

# *Acta Medica Okayama*

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*Volume 41, Issue 4*

1987

*Article 2*

AUGUST 1987

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## Synergism between human tumor necrosis factor and human interferon-alpha: effects on cells in culture.

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# Synergism between human tumor necrosis factor and human interferon-alpha: effects on cells in culture.\*

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## Abstract

The cytostatic and cytotoxic effects of highly purified natural human tumor necrosis factor (HuTNF-alpha) and natural human interferon-alpha (HuIFN-alpha) on 23 cell lines were studied in vitro. Natural HuTNF-alpha showed cytostatic and cytotoxic effects on PC-9, KHG-2, HT-1197, KG-1 and L-929 cells, and HuIFN-alpha showed both effects on KHG-2 and Daudi cells. A mixture of HuTNF-alpha and HuIFN-alpha (1:1, by unit) showed cytostatic and cytotoxic effects on HuTNF-alpha- or HuIFN-alpha-resistant cell lines such as KB, KATO-III, HEP-2, P-4788, as well as on HuTNF-alpha- or HuIFN-alpha-susceptible cells. Thus, the combined preparation of HuTNF-alpha and HuIFN-alpha expanded the spectrum of sensitive cells. The dosage of the mixed preparation required to produce 50% inhibition of cell growth was less than 20% of that of HuTNF-alpha or HuIFN-alpha alone. These results indicate that the cytostatic and cytotoxic effects of HuTNF-alpha and HuIFN-alpha are synergistically enhanced when they are administered together.

**KEYWORDS:** synergistic enhancement, cytostatic effect, cytotoxic effect, HuTNF-?, HuIFN-?

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\*PMID: 3661240 [PubMed - indexed for MEDLINE]

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## Synergism between Human Tumor Necrosis Factor and Human Interferon- $\alpha$ : Effects on Cells in Culture

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The cytostatic and cytotoxic effects of highly purified natural human tumor necrosis factor (HuTNF- $\alpha$ ) and natural human interferon- $\alpha$  (HuIFN- $\alpha$ ) on 23 cell lines were studied *in vitro*. Natural HuTNF- $\alpha$  showed cytostatic and cytotoxic effects on PC-9, KHG-2, HT-1197, KG-1 and L-929 cells, and HuIFN- $\alpha$  showed both effects on KHG-2 and Daudi cells. A mixture of HuTNF- $\alpha$  and HuIFN- $\alpha$  (1:1, by unit) showed cytostatic and cytotoxic effects on HuTNF- $\alpha$ - or HuIFN- $\alpha$ -resistant cell lines such as KB, KATO-III, HEp-2, P-4788, as well as on HuTNF- $\alpha$ - or HuIFN- $\alpha$ -susceptible cells. Thus, the combined preparation of HuTNF- $\alpha$  and HuIFN- $\alpha$  expanded the spectrum of sensitive cells. The dosage of the mixed preparation required to produce 50% inhibition of cell growth was less than 20% of that of HuTNF- $\alpha$  or HuIFN- $\alpha$  alone. These results indicate that the cytostatic and cytotoxic effects of HuTNF- $\alpha$  and HuIFN- $\alpha$  are synergistically enhanced when they are administered together.

*Key words* : synergistic enhancement, cytostatic effect, cytotoxic effect, HuTNF- $\alpha$ , HuIFN- $\alpha$

Tumor necrosis factor (TNF- $\alpha$ ) was recognized by Carswell *et al.* (1) as a factor with anti-tumor activity in the serum of mice which had been infected with bacillus Calmette-Guérin (BCG) as the priming agent and subsequently injected with endotoxin as the eliciting agent. TNF- $\alpha$  induces *in vivo* hemorrhagic necrosis of certain types of mouse sarcomas and exhibits *in vitro* cytostatic and cytotoxic effects on some tumor cell lines (1-4).

Interferon (IFN), one of the most investigated lymphokines, was discovered independently as an anti-viral protein by Nagano (5) and Isaacs *et al.* (6). The multiple biological activities of IFN including *in vitro* and *in vivo* antitumor activities are well known. IFNs have been applied to the treat-

ment of different types of cancers (7). Recent studies indicate that IFNs synergistically enhance the target cell killing activity of TNF- $\alpha$  (8,9) and lymphotoxin (TNF- $\beta$ ) (10-13).

We have reported the simultaneous production of HuTNF- $\alpha$  and HuIFN- $\alpha$  by BALL-1 cells, a human B-cell line originated from acute B-cell leukemia, stimulated with hemagglutinating virus of Japan (HVJ) (14,15).

This paper reports the synergistic effect of highly purified HuTNF- $\alpha$  and HuIFN- $\alpha$  which were cytostatic and cytotoxic to cell lines derived from cancer and fetal tissues.

### Materials and Methods

*Cell lines.* BALL-1 cells were kindly sup-

plied by the Second Department of Internal Medicine of Okayama University Medical School. FL cells (5-1 strain) were obtained from Dr. S. Yamazaki (NIH of Japan, Tokyo, Japan). Mouse L-929, KB, WI-38, Flow-4000, Flow-11000 and Intestine-407 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). RPMI-1788, Daudi, Raji, EB-3, ARH-77, CCRF-CEM, CCRF-HSB-2, KG-1 and THP-1-O cells were kindly provided by Dr. J. Minowada (Roswell Park Memorial Institute, New York, USA). KATO-III, PC-9, PC-10, HEP-2, HT-1197 and P-4788 cells were from the stock of the First Department of Surgery of Okayama University Medical School. The origins of the cell lines are listed in Table 1. These cell lines were cultured in RPMI-1640 medium or Eagle's minimal essential medium (MEM) supplemented to a concentration of 10% with heat-inactivated fetal calf serum (FCS).

**TNF assay.** TNF activity was assayed by the dye uptake microtiter method using mouse L-929 cells treated with actinomycin D (Pharmacia, Sweden) as described previously (16). The concentration of the TNF preparation at which 50% of the cells were killed was defined as 50 units/ml. TNF activity was determined and expressed in units (u) using, as the standard, a house reference (Lot 81-B,  $1.5 \times 10^4$  u/ml) prepared from highly purified HuTNF- $\alpha$ .

**IFN assay.** IFN activity was determined by the method reported by Johnston *et al.* (17). The titer was expressed in international units (IU) using an international reference for human lymphoblastoid IFN- $\alpha$  (Ga 23-901-532).

**Preparation of HuTNF- $\alpha$  and HuIFN- $\alpha$ .** Production, purification and characterization of HuTNF- $\alpha$  and HuIFN- $\alpha$  from BALL-1 cells were previously reported (14, 15). Briefly, BALL-1 cells were propagated in immunosuppressed hamsters, and simultaneous production of HuTNF- $\alpha$  and HuIFN- $\alpha$  was induced by stimulating BALL-1 cells with HVJ. HuTNF- $\alpha$  and HuIFN- $\alpha$  were separately purified using affinity columns with monoclonal antibodies to HuTNF- $\alpha$  and HuIFN- $\alpha$ , respectively. The HuIFN- $\alpha$  preparation was found to be purified about 500-fold and had specific activity of  $2 \times 10^8$  IU/mg of protein with no TNF activity. HuTNF- $\alpha$  was purified about 2000-fold with a specific activity of  $1 \times 10^9$  u/mg of protein

and with no IFN activity. The purity of the HuTNF- $\alpha$  and HuIFN- $\alpha$  was found to be over 90% as judged by SDS-polyacrylamide gel electrophoresis. The purified HuTNF- $\alpha$  and HuIFN- $\alpha$  preparations were used as such, or as a 1:1 mixture (u/IU).

**Determination of cytostatic and cytotoxic activities.** Cells were plated in a 24-well microplate (No. 3047, Falcon Labware, Oxnard, CA.) at  $5 \times 10^4$  cells/well. Adherent cells were treated with 0.05% trypsin and 0.02% (w/v) EDTA to suspend the cells. After 20 h of culture, 0.1 ml of a solution of HuTNF- $\alpha$  ( $1 \times 10^5$  u/ml), HuIFN- $\alpha$  ( $1 \times 10^5$  IU/ml), or a mixture of these preparations (HuTNF- $\alpha$ ,  $1 \times 10^5$  u/ml + HuIFN- $\alpha$ ,  $1 \times 10^5$  IU/ml) was added to each well. The number of viable cells was determined by the erythrosin B dye exclusion test 4 days after the addition of HuTNF- $\alpha$ , HuIFN- $\alpha$  or the mixed preparation. Cell growth of treated cultures in comparison with that of the control culture was determined, and the cytostatic effect was expressed as a percent according to the following equation:

$$\text{Cytostatic effect (\%)} = \frac{\text{Number of cells in the control} - \text{Number of cells in the test}}{\text{Number of cells in the control} - \text{Number of cells seeded}} \times 100$$

The decrease in the viability of the cells due to treatments was determined, and the cytotoxic effect was calculated as follows:

$$\text{Cytotoxic effect (\%)} = \frac{\text{Viable cells in the control} - \text{Viable cells in the test}}{\text{Viable cells in the control}} \times 100$$

**Determination of dosage required to produce 50% inhibition of cell growth.** Cells were suspended at a concentration of  $2 \times 10^4$  cells/ml in RPMI-1640 or MEM supplemented to a concentration of 10% with FCS, and 0.1 ml of the cell suspension was seeded into each well of 96-well flatbottomed microtiter plates (No. 167008, Nunc, Roskilde, Denmark). Fifty  $\mu$ l aliquots of 2-fold serially diluted HuTNF- $\alpha$ , HuIFN- $\alpha$  or the 1:1 mixture of these preparations were added to each well. After 3 days of incubation at 37°C in an atmosphere of 5% CO<sub>2</sub>-95% air, the number of viable adherent cells was determined by the dye

uptake method (17), or the number of viable non-adherent cells by T. Mosmann's method (18). The 50% inhibition dosage (ED<sub>50</sub>) was defined as the dosage of either HuTNF- $\alpha$  or HuIFN- $\alpha$  that caused 50% inhibition of cell growth compared to that of the untreated control. In this paper, ED<sub>50</sub> values are expressed as TNF activity/ml for HuTNF- $\alpha$  and IFN- $\alpha$  activity/ml for HuTNF- $\alpha$  preparations, and as both TNF and IFN- $\alpha$  activities/ml for the mixture of these preparations.

## Results

*Cytostatic and cytotoxic effects of HuTNF- $\alpha$  and HuIFN- $\alpha$  preparations, and their synergistic effect.* Cytostatic and cytotoxic effects of the mixture of HuTNF- $\alpha$  and HuIFN- $\alpha$  preparations were tested on 23 cell lines

established from cancer and fetal tissues and compared with those using either HuTNF- $\alpha$  or HuIFN- $\alpha$  alone. The results are summarized in Table 1.

The natural human TNF- $\alpha$  preparation had a cytostatic effect on KB, PC-9, PC-10, KHG-2, HT-1197, KG-1 and mouse L-929 cells and a cytotoxic effect on PC-9, KHG-2, KG-1 and L-929 cells, while natural human IFN- $\alpha$  had a cytostatic effect on KB, PC-10, KHG-2, HEp-2, Flow-4000, Daudi and ARH-77 cells and a cytotoxic effect on KHG-2 and Daudi cells. The mixture of highly purified HuTNF- $\alpha$  and HuIFN- $\alpha$  preparations had cytostatic or cytotoxic effects on the cell lines that were resistant to either HuTNF- $\alpha$  or HuIFN- $\alpha$ , KATO-III, P-4788, Flow-

**Table 1** Cytostatic and cytotoxic effects of natural human TNF- $\alpha$  (HuTNF- $\alpha$ ), natural human IFN- $\alpha$  (HuIFN- $\alpha$ ) and a mixture of the two on cells in culture<sup>a</sup>

Target cells	The mixed preparation <sup>b</sup>		HuTNF- $\alpha$		HuIFN- $\alpha$	
	Cytostatic	Cytotoxic	Cytostatic	Cytotoxic	Cytostatic	Cytotoxic
KB (nasopharyngeal cancer)	+++++ <sup>c</sup>	+++	+++	±	+++	±
KATO-III (gastric cancer)	++++	++	-	-	+	-
PC-9 (lung cancer)	+++++	++	+++	++	+	-
PC-10 (lung cancer)	+++++	++	++	-	+++	±
KHG-2 (glioma)	+++++	+++++	+++++	+++	+++++	+++
HEp-2 (laryngeal cancer)	+++++	+++++	+	±	+++	+
HT-1197 (bladder cancer)	+++++	+++++	+++++	+++	±	±
P-4788 (colon cancer)	+++++	+++	+	±	+	±
WI-38 (fetal lung)	-	-	±	-	-	-
Flow-4000 (fetal kidney)	+++	±	-	-	++	±
Flow-11000 (fetal small intestine)	+++++	+	-	-	±	-
Intestine 407 (fetal small intestine)	+++	±	-	-	+	-
RPMI 1788 (normal B cell)	-	-	-	-	-	-
Daudi (BL)*	++++	+++	-	-	++++	+++
Raji (BL)*	-	-	-	-	-	-
EB-3 (BL)*	±	-	±	-	-	-
BALL-1 (ALL)*	+	-	+	-	+	-
CCRF-CEM (ALL)*	++	-	±	-	-	-
CCRF-HSB-2 (ALL)*	±	±	++	-	-	-
ARH-77 (MM)*	++	-	±	-	++	±
KG-1 (AML)*	+++++	+++++	+++++	+++++	-	-
THP-1-O (AML.O)*	++	+	±	-	-	-
L-929 (mouse fibroblast)	+++++	+++++	+++++	+++++	-	-

*a:* Cytostatic and cytotoxic effects were determined *in vitro* by incubating cells for 4 days in the presence of HuTNF- $\alpha$  ( $1 \times 10^5$  u/ml), HuIFN- $\alpha$  ( $1 \times 10^5$  IU/ml) or a mixture of the two.

Cytostatic and cytotoxic effects were determined by the methods described in Materials and Methods.

*b:* A mixture of HuTNF- $\alpha$  ( $1 \times 10^5$  u/ml) and HuIFN- $\alpha$  ( $1 \times 10^5$  IU/ml).

*c:* The grading of cytostatic and cytotoxic effects is as follows: 0~10%, -; 10~20%, ±; 20~30%, +; 30~40%, ++; 40~50%, +++; 50~60%, ++++ and over 60%, +++++.

\*ALL, Acute lymphoblastic leukemia; BL, Burkitt lymphoma; MM, Multiple myeloma; AMOL, Acute monocyte leukemia; AML, Acute myeloblastic leukemia.

11000, Intestine 407 and CCRF-CEM cells, as well as the cell lines susceptible to either HuTNF- $\alpha$  or HuIFN- $\alpha$ . Furthermore, the cytostatic and cytotoxic effects of the mixed preparation on the cell lines susceptible to either HuTNF- $\alpha$  or HuIFN- $\alpha$  preparations, such as KB, PC-9, PC-10, HEP-2 and Flow-4000, were found to be markedly augmented. These results indicate that when HuTNF- $\alpha$  and HuIFN- $\alpha$  are administered in combination, the spectrum of their cytostatic and cytotoxic effects extends to cell lines which are not susceptible to singly administered HuTNF- $\alpha$  or HuIFN- $\alpha$ . Furthermore, the intensities of cytostatic and cytotoxic effects of HuTNF- $\alpha$  and HuIFN- $\alpha$  were markedly enhanced on the cell lines susceptible to HuTNF- $\alpha$  or HuIFN- $\alpha$ . The mixed preparation had a cytostatic effect on the Flow-4000, Flow-11000 and Intestine 407 cell lines, established from fetal kidney and small intestine.

*ED<sub>50</sub> of HuTNF- $\alpha$ , HuIFN- $\alpha$  and the mixture of the two agents.* In order to confirm the synergistic cytostatic effect between HuTNF- $\alpha$  and HuIFN- $\alpha$ , the respective ED<sub>50</sub> values of the preparations of HuTNF- $\alpha$ , HuIFN- $\alpha$  and their mixture were determined using KG-1, Daudi, KB, KATO-III and P-4788 cells. Representative results are sum-

**Table 2** ED<sub>50</sub> of HuTNF- $\alpha$ , HuIFN- $\alpha$  and a mixture of the two<sup>a</sup>

Target cells	ED <sub>50</sub>		
	HuTNF- $\alpha$ (u/ml)	HuIFN- $\alpha$ (IU/ml)	The mixture <sup>b</sup> (u/ml + IU/ml)
KG-1	50	> 5,000	20+20
Daudi	> 5,000	5	5+5
KB	> 5,000	3,500	400+400
KATO-III	> 5,000	> 5,000	250+250
P-4788	3,500	> 5,000	600+600
PC-9	> 5,000	> 5,000	700+700

*a:* After a 3-day incubation at 37° C in an atmosphere of 5% CO<sub>2</sub>-95% air, the number of viable KG-1 and Daudi cells was determined by Mosmann's method (18) and that of KB, KATO-III, P-4788 and PC-9 cells by the dye uptake method.

*b:* See the legend to Table 1.

marized in Table 2. Some of the results are illustrated in Fig. 1.

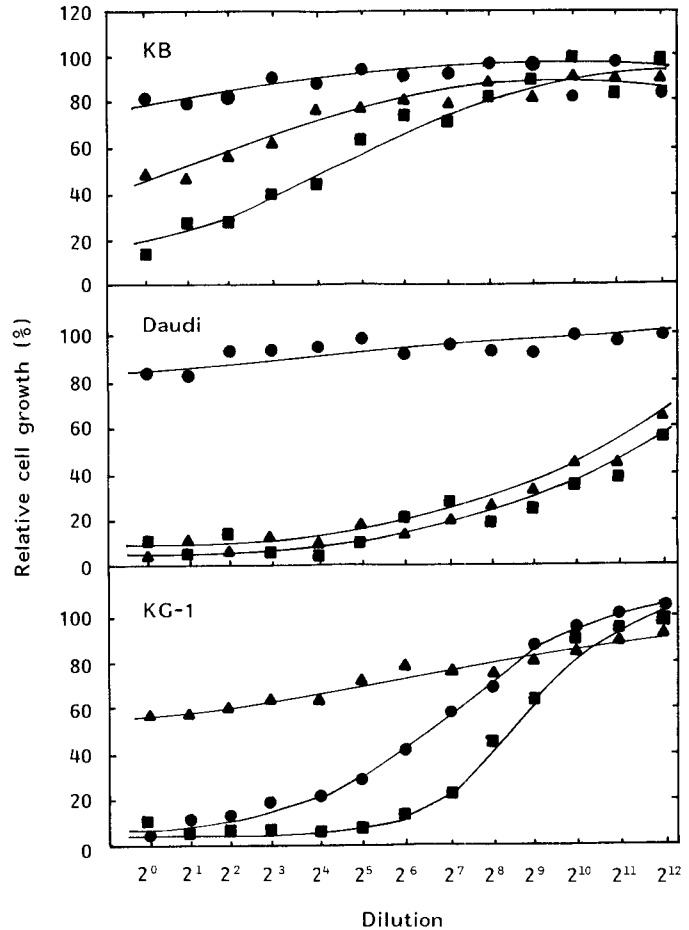
As is apparent from Table 2, HuTNF- $\alpha$  and HuIFN- $\alpha$  showed high ED<sub>50</sub> values to KB, KATO-III and PC-9 (ED<sub>50</sub>  $\geq$  3500). These cell lines were not susceptible to either HuTNF- $\alpha$  or HuIFN- $\alpha$ . The ED<sub>50</sub> values of the mixed preparation against KB, KATO-III and PC-9 decreased to less than 20% of those of HuTNF- $\alpha$  or HuIFN- $\alpha$  administered singly. Particularly, the ED<sub>50</sub> values of the mixed preparation to KATO-III decreased to less than 5% of the ED<sub>50</sub> value of HuTNF- $\alpha$  or HuIFN- $\alpha$ . These results indicate that the cytostatic effects of HuTNF- $\alpha$  and HuIFN- $\alpha$  are synergistically enhanced by combining the two agents.

HuTNF- $\alpha$  showed a very low ED<sub>50</sub> value (50 u/ml) to KG-1 cells, and HuIFN- $\alpha$  a low ED<sub>50</sub> value (5 IU/ml) to Daudi cells. The ED<sub>50</sub> values of the mixed preparation, however, were not higher than those of HuTNF- $\alpha$  or HuIFN- $\alpha$  administered singly to KG-1 and Daudi cells which were highly sensitive to HuTNF- $\alpha$  and HuIFN- $\alpha$ , respectively.

## Discussion

Studies on the synergistic enhancement of the cytostatic and cytotoxic effects of TNF- $\alpha$  or lymphotoxin (TNF- $\beta$ ) by the simultaneous use of IFNs have been reported (8-13). In most of these studies, TNF- $\alpha$  or lymphotoxin were investigated in combination with IFN- $\gamma$ . In this paper, some evidence of the synergistic cytostatic and cytotoxic activities between HuTNF- $\alpha$  and HuIFN- $\alpha$  preparations was presented. Furthermore, when HuTNF- $\alpha$  and HuIFN- $\alpha$  were administered in combination, the spectrum of their effects extended to HuTNF- $\alpha$ -resistant cell lines and HuIFN- $\alpha$ -resistant cell lines. Thus, higher efficacies are attainable on some cultured cells by using different lymphokines in combination than by using an increased dosage of a single

**Fig. 1** ED<sub>50</sub> values of HuTNF- $\alpha$  (●), HuIFN- $\alpha$  (▲) and a mixture of the two (■) on KB, Daudi and KG-1 cells. Cells were incubated with 50  $\mu$ l of 2-fold serially diluted preparations of HuTNF- $\alpha$  ( $1 \times 10^5$  u/ml), HuIFN- $\alpha$  ( $1 \times 10^5$  IU/ml) or the mixture (HuTNF- $\alpha$ ,  $1 \times 10^5$  u/ml + HuIFN- $\alpha$ ,  $1 \times 10^5$  IU/ml). After 3 days of incubation, the numbers of viable cells were determined by Mosmann's method (18) or the dye uptake method.



lymphokine.

In clinical treatment of cancer, it is very important to use drugs with anti-tumor activities of wide spectrum and high potency. Therefore, the combined use of lymphokines may provide a new approach to cancer treatment. Clinical use of the mixed preparation of HuTNF- $\alpha$  and HuIFN- $\alpha$  is under investigation.

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Received: November 5, 1986  
Accepted: May 26, 1987

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