Recombinant Human Tumor Necrosis Factor-α: Effects on Proliferation of Normal and Transformed Cells in Vitro

Abstract. *Modulation of the growth of human and murine cell lines in vitro by recombinant human tumor necrosis factor-α (rTNF-α) and recombinant human interferon-γ (rIFN-γ) was investigated. rTNF-α had cytostatic or cytolytic effects on only some tumor cell lines. When administered together with rIFN-γ, rTNF-α showed enhanced antiproliferative effects on a subset of the cell lines tested. In contrast to its effects on sensitive tumor cells, rTNF-α augmented the growth of normal diploid fibroblasts. Variations in the proliferative response induced by rTNF-α were apparently not due to differences in either the number of binding sites per cell or their affinity for rTNF-α. These observations indicate that the effects of rTNF-α on cell growth are not limited to tumor cells, but rather that this protein may have a broad spectrum of activities in vivo.*

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Stimulation of leukocytes with mitogens results in the secretion of a class of proteins called cytokines. These immunomodulatory proteins, which include interleukin-2, interferon-γ, and various other factors, have multiple effects on sensitive cells (*1*). One particular cytokine, named tumor necrosis factor (TNF), was originally identified as an activity present in the sera of mice treated sequentially with the *Mycobacterium bovis* strain Bacillus Calmette-Guerin and endotoxin (*2*). This activity, thought to be produced by macrophages, caused hemorrhagic necrosis of Meth A sarcomas in vivo as well as inhibition of the growth of certain murine and human tumor cells in vitro (*2–4*). Subsequent experiments in vitro showed that antibodies directed against a highly purified cytotoxic polypeptide secreted from a lymphoblastoid cell line (RPMI 1788) could neutralize a TNF-like activity derived from peripheral blood lymphocytes but not macrophages (*5*). This implied that at least two distinct cytotoxic factors are produced by leukocytes. Additional studies showed that mitogen-stimulated macrophages produce TNF-α (*6*), whereas TNF-β (*7*) is secreted by mitogen-stimulated lymphocytes (*8*).

The genes encoding both TNF-α and TNF-β have been cloned, and the corresponding proteins have been expressed in *Escherichia coli* and purified to homogeneity (*9, 10*). Even though a comparison of the primary structure of these proteins reveals that they are 50 percent homologous (*6*), they appear to have comparable cytostatic and cytolytic properties in vitro (*6, 8*). Here we describe the response of 39 human and murine cell lines to treatment with recombinant TNF-α (rTNF-α) in vitro. The results indicate that these cell lines can be subdivided into three groups on the basis of their response to rTNF-α (Table 1). Several cell lines from each of these groups were examined in more detailed studies. Since IFN-γ has been reported to enhance the anticellular effects of TNF-β (*11*), we compared the growth of eight cell lines for their response to rTNF-α alone, recombinant IFN-γ (rIFN-γ) alone, and combinations of both cytokines. We also determined whether the type of cellular response to rTNF-α could be correlated with the number of binding sites or ligand affinity.

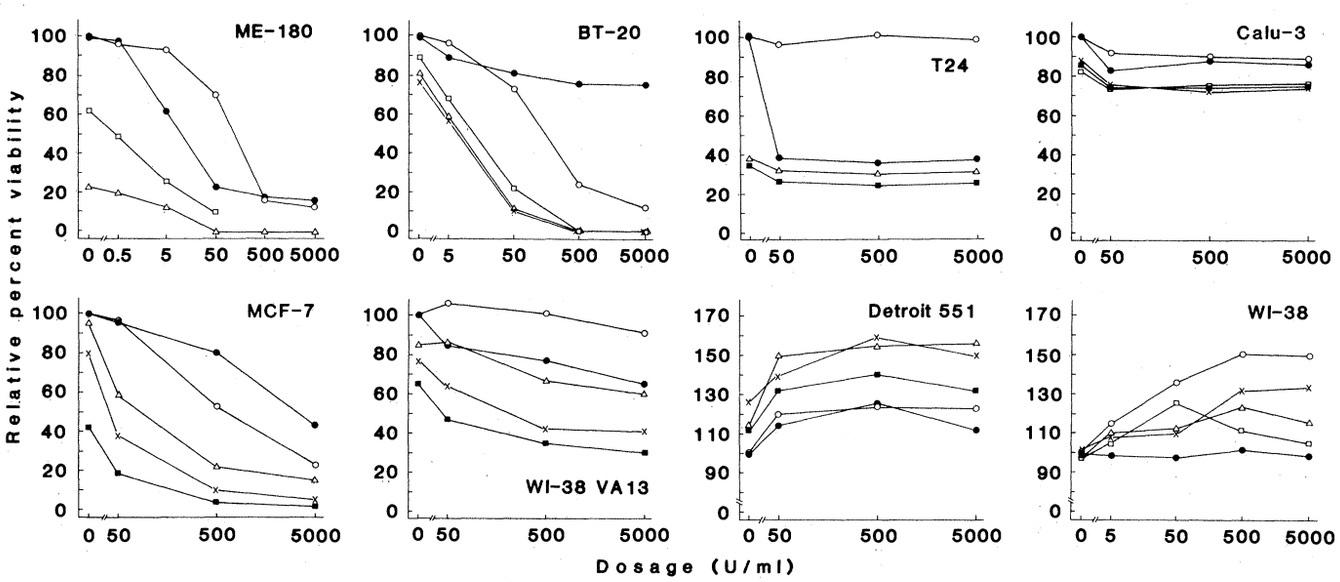

Fig. 1. Response of human cell lines to treatment with rTNF-α and rIFN-γ in vitro. Changes in the viability of eight cell lines after treatment with either cytokine alone or with the two cytokines in combination. Symbols: (○) rTNF-α only; (●) rIFN-γ only; (□) rIFN-γ (5 U/ml) plus various doses of rTNF-α; (△) rIFN-γ (50 U/ml) plus various doses of rTNF-α; (X) rIFN-γ (500 U/ml) plus various doses of rTNF-α; (■) rIFN-γ (5000 U/ml) plus various doses of rTNF-α. The specific activity of rTNF-α was 3.7×10^7 U/mg (6.3×10^8 U/μmol) and rIFN-γ was 4.0×10^7 U/mg (6.8×10^8 U/μmol). The assays used to determine the specific activity of each cytokine have been described (*6, 8, 21*). To determine the relative percent viability, we used a modification of a published procedure (*11*). Portions (100 μl) of cells (10^5 per milliliter) were dispensed into 96-well microtiter plates. Appropriate amounts of rTNF-α and rIFN-γ were added to a final volume of 200 μl. Cells were incubated for 72 hours, washed three times with phosphate-buffered saline, fixed, and stained with 20 percent methanol containing 0.5 percent crystal violet. The dye was eluted with 0.1*M* sodium citrate (*p*H 4.2) and 50 percent ethanol for 30 minutes at room temperature and the absorbance (OD) at 540 nm was measured. The relative percent viability (RPV) was calculated as follows: RPV = [mean OD (drug-treated)/mean OD (non–drug-treated)] × 100 where the mean absorbance represents the average OD from six replicates (the coefficient of variance ranged from 0.5 to 4 percent).

In the presence of rTNF-α, the viability of three human carcinoma cell lines, ME-180 (cervical), BT-20 (breast), and MCF-7 (breast), was reduced by 50 percent at doses of 120 U/ml, 150 U/ml, and 500 U/ml, respectively (Fig. 1). ME-180 cells were also susceptible to growth inhibition by rIFN-γ (50 percent inhibition at 10 U/ml), whereas MCF-7 cells (50 percent inhibition at about 3500 U/ml) and BT-20 cells (25 percent inhibition at 5000 U/ml) were only slightly sensitive to its antiproliferative effects. These data indicate that there is no correlation between the sensitivity of these cells to the antiproliferative effects of rTNF-α and rIFN-γ.

Table 1. Summary of the responses of 39 human and murine cell lines to treatment with rTNF-α in vitro. Sensitivity to rTNF-α (at concentrations of rTNF-α ≤ 10,000 U/ml) was determined by the crystal violet staining procedure outlined in Fig. 1. Cell lines were obtained as described (*19*).

Cell lines
Human cells
Growth enhancement
CCD-18Co (normal colon)
Detroit 551 (normal fetal skin)
LL24 (normal lung)
WI-38 (normal fetal lung)
WI-1003 (normal lung)
*Null response**
A549 (lung carcinoma)
Calu-3 (lung carcinoma)
G-361 (melanoma)
HeLa (cervical carcinoma)
HT-1080 (fibrosarcoma)
KB (oral epidermoid carcinoma)
LS174T (colon carcinoma)
RD (rhabdosarcoma)
Saos-2 (osteogenic sarcoma)
SK-CO-1 (colon carcinoma)
SK-LU-1 (lung carcinoma)
SK-OV-3 (ovarian carcinoma)
SK-UT-1 (uterine carcinoma)
T24 (bladder carcinoma)
WI-38 VA13 (SV40-transformed WI-38)
Antiproliferative response†
BT-20 (breast carcinoma)
BT-475 (breast carcinoma)
MCF-7 (breast carcinoma)
ME-180 (cervical carcinoma)
SK-MEL-109 (melanoma)
SK-OV-4 (ovarian carcinoma)
WiDr (colon carcinoma)
Murine cells
*Null response**
B16F10 (melanoma)
CMT-93 (rectal carcinoma)
S49 (lymphoma)
Antiproliferative response†
B6MS2 (sarcoma)
B6MS5 (sarcoma)
CMS4 (sarcoma)
CMS16 (sarcoma)
L929 (fibroblast)
Meth A (sarcoma)
MMT (breast carcinoma)
SAC (Moloney-transformed 3T3)
WEHI-164 (sarcoma)

*Less than 25 percent cytostasis or cytotoxicity. †Greater than or equal to 25 percent cytostasis or cytotoxicity.

Treatment of ME-180, BT-20, and MCF-7 cells with rTNF-α and rIFN-γ in combination, at concentrations that are ineffective when the cytokines are administered alone, resulted in an enhanced antiproliferative response. With concentrations of 5 U/ml for rTNF-α and 5 U/ml for rIFN-γ, the relative cytotoxicity to ME-180 cells was 75 percent (Fig. 1). At these concentrations the additive effect predicted by treatment with either protein alone would have been 45 percent (Fig. 1). To determine if this augmented antiproliferative response was the result of synergistic interactions between rTNF-α and rIFN-γ, we subjected the data to an isobologram analysis (*12*). Figure 2 shows those concentrations of rTNF-α or rIFN-γ, or both, which resulted in a 40 percent decrease in cell viability. Marked departure below the hyphenated diagonal is indicative of synergistic interactions (*12*). These results show that synergism between rIFN-γ and rTNF-α is not uniform throughout the range of tested concentrations. It was optimum between 10 and 60 U/ml for rTNF-α combined with 0.5 to 3.5 U/ml of rIFN-γ. A similar finding was observed for BT-20 and MCF-7 cells (Fig. 1). The enhanced cytotoxicity observed in the presence of these two cytokines suggests that they may act through convergent cellular response pathways.

Three cell lines tested in detail were relatively insensitive to the antiproliferative effects of rTNF-α. These include WI-38 VA13 (subline 2RA), an SV40-transformed derivative of WI-38, T24 bladder carcinoma, and Calu-3 lung carcinoma (Fig. 1). In each of these lines the number of viable cells decreased 0 to 10 percent in the presence of rTNF-α up to a concentration of 5000 U/ml. In contrast, the response of these cell lines to rIFN-γ was variable. Growth of WI-38 VA13 (subline 2RA) was reduced by 35 percent at 5000 U/ml; viability of T24 bladder carcinoma cells was decreased by 60 percent at a dose of only 50 U/ml, and did not change with increasing doses of rIFN-γ; and a 15 percent loss in viability was observed with Calu-3 in the range of 500 to 5000 U of rIFN-γ per milliliter. Treatment of either T24 or Calu-3 cells with both cytokines in combination did not result in a synergistic antiproliferative response. However, treatment of WI-38 VA13 (subline 2RA) cells with both rTNF-α and rIFN-γ resulted in synergistic growth inhibition (Fig. 1). Therefore, lack of responsiveness to rTNF-α does not preclude synergism between rTNF-α and rIFN-γ.

The response of normal diploid skin (Detroit 551) and lung (WI-38) fibroblasts to treatment with rTNF-α appears to be affected in a manner completely different from other cell lines examined. Their growth was stimulated in the presence of

Table 2. A comparison of rTNF-α binding to ME-180, WI-38, and T24 cells. The rTNF-α binding assay was done as described (*20*). Confluent monolayers (~2 × 10^5 cells) grown in 12-well plates were incubated with 2 × 10^5 counts per minute of ^{125}I-labeled rTNF-α (~850 μCi/nmol) and various concentrations of unlabeled rTNF-α at 37°C for 2 hours. After incubation, the cells were washed three times with the incubation medium (McCoy's 5A medium plus 10 percent fetal bovine serum), solubilized with 2 percent sodium dodecyl sulfate, and counted for bound radioactivity. Binding affinities and number of binding sites were determined from Scatchard analysis. Data were corrected for nonspecific binding in the presence of a 1000-fold excess of unlabeled rTNF-α. Results are the average of triplicate determinations ± standard error of the mean.

Cell line	K_d (n*M*)	Average number of binding sites per cell
ME-180	0.31 ± 0.055	2250 ± 128
WI-38	0.22 ± 0.051	2172 ± 672
T24	0.25 ± 0.133	1512 ± 205

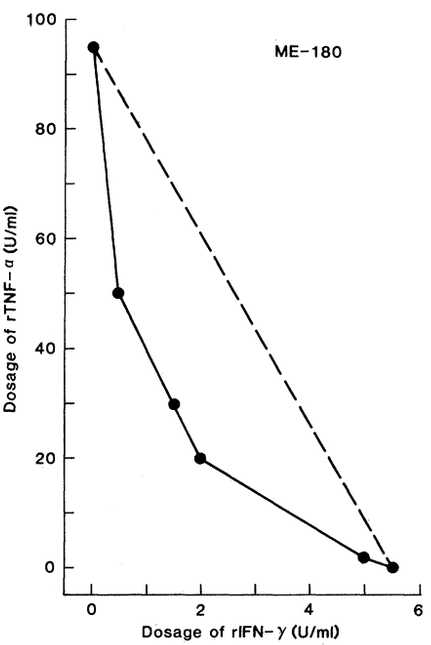

Fig. 2. An isobologram depicting synergism between rTNF-α and rIFN-γ. The solid line represents doses of cytokines which cause a 40 percent reduction in ME-180 viability. The hyphenated line represents the hypothetical amounts of rTNF-α and rIFN-γ required to cause this same decrease in viability if the interactions were additive (*12*). Results are the average of quadruplicate determinations (the range of the coefficient of variance was from 0.5 to 5 percent).

rTNF-α. There was a 20 percent increase in the number of Detroit 551 cells in the presence of 50 to 5000 U of rTNF-α per milliliter compared to untreated cells (Fig. 1). WI-38 cells showed a more marked dose-dependent effect. There was a 50 percent increase in growth at 500 U of rTNF-α per milliliter. The proliferative response of each of these cell lines in the presence of rIFN-γ alone was also different. WI-38 cell growth was neither enhanced nor diminished, whereas Detroit 551 showed a slight increase (10 to 20 percent) in the presence of rIFN-γ (Fig. 1). Treatment of Detroit 551 with both cytokines resulted in further growth enhancement. WI-38 cells, when treated with various combinations of both cytokines, showed an increase in cell number intermediate between the levels observed with either rTNF-α or rIFN-γ alone. Thus, in contrast to the growth inhibitory properties of these proteins, their growth-enhancing properties did not appear to be synergistic.

The enhancement of growth by rTNF-α was confirmed by monitoring changes in DNA synthesis as assayed by [^{3}H]thymidine uptake. Briefly, WI-38 cells were treated in vitro with various concentrations of rTNF-α for 24, 48, and 72 hours and the amount of [^{3}H]thymidine incorporated was compared between treated and untreated cultures (Fig. 3). The amount of [^{3}H]thymidine incorporated into WI-38 cells increased with the concentration of rTNF-α and appeared to plateau between 50 and 500 U/ml. This response was completely abrogated (Fig. 3, solid bars) when rTNF-α was preincubated with a monoclonal rTNF-α antibody which by itself had no effect on cell growth. Polymyxin B, which binds bacterial endotoxin and thus inhibits its effect on growth (*13*), had no effect on rTNF-α enhanced proliferation of WI-38 cells (*14*). These data substantiate the conclusion that rTNF-α is a positive growth regulator for WI-38 cells. The bifunctional regulatory nature of rTNF-α is unusual among protein hormones, although transforming growth factor-β (*15*) and tumor cell growth–inhibiting factors (*16*) are reported to possess both growth-enhancing and inhibitory properties.

To determine whether the different cellular responses to rTNF-α could be attributed to some heterogeneity in rTNF-α binding sites, we examined the binding of radiolabeled rTNF-α to ME-180, T24, or WI-38 cells. These data (Table 2) indicate that an equivalent number of rTNF-α binding sites (about 2000) was present on the surface of these cells, and that the binding sites had a similar affinity for the ligand (approximately 0.3 n*M*). Thus, the diverse proliferative responses of these three cell lines is not reflected in variations of binding site number or affinity. However, this does not exclude the possibility that there are other classes of cells that show no response to rTNF-α because they have a deficient number of binding sites.

These data demonstrate the existence of three classes of cells that can be distinguished by their response to rTNF-α. The effect of rTNF-α on cells may be antiproliferative (for example, ME-180) or growth-enhancing (for example, WI-38). rTNF-α has a minimal modulatory effect on the growth of cells of the third class. In the case of the T24 bladder carcinoma cell line, the resistance to rTNF-α is not due to a lack of rTNF-α binding sites (Table 2), but could be due to the absence of a subsequent biochemical activity elicited in sensitive cells after rTNF-α binds to its receptor. Such a finding has been reported for insulin resistance in obese mice (*17*).

Recombinant TNF-α affects multiple biological functions, including regulation of granulocyte function (*1*) and lipid metabolism (*18*). Our results show that rTNF-α has divergent effects on cell growth. It is unclear how rTNF-α can stimulate the growth of certain cell lines while inhibiting the growth of others. In this regard, the use of tumor cell variants resistant to rTNF-α will allow definition of regulatory pathways which mediate the divergent effects of rTNF-α on cell growth.

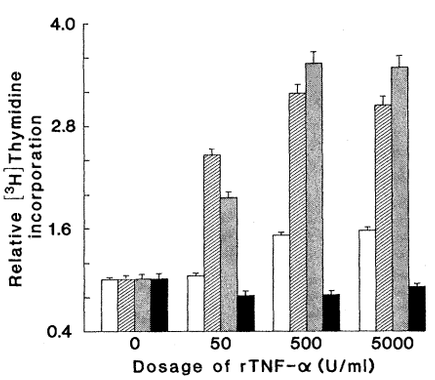

Fig. 3. Proliferation of WI-38 cells in the presence or absence of rTNF-α. Cells were exposed to various concentrations of rTNF-α in the presence or absence of a neutralizing mouse monoclonal antibody to rTNF-α. Cell growth was measured by using a standard [^{3}H]thymidine uptake assay (*22*). Changes in the growth of WI-38 cells treated with rTNF-α (0, 50, 500, or 5000 U/ml) after 24, 48, or 72 hours are reflected by differences in the amount of [^{3}H]thymidine incorporated. Cells treated with rTNF-α for 24 hours are depicted by the open bars, 48 hours by the diagonally hatched bars, and 72 hours by the stippled bars. Changes in the relative amount of [^{3}H]thymidine incorporated into WI-38 cells treated for 72 hours with rTNF-α (0, 50, 500, or 5000 U/ml) in the presence of 10,000 U of a neutralizing monoclonal antibody per milliliter (2700 anti-rTNF-α units/μg) are represented by the solid bars. The mean (± standard error of the mean) for each experiment represents the average number of counts per minute incorporated from 12 replicates. The relative amount of [^{3}H]thymidine incorporated represents the ratio of the means for the treated samples to untreated cells for each experiment.

References and Notes

1. G. Trinchieri and B. Perussia, *Immunol. Today* **6**, 131 (1985); C. H. Evans, *Cancer Immunol. Immunother.* **12**, 181 (1982); M. R. Shalaby *et al.*, *J. Immunol.* **135**, 2069 (1985); M. J. Clemens and M. A. McNurlan, *Biochem. J.* **226**, 345 (1985).
2. E. A. Carswell *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3666 (1975).
3. K. Haranaka and N. Satoris, *Jpn. J. Exp. Med.* **51**, 191 (1981); F. C. Kull and R. Cuatrecacas, *J. Immunol.* **126**, 1279 (1981); D. N. Mannel, M. S. Meltzer, S. E. Mergenhagen, *Infect. Immun.* **28**, 204 (1980).
4. B. D. Williamson, E. A. Carswell, B. Y. Rubin, J. S. Prendergast, L. J. Old, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5397 (1983).
5. D. S. Stone-Wolff *et al.*, *J. Exp. Med.* **159**, 828 (1984).
6. B. B. Aggarwal *et al.*, *J. Biol. Chem.* **260**, 2345 (1985).
7. This factor, formerly called lymphotoxin, was renamed tumor necrosis factor-β because of its structural homology to rTNF-α and its ability to induce hemorrhagic necrosis of Meth A sarcoma in vivo.
8. B. B. Aggarwal, B. Moffat, R. N. Harkins, *J. Biol. Chem.* **259**, 686 (1984); B. B. Aggarwal, W. J. Henzel, B. Moffat, W. J. Kohr, R. N. Harkins, *ibid.* **260**, 2334 (1985).
9. D. Pennica *et al.*, *Nature (London)* **312**, 724 (1984).
10. P. W. Gray *et al.*, *ibid.*, p. 721.
11. S. H. Lee, B. B. Aggarwal, E. Rinderknecht, F. Assisi, H. Chiu, *J. Immunol.* **133**, 1083 (1984).
12. M. A. Sande and G. L. Mandell, in *The Pharmacological Basis of Therapeutics*, A. G. Gilman, L. S. Goodman, A. Gilman, Eds. (Macmillan, New York, 1980), pp. 1080–1105.
13. G. T. Spear and M. Teodorescu, *J. Immunol. Meth.* **73**, 321 (1984).
14. B. J. Sugarman, I. S. Figari, M. A. Palladino, H. M. Shepard, unpublished observation.
15. R. F. Tucker, M. E. Volkenant, E. L. Branum, H. L. Moses, *Cancer Res.* **43**, 1581 (1983); A. B. Roberts *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 119 (1985).
16. K. K. Iwata, C. M. Fryling, W. B. Knott, G. J. Todaro, *Cancer Res.* **45**, 2689 (1985); C. M. Fryling, K. K. Iwata, P. A. Johnson, W. B. Knott, G. J. Todaro, *ibid.*, p. 2695.
17. Insulin binds to its receptor with the same affinity in both insulin-resistant and insulin-sensitive mice. However, the tyrosine kinase activity associated with the receptor is defective in insulin-resistant mice [Y. L. Marchand-Brustel, T. Gremeaux, R. Ballotti, E. Van Obberghen, *Nature (London)* **315**, 676 (1985)].
18. Evidence suggesting that rTNF-α affects lipid metabolism comes from studies with cachectin. This monokine, whose NH_2-terminal sequence has strong homology to the sequence of human rTNF-α, can suppress lipoprotein lipase activity in 3T3-L1 cells and affect adipocyte gene expression [B. Beutler, J. Mahoney, N. Le Trang, P. Pekala, A. Cerami, *J. Exp. Med.* **161**, 984 (1985); B. Beutler *et al.*, *Nature (London)* **316**, 552 (1985); F. M. Torti, B. Dieckmann, B. Beutler, A. Cerami, G. M. Ringold, *Science* **229**, 867 (1985)].
19. All cell lines were either purchased from the American Type Culture Collection or were a gift from L. J. Old of the Memorial Sloan-Kettering Cancer Center.
20. P. E. Hass, A. J. Hotchkiss, M. A. Mohler, B. B. Aggarwal, *J. Biol. Chem.* **260**, 12214 (1985).
21. E. Rinderknecht, B. H. O'Connor, H. Rodriguez, *ibid.* **259**, 6790 (1984).
22. J. N. Ihle *et al.*, *J. Immunol.* **129**, 1377 (1982).

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