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Synergy of antiviral actions of TNF and IFN-γ: evidence for a major role of TNF-induced IFN-β

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Summary

Tumor necrosis factor (TNF) potentiates the antiviral activity of interferon-gamma (IFN-γ) directly and through induction of IFN-β in HEp-2 cells. Antibody to IFN-β inhibits a large component of the TNF and IFN-γ synergy and immunoreactive IFN-β can be detected in the supernatant fluids of TNF-treated HEp-2 cells. Furthermore, the kinetics of development of this synergy and its susceptibility to inhibition by antibody to IFN-β is demonstrated.

TNF; IFN-γ; Potentiation

Introduction

Tumor necrosis factor (TNF) was initially found in the serum of BCG-primed mice after endotoxin administration and was shown to have tumor-specific cytocidal activity. It is now known that the major source of TNF in these animals and in tissue culture is the monocyte–macrophage (Carswell et al., 1975). TNF has been shown to cause preferential necrosis of some tumor cells, but not normal cells, both in vitro and in vivo (Old, 1985). It has also been shown that TNF mediates the activity of interferon-gamma (IFN-γ)-induced macrophage cytotoxicity against several tumor cell lines (Philip and Epstein, 1986). In these macrophages, TNF has also been shown to act in an autocrine fashion (i.e., it will induce itself and enhance cytotoxic activity). Additionally, TNF will lyse virus-infected cells in vitro (Wong and Goeddel, 1986) and will induce an antiviral state in some cells if they

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are treated with TNF prior to viral infection. Recent contradictory evidence has led various groups to conclude that (a) the antiviral activity is intrinsic to TNF (Wong and Goeddel, 1986), or (b) the antiviral activity is due to the induction of IFN-β_2 by TNF (Kohase et al., 1986), or (c) some combination of these two hypotheses is operating (Mestan et al., 1986).

The antiviral activity of IFN-α, β, and γ has been well documented (Baron et al., 1987). It has also been shown that some IFN combinations interact synergistically (Fleischmann et al., 1979). For example, a potentiation of antiviral activity occurs if one combines IFN-α and/or β with IFN-γ. This potentiation, however, does not occur between IFN-α and β. Recent evidence indicates that IFN-γ will also potentiate the antiviral activity of TNF (Old, 1985). In light of the above findings, it would be important to determine whether IFN-γ is directly synergizing with TNF, with IFN induced by TNF, or both. Here, we present evidence that the majority of the synergy appears to be between IFN-γ and IFN-β. The implications of these findings are further discussed.

Materials and Methods

Cells

Human HEp-2, WISH and mouse L-929 cells were obtained from the American type culture collection. Cells were routinely grown to confluency in 32 oz plastic culture flasks or microtiter plates (Costar, Cambridge, MA) in Eagle's minimal essential medium (EMEM) with Earle's salts supplemented with 10% fetal calf serum, penicillin (100 U), streptomycin (100 μg/ml), and gentamycin (100 U) (EMEM 10%). Confluent cells were maintained in the same media supplemented with only 2% fetal calf serum (EMEM 2%).

Virus

Vesicular stomatitis virus (Indiana strain, VSV) was routinely propagated in primary chick embryo fibroblasts in our laboratory and stored at -70°C.

Reagents

Recombinant human TNF-α (specific activity 10^7 U/mg protein, 0.13 ng/mg LPS) were obtained from Suntory, Tokyo, Japan. Recombinant human IFN-γ (specific activity 10^8 U/mg protein) was obtained from Biogen, Cambridge, MA. Rabbit antibody to IFN-β was obtained from Lee Biomolecular, San Diego, CA. Polyclonal antibodies to IFN-β_1 have been shown to crossreact with IFN-β_2 (Zilberstein et al., 1986).

Antiviral and synergy assays of TNF and IFN-γ

The antiviral activities of TNF and IFN-γ were determined as follows: HEp-2 cells, seeded at 5.0×10 cells/well in EMEM 10%, were grown for 24 h at 37°C in a 96-well culture plate. After cells had reached confluency (24 h), recombinant TNF was added. Following 24 h incubation, the supernatant fluids were decanted

and the cells infected with VSV at a multiplicity of infection (MOI) of 0.5 in 0.1 ml EMEM 2%. Virus was adsorbed for 1 h at 37°C, after which the monolayer was decanted and refed with 0.1 ml EMEM 2%. Twenty-four hours later, virus was harvested and the yields were quantified on WISH cells using a microplaque reduction assay (Campbell et al., 1975). IFN-γ was assayed in a similar manner.

To perform synergy assays, HEp-2 cells were grown as above. Twenty-four hours later, rTNF and rIFN-γ were added at the desired concentrations. Following 24 h incubation the cells were washed lightly one time with EMEM 2% and infected with VSV (MOI 0.5). Yields of virus were quantified as before.

Results

Determination of TNF's antiviral activity on HEp-2 cells and its synergy with IFN-γ

In order to determine the optimum conditions and concentration of TNF and IFN-γ needed for synergy to occur, a crossed titration of recombinant TNF and IFN-γ was performed. Results of a typical experiment are presented in Fig. 1. When TNF in dosages ranging from 3–3000 U/ml was titrated on HEp-2 cells in the absence of IFN-γ, a dose-dependent inhibition of virus replication was seen. The maximum inhibition obtained was approximately 30-fold. However, a combination of TNF and IFN-γ at the highest concentrations used (3000 and 100 U/ml, respectively) resulted in a greater than 10000-fold inhibition of virus yield. Thus, the expected additive level of antiviral activity between TNF and IFN-γ (which would be 43-fold) is potentiated greater than 230 times. At the next lower concentration of TNF and IFN-γ (1000 and 10 U/ml, respectively), a greater than 500-fold inhibition of virus was observed. This represents a potentiation of the antiviral activity of more than 60-fold beyond additivity (9-fold). For the remainder of these experiments, we chose to use 1000 U/ml of TNF in combination with 33 U/ml IFN-γ. This combination gives a marked synergistic effect while minimizing the independently detectable antiviral activity of either cytokine alone. In addition, effects due to residual LPS remaining in the TNF preparation would be highly unlikely since at the dilution used, 1.69×10^{-7} ng/ml would be present (see Materials and Methods).

A large component of the synergy between TNF and IFN-γ is due to IFN-β

Recent studies have indicated that TNF in aged fibroblasts mediates its antiviral activity through the induction of IFN-β_2 (Kohase et al., 1986). Other studies, however, have concluded that IFN-β_2 has little, or no involvement in the antiviral effect seen in this system (Mestan et al., 1986; Wong and Goeddel, 1986). In light of the synergy seen between IFN-γ and IFN-β (Fleischmann et al., 1979), we tested the possibility that IFN-γ was in fact synergizing with an IFN which was being induced by TNF. To perform these experiments, we treated HEp-2 cells with TNF and IFN-γ (1000 and 33 U/ml, respectively) in the presence of 250 U/ml of antibody to IFN-β. One unit of antibody neutralizes the antiviral activity of 3 U of

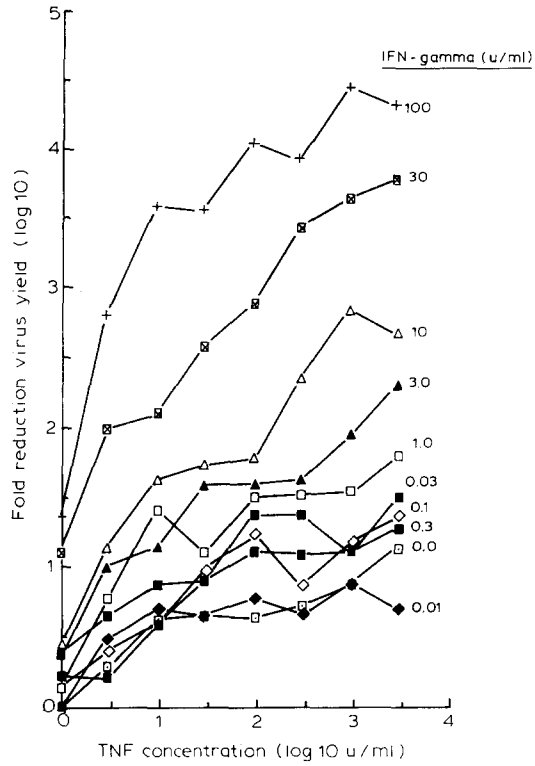

Fig. 1. Synergy between the antiviral activities of IFN-γ and TNF. HEp-2 cells (50000/well) were grown in microtiter plates (Costar, Cambridge, MA) at 37°C, 4% CO_2 in EMEM 10% to confluency. Supernatant fluids were decanted and replaced with recombinant TNF-α (Suntory, Tokyo, Japan) and/or recombinant IFN-γ (Biogen, Inc.) at the indicated concentrations in EMEM 2%. Twenty-four hours later, fluids were decanted and the cells were infected with VSV (MOI = 0.5). Virus was harvested 24 h later and yields were quantified by microplaque reduction assay. One U/ml of TNF is defined as the reciprocal of the dilution of TNF-α that is required to lyse 50% of approximately 10000 actinomycin D (5 μg/ml) treated mouse L-929 cells in a standard in vitro cytotoxic assay (Flick and Gifford, 1984).

IFN-β. Antibody to IFN-β has been shown to crossreact with IFN-β_2 (Zilberstein et al., 1986). As shown in Table 1, TNF alone resulted in 31.6-fold inhibition of virus yield while IFN-γ alone gave an 7.9-fold inhibition. The combination of TNF with IFN-γ would thus result in a 39.5-fold inhibition of virus yield if the two cytokines behave in an additive manner. When TNF and IFN-γ were combined, however, a 446683.6-fold reduction of virus yield was observed (11308.4-fold beyond additivity). When anti-IFN-β was included with the synergizing mixture, the synergy between IFN-γ and TNF was reduced to 4466.8-fold or 113.1-fold beyond additivity. Thus, approximately 99% of the synergistic antiviral action between TNF and IFN-γ in this system was negated by the addition of the anti-IFN-β preparation. In a separate experiment, antibody specific for IFN-β_1 (kindly provided by Dr. J. Van Damme) was used (Van Damme et al., 1987) which resulted in a 90%

TABLE 1

A large component of the synergy between TNF and IFN-γ is due to IFN-β in HEp-2 cells

Treatment[a]	Fold reduction virus yield		Fold potentiation antiviral activity	Decrease of potentiation (%)
	Observed	Expected		
TNF alone	7.9	–	–	–
IFN-γ alone	31.6	–	–	–
TNF + IFN-γ	446,683.6	39.5	11,308.4	–
TNF + IFN-γ + anti-IFN-β[b]	4,466.8	–	113.1	(98.9)
TNF + anti-IFN-β	13.6	–	–	–

[a] HEp-2 cells (5.0×10^4/well) were grown to confluency in 96-well culture plates in EMEM 10% at 37°C in 4% CO_2. Recombinant human TNF-α (1000 U/ml) and/or recombinant human IFN-γ (33 U/ml) were then added in 0.1 ml volumes to the wells in duplicate cultures in EMEM 2%. In addition, rabbit anti-human IFN-β (250 U/ml) was added as indicated above. Following 24 h of incubation, the supernatant fluids were decanted and the cells washed 3 × with EMEM 2%. Following wash, cells were infected with VSV in 0.1 ml volumes (MOI = 0.5). After 1 h adsorption, non-attached virus was decanted and the cells were refed with 0.1 ml EMEM 2%. Twenty-four hours later, virus from duplicate cultures was harvested and yield was determined by a microplaque assay as described in Campbell et al. (1975).

[b] Rabbit anti-human IFN-α antiserum was used as a control and had no effect.

reversal of the potentiation between IFN-γ and TNF. The most likely interpretation of these observations is that IFN-γ is synergizing with a component(s) neutralizable by both anti-IFN-β preparations, possibly even IFN-β_2. To our knowledge, such a synergy between IFN-γ and IFN-β_2 has not been observed.

Detection of immunoreactive IFN-β in the supernatant fluids of TNF-treated HEp-2 cells

We next attempted to directly demonstrate the presence of IFN-β activity in the supernatant fluids from TNF treated HEp-2 cells. To perform these experiments,

TABLE 2

Lack of detection of IFN activity in the supernatant fluids of TNF-treated HEp-2 cells.

Treatment[a]	Fold reduction of virus yield ($\log_{10}$)
Media	0.0
TNF (1300U/ml) × 8 h	0.4 (NS)[b]
TNF (1300U/ml) × 24 h	0.0
TNF (1300U/ml) + IFN-γ (33 U/ml) × 24 h	0.0

[a] HEp-2 cells were grown to confluency in 150 cm^2 plastic tissue culture flasks (Costar, Cambridge, MA) at 37°C, 4% Co_2. Upon confluency, the supernatant fluids were replaced with EMEM 2% or recombinant TNF at the indicated concentrations. Following 8 or 24 h incubation, the cells were washed 3 times with EMEM 2% and incubated another 24 h in medium alone. Supernatant fluids were then checked for antiviral activity in a standard IFN yield reduction assay on human WISH cells. Residual IFN-γ was acid inactivated at a pH of 2 for 24 h prior to assay of fluids.

[b] NS = not significant.

HEp-2 cells were treated with TNF for 8 or 24 h, washed extensively and refed. The supernatant fluids were harvested 24 h later and tested for antiviral activity. As shown in Table 2, these fluids did not yield detectable antiviral activity when measured in a standard IFN assay on human WISH cells. However, if one tests these fluids for immunoreactive IFN-β by Western analysis, a positive reaction is shown (see Fig. 2). Thus, it appears that immunoreactive IFN-β is present in these

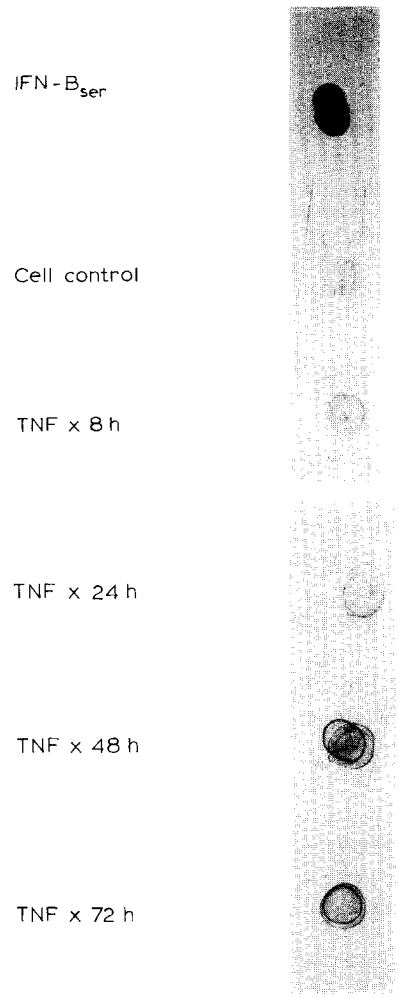

Fig. 2. Dection of IFN-β in TNF treated HEp-2 cell supernatant fluids by Western blot. HEp-2 cells (2×10^7 cells/150 cm^2 flask) were treated with 8 ml TNF (1300 U/ml) for the indicated periods of time. Following treatment, non-diluted supernatant fluid (7.5 μl × 2) was blotted on nitrocellulose paper and subjected to Western analysis using rabbit anti-human IFN-β (Lee Biomolecular, San Diego, CA) as a primary probe. Biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories, Burlingame, CA) was used as the secondary antibody. Avidin-horseradish peroxidase and 4-chloro-1-naphthol for a substrate were used as the indicator system. IFN-β_{ser} (1000 U/ml, Triton, San Francisco, CA) was used as a positive control.

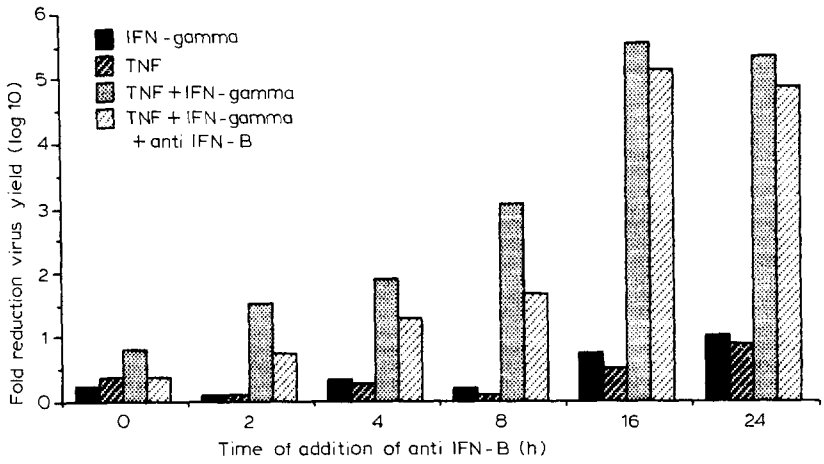

Fig. 3. Kinetics of induction of synergy between IFN-γ and TNF and its sensitivity to anti-IFN-β. HEp-2 cells were grown in microtiter plates as before. Twenty-four hours later, TNF-α (1000 U/ml) and/or IFN-γ (33 U/ml) were added. At the indicated times, the cells were washed 3 × with EMEM 2% and refed with or without anti-IFN-β (250 U/ml) was added. Following 24 hours incubation, cells were infected with VSV and virus yields were quantified as before.

supernates but that its direct antiviral activity is negligible. This result would be expected if the immunoreactive IFN-β being produced in these cultures is in fact IFN-β_2, since IFN-β_2 is known to be a much less potent antiviral molecule than IFN-β_1. Finally, we found that anti-IFN-β antibody had no significant effect on the direct antiviral activity of TNF (Table 1).

Kinetics of the induction of TNF and IFN-γ antiviral synergy and its abrogation by anti-IFN-β

We next attempted to determine the kinetics of induction of TNF and IFN-γ antiviral synergy and its sensitivity to anti-IFN-β. To perform these experiments, TNF (1000 U/ml) and IFN-γ (33 U/ml) were placed on HEp-2 cells for periods of 0, 1, 2, 4, 8, 16 and 24 h. Following these time periods, the cells were washed extensively and refed with EMEM 2% alone or containing 250 U/ml of anti-IFN-β. As shown in Fig. 2, maximal synergy developed within 16 h of incubation of TNF and IFN-γ. This synergy could be significantly reversed up to 8 h after initiation of culture by the addition of anti-IFN-β. Thus, it appears that IFN-γ and TNF require 8–16 h to maximally synergize and that this synergy can be significantly abrogated by anti-IFN-β up to 8 h after initiation.

Discussion

These experiments were designed to investigate the possibility that a component of the antiviral synergy that occurs between TNF and IFN-γ is due to the induc-

tion of IFN-β by TNF. Taken together, the present findings demonstrate that, (a) the antiviral activity of recombinant human TNF can be demonstrated on HEp-2 cells, (b) synergy between recombinant human TNF and recombinant human IFN-γ occurs in these cells, (c) a component of this synergy is due to IFN-γ synergizing with TNF-induced immunoreactive IFN-β even though the IFN-β is at biologically subdetectable levels in terms of antiviral activity, (d) optimum synergy occurs between 8 and 16 h after exposure of cells to TNF and IFN-γ, and (e) inhibition of the TNF-induced IFN-β component by anti-IFN-β can occur as late as 8 h after initiation.

Thus, it appears that synergy of TNF and IFN-γ antiviral activities is a multicomponent process occurring on one hand between TNF and IFN-γ and on the other between TNF-induced IFN-β and IFN-γ. Although we have only been able to detect the IFN-β (presumably β_2) indirectly through the use of antibody, there is precedence for its induction by TNF in HEp-2 cells (Mestan et al., 1986). The finding that significant synergy can be demonstrated in the absence of detectable immunoreactive IFN-β at 16 h appears paradoxical (Figs. 2, 3). However, it has been reported in the past that subdetectable levels of IFN can have significant antiviral activity (Dianzani and Baron, 1975; Buckler et al., 1968) and thus may also be able to synergize with a second IFN. This may be a demonstration of IFN-β_2 synergizing with IFN-γ, but this remains to be determined. In contrast to other reports (Wong and Goeddel, 1986), therefore, a large component of the synergy between TNF and IFN-γ antiviral activities develops as a consequence of the induction of a second IFN. Some synergy also appears to occur directly between IFN-γ and TNF since anti-IFN-β failed to completely ablate the synergistic response (Table 1). However, the possible contribution of a non-neutralizable IFN-β component (i.e., in antibody-inaccessible spaces such as intracellular or in tight intercellular junctions) cannot be ruled out from the present study. If a non-neutralizable IFN-β component is present, it would imply that the relative contribution of the direct TNF/IFN-γ interaction is negligible.

We have detected IFN-β in this system using two techniques: through the use of antibody in cell culture and Western blot analysis. These data are consistent with the reports of the induction of IFN-β_2 by TNF in HEp-2 cells (Mestan et al., 1986). Whether the IFN which we detect is IFN-β_1 or β_2 is unknown. Recent studies have indicated that TNF induces the production of IFN-β_1, in addition to IFN-β_2, although at a much lower level (Van Damme et al., 1987). Since IFN-β_2 has a theoretical specific antiviral activity of $\leq 10^4$ U/mg protein (Van Damme et al., 1987), it would take about 10000-fold less IFN-β_1 (specific activity = approximately 10^8 U/mg protein) to produce an equivalent amount of antiviral activity. It is not known if the synergistic potential of IFNs is proportional to their relative antiviral activities. Hence, we cannot definitively determine if only one of the TNF-induced IFN-β species is interacting with IFN-γ. Attempts are currently underway to directly detect synergy between IFN-γ and IFN-β_2.

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