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Development of Tumour Cell Resistance to Tumour Necrosis Factor Does Not Confer Resistance to Cytotoxic Drugs

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Abstract—*Tumour necrosis factor (TNF) is a protein product of macrophages with potential anti-cancer activity. As with other anti-cancer agents, tumour cells can develop resistance to the cytotoxic effects of TNF. The aim of his study was to see whether development of resistance to TNF resulted in a concomitant resistance to other anti-cancer agents, in particular those associated with multidrug resistance.*

Three TNF-susceptible tumour cell lines (L929, U937 and RK13) and their TNF-resistant sublines were compared for susceptibility in vitro *to several cytotoxic drugs. The TNF-resistant sublines were not significantly more resistant to these drugs. In addition, an L929 subline selected for resistance to actinomycin D retained its susceptibility to TNF. These observations show that tumour cell resistance to TNF develops independently of resistance to cytotoxic drugs.*

INTRODUCTION

A MAJOR PROBLEM in the chemotherapy of cancer is the development of resistance to anti-cancer drugs. Development of resistance to a particular anti-cancer drug frequently leads to resistance to other, unrelated agents to which the cancer cells have not been exposed. Cross resistance is seen with drugs which have quite distinct structures and apparently different modes of action [1]. Recently, a new potential anti-cancer agent, tumour necrosis factor (TNF), has entered clinical trials. TNF is a 17 kD protein product of macrophages and its action on cancer cells *in vivo* may be either direct or mediated via activation of an inflammatory response [2–4]. *In vitro* TNF is growth inhibitory or cytolytic to about 25% of tumour cell lines (reviewed in [5]). Tumour cell resistance to the effects of TNF can develop *in vitro* as with other cancer agents. The purpose of this study was to see if development of tumour cell resistance to TNF resulted in the acquisition of resistance to other cancer agents.

MATERIALS AND METHODS

Cell lines

Three TNF susceptible cell lines were employed (mouse fibroblastoid L929, rabbit kidney RK13 and a variant of human myelomonocytic U937) along with their TNF resistant sublines (L929/R, RK13/R and U937/R) [6, 7]. All cell lines are plastic adherent and were maintained in RPMI 1640 medium with 5% foetal calf serum. L929 cells resistant to actinomycin D (L929/ADR) were selected by treating L929 cells with 40 ng/ml actinomycin D for 1 week followed by a week in normal growth medium. After four such cycles the cells were grown continuously in 50 ng/ml actinomycin D for 4–6 months before use in the experiments described here.

Tumour necrosis factor

TNF was purified from the serum of rabbits with endotoxic shock [8] and the same batch (10^5 U/ml) was used throughout. A unit is defined as the minimal amount of TNF necessary to kill 50% of the L929 cells in a 3-day microplate assay.

Microplate cytotoxicity assay

The assay is long established in our and other laboratories, has been described in detail [9] and correlates well with other assays [10]. Briefly, 75 μl L929 cells at 10^5/ml were plated in 96-well microtitre trays. After allowing the cells to adhere, 75 μl amounts of drug dilution were added in triplicate and the trays were incubated for 3 days at 37°C.

After incubation, the culture medium containing the dead cells was flicked off and the remaining,

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adherent viable cells were fixed with formaldehyde and stained with crystal violet. The amount of dye uptake is proportional to the number of remaining cells and was quantitated photometrically at 540 nm by a Titertak Multiskan ELISA reader. Percentage cytotoxicity was calculated from the formula 100 $(a-b)/a$ where a and b are respectively the mean absorbances of triplicate wells without, or with drug.

Clonogenic assay

Cells were harvested from exponentially growing cultures, resuspended in fresh growth medium and 100 cells were plated in 2-ml volumes in 2.4-cm wells of Linbro 12-well cluster plates. After overnight establishment, the appropriate amount of anti-cancer agent was added and the cells were incubated at 37°C for a further 24 h. The agent was removed, the cells were washed once with fresh medium and then replenished with 2 ml fresh medium before incubation for an additional 6–7 days. The cells were fixed with formaldehyde and stained with crystal violet as above and colonies were counted. Each agent was tested at three concentrations with three replicates and the concentration required for 50% reduction of the colony count was determined graphically.

Enzyme assays

Tissue homogenates and assays for catalase and glutathione peroxidase were performed as described [11] except that in the catalase assay H_2O_2 consumption was measured spectrophotometrically at 234 nm. Glutathione-*S*-transferase was measured by an established method [12].

RESULTS

When tested in the conventional microplate assay for TNF susceptibility, L929/R cells were approx. 1000-fold more resistant to killing by TNF than the parental L929 line. The two cell lines were also compared for susceptibility to a number of cytotoxic drugs implicated in multidrug resistance. Initially the microplate assay was employed and the cells were continuously exposed to the drugs for 3 days (Fig. 1). In contrast to their differing susceptibilities to TNF, L929 and L929/R had comparable susceptibilities to the cytotoxic drugs, although L929/R did exhibit some increase in resistance to actinomycin D. These experiments were repeated on three additional occasions with similar results. As this microplate assay is not normally used for assessment of susceptibility to cytotoxic drugs, the more conventional clonogenic assay was also employed and the results of a representative assay are shown in Table 1. In the clonogenic assay, L929 and L929/R had comparable resistance to the cytotoxic drugs. These results were confirmed on two other occasions. Although L929/R showed an apparently increased resistance to actinomycin D and colchicine this difference is not significant in the context of this assay.

Two other TNF-susceptible cell lines, U937 and RK13, and their TNF-resistant counterparts were also tested for susceptibility to anti-cancer drugs. The simpler microplate assay was used in these studies as it gave corresponding results to the clonogenic assay when comparing L929 and L929/R. Table 2 shows that U937 and its TNF-resistant subline U937/R had comparable susceptibilities to cytotoxic drugs, as did RK13 and RK13/R. Thus in all three cases tested, with tumour cell lines from three different species, development of resistance to TNF-induced cytolysis did not result in the development of multi-drug resistance.

The data above show that development of resistance to TNF does not result in multidrug resistance. However it is also important to know whether the converse is true, i.e. does development of resistance to cytotoxic drugs lead to TNF resistance? To test this it is necessary to start with a TNF-susceptible line and select for resistance to cytotoxic drugs. For 2 years we have tried to select actinomycin D resistant L929 cells using various regimes but have only succeeded in developing a line with about 10-fold resistance to actinomycin D as measured in the

Table 1. Comparison of L929, L929/R and L929/ADR cells by clonogenic assay for susceptibility to cytotoxic drugs

Cytotoxic agent	Concentration for 50% inhibition for colony number (ng/ml) L929	L929/R	L929/ADR
Actinomycin D	88	150	1000
Daunomycin	63	67	47
Colchicine	73	145	123
Vincristine	40	30	400
Mitomycin C	32	44	25

Table 2. Comparison of U937 and RK13 cells with their TNF resistant sublines for susceptibility to cytotoxic drugs in the microplate assay

Cytotoxic agent	Concentration* (ng/ml)	Percentage cytotoxicity† U937	U937/R	RK13	RK13/R
TNF	10,000	—	—	75 ± 1	24 ± 7
	1000	—	—	65 ± 3	23 ± 4
	100	95 ± 2	15 ± 2	39 ± 7	24 ± 8
	10	58 ± 4	5 ± 7	—	—
	1	−1 ± 2	−9 ± 3	—	—
Actinomycin D	50	80 ± 2	78 ± 1	80 ± 1	74 ± 3
	5	81 ± 3	76 ± 5	26 ± 2	26 ± 4
	0.5	73 ± 5	60 ± 4	3 ± 3	9 ± 7
Daunomycin	5000	100 ± 2	100 ± 2	95 ± 1	96 ± 2
	500	78 ± 1	78 ± 2	20 ± 4	37 ± 3
	50	56 ± 1	42 ± 6	−6 ± 2	13 ± 9
Vincristine	500	79 ± 4	73 ± 3	75 ± 3	56 ± 6
	50	80 ± 5	65 ± 5	3 ± 3	−11 ± 4
	5	59 ± 11	33 ± 7	—	—

*Except for TNF where concentration is given in units.
†Results expressed ± S.D.

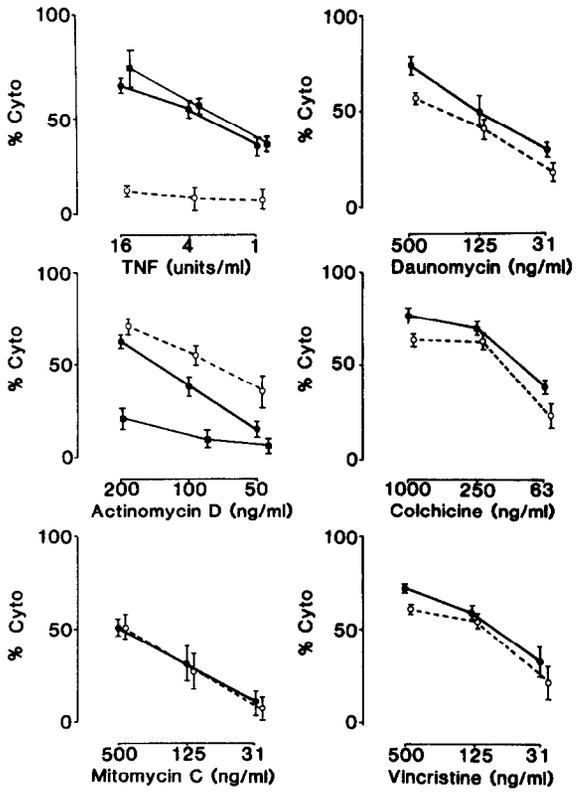

Fig. 1. Microplate assay for susceptibility to TNF and cytotoxic drugs of L929 (—●—), L929/R (—○—) and L929/ADR (—■—) cells.

microplate assay (Fig. 1). This line, termed L929/ADR, exhibited similar resistance in the clonogenic assay to actinomycin D and also to vincristine but not to the other agents tested (Table 1). Despite the admittedly modest degree of resistance to these cytotoxic drugs, L929/ADR was comparable to the parental L929 line in susceptibility to TNF (Fig. 1).

At least in the terminal stages of TNF-induced cell damage, free radical-induced mechanisms are involved [13]. Therefore it is possible that TNF-resistant cells have increased amounts of enzymes such as catalase, glutathione-*S*-transferase and glutathione peroxidase which can combat free radical-induced damage: increased expression of glutathione-*S*-transferase has also been implicated as a mechanism of multi-drug resistance. However, as shown in Table 3 there was no major difference between the concentrations of these enzymes in TNF susceptible and resistant cell lines.

DISCUSSION

With both of the methods employed, murine L929 and L929/R cells had comparable susceptibilities to cytotoxic drugs although exhibiting a 1000-fold difference in their resistance to TNF. Similarly, human U937 and rabbit RK13 cells did not exhibit a significant increase in resistance to cytotoxic drugs on development of TNF resistance *in vitro*. In addition, L929 cells which had developed a 10-fold resistance to actinomycin D and vincristine did not develop a corresponding degree of resistance to TNF. These observations suggest that the mechanism of resistance to TNF cytolysis is different from that of multidrug resistance. In sup-

Table 3. Concentrations of catalase, glutathione-S-transferase and glutathione peroxidase in TNF-susceptible and -resistant cell lines

Cell line	Catalase (U/mg)	Glutathione-S-transferase (U/mg)	Glutathione peroxidase (mU/mg)
L929	2.2	1.40	80
L929/R	3.6	0.84	70
U937	4.7	1.03	30
U937/R	6.2	0.84	26
RK13	4.3	0.24	N.D.
RK13/R	4.5	0.25	N.D.

N.D. = not detectable.

port of this, glutathione-S-transferase concentrations were not raised in TNF-resistant cells but frequently are in cells which develop multidrug resistance [14, 15]. In other examples of resistance to cytotoxic drugs, resistance also correlates with increased concentrations of certain other proteins [16, 17]. In contrast two lines of evidence suggest that the development of resistance to TNF cytolysis is associated with protein loss. Firstly, despite numerous attempts we have been unable to transfer TNF resistance to susceptible cells by either DNA transfection or cell fusion (Daniels and Matthews, unpublished). Secondly, activation of arachidonate metabolism appears to be an essential step in TNF-induced cytolysis but on development of TNF resistance cells no longer activate arachidonic acid metabolism on TNF challenge although they retain TNF receptors [3, 18].

TNF acts syncrgistically with some cytotoxic agents, notably actinomcyin D and vinblastine [19, 20] and should TNF have a role in cancer therapy it may well be in combination with cytotoxic drugs. From this viewpoint it is encouraging that there is no cross-resistance between TNF and the various cytotoxic agents used in this study.

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