9 (47)	21.8	18.2 ± 2.6

[a]Mean of triplicate experiments with SD ≤ 10%. RI: index of resistance.
[b]Mean of triplicate experiments ± SD.

phenotype of these cell lines included also the over-expression of P-glycoprotein, detected by immunocytochemistry or immunoblotting.[10,12]

Sensitivity of the cell lines to the antiproliferative effects of IFN-γ

The effect of IFN-γ on the *in vitro* growth of the cell lines exposed for 72 h to the cytokine is shown in Figure 1. B16 and B16/DXR were quite sensitive to IFN-γ which was active in the range of a limited number of U/ml (Figure 1A). It should be noted that B16 and B16/DXR were cultured as adherent cells and that IFN-γ had been present from the onset of the culture. Therefore, there was the possibility that the marked antiproliferative effect of the cytokine on the two tumors was due to its interference with cell attachment on the plates. However, this was not the case because, when the cells were precultured in the absence of IFN-γ for 24 h and then exposed to it for 72 h (experiment not shown), the concentrations of IFN-γ inhibiting the growth of B16 and B16/DXR did not differ from those found in the experiment in Figure 1A in which IFN-γ was present throughout the whole culture period.

The response to the cytokine of FLC and FLC/DXR was not as good as that seen in B16 and B16/DXR. It was also dissimilar in the two leukemias (Figure 1B). In fact, the antiproliferative effect of IFN-γ progressively increased in FLC, reaching the concentration inhibiting the growth of 50% (IC_{50}) between 100 and 250 U/ml, and with no further variation by escalating the doses up to 10 000 U/ml, while in the case of FLC/DXR a steady inhibition of the growth approximately equal to 20% was observed over the whole range of concentrations tested.

Both K562 and K562/DXR were marginally sensitive to IFN-γ which produced slight decreases in their growth over the whole range of the concentrations tested (Figure 1C).

Effect of BSO

The antiproliferative activity of IFN-γ was also studied in combination with glutathione-depleting concentrations of BSO (Figure 2). In these experiments the cells were seeded in the presence of a fixed concentration of BSO; 24 h later, various concentrations of IFN-γ were added and the cells were counted after a further 48 h.

The basal glutathione content was 35.4 ± 2.6 nmol/mg protein in B16 and 47.8 ± 1.1 nmol/mg protein in B16/DXR (Table 1); BSO 50 μM for 72 h reduced the glutathione content to 1.0 ± 0.3 (2.8% of the basal content) in B16 and to 1.0 ± 0.2 (2.0% of the basal content) in B16/DXR. After 72 h the same concentration of BSO lowered the growth of B16 by 20% and that of B16/DXR by 32% (not shown); in plotting the combination data (Figure 2A and A*), these effects of BSO alone were compensated by making these equal to 100%. As illustrated in Figure 2A and A*, the effects of BSO 50 μM and IFN-γ on the growth of B16 and B16/DXR were slightly, but consistently, synergistic.

The basal glutathione content was 8.8 ± 0.5 nmol/mg protein in FLC and 23.6 ± 0.9 nmol/mg protein in FLC/DXR (Table 1); BSO 100 μM for 72 h lowered this level to 2.1 ± 0.6 (23.8% of the basal content) in FLC and to 1.9 ± 0.2 (8.0% of the basal content) in FLC/DXR. After 72 h the same concentration of BSO lowered the growth of FLC by 9% and that of FLC/DXR by 6% (not shown); in presenting the combination data (Figure 2B and B*), these effects of BSO alone were compensated by making these equal to 100%. The combination of BSO 100 μM and IFN-γ produced slightly synergistic effects on the growth of FLC (Figure 2B) and substantially additive ones on that of FLC/DXR (Figure 2B*).

The basal content of glutathione was 22.0 ± 1.4 in K562 and 18.2 ± 2.6 in K562/DXR (Table 1). BSO 100 μM for 72 h lowered this level to 0.3 ± 0.2 (1.3% of the basal content) in K562 and to 0.3 ± 0.1 (1.6% of the basal content) in K562/DXR, and had no effect on the growth of the two cell lines (not shown). In the same lines, BSO 100 μM did not modify the antiproliferative effects seen with IFN-γ alone (Figure 2C and C*).

Effect of IFN-γ on the sensitivity to DXR of the cell lines

Finally, we studied the activity of DXR in combination with IFN-γ (Figure 3). In these experiments, the cells were seeded in the presence of a fixed slightly cytotoxic concentration of IFN-γ; 24 h later, a range of concentrations of DXR was added and the cells were counted after an additional 48 h.

For B16 and B16/DXR the doses of IFN-γ used were 0.5 and 2.0 U/ml, which, alone, reduced the growth of both tumors after 72 h by about 20% (0.5 U) and 25% (2.0 U) (as in the experiment of

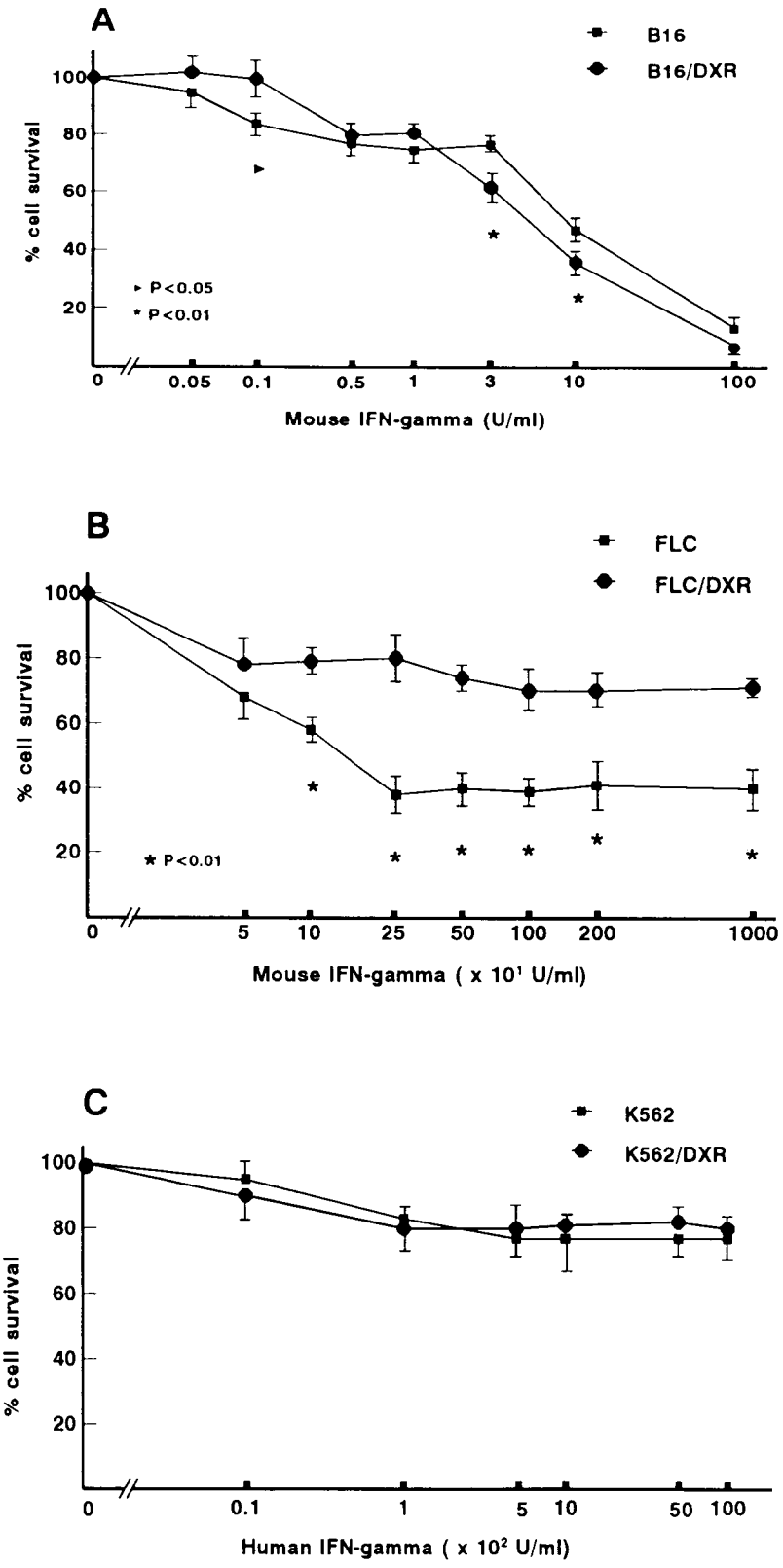

Figure 1. Effect of IFN-γ on the growth of B16 and B16/DXR cells (A), FLC and FLC/DXR cells (B), and K562 and K562/DXR cells (C) exposed for 72 h to the biological agent. Results are the mean $\pm$ SD of three independent experiments.

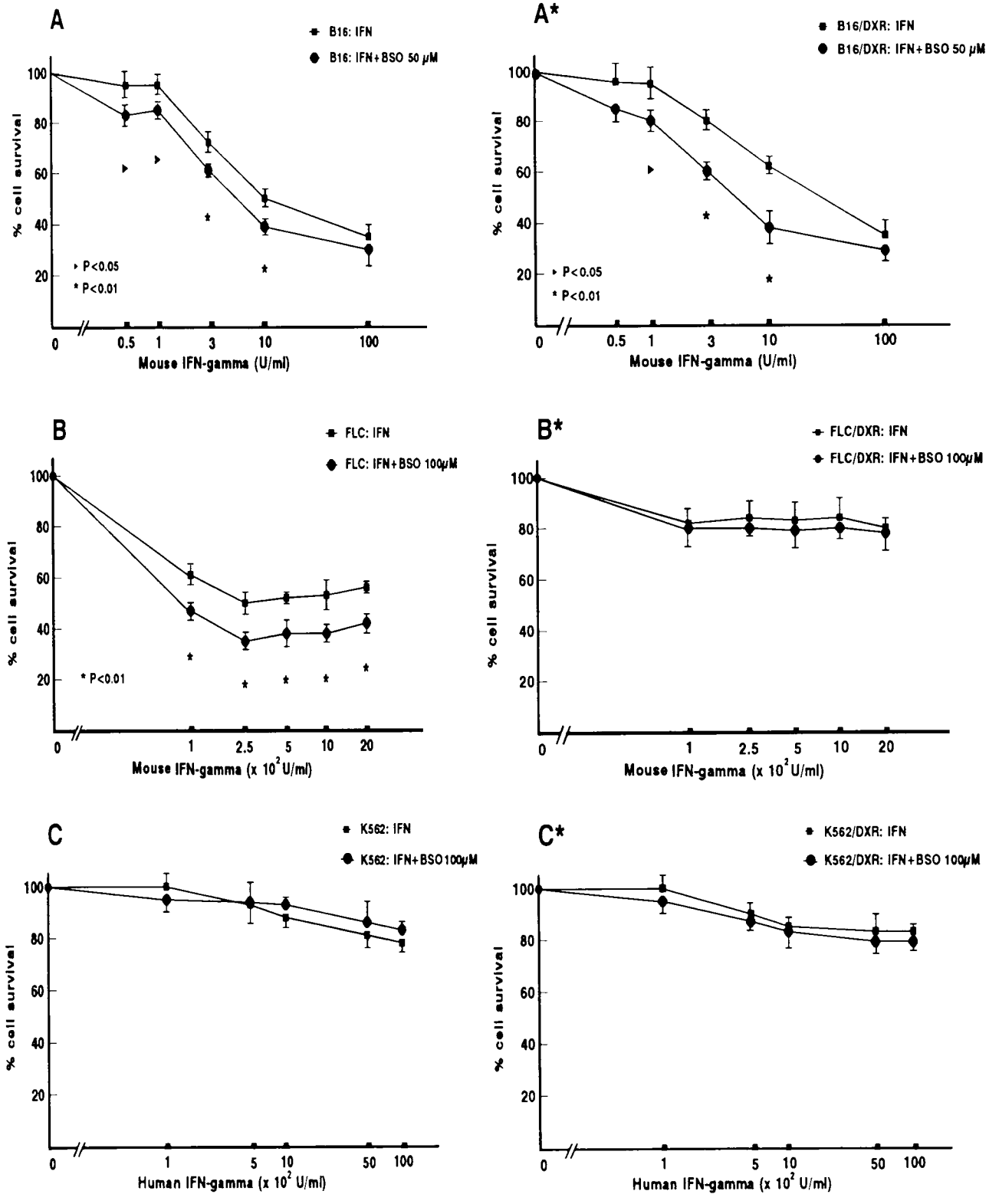

Figure 2. Effect of IFN-γ ± BSO on the growth of B16 (A) and B16/DXR (A*) cells, FLC (B) and FLC/DXR (B*) cells, and K562 (C) or K562/DXR (C*) cells. The cells were seeded in the presence of BSO or not. After 24 h, various concentrations of IFN-γ were added and the cells were counted after a further 48 h. In (A), (A*), (B) and (B*), combined IFN-γ/BSO data are normalized to account for the cell survival inhibitory effect of BSO alone (see Results). Results are the mean ± SD of three independent experiments.

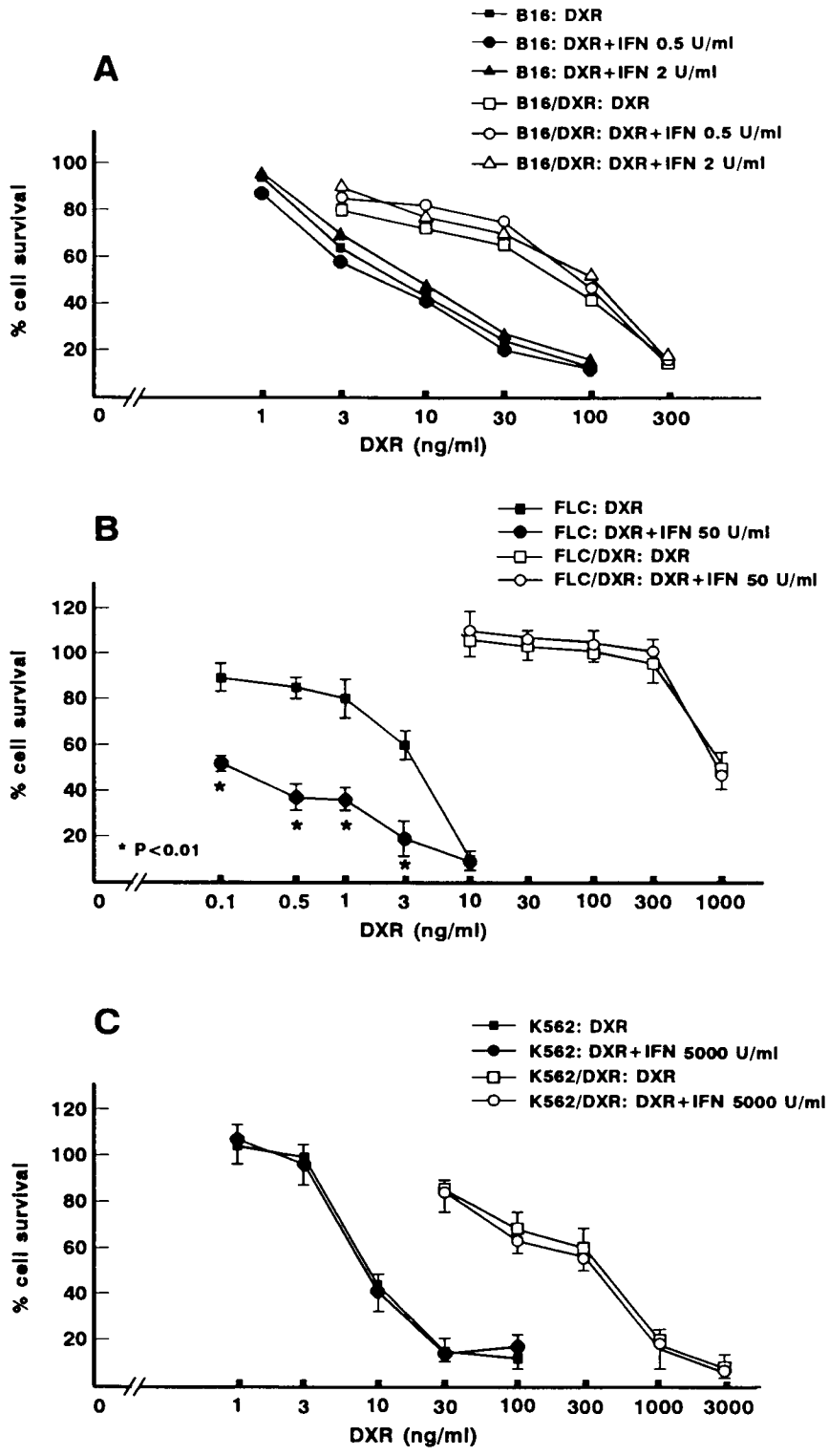

Figure 3. Effect of DXR ± IFN-γ on the growth of B16 and B16/DXR cells (A), FLC and FLC/DXR cells (B), and K562 and K562/DXR cells (C). The cells were seeded in the presence of IFN-γ or not. After 24 h, various concentrations of DXR were added and the cells were counted after a further 48 h. Combined DXR/IFN-γ data are normalized to account for the cell survival inhibitory effect of IFN-γ alone (see Results). Data are the mean ± SD of three independent experiments. In (A), SD ≤ 10.7%.

Figure 1A); in plotting the combination data (Figure 3A), these effects of IFN-γ alone were compensated by making these equal to 100%. The combination of either dose of IFN-γ with DXR produced substantially additive effects on the growth of B16 and B16/DXR (Figure 3A).

For FLC and FLC/DXR the dose of IFN-γ used was 50 U/ml, which, alone, reduced the growth of FLC after 72 h by about 30% and that of FLC/DXR by about 20% (see also the experiment of Figure 1B); in plotting the combination data (Figure 3B) these effects of IFN-γ alone were compensated by making these equal to 100%. The combination of this dose of IFN-γ with DXR produced clear and reproducible synergistic effects in FLC and additive ones in FLC/DXR (Figure 3B). The same kinds of interaction were obtained when the combination of a higher dose of IFN-γ (200 U/ml) with appropriate concentrations of DXR was tested in FLC and FLC/DXR (not shown).

For K562 and K562/DXR the doses of IFN-γ used were 100, 1000 or 5000 U/ml, which, alone, produced slight inhibitions of the growth of the two cell lines after 72 h (as in the experiment of Figure 1C); in plotting the combination data, these effects of IFN-γ alone were compensated by making these equal to 100%. The combination of 5000 U of IFN-γ with DXR produced merely additive effects on the growth of K562 and K562/DXR (Figure 3C). Also, in the other experiments (not shown), in which the doses of IFN-γ were 100 or 1000 U/ml, the addition of the cytokine to DXR did not produce anymore than the sum of the antiproliferative effects of the two agents given separately.

Discussion

In the present paper we have studied the effects of a possibly useful therapeutic cytokine, IFN-γ, on the *in vitro* growth of three tumor cell lines, the mouse B16 melanoma and Friend erythroleukemia, and the human K562 erythroleukemia, as DXR-sensitive and -resistant (MDR) variants. The responsiveness to IFN-γ was fairly good in B16 and B16/DXR, as judged by their sensitivity to a limited number of U ml of the cytokine; it was slight in K562 and K562 DXR. The chemosensitive variant of Friend erythroleukemia, FLC, showed an intermediate response, which was greater than that seen in its chemoresistant counterpart, FLC DXR.

With reference to this last result, it is noteworthy that decreases in the sensitivity to the antiproliferative effects of IFN-γ have already been described in other MDR variants.[5,6] Thus, our results and those of others suggest that the acquisition of an MDR phenotype may in some cases alter the response to this biological agent, a notion that could be relevant in the clinical context.

The different responsiveness of our cell lines to IFN-γ might represent a model which is suitable to study the determinants which influence the cell response to the direct antiproliferative effects of the cytokine. Clearly, many factors have been put forth[3,15-17] and among these it has been suggested that an enhanced free radical generation and, counteracted to it, the efficiency of the glutathione-related antioxidant activities may be involved, other than in the host-mediated,[18] in the direct antitumor effects of this biological agent.[13] Thus, we studied the glutathione content of the cell lines (Table 1) as well as the antiproliferative effects of IFN-γ on the cells treated with glutathione-depleting doses of BSO. The analysis of the data did not show any apparent straightforward relationship between low glutathione levels and sensitivity to the antiproliferative effect of IFN-γ; for example, the glutathione content of K562 and K562/DXR, which responded slightly to IFN-γ, was lower than that of B16 and B16/DXR, which were the most responsive. On the other hand, glutathione-depleting concentrations of BSO synergized with IFN-γ, but this occurred only in those cells which responded better to the cytokine alone, i.e. B16, B16/DXR and FLC. In the other tumor lines some causes, of which we are not currently aware, probably prevented the antiproliferative effects of the cytokine and the mechanisms related to it from taking place completely; these causes could involve alterations in the number or function of cell surface receptors to IFN-γ as well as post-receptor events.[3,5,15] Also, in a previous *in vivo* study of ours on mouse P388 leukemia, as a DXR-sensitive or -resistant (MDR) variant, IFN-γ produced very poor, probably mostly direct, antiproliferative effects on both the lines and these effects were not enhanced by co-treatment with BSO.[6] Part of our present results are in agreement with the observations of others where a synergism of the combination of BSO and IFN-γ occurred;[13] however, they also indicate that glutathione depletion with BSO should not be viewed as a universal tool to specifically increase the direct antiproliferative activity of the biological agent.

Finally, it has been proposed that type I IFNs, at slightly cytotoxic concentrations, might favorably modulate the cell responsiveness to DXR in MDR tumors and not in the chemosensitive ones,

perhaps by interacting at the P-glycoprotein level.[8,9] However, this was not the case in our MDR cell lines treated with IFN-γ and DXR. An encouraging synergistic antiproliferative effect of this combination was seen only in the Friend erythroleukemia chemosensitive variant, FLC; additive interactions occurred in all the other cell lines and, in the case of the MDR variants, they are probably of scholastic interest because they were observed at the high DXR concentrations necessary to affect the growth of these chemoresistant cells.

The possibility of additive or synergistic antiproliferative effects of the combination of IFN-γ with DXR has already been noticed on cell lines not selected for resistance to DXR.[19-21] On the other hand, Di Cicco *et al.*[22] have reported that treatment with IFN-γ does not alter cellular resistance to DXR in murine P388 leukemia cells sensitive or resistant to the drug. Also, our results are not promising with regard to the possible modulatory actvity of IFN-γ on resistance to DXR.

In conclusion, our data extend the observations on the important chapter of the interactions between biological agents and tumor cell populations, particularly drug-resistant ones. Of course, we have taken into account only one of the possible aspects of the antitumor activity of IFN-γ, i.e. that of its direct effects at the cell level. Certainly, it is worth carrying out further studies in more complex, especially *in vivo* models, in order to fully assess the therapeutic possibilities of IFN-γ against drug-sensitive and -resistant cancer.

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(Received 3 December 1992; accepted 25 January 1993)