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Characterization of MBT-2 tumour cell "variant" resistant to tumour necrosis factor

S. A. Kadhim, J. Y. Wang, B. McLean, and J. L. Chin

Division of Urology, Department of Surgery, University Hospital, University of Western Ontario, London, Ontario, Canada

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Summary. Previously we reported sensitivity of MBT-2 murine bladder tumour to tumour necrosis factor (TNF) in vivo and in vitro [8]. We showed that with prolonged exposure of cultured MBT-2 tumour cells to TNF, a resistant MBT-2 "variant" tumour cell population emerged in vitro. This concurred with the finding of transient in vivo cytotoxic effect of TNF against MBT-2 tumour. Herein, we delineate phenotypic changes in MBT-2 cells associated with TNF resistance. Parent MBT-2 (MBT-2P) and the TNF-resistant "variant" MBT-2R cells were compared in terms of in vitro sensitivity to TNF, DNA profile, karyotype and in vitro growth kinetics. We conclude that acquisition of resistance to TNF may be due to cell cycle derangement and differences in in vitro growth characteristics. DNA indices and karyotype of "variant" MBT-2R cells were not altered, indicating the anti-tumour action of TNF is not-mutagenic.

Key words: Tumour necrosis factor (TNF) – Mouse bladder tumour (MBT-2) – TNF-resistance – Growth characteristics – DNA profile – Karyotype

Tumour Necrosis Factor (TNF) is a cytokine that causes rapid haemorrhagic tumour necrosis of a variety of animal and human tumours [3, 6]. It can also mediate a wide spectrum of other activities including immunoregulation of human T cell responses [14] and activation of polymorphonuclear neutrophil functions [15]. TNF is cytotoxic for some tumour cell lines [12] (TNF-sensitive targets) and cytostatic for others [7]. A number of tumour cell lines, however, are resistant to TNF in vitro [17]. Presently, there is no satisfactory explanation for this diverse pattern of in vitro sensitivity. In our previous study [8] we showed that the in vivo response of MBT-2 tumours to TNF treatment was transient and in vitro cell lysis by TNF parallelled the in vivo response. We also showed that resistance to TNF can be induced by serial culturing of MBT-2 cells in higher TNF concentrations. The emergence of in vitro resistance to TNF could account for the "rebound" tumour growth after the initial growth impedance.

In an attempt to elucidate the mechanism of TNF cytotoxicity we characterize the changes in MBT-2 cells associated with TNF resistance in terms of growth characteristics, cell cycle distribution and karyotype.

Materials and methods

Cell lines

In vitro MBT-2 tumour cells (originally derived by Soloway et al. [16] and L929 murine fibroblasts (standard control for TNF sensitivity) were maintained using standard culture media and techniques [8]. A TNF-resistant MBT-2R cell population was derived by sequential culturing of the parental cloned tumour cells (cloned by limiting dilution) in continuous presence of progressively higher concentrations (from 50 to 250 Lytic Units, see below) of TNF in the culture medium as described elsewhere [8].

Tumour necrosis factor

Recombinant human TNF alpha/cachectin was obtained from Amgen Biologicals, Canada Inc. (specific activity 1×10^6 units/mg protein). Biological assay was measured by cytolysis of mouse L-929 cells incubated with TNF for 48 h in the presence of actinomycin D. The concentration required to yield 50% lysis of L929 cells was defined as one TNF Lytic Unit (1 L.U.).

Assessment of tumour cell sensitivity to TNF

To assess the relative sensitivity of the MBT-2 cell lines to TNF treatment, cultured MBT-2P parental and MBT-2R resistant cells were treated with various concentrations of TNF using either the [^{3}H]-thymidine proliferation assay (described elsewhere [8]) or standard cytotoxicity assay measuring the relative percent viability. For the [^{3}H]-thymidine assay, 1.5×10^5 MBT-2 or control L929 cultured cells were pulsed with 25 μci of [^{3}H]-thymidine for 18 h and thereafter detached with trypsin-EDTA, resuspended in RPMI 1640 plus 10% FCS at 8×10^4 cells/ml and plated in 96 well flat bottom plates in an admixture with 100 μl of TNF per well. Doubling

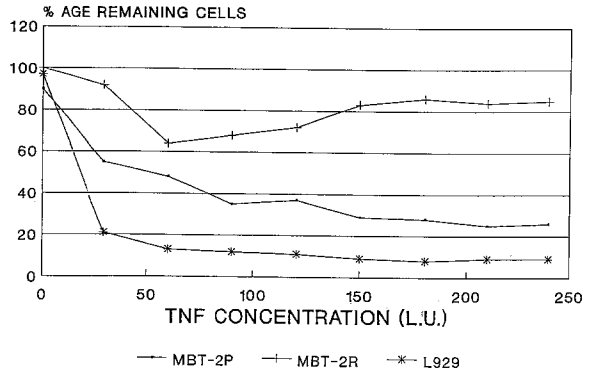

Fig. 1. Sensitivity of MBT-2R tumour cells to treatment with TNF of various concentrations ([^{3}H]-thymidine proliferation assay)

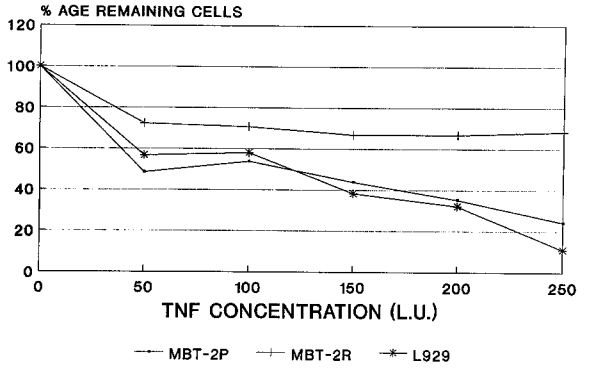

Fig. 2. Sensitivity of MBT-2R tumour cells to treatment with TNF of various concentrations (cytotoxicity assay)

dilutions of TNF ranged from highest (250 L.U.) to lowest (1 L.U.). The plates were then incubated for 48 h at 37°C in 5% CO_2 in air and after incubation the adherent cells were detached with 50 µl trypsin-EDTA per well, collected on filters using the cell harvester and radioactivity counted in a liquid scintillation β-counter. The percent remaining (proliferating) cells (% R.C.) was then estimated using the formula:

% R.C. = TNF treated − background/Controls − background

For the cytotoxicity assay 8 × 10^4 cells/ml of either MBT-2 or control L929 cell line were similarly plated in 96 well flat bottom plates in an admixture with 100 µl of TNF per well using the same range of TNF doubling dilutions. Following incubation for 48 h, cell monolayers were stained with 0.5% crystal violet in 20% ethanol for 10 min at room temperature. The plates were then rinsed in tap water, dried, and the dye eluted by adding 0.1 ml of 1% SDS at room temperature for 15 min. The absorbance (OD) of each well was then read at 590 nm on Titerteck Multiscan plate reader. To illustrate the relative sensitivity of tumour cells to TNF, the TNF concentration was plotted on the abscissa, and the percentage of remaining cells on the ordinate (Figs. 1 and 2).

Flow cytometric analysis

For DNA staining, parent MBT-2P or "variant" MBT-2R tumour cells in single cell suspensions were labelled with propidium iodide (50 µg/ml in 1.12% sodium citrate) and the DNA histograms and cell distribution analyzed on the Epics II dual laser Flow Cytometer. Fluorescence intensity data were collected on an IBM computer in List mode and a convolution/deconvolution curve fitting routine (cytology program Coulter Electronics) was used to analyze the percentage of cells in G_0/G_1, S, and G_2/M, and the coefficients of variation of each phase. Mouse spleen cells were used as the diploid standard.

Cell growth curves

To construct growth curves of MBT-2P and MBT-2R cell lines 2 × 10^4 tumour cells were plated in 24 well Costar plates and the cells were harvested and counted daily (total-viable counts) for 7 days. Growth curves were then constructed by plotting the total counts per ml on the abscissa and the time (days in culture) on the ordinate. Doubling times (t_D) for the parent and resistant line were calculated by the formula $t_D = 0.693\ t/\ln N/N_0$ where t = elapsed time, N_0 = starting number of cells and N = final number of cells.

Karyotype analysis

Karyotype analysis of MBT-2 tumour cells was done by the Quinacrine stain fluorescent banding technique [11] which causes specific fluorescence of the adenine-thymine (A-T) regions in the chromosome (Quinacrine binds to these regions by intercalating between the DNA strands).

Results

Assessment of tumour cell sensitivity to TNF

Assessment of cell cytotoxicity by [^{3}H]-thymidine proliferation assay revealed that ninety percent of the control L929 cells were killed by the minimum TNF concentration (25 TNF L.U.'s); 75% of the parental MBT-2P cells were killed by the same TNF concentration and only 17% of MBT-2R tumour cells were killed, indicating that the surviving MBT-2R cells had acquired TNF resistance (Fig. 1). However, this acquired phenotypic change in the MBT-2R cells was stable only for 3 days. When cultured in the absence of TNF for 3 days and then assessed by short exposure to TNF (by [^{3}H]-thymidine incorporation) the resistant MBT-2R cells reverted to become TNF-sensitive. Measurement of TNF cell kill by a direct cytotoxicity assay revealed a similar pattern of TNF sensitivity but with a higher percentage cell kill of MBT-2R cells at higher TNF concentrations (Fig. 2). Some clones of parent MBT-2P cells showed a variable degree of TNF sensitivity without prior selection by continuous exposure to TNF, indicating possible pre-existing tumour cell heterogeneity (unpublished data).

Cell growth curves

Growth curves of the parent MBT-2P and variant MBT-2R cells showed different growth patterns with relatively longer "lag" and "log" phases for MBT-2R "variant" cells compared to parent MBT-2P cells. Tumour cell doubling time (t_D) during the "log" phase of cell growth was 20.6 h for MBT-2R cells compared to 8.4 h for parent MBT-2P cells (Fig. 3). The cell growth rate during the confluence

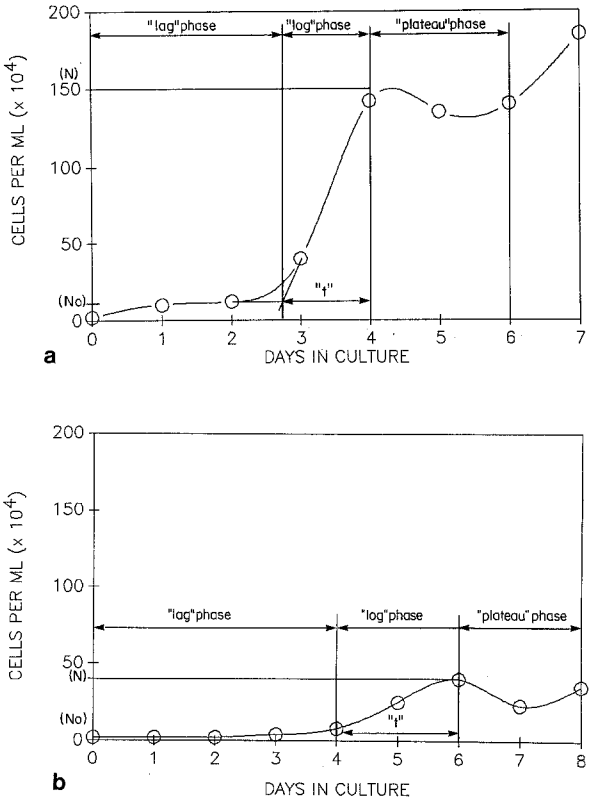

Fig. 3a, b. Tumour cell growth curves of a MBT-2P (parent) and b MBT-2R (TNF-resistant) cells. Tumour cell doubling time (t_D) of cells in the "log" phase was calculated by the formula $t_D = 0.693\ t/\ln N/N_0$ where t = elapsed time, N_0 = starting number of cells and N = final number of cells. For the parent MBT-2P cells: t = 30 h; $N_0 = 12.5 \times 10^4$ cells; $N = 150 \times 10^4$ cells and $t_D = 0.693 \times 30/\ln 150/12.5 = 8.4$ h. For the "variant" MBT-2R cells: t = 48 h; $N_0 = 8 \times 10^4$ cells; $N = 40 \times 10^4$ cells and $t_D = 0.693 \times 48/\ln 40/8 = 20.6$ h

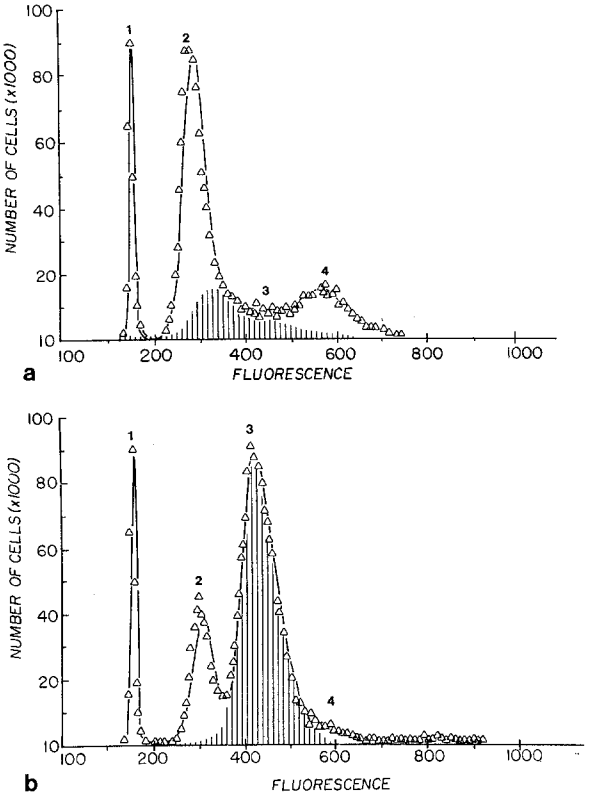

Fig. 4a, b. DNA histograms of a MBT-2P (parent) and b MBT-2R (TNF-resistant) tumour cells. "1" is the diploid (G_1) peak of tumour cell populations, likely stromal cells. "2" is the aneuploid (G_1') peak of tumour cells. "3" (shaded area) is the S phase fraction and "4" is the G_2/M peak of tumour cells

("plateau") phase was similar for both tumour cell populations.

Flow cytometric analysis

Flow cytometric analysis of both MBT-2P and MBT-2R cells showed a DNA index of 1.8 (Fig. 4). However, the 2 populations were quite different in the relative distribution of cells in the cell cycle phases. MBT-2P cells showed 52% in G_0/G_1, 27% in S and 21% in G_2, whereas MBT-2R cells had 20%, 76% and 4% cells respectively. Intermittent TNF exposure of MBT-2P cells for 20 h resulted in similar alteration of their DNA profile and cell cycle changes with cells accumulating in the S phase (Fig. 5). Thus it appears that either prolonged or intermittent TNF exposure of MBT-2 cells results in arrest of cells in the S phase with decrease of cell entrance into G_2 and M phases.

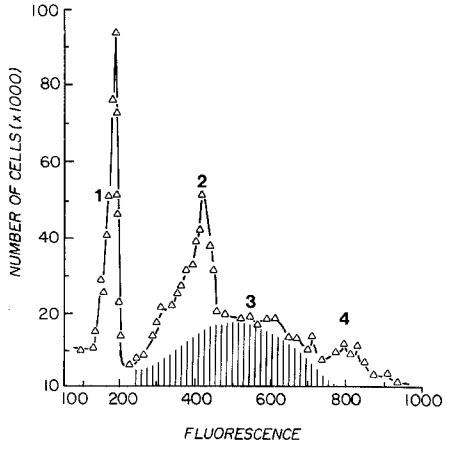

Fig. 5. Intermittent TNF exposure of parent MBT-2P cells for 20 h. DNA histogram: "1" is the diploid (G_1) peak tumour cells. "2" is the aneuploid (G_1') peak of tumour cells. "3" (shaded area) is the S phase fraction and "4" is the G_2/M peak tumour cells

Karyotype analysis

The karyotype analysis revealed both populations of MBT-2P and MBT-2R cells had a modal count of 62 chromosomes (consistent with the flow cytometric DNA index of 1.8) and similar number of marker chromosomes (Fig. 6).

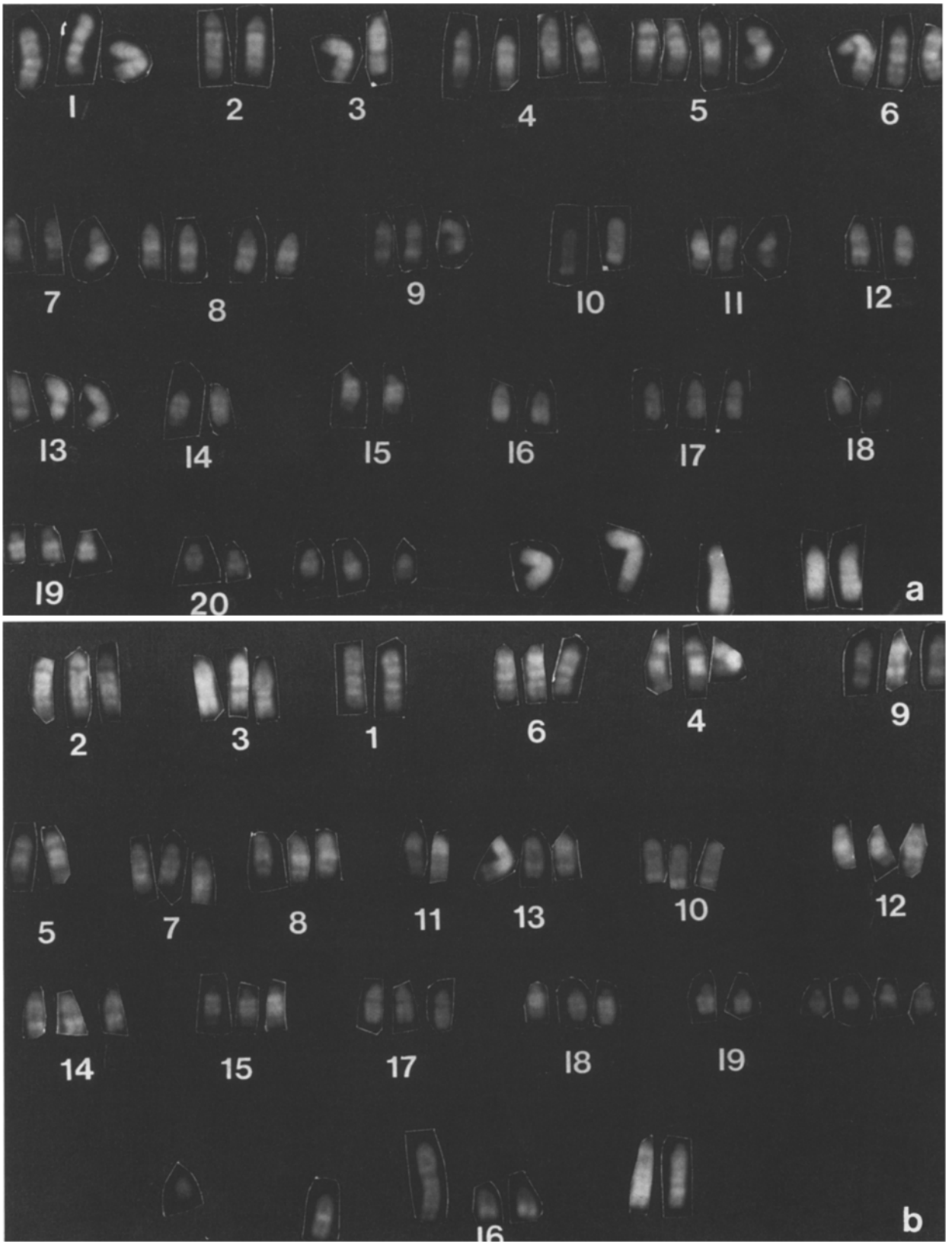

Fig. 6a, b. Karyotype of **a** MBT-2P (parent) and **b** MBT-2R (TNF-resistant) tumour cells. Both have a modal number of 62 chromosomes and a similar number of marker chromosomes

Discussion

Cancer chemotherapeutic agents may contribute to the induction of tumour resistance as a result of their mutagenic and epigenetic effects [9]. Tumour cell variants arising from subpopulations refractory to chemotherapy confer a selective growth advantage to tumours and may represent the initial step in the progression and metastasis of primary tumours [13].

Several human tumour cell lines including transitional cell bladder carcinoma, melanoma, lung and pancreatic carcinomas, have been shown to be resistant to the cytotoxic and cytostatic effects of TNF [4, 17]. One possible mechanism for the emergence of TNF resistant cell lines involves a decrease in the number of TNF binding sites on the tumour cell surface [1]. In addition to receptor down-regulation, other mechanisms appear to contribute to TNF resistance. Wallach et al. [18] showed that a 3 hour pre-treatment of SV80 and HeLa tumour cell line with cytotoxins (containing both TNF and lymphotoxin) in the absence of protein synthesis inhibitors resulted in a decreased responsiveness of cells to the TNF cytotoxic effect despite the recovery of TNF receptors. Since both TNF sensitive and TNF resistant cell types seem to have a similar number of high-affinity TNF binding sites, it is unlikely that alterations in the number or the affinity to TNF receptors is a predominant mechanism of resistance induction [17]. Another possible mechanism of TNF resistance is the inability to transduce a cellular signal subsequent to the binding of TNF to the receptor. Such altered signal transduction has been observed for both insulin [10] and the epidermal growth factor (EGF) receptors [2]. However, until the TNF receptor is fully characterized, it would not be possible to determine if this type of defect occurs among TNF resistant tumour cells.

In our earlier study, we had demonstrated in vitro correlation with in vivo sensitivity to TNF [8]. We also showed that when MBT-2 cells were cultured in progressively higher concentrations of TNF, a "variant" cell population relatively resistant to TNF can emerge. Compared to the standard L929 cells where there was a 90% cell kill as measured by the [^{3}H]-thymidine incorporation assay, the parental MBT-2 cells had a 75% cell kill when initially exposed to TNF. The "variant" cell population derived by the method described only exhibited 17% cell kill. Measurement of TNF cell kill by direct cytotoxicity assay revealed a similar pattern of TNF sensitivity, although the percentage of cell kill for MBT-2R cells was higher. This may reflect the differences in the experimental conditions employed in the [^{3}H]-thymidine proliferation assay compared to cytotoxicity assay. The cells in the [^{3}H]-thymidine proliferation assay are allowed to proliferate into the "log" phase for [^{3}H]-thymidine labelling before TNF exposure, whereas in the cytotoxicity assay tumour cells are not confined in the "log" phase and are exposed to TNF directly. Thus it appears that MBT-2R cells undergoing active proliferation are more resistant to TNF exposure than non-proliferating cells.

Results of our growth kinetics experiments illustrate that the TNF resistant MBT-2R cells have relatively longer "lag" and "log" phases but a similar "plateau" in comparison to parent MBT-2P cells. This is due to the fact that "variant" MBT-2R cells remain moderately susceptible to high concentrations of TNF (as shown by our [^{3}H]-thymidine proliferation and cytotoxicity results) and thus require a longer recovery time before exponentially increasing in cell number. However, once these cells survive the TNF effect and emerge from the "log" phase they become adapted to its continuous presence. Thus the two tumour cell populations have similar "plateau" phases.

Our flow cytometric (FCM) analysis data suggest that the resistant population has a cell kinetic picture different from that of the parental cell line. FCM analysis of the MBT-2 parental line shows a DNA index of 1.8 which is in agreement with findings of Mickey et al. who reported a hypertriploid karyotype with 64 chromosomes and 3 marker chromosomes [11]. FCM analysis of the TNF-resistant cell population (continuous exposure to TNF with repeated cultures) shows the same G_0/G_1 population with a DNA index of 1.8. However, the distribution of the cells in the cell cycle was drastically different for the MBT-2R cells, with the majority of cells residing in the S phase. This was also observed with an intermittent 20 hour exposure to TNF. Thus it would appear that either prolonged continuous or intermittent exposures to TNF in this experimental setup can induce arrest of cells in the S phase with decrease in the rate of cell entrance into G_2 phase, thus limiting the efficacy of TNF on MBT-2 cells. Previous studies by Darzynkiewicz et al. have shown that single exposure of L-cells to TNF causes cytostasis, with arrest of cells in G_2 phase [5]. The different experimental conditions and different cell lines used in this study may explain the different findings. Nevertheless, the mere change in cell cycle kinetics and the preservation of the modal chromosome numbers suggest that TNF was not mutagenic in its action.

The TNF resistance induced by in vitro exposure of MBT-2 cells may explain the transient nature of our observed TNF-mediated tumour regression in vivo [8]. Such TNF-resistant cells could account for the surviving cells and continued tumour growth in mice treated with TNF. Alternatively it is possible that such TNF resistance may reflect a pre-existing tumour cell heterogeneity rather than adaptation to TNF exposure. The emergence of resistance to TNF in this tumour model may have a bearing on the way this cytokine can be applied in therapy.

Combination therapy with other biological response modifiers such as Interferon-gamma and Interleukin-2 may increase the efficacy of TNF and may also prove to be beneficial in circumventing TNF resistance. The growth characteristics and the eventual reversion of MBT-2R cells to become TNF-sensitive may be exploited in designing more effective treatment regimens in the murine model.

References

1. Baglioni C, McCandless S, Tavernier J, Fiers W (1985) Binding of human tumour necrosis factor to high affinity receptors on HeLa and lymphoblastoid cells sensitive to growth inhibition. J Biol Chem 260:13395

2. Carlin CR, Philipps PD, Knowles BB, Cristofalo VJ (1983) Diminished in vitro tyrosine kinase activity of the EGF receptor of senescent human fibroblasts. Nature 306:617
3. Carswell EA, Old LJ, Kasse RL, Green S, Fiore N, Williamson B (1975) An endotoxin-induced serum factor that causes necrosis of tumours. Proc Natl Acad Sci USA 72:3666
4. Creasey AA, Doyle LM, Reynolds MT, Wang AM, Lin LS, Vitt CR (1987) Biological effect of recombinant human tumour necrosis factor and its novel muteins on tumour and normal cell lines. Cancer Res 47:145
5. Darzynkiewicz Z, Williamson B, Carswell EA, Old LJ (1984) Cell cycle-specific effects of tumour necrosis factor. Cancer Res 44:83
6. Haranaka K, Satomi N, Sakurai A (1984) Anti-tumour activity of murine tumour necrosis factor (TNF) against transplanted murine tumours and heterotransplanted human tumours in nude mice. Int J Cancer 34:263
7. Heicappell R, Naito S, Ichinose Y, Creasey AA, Lin LS, Fidler IJ (1987) Cytostatic and cytolytic effects of human recombinant tumour necrosis factor on human renal cell carcinoma cell lines derived from a single surgical specimen. J Immunol 136:1634
8. Kadhim SA, Chin JL (1988) Anti-tumour effect of tumour necrosis factor and its induction of tumour variant of MBT-2 transitional carcinoma of the bladder. J Urol 139:1091
9. Kerbel RS, Davies AJS (1982) Facilitation of tumour progression by cancer therapy. Lancet II:977
10. Marchand-Brustel YL, Gremeaux T, Ballotti R, Van Obberghen E (1985) Insulin receptor tyrosine kinase is defective in skeletal muscle of insulin-resistant obese mice. Nature 315:676
11. Mickey DD, Mickey GH, Murphy WM, Neill HB, Soloway MS (1982) In vitro characterization of four N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) induced mouse bladder tumour. J Urol 127:1233
12. Philip R, Epstein LB (1986) Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, gamma-interferon and interleukin-1. Nature 323:86
13. Poste G, Fidler IJ (1980) The pathogenesis of cancer metastasis. Nature 283:139
14. Scheurich BB, Thoma U, Pfizenmaier K (1987) Immunoregulatory activity of recombinant human tumour necrosis factor (TNF-α): induction of TNF receptors on human T cells and TNF-α-mediated enhancement of T cell responses. J Immunol 138:1786
15. Shalaby MR, Paladino MA, Hirabayashi SE, Eassalu TE, Lewis GD, Shepard HM, Aggarwal BB (1987) Receptor binding and activation of polymorphonuclear neutrophils by tumour necrosis factor-alpha. J Leuk Biol 41:196
16. Soloway MS, de Kernion JB, Rose D, Persky L (1973) Effect of chemotherapeutic agents on bladder cancer: a new animal model. Surg Forum 13:542
17. Sugarman BJ, Aggarwal BB, Hass PE, Figari SI, Palladino MA, Shepard HM (1985) Recombinant human tumour necrosis factor – alpha: effects on proliferation of normal and transformed cells in vitro. Science 230:943
18. Wallach D, Holtmann H, Hahn T, Israel S (1987) Interactions of tumour necrosis factor, interferon and interleukin-1 in cell killing. In: Cantell K, Schellekens H (eds) Nijhoff, Boston, p 223

S. Kadhim, PhD
Division of Urology
Department of Surgery
University of Western Ontario
London, Ontario
Canada N6A 5A5