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Recombinant Tumor Necrosis Factor: its Effect and its Synergism with Interferon-γ on a Variety of Normal and Transformed Human Cell Lines

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Abstract—*Tumor Necrosis Factor (TNF), released by induced macrophages, causes tumor necrosis in animals, and preferentially kills transformed cells* in vitro. *Using pure, recombinant human TNF, we report here its cytotoxic action on several human transformed and non-transformed cell lines. Furthermore, remarkable synergism between TNF and interferon-γ (IFN-γ) was observed in a large number of human cell lines, especially breast, cervix and colon carcinomas. Some other human cell lines, not sensitive to TNF alone, became highly sensitive when IFN-γ was present as well. We could not demonstrate a synergism between TNF and IFN-γ on any of the lymphoma/leukemia cell lines tested. All normal human, non-transformed diploid cell lines were insensitive to TNF even in the presence of IFN-γ. This study also confirms the observation that inhibition of protein synthesis by metabolic drugs (e.g. actinomycin D) remarkably enhances the sensitivity of several target cell lines to cytolysis by TNF.*

INTRODUCTION

CARSWELL *et al.* [1] first reported that sera from endotoxin-treated mice, rabbits and rats previously sensitized with an immunostimulant such as Bacillus Calmette Guerin or *Corynebacterium parvum* contained a substance defined as Tumor Necrosis Factor (TNF). When injected into mice harboring methyl-cholantrene-induced sarcomas, TNF causes hemorrhagic necrosis of the tumor but apparently has no effect on normal tissue [1, 2]. *In vitro*, partially purified murine TNF is cytostatic or cytolytic to many mouse and human transformed cell lines, but has no or little cytotoxic effect on non-transformed cell lines [1, 3–6].

The availability of recombinant TNF (rTNF) allows further characterization of both the *in vitro* and the *in vivo* biological activity of this protein. Especially intriguing are its mechanism of action, the molecular basis for its apparently selective toxicity to transformed cells *in vitro*, and its tumor-necrotizing activity in mice. Here, we describe the results of our study on the *in vitro* action of recombinant human TNF (r-hTNF) [7] on a set of well-characterized human transformed and non-transformed cell lines. The remarkable synergism between interferon-γ (IFN-γ) and TNF is also clearly documented.

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MATERIALS AND METHODS

In vitro *TNF cell-killing assay*

The TNF cell-lysis assay was performed both in the presence and in the absence of actinomycin D (actD), essentially as described by Ruff and Gifford [3]. Serial dilutions of the sample were made in microtitre plates. Target cells were added to their cell-specific growth medium at a concentration of 1.5–2 × 10^4 cells/well in the assay made in the absence of actD or at a concentration of 4 × 10^4 cells/well when actD (at a final concentration of 1 μg/ml) was also present. The cells were incubated for 48 hr or 18 hr in the absence or in the presence of actD, respectively. Adherent cells were fixed and stained for 10 min (0.5% crystal violet, 8% (v/v) formaldehyde (40%), 0.17% NaCl, 22.3% (v/v) ethanol). The wells were thoroughly washed with tap water and adherent cells were then dissolved in 33% acetic acid (0.1 ml/well). The released dye was measured spectrophotometrically at 577 nm, using a Kontron spectrophotometer (SLT 210).

In vitro *TNF cell-growth-inhibition assay*

Twenty-four hours before treatment, adherent cells were plated out in microtitre plates at a concentration of 5–10 × 10^3 cells/well in their cell-specific growth medium. Then, serial dilutions of TNF, IFN-γ, or a combination of both, were added. After an incubation period of 72 hr at 37°C, the cells were fixed, stained, and measured spectrophotometrically, as described for the cell-killing assay.

Non-adherent cells, at a concentration of 0.5–1.5 × 10^4 cells per 100 μl per well, were mixed with serial dilutions of TNF, IFN-γ, or a combination of both. After 72 hr at 37°C, 0.5 μCi [^{3}H]-thymidine (21 Ci/mmol; Amersham) in 50 μl medium was added to each well. Five hours later, the cell suspension was filtered through glass-fiber filters (Type AIE; Gelman Science, Michigan, IL). The cups and filters were washed with distilled water and ethanol, and the incorporated radioactivity was counted.

One TNF unit (U)/ml is defined as the reciprocal of the dilution required to reduce cell survival of L–929 cells (40,000 cells/0.3 cm^2 well; 200μl Dulbecco's modified Eagle's medium – 10% new-born calf serum) by 50% in the 18-hr cell-lysis assay made in the presence of actD.

TNF- and IFN-preparations

Bacterial r-hTNF, produced in amounts of up to 30% of the total bacterial protein mass by the *E. coli* strains NF–1 or MC–1061, which harbor the expression plasmid $pP_Lc236mu$-hTNF1 [7], and purified to apparent homogeneity (Tavernier *et al.*, unpublished results), was used in the different biological assays. The protein preparation was at least 99% pure, migrated as a 36 Kd protein upon gel filtration, as a 17 Kd band on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, and had a specific activity of 2–3 × 10^7 U/mg. Pure, glycosylated human IFN-γ [8, 9], produced in cultures of Chinese hamster ovary cells transformed with a combination of plasmids coding for dihydrofolate reductase and interferon gamma (IFN-γ), was used. The concentration of IFN-γ is expressed in international units (IU)/ml. Immuneron®, human interferon γ synthesized in *E. coli*, is a product of Biogen.

Target cell lines

The human cell lines ME–180 (cervix carcinoma), SK–BR–3, MCF–7, and BT–20 (breast carcinoma), SK–OV–3 (ovary carcinoma), HT–29, COLO 320 DM, and SK–CO–1 (colon adenocarcinomas), A–549 (lung carcinoma), VA–10 and SV–80 (SV40-transformed fibroblasts), KB and CALU–I (epidermal carcinomas), WI–38 (diploid embryonic lung), RPMI–7951 (melanoma), MG–63 and HOS (osteosarcoma), MNNG–HOS (chemically transformed osteosarcoma), Raji (Burkitt lymphoma), WISH (amnion), were obtained from the American Type Culture Collection (Rockville, MD). The human urothelial cell lines (HCV 29, HCV 29 T, Hu 609, Hu 609 T, Hu 456, T–24, Hu 961a, Hu 1703He) [10] and the glioma cell line (SA–4) [11], were supplied by Dr. M. Mareel (University Hospital, Ghent, Belgium). The two hepatoma cell lines PLC/PRF/5 and McG(30–80)6 were a kind gift of Dr. M. F. Bassendine (University of Newcastle, U.K.). Hela D98/AH_2 [12] and Hela H21 (human cervix carcinoma sublines), Jurkat C and CEM WT 053 (human T-cell lymphomas), FS–4 (human diploid fibroblast), were obtained from the cell line collection of the Laboratory of Molecular Biology (University of Ghent, Belgium). The mouse cell line L–929 (fibrosarcoma) and the human diploid fibroblast line E_1SM were obtained from the Rega Insitute (Louvain, Belgium). Jurkat A (human T-cell lymphoma) was obtained from Biogen S.A. (Geneva, Switzerland). (CCL 23)HEP (human epidermoid carcinoma) was provided by Dr. C. Weissman (Zurich, Switzerland).

RESULTS

Sensitivity of human tumor cell lines to TNF in a cytolytic assay

The cytolytic/cytostatic effect of r-hTNF on different human cancer cell lines was examined. Two human diploid fibroblast cell lines (FS–4 and E_1SM) and a human diploid lung cell line (WI–38) were used as normal cell controls. It had previously been shown that TNF often acts synergistically with actD in killing "susceptible" cell lines. Cytotoxicity is enhanced and the cells are killed much earlier, cell death being already apparent about 8 hr after exposure [3]. The susceptibility to rTNF of a large number of human tumor cell lines was tested in a "cell-lytic" assay both in the absence (–actD) and in the presence (+actD) of actD. Cell survival was measured after an incubation period of 48 hr (–actD) or 18 hr (+actD) as described under "Methods". Results of both assays (– and +actD) are summarized in Table 1. Sensitivity of the target cells to r-hTNF is expressed as the concentration of TNF, indicated in U/ml, needed to reduce cell survival by 50%.

We observed that in the 48-hr cell-lysis assay performed in the absence of actD, only very few cell lines (e.g. Hela D98/AH2 and Hela H21 subclones) expressed a 50% cytolytic sensitivity. However, this does not mean that the other malignant cell lines tested were totally insensitive to the cytolytic action of TNF; it only shows that in the latter target cell lines no 50% cytotoxicity value

Table 1. r-hTNF sensitivity of human malignant and non-malignant cell lines

Cell line	TNF-sensitivity*		Growth-inhibition assay†							Growth-charact.
	+act.D	−act.D	TNF-sensitivity			Synergism			Growth inh. by IFN-γ	
			50%	25%	10%	50%	25%	10%		
Cervix carcinoma										
C4-II	N.D.	N.D.	−	−	10^4	−	+	+	+++	adh.
ME-180	0.25	−	125	30	5	+	+	+	++++	adh.
HeLa-H21	10	−	3300	40	3	+	+	+	+++	adh.
HeLa D98/AH2	10	2200	100	30	1.5	+	+	+	+++	adh.
Ovary carcinoma										
SK-OV-3	−	−	−	−	<15	+	+	+	++	adh.
Breast carcinoma										
BT-20	−	−	40	5	1	+	+	+	++	adh.
MCF-7	20	−	80	1.5	1	+	+	+	++++	adh.
SK-BR-3	−	−		15	<15	+	+	+	++	adh.
Epidermoid carcinoma										
KB	N.D.	N.D.	−	−	−	−	−	+	++++	adh.
CALU-1	−	−	−	40	5	−	−	−	−	adh.
CCL3 (HEP)	50	−	−	−	3300	−	−	+	+++	adh.
Lung carcinoma										
A-549	−	−	−	−	500	−	+	+	+	adh.
Colon adenocarcinoma										
HT-29	−	−	−	−	250	+	+	+	++	adh.
COLO 320 DM	N.D.	N.D.	−	−	−	−	−	−	++++	non-adh.
SK-CO-1	−	−	−	200	<15	+	+	+	+++	adh.
Hepatoma										
McG(30-80)6	−	−	−	5	1.5	+	+	+	++	adh.
PLC/PRF/5	N.D.	N.D.	−	−	125	−	+	+	−	adh.
Melanoma										
RPMI-7951	200	−	−	3300	20	−	+	+	+	adh.
Osteosarcoma										
MG-63	10	−	−	−	50	−	+	+	+++	adh.
HOS	150	−	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	adh.
MNNG-HOS	250	−	−	3300	2200	−	+	+	++++	adh.
Glioma										
SA-4	0.25	−	−	800	50	−	+	+	+++	adh.
Leukaemia/lymphoma										
Jurkat A	N.D.	N.D.	−	−	200	−	−	−	−	non-adh.
Jurkat C	N.D.	N.D.	300	10	<10	−	−	−	−	non-adh.
CEM WT 053	N.D.	N.D.	<10	<10	<10	−	−	−	−	non-adh.
Raji	N.D.	N.D.	−	−	−	−	−	−	−	non-adh.
SV40-transf. fibrobl.										
SV-80	200	−	−	−	−	−	−	+	+	adh.
VA-10	10	−	−	−	40	−	+	+	+	adh.
Amnion.										
WISH	15	−	−	10	1	−	+	+	++++	adh.
Non-transformed diploid										
WI-38	5	−	−	−	−	−	−	−	−	adh.
ElSM	110	−	−	−	−	−	−	−	−	adh.
FS-4	−	−	−	−	−	−	−	−	−	adh.

*Sensitivity to TNF in the cell-lysis assay in the presence (+actD) or in the absence of actD (−actD). Sensitivity is expressed as U/ml TNF required to reduce cell survival to 50% N.D.: not done; (−): no 50% sensitivity could be obtained at TNF concentrations up to 3300 U/ml.

†Sensitivity of the cells to growth in the presence of different concentrations of TNF, IFN-γ or a combination of both. TNF sensitivity is expressed as U/ml TNF required to induce an antiproliferative effect of 50, 25 or 10%. (−) indicates that this effect was not present even when using a TNF concentration of 3300 U/ml. The presence (+) or absence (−) of a synergistic cytotoxic/cytostatic effect of TNF and IFN-γ is indicated at 50, 25 and 10% sensitivity of the cells to IFN-γ alone. The growth-inhibitory effect of IFN-γ (at a concentration of 18,000 IU/ml) on the cells is expressed as no reduction (−), or a reduction of 10 (+), 20 (++), 40 (+++) or 60% or more (++++) of the treated cells compared with the cell number present in the control.

could be obtained within 2 days with TNF doses of up to 3000 U/ml. When the assay was performed in the presence of actD, most of the cell lines became sensitive, so that a 50% lytic value was attained at cell-specific TNF concentrations. These results show that actD greatly enhances the sensitivity of many target cell lines to the cytolytic activity of TNF. The question can be raised whether treatment of the cells with actD does not block a mechanism which allows the cells to avoid or counteract TNF-induced killing, as the apparent tumor-cell specificity of the TNF activity seems less stringent or even partly lost in the presence of actD. Only one of the diploid control cell lines (FS–4) remained insensitive to the cytolytic action of TNF, even when up to 1.6×10^4 U/ml TNF were used. The two other non-transformed cell lines tested (WI–38) and E_1SM) became clearly sensitive to TNF by treatment with actD.

Synergism of hIFN-γ with the cytolytic activity of TNF

We next evaluated the synergistic action of IFN-γ and TNF on a set of cell lines known to be tumorigenic or at least transformed, as well as on a set of non-transformed cell lines. Preliminary results regarding this synergism were obtained with natural hTNF from 12–O–tetradecanoyl phorbol 13-acetate (TPA)-induced U-937 cells, on two cervix carcinoma cell lines, ME-180 and Hela H21, and on one breast-carcinoma cell line, BT–20. All these cell lines became sensitive or more sensitive to the cytotoxic action of TNF by addition of hIFN-γ, and this synergy was nearly indistinguishable for natural hTNF or r-hTNF. Table 1 summarizes the results obtained by testing different cell lines with r-hTNF and/or hIFN-γ in the growth-inhibition assay. Data on the sensitivity of the target cell lines to rTNF in the absence of IFN-γ are presented for the 50, 25 and 10% cytotoxicity levels. The 50 and 25% values were chosen to allow an evaluation of the cytolytic effect as well as the comparison of the TNF sensitivity of the different target cells. The 10% value of cytotoxicity was used to differentiate between cell lines that are completely insensitive and cell lines that are only slightly sensitive to TNF. Furthermore, the extent of growth inhibition of cells treated with 18,000 IU of IFN-γ/ml is indicated. Finally, the presence of a synergism between hIFN-γ and r-hTNF, attaining 50, 25 or 10% of the cytolytic activity at a given IFN-γ concentration, is also shown (this activity refers to the cytolytic/antiproliferative effect of the combined treatment with TNF and IFN-γ compared with the effect obtained by treatment with IFN-γ alone).

The data allow a classification of the cell lines into different groups, depending on their sensitivity to cytolysis by TNF alone or in combination with hIFN-γ. A first group of target cell lines is characterized by the fact that they are sensitive to treatment with TNF alone, as 50% cytolysis occurs at a specific TNF concentration, but they become clearly more sensitive when even low doses of IFN-γ are added. The *in vitro* TNF-induced cell-killing is significantly enhanced by a combined treatment of these target cell lines with TNF and IFN-γ (Table 2 and Fig. 1). This synergy is dose-dependent and can be observed with as little as 10 IU/ml of IFN-γ; it reaches a plateau value at 200 IU/ml. This group includes the cervix carcinoma cell lines (ME–180, Hela–H21 and Hela D98/AH2) and two breast carcinoma cell lines (BT–20 and MCF–7). A second group of malignant cell lines (SK–BR–3, SK–CO–1, McG (30–80)6, SK–OV–3 and HT–29) are characterized by a relatively low sensitivity to treatment with TNF alone, attaining a cytolytic effect of at most 25% or even 10%, but by a remarkable enhancement of this sensitivity by synergism with IFN-γ. At approx. 200 IU/ml of IFN-γ, about 100 U/ml of TNF is sufficient to attain a 50% cytolytic effect (Table 2). A third group of cell lines tested (PLC/PRF/5, MG–63, MNNG–HOS, SA–4 and C4–II) shows the same relatively low sensitivity to TNF treatment, but synergism with IFN–γ is less pronounced than in the second group. Even with relatively high doses of IFN–γ, a 50% cytolytic effect could not be obtained. The three TNF-sensitive leukemia cell lines (CEM WT 053 and Jurkat A and C) are not susceptible to synergism with IFN–γ. Finally, all non-transformed fibroblast cell lines tested (FS–4, WI–38 and E_1SM), and also two non-adherent malignant cell lines (Raji and COLO 320 DM), are completely resistant to the cytolytic activity of TNF even at IFN–γ concentrations as high as 18,000 IU/ml.

Synergism can also be demonstrated by quantifying the percentage of cells resistant to treatment with TNF alone or to a combination of TNF and IFN–γ. The number of resistant cells remaining after treatment with high doses of TNF and IFN-γ is always remarkably lower than the number of cells present after treatment with each of these proteins alone (data not shown). Even at IFN-γ concentrations too low to induce any antiproliferative effect on their own, the cytostatic/cytolytic effect of TNF is already considerably enhanced. This again illustrates the remarkable synergism between TNF and IFN–γ.

Correlation between malignancy and TNF-sensitivity

In order to study in more detail the apparent specificity of TNF in killing transformed cell lines, a number of human urothelial cell lines of non-malignant as well as of malignant origin were tested for their TNF-sensitivity and susceptibility

Table 2. Synergism of r-hTNF and hIFN-γ on different human cell lines

hIFN-γ IU/ml	Cell line: HeLa D98	HeLa H21	BT-20	MCF-7	ME-180	HT-29	SK-BR-3	SK-CO-1	SK-OV-3	McG (30-80)6	FS-4 WI-38 E,SM
	U/ml TNF										
0	100	3300	30	80	370	>3300	>33300	>33300	>33300	>3300	>3300
0.9	100	2200	30	80	370	>3300	>33300	>33300	>33300	>3300	>3300
2.7	100	3300	30	80	370	>3300	>33300	>33300	>33300	>3300	>3300
8	30	3300	30	80	370	2200	>33300	140	>33300	2220	>3300
25	10	1110	30	80	2	35	>33300	140	>33300	1110	>3300
75	12	750	20	80	2	9	270	–	>33300	–	>3300
220	10	250	9	60	2	5	–	100	–	30	>3300
670	5	125	5	30	2	3	100	–	11100	10	>3300
2000	7	80	5	30	10	9	270	15	11100	10	>3300
6000	5	60	5	30	10	9	400	–	–	30	>3300
18000	7	35	3	30	10	5	400	15	1250	30	>3300

Cells were tested using a "growth-inhibitory" assay as described in "Materials and Methods".

Sensitivity is expressed as U/ml hTNF required to result in an antiproliferative effect of 50% of the number of cells present when treated with hIFN-γ alone.

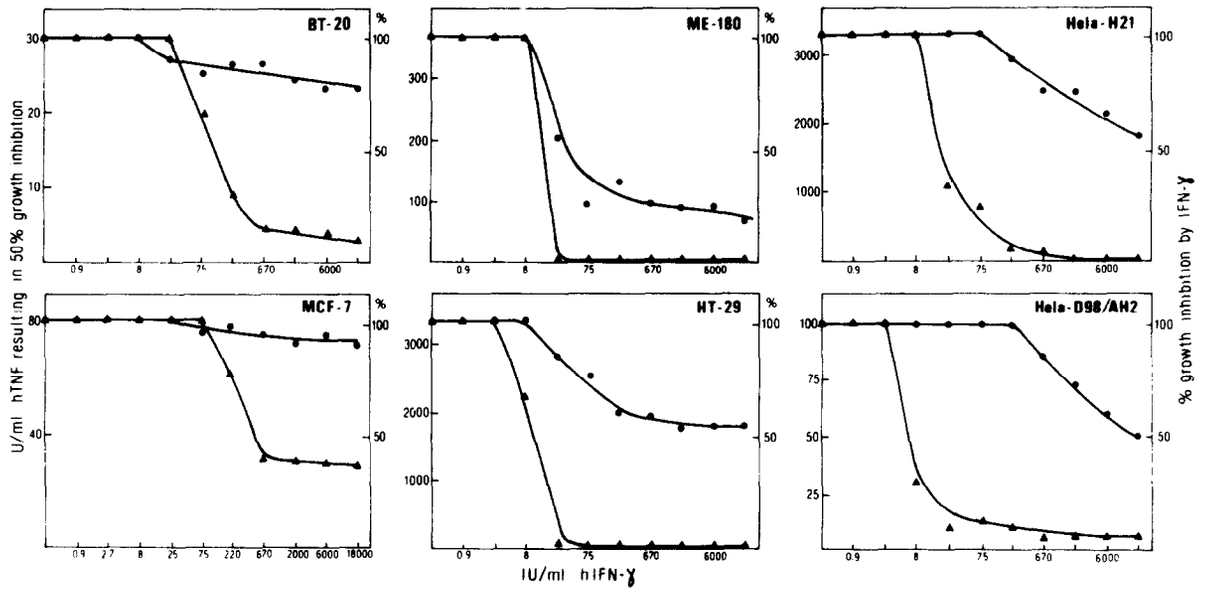

Fig. 1. Synergism of h-rTNF and hIFN-γ on different human malignant cell lines. TNF sensitivity (▲–▲) is expressed as U/ml *r-hTNF (left ordinate) required to obtain* 50% *cell-growth inhibition in the presence of the corresponding IFN-γ concentration. Growth inhibition by hIFN-γ (●–●; right ordinate) is expressed as a percentage of the untreated control.*

to the synergistic effect with IFN-γ. As these cell lines have been exceptionally well characterized and also classified into different transformation grades (TGr) [10], we have investigated a possible correlation between the grade of transformation and TNF-susceptibility. TGrII cells differ from TGrI in having an infinite life span, but both classes of cells are non-tumorigenic in nude mice and non-invasive *in vitro*. TGrIII cells have the ability to produce tumors in nude mice and are invasive *in vitro* (destruction of fragments of embryonic heart tissue) [13]. The results of our study are summarized in Table 3. Hu 609 T and HCV 29 T cells are spontaneously transformed sub-lines of Hu 609 and HCV 29, respectively, and these couples may therefore be considered to be pairs of closely related cell lines with different transformation characteristics. The Hu 609 T cell line shows some TNF-sensitivity (10% cytolysis with 80 TNF U/ml), which can be enhanced by a

combined treatment with IFN-γ (up to 25% cytolysis). The HCV 29 T cell line, on the other hand, is not sensitive to the cytotoxic activity of TNF, but shows sensitivity to the growth-inhibitory effect of IFN-γ. Other cell lines tested, classified as TGrIII, respond variously to treatment with TNF. Two cell lines (Hu 961a and Hu 1703He) are sensitive to cytolysis by TNF but are not susceptible to synergism with IFN-γ. Another cell line (Hu 456) is only slightly sensitive to treatment with TNF (up to 25% cytolysis), but the sensitivity can be somewhat increased by simultaneous treatment with IFN-γ. T-24 is slightly sensitive to TNF (up to 10% cytolysis) only when treated with IFN-γ.

DISCUSSION

This study documents the *in vitro* effect of r-hTNF on a broad range of human transformed and non-transformed cell lines. hTNF is cytolytic to a number of malignant cell lines and has a cytostatic/cytotoxic effect synergistic with the growth-inhibitory effect of IFN-γ. This effect was clearly in evidence when tested on different cervix and breast carcinoma cell lines. Remarkable enhancement of the cytotoxicity of TNF could be detected with as little as 10 IU/ml of IFN-γ, the saturation concentration being 200 IU/ml, while other cell lines tested needed higher IFN-γ concentrations to show synergism with the cytotoxic effect of TNF. Some cell lines, not sensitive to TNF alone, became highly sensitive in the presence of IFN-γ. All human, non-transformed, diploid cell lines tested were insensitive to TNF even in the presence of IFN-γ. The enhancement of the sensivity of many target cell lines to TNF-cytolysis by treatment with actD was also clearly demonstrated. In contrast to the synergism with IFN-γ, however, some normal cell lines in the presence of actD also became sensitive to cytolysis by TNF. In order to simulate natural conditions, all experiments presented here were performed with glycosylated hIFN-γ. But synergism was also tested on a number of human cell lines with unglycosylated human recombinant IFN-γ (Immuneron, Biogen) and, within the accuracy of the assay, the results were indistinguishable from those obtained with glycosylated hIFN-γ.

Our results, obtained by measuring TNF-sensitivity of a number of human urothelial cell lines classified into different transformation grades, suggest that TNF-sensitivity does not automatically correlate with an increased degree of malignancy. Clearly, not all cells displaying well-defined transformation characteristics are susceptible to cytolysis by TNF or become so through synergism with IFN-γ. On the other hand, several cell lines classified as TGrIII are sensitive to cytolysis by TNF. The reason for the lack of response to TNF of some tumor cells and for the cytotoxic or only cytostatic response of other malignant cells is not yet known. Some lymphoblastoid cell lines do not respond to TNF, and indeed, some lack a significant number of TNF receptors [14]. Therefore, the resistance of at least some cell lines may be due to the (near) absence of specific cell-surface receptors to which TNF must bind to exert its cytostatic and/or cytolytic activity. However, some other cell lines resistant to the cytotoxic action of TNF can be killed when also treated with metabolic blockers such as actD. This makes it unlikely that these cells lack the ability to interact with TNF.

The molecular mechanism of the enhancement of TNF cytotoxicity of IFN–γ or actD is at present unknown. It was previously shown [3], and the present study confirms this, that inhibition of protein synthesis in the target cell line by certain metabolic drugs causes a remarkably accelerated TNF-induced cytolysis. The hypothesis that a mechanism, required for repairing a TNF-induced defect, is inhibited still needs to be proved. Using similar assay conditions, we could find no correlation between the enhancement of the sensitivity of the target cell and the treatment with actD or with IFN-γ. Presumably, actD and IFN-γ influence different steps in the complex process of TNF-induced cell lysis. While metabolic blockers such as actD possibly increase the sensitivity of the cell by interfering with some protective or repair mechanism, IFN-γ may enhance the sensitivity of the cells by inducing (more) TNF-receptors or changes in the membrane structure. Studies on the effect of IFN-γ on the target cells have shown that, besides an antiproliferative effect, an alteration of the membrane structure could also be observed [15]. The cell lines tested varied in their sensitivity to the antiproliferative effect of IFN-γ, added as a single effector, but no correlation between the degree of cell-growth inhibition and the sensitivity to TNF-cytolysis induced by synergism with IFN-γ could be detected (Table 1). Schreiber *et al.* [16] have shown, by using monoclonal antibodies, that IFN-γ contains topologically distinct functional domains responsible for inducing different responses. Therefore, the antiproliferative effect of IFN-γ may perhaps not be directly correlated with its synergistic action on cell lysis by TNF. We were not able to demonstrate synergy between IFN-γ and TNF on any of the lymphoma/leukemia cell lines tested. Indeed, some lymphoblastoid cell lines do not carry membrane receptors for IFN-γ [17].

By using the growth-inhibition assay it is difficult to prove unambiguously whether cytostasis or cytolysis occurs during treatment with the combination of TNF and IFN-γ. By comparing the number of cells present before and after the 3-day

Table 3. TNF sensitivity of different human malignant urothelial cell lines

	Classification	TNF sensitivity			Synergism with IFN-γ			Growth inhibition by IFN-γ
		50%	25%	10%	50%	25%	10%	
Hu 609	TGr II	–	–	–	–	–	–	+++
HCV 29	TGr II	–	–	–	–	–	–	–
Hu 609 T	TGr III	–	–	80	–	+	+	++
HCV 29 T	TGr III	–	–	–	–	–	–	+++
Hu 961 a	TGr III	80	1	1	–	–	–	++
Hu 1703 He	TGr III	40	3	1	–	–	–	+++
Hu 456	TGr III	–	2000	80	–	+	+	+++
T-24	TGr III	–	–	–	–	–	+	+++

Legend: see Table 1.

treatment, it is possible to get an idea as to which effect predominates. These data suggest that cytolysis and cytostasis occur simultaneously, and that the balance depends on the specific sensitivity of the target cell line. Furthermore, the growth-inhibitory effect of IFN-γ is not only additive but clearly synergistic with the cytostatic effect of TNF itself. But further studies, using techniques which allow quantitation of the cells which actually lyse in the growing culture, may be needed for more definite conclusions.

The known *in vitro* and *in vivo* biological activities of TNF and lymphotoxin (LT) are very similar. LT is produced by B-cells, while TNF is secreted by macrophage-type cells. LT has also been reported to act synergistically with IFN [18, 19]. Even the synergism between TNF and IFN, first described by Williamson *et al.* [20], most probably refers to a synergism between LT and IFN, as the cytotoxic product was produced by a B-cell line. Sequence analysis of cloned TNF and LT has shown that there is about 30% homology between the amino acid sequences of the two proteins. In addition, LT activity on L–929 cells is not inhibited by TNF-specific antibodies (data not shown) [21]. TNF and LT may interact with a common membrane receptor or, alternatively, the two products may share common biological activities acting on different targets.

Since TNF is cytotoxic to a broad range of human tumor cell lines, especially in combined treatment with IFN-γ, it could prove to be an important tool in cancer therapy, for limiting tumor growth, and, hopefully, also for preventing invasion and metastasis. The use of TNF in a combined treatment with other clinical cytotoxic drugs, now used in cancer therapy, should also be evaluated.

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