*Antiviral Research*, 22 (1993) 15–29

0166-3542/93/$06.00

AVR 00646

Antiviral Research

# Synergistic anti-HSV effect of tumor necrosis factor alpha and interferon gamma in human corneal fibroblasts is associated with interferon beta induction

Shun-Hua Chen, John E. Oakes and Robert N. Lausch

*From the Department of Microbiology and Immunology, College of Medicine, University of South Alabama, Mobile, AL 36688, USA*

(Received 10 March 1993; accepted 4 May 1993)

## Summary

HSV-1 (17) replicated to high titer in human corneal fibroblasts ($>10^8$ PFU/$10^5$ cells) following infection at one PFU per 100 cells. Pretreatment of the cells for 24 h with 50 U/ml recombinant human tumor necrosis factor alpha (TNF-$\alpha$) or 5 IU/ml of human interferon gamma (IFN-$\gamma$) resulted in only modest reduction (2- to 19-fold) in virus yield. However, when the two cytokines were combined the antiviral effect was dramatically increased. There was >1000-fold reduction in virus titer in 8 of 8 trials. In contrast, the combinations of 50 U/ml TNF-$\alpha$ with 5 IU/ml IFN-$\alpha$ or IFN-$\beta$ did not produce a synergistic effect. The pronounced synergistic antiviral activity of TNF-$\alpha$+IFN-$\gamma$ could be demonstrated in fibroblast cultures from different donors, and HSV-2 as well as HSV-1 strains were inhibited. There was no evidence that dual cytokine treatment was toxic for uninfected or HSV-infected cells. Insight into the mechanism responsible for the synergistic effect was provided by the observation that TNF-$\alpha$+IFN-$\gamma$ induced IFN-$\beta$. In addition, anti-IFN-$\beta$ but not anti-IFN-$\alpha$ antibodies could reverse the antiviral effect, and reconstitution with IFN-$\beta$ could duplicate the phenomenon. We conclude that the combination of TNF-$\alpha$ and IFN-$\gamma$ at low concentrations can exert a powerful anti-herpes effect in human corneal fibroblasts which can be chiefly attributed to the induction of IFN-$\beta$.

*Correspondence to:* R.N. Lausch, Ph.D. Department of Microbiology and Immunology, College of Medicine, University of South Alabama, Mobile, AL 36688, USA.

HSV-1; Human corneal fibroblasts; Tumor necrosis factor alpha; Interferon gamma; Interferon alpha

## Introduction

Herpes simplex virus type 1 (HSV-1) infections can result in the synthesis and release of cytokines which can have antiviral, proinflammatory, and immunoregulatory properties. In the inbred mouse $\alpha$, $\beta$, and $\gamma$ interferons (IFNs) have been associated with herpetic eye infection (Su et al., 1990; Hendricks, 1991). The importance of endogenously produced IFN is evidenced by the fact that passive transfer of anti-IFN-$\alpha/\beta$ antibody enabled avirulent HSV strains to replicate in the cornea for a prolonged period and induce stromal keratitis (Su et al., 1990; Hendricks et al., 1991). Anti-IFN-$\gamma$ treatment enhanced mortality in mice infected intraperitoneally with HSV-1 (Stanton et al., 1987). Furthermore, IFN treatment could protect mice, rabbits, or monkeys from lethal intraperitoneal or ocular challenge with virulent HSV strains (Sanitato et al., 1984; Pinto et al., 1990). These observations established that the IFNs can play a significant role in limiting HSV infection in the experimental host.

Tumor necrosis factor alpha (TNF-$\alpha$) is a proinflammatory cytokine produced by a variety of cell types (Beutler and Cerami, 1989). It can mediate a diverse array of biological activities including antiviral effects (Larrick and Wright, 1992). Indeed, passive transfer of this cytokine has been found to protect mice from intraperitoneal HSV-1 infection (Rossol-Voth et al., 1991). This cytokine has been reported to induce the synthesis of IFNs and may interact synergistically with these proteins to inhibit virus replication in selected transformed cell lines (Jacobsen et al., 1989; Wong and Goeddel, 1986; Mestan et al., 1986; Ito and O'Malley, 1987).

HSV-1 is an important ocular pathogen in humans (Kaufman, 1981). It is not known precisely how this virus is cleared from infected corneal tissue. HSV-1 infection can be expected to induce various cytokines some of which have the potential to affect virus replication in the cornea (Hooks et al., 1988). The objective of the present study was to determine how effectively the proinflammatory cytokine TNF-$\alpha$ by itself, and in combination with different types of IFN, could inhibit HSV-1 replication in fibroblasts derived from human corneas. We observed a remarkable synergistic antiviral effect following pretreatment of human corneal fibroblasts (HCF) with low dose TNF-$\alpha$ and IFN-$\gamma$. Experiments to identify the mechanism responsible for the profound inhibition in virus replication were carried out.

## Materials and Methods

*Virus.* HSV-1 strain 17 and HSV-2 strain 333 were grown in Vero cells after being plaque purified. Infectious titers of virus preparations were determined by standard plaque assay on Vero cells. Vesicular stomatitis virus was grown and titered on L-929 fibroblasts.

*Cells.* L-929 cells (a gift from Dr. Jenifer Turco, University of South Alabama), Vero cells (Flow Laboratories), and A549 cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% calf serum, 0.15% sodium bicarbonate, 10 mM Hepes buffer solution, and antibiotics.

*Reagents.* Recombinant human TNF-$\alpha$ ($2 \times 10^7$ units/mg; $2 \times 10^5$ units/ml) and TNF-$\beta$ ($3 \times 10^7$ U/mg; $1.5 \times 10^5$ U/ml) were purchased from Genzyme (Cambridge, MA). Recombinant human IFN-$\alpha$ and IFN-$\beta$ were obtained from Lee BioMolecular (San Diego, CA), and recombinant human IFN-$\gamma$ was purchased from Alpha Therapeutic Corporation (Los Angeles, CA). Interferon activity was calibrated using the World Health Organization international reference preparations of human interferons provided by NIH. Anti-human TNF-$\alpha$ monoclonal antibody was purchased from Endogen (Boston, MA). Sheep antiserum to human IFN-$\alpha$ and rabbit antiserum to IFN-$\gamma$ were obtained from the NIH. Anti-human IFN-$\beta$ monoclonal antibody was purchased from Boehringer-Mannheim (Indianapolis, IN).

*Preparation of human corneal fibroblast (HCF) culture.* Human corneas were obtained from the National Disease Research Interchange (Philadelphia, PA), or the Alabama Eye and Tissue Bank (Mobile, AL), and placed in RPMI-1640 medium (Irving Scientific, Santa Anna, CA) with 1% Fung-Bact Solution (Irving Scientific, Santa Anna, CA). After trimming off the cornealscleral rims and peeling off the endothelial cell layer, the corneas were washed in RPMI-1640 medium. To dissociate the epithelial cell layer from the stroma, the concave side of the cornea was placed on a drop of Dispase, grade II (Boehringer-Mannheim, Indianapolis, IN) containing 25 caseinolytic units/ml Dispase and 20 $\mu$g/ml gentamicin in Hanks Balanced Salt Solution. Following incubation with Dispase at 4°C for 24 h in a humidified environment, the epithelial cell layer was lifted from the stroma with forceps. The stroma was cut into small pieces and digested with 300 U/ml of type I collagenase (Sigma, St. Louis, MO) at 37°C for 1 h. DMEM supplemented with 10% fetal bovine serum, 0.15% sodium bicarbonate, 10 mM Hepes buffer solution and antibiotics was used to wash and seed the stromal pieces in one 75 $cm^2$ flask in a 2 ml vol. The tissue pieces were allowed to adhere to the bottom of the flask in 5% $CO_2$ at 37°C. 2 days later, additional medium was added and incubation was continued. After the cells became confluent, the cultures were trypsinized and passed to 225 $cm^2$ flasks. The cultures consisted entirely of

fibroblasts as assessed by morphology and immunofluorescent studies (Cubitt et al., 1993).

*Assay for the effect of cytokine pretreatment on HSV replication in HCF.* To assess the growth of HSV in HCF after cytokine treatment, confluent monolayers in 12-well plates ($1.3 \times 10^5$ cells/per well) were exposed to the desired cytokines. 24 h later, the supernatants were removed and then the cells were infected with HSV-1 at a multiplicity of infection of 0.01. After virus adsorption for 1 h at room temperature, the infected cells were washed 3 times with medium, incubated in fresh medium for 24 h, and then the infected monolayers were collected by scraping with a rubber policeman and frozen at $-70$°C. The samples were subsequently thawed and briefly sonicated for 10 to 20 s using a Sonic 300 dismembrator (Arteck System Incorporated, Farmingdale, NY). The cell lysates were then titered for infectious virus on Vero monolayers in a 48 h plaque assay. The fold reduction of virus growth was calculated using the formula: fold reduction = (virus titer of untreated sample/virus titer of cytokine-treated sample). To evaluate the combined effects of two cytokines in cell culture, the criteria for drug combinations described by Weiss (1989) were used. The relative decrease of the virus control titer ($Y_A$) caused by cytokine A is defined as the reduction in virus titer induced in cultures in the presence of cytokine A, relative to the virus titer in the untreated sample ($Y_A$ = virus titer of untreated sample – virus titer of cytokine A treated sample). Similarly, $Y_{AB}$ is the observed decrease of the virus control titer in cultures treated with both cytokines, A and B. The calculated decrease of the virus control titer ($Y_C$) for an additive effect is the product of the observed decrease of the virus control titer induced by each cytokine alone ($Y_C = Y_A \times Y_B$, and $\log_{10} Y_C = \log_{10} Y_A + \log_{10} Y_B$). A synergistic effect is defined by $Y_{AB} > Y_C$.

*Trypan blue exclusion test of cell viability.* To determine the number of viable cells, the cell monolayers were trypsinized and then centrifuged 10 min at $100 \times g$. After the centrifugation, the supernatant was discarded and the pellet was resuspended in 1 ml medium. A 0.5 ml vol of the cell suspension was mixed with 0.5 ml of 0.4% trypan blue, and the unstained (viable) cells were counted.

*IFN bioassay.* To measure the IFN concentration in cell lysates, a modification of the method of Havell and Vilcek (1972) was used. Briefly, the assay involved protection of A549 cells against infection with vesicular stomatitis virus as assessed by inhibition of the cytopathic effect. The test samples were diluted in a series of 2-fold dilutions in 96-well plates. Each well was then seeded with $4 \times 10^4$ A549 cells. After 24 h of incubation, the monolayers were infected with $10^3$ PFU of vesicular stomatitis virus. The cultures were then incubated until the virus control showed complete destruction of the A549 monolayers (usually 48 h). The cultures were then stained with 2% crystal violet, and the titrations were scored microscopically.

The highest dilution of the titrated sample providing at least 50% protection of the cells was taken as the endpoint. An internal human IFN-$\beta$ standard from the NIH was included with each titration and served as the reference for calculating IFN-$\beta$ units in each test sample. IFN-$\beta$ activity was confirmed by neutralization with anti-human IFN-$\beta$ antibody.

## Results

*Antiviral effect of TNF-$\alpha$ and interferons. Evidence for synergism.* TNF-$\alpha$ and the three types of interferon, $\alpha$, $\beta$, and $\gamma$, were tested for their capacity to inhibit HSV-1 replication in cultured HCF. When tested individually at a physiological concentration, IFN-$\beta$ proved to be the most effective inhibitor. In the experiment depicted in Fig. 1, IFN-$\beta$ at 5 IU/ml reduced virus replication by some 380-fold whereas the same concentration of IFN-$\alpha$ and IFN-$\gamma$ inhibited HSV-1 growth by 6- and 11-fold, respectively. A 19-fold reduction in virus titer was observed when the cells were pretreated with 50 U/ml TNF-$\alpha$ and higher concentrations did not noticeably increase its antiviral effect.

Strikingly more pronounced inhibition was seen when the combination of

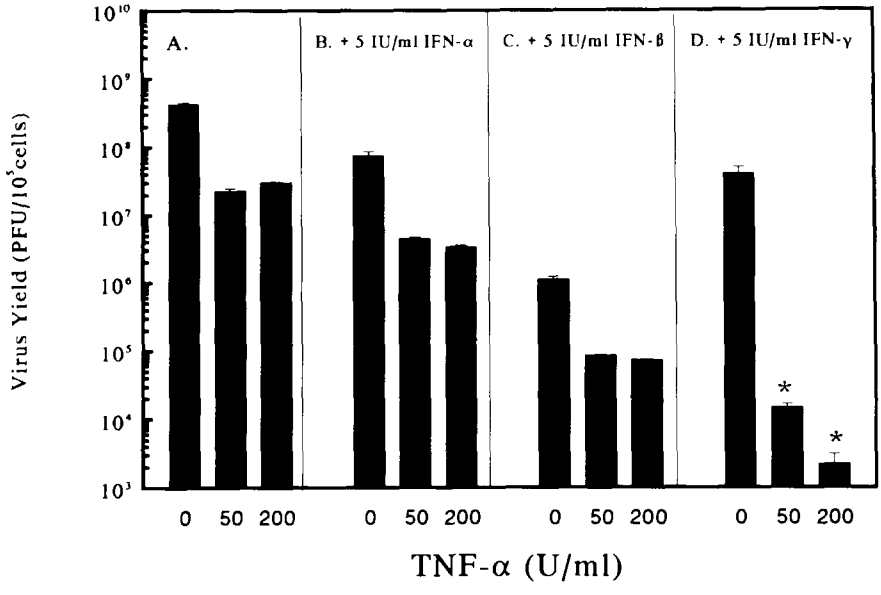


Fig. 1. Antiviral effect of TNF-$\alpha$ and IFNs on HSV-1 replication in HCF. Fibroblasts were exposed to (A) TNF-$\alpha$ or left untreated, (B) 5 IU/ml IFN-$\alpha$ or the combination of IFN-$\alpha$ + TNF-$\alpha$, (C) 5 IU/ml IFN-$\beta$ or the combination of IFN-$\beta$ + TNF-$\alpha$, (D) 5 IU/ml IFN-$\gamma$ or the combination of IFN-$\gamma$ + TNF-$\alpha$. 24 h later, the cells were infected with HSV-1. Following an additional 24 h incubation period, the samples were harvested and virus titers were determined. Each bar represents the mean infectious titer of samples titrated in triplicate. Error bars indicate standard errors of the mean. *The virus titers of the combined treatment of IFN-$\gamma$ and TNF-$\alpha$ were significantly lower than the virus titers of the combined treatment of IFN-$\alpha$ + TNF-$\alpha$ or IFN-$\beta$ + TNF-$\alpha$ ($P < 0.01$).

IFN-$\gamma$ (5 IU/ml) and TNF-$\alpha$ (50 U/ml) was tested. Now the virus yield was reduced by >28 000-fold (Fig. 1, Panel D) or far greater than the expected additive effect which would be 209-fold. This combination of cytokines was consistently synergistic in repeated experiments using HCF from different donors, and was also synergistic when HSV-2 (strain 333) served as the challenge virus (data not shown). The combinations of TNF-$\alpha$ + IFN-$\alpha$ and TNF-$\alpha$ + IFN-$\beta$ also led to a greater reduction in virus titer than when the individual cytokines were tested alone but the decrease was not as pronounced. Panel B in Fig. 1 shows that the combination of TNF-$\alpha$ + IFN-$\alpha$ was additive rather than synergistic. The combination of TNF-$\alpha$ + IFN-$\beta$ was less than additive (Fig. 1, Panel C). The fold reduction produced by single or dual cytokine treatment was found to vary from experiment to experiment. However, IFN-$\beta$ was always the most inhibitory individual cytokine, and TNF-$\alpha$ + IFN-$\gamma$ was invariably the most potent combination.

Fig. 2 shows that HSV-1 replication was not significantly impaired when TNF-$\alpha$ + IFN-$\gamma$ treatment was delayed until after infection. Thus, to obtain a synergistic inhibitory effect it was necessary to pretreat the target cells with the two cytokines.

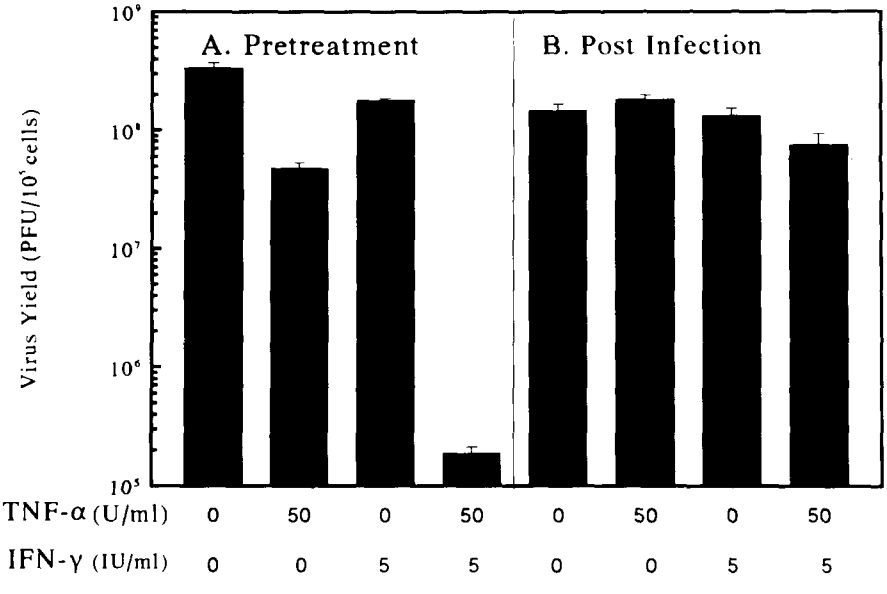


Fig. 2. Effect of time of cytokine addition on HSV-1 replication in HCF. (A) Fibroblasts were pretreated with 50 U/ml of TNF-$\alpha$, 5 IU/ml of IFN-$\gamma$, both, or left untreated for 24 h prior to HSV-1 infection. After virus adsorption for one h at room temperature, the infected cells were washed 3 times. Following an additional 24 h incubation period in fresh medium, the samples were harvested and virus titers were determined. (B) Fibroblasts were infected with HSV-1 without cytokine pretreatment. After adsorption for 1 h at room temperature, the infected cells were washed 3 times with medium and then incubated in medium alone or in medium containing 50 U/ml of TNF-$\alpha$, 5 IU/ml of IFN-$\gamma$, or both. 24 h later, the samples were harvested and the virus titers were determined. Each bar represents the mean infectious titer of samples titrated in triplicate. Error bars indicate standard errors of the mean.

TABLE 1

Effect of TNF-α and IFN-γ pretreatment on the viability of HCF*

| | Number of viable cells per sample | |
|---|---|---|
| | Uninfected | Infected |
| Untreated | $5.9 \times 10^5$ | $4.1 \times 10^5$ |
| TNF-α | $5.6 \times 10^5$ | $5.0 \times 10^5$ |
| IFN-γ | $4.8 \times 10^5$ | $5.0 \times 10^5$ |
| TNF-α + IFN-γ | $4.9 \times 10^5$ | $4.7 \times 10^5$ |

*Fibroblasts ($1.3 \times 10^5$ cells) were pretreated with 800 U/ml of TNF-α, 10 IU/ml of IFN-γ, both, or left untreated for 24 h. The cytokine treated cells and untreated cells were mock infected or infected with HSV-1. After a 24 h incubation period, the cells in each sample were trypsinized and counted. Cell viability was determined by trypan blue exclusion.

*Viability of HCF after cytokine treatment.* There was a possibility that cytokine pretreatment was toxic to the HCF (Koff and Fann, 1986; Dijkmans et al., 1989), and this was why the virus yield was sharply reduced. To address this issue, the cells were pretreated with TNF-α + IFN-γ, and then infected or mocked-infected with HSV-1. Cell viability was determined 24 h later via trypan blue exclusion. Table 1 shows that the number of viable cells in HSV-infected and uninfected samples after cytokine pretreatment was comparable to that of the untreated samples. Furthermore, we found that uninfected cells after cytokine treatment continued to grow as well as untreated cells (data not shown). Similar results were obtained in a second independent experiment using HCF cultures established from a different donor. These results indicated that dual cytokine pretreatment even at concentrations 2–16-fold higher than that producing synergism was not toxic for uninfected or virus-infected HCF.

*Antiviral effect of TNF-β and interferon.* TNF-β and TNF-α share many properties. Both are produced by lymphocytes, bind to the same receptors, and mediate similar biological activities including antiviral effects (Trinchieri, 1992; Wong and Goeddel, 1986; Aderka et al., 1985). Thus, it was of interest to test whether the combination of TNF-β and IFN-γ could also exert a synergistic antiviral effect. The results of two experiments are shown in Fig. 3. It was found that the combination of TNF-β + IFN-γ did inhibit HSV-1 in a synergistic manner. However, the reduction in virus titer was 75-fold less in experiment 1 and 16-fold less in experiment 2 than that produced by TNF-α + IFN-γ. Thus, TNF-β was not as effective as TNF-α in combining with IFN-γ to produce synergism. Additional studies showed that the combinations of TNF-β + IFN-α and TNF-β + IFN-β were also less inhibitory than TNF-α + IFN-α and TNF-α + IFN-β (data not shown).

*TNF-α + IFN-γ treatment induces IFN-β.* Literature reports have indicated that TNF-α can induce the synthesis of selected cytokines including IFN-β

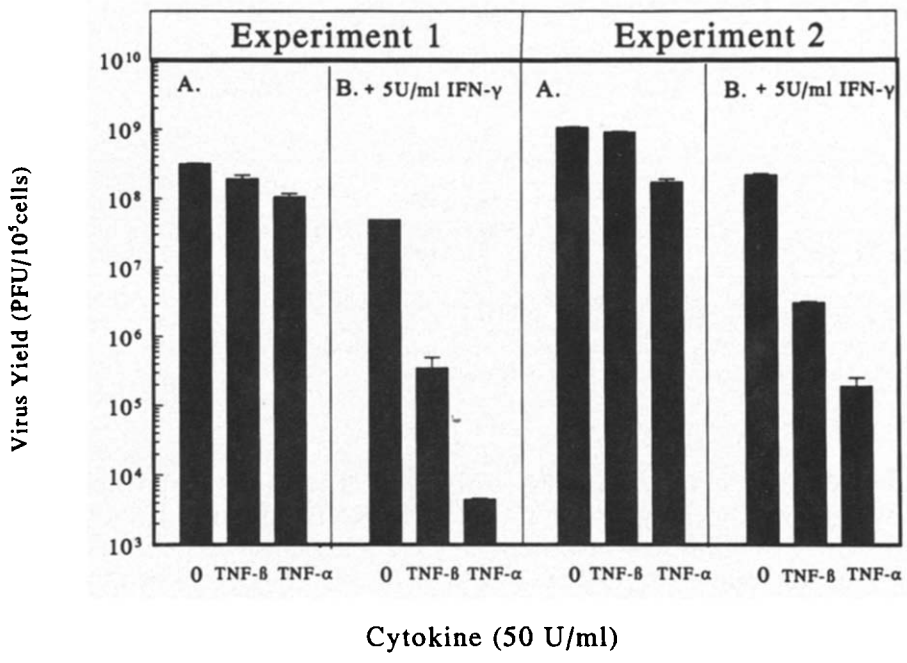


Fig. 3. Comparison of TNF-$\beta$ + IFN-$\gamma$ vs. TNF-$\alpha$ + IFN-$\gamma$ treatment on HSV-1 replication. Fibroblasts were exposed to TNF-$\beta$, TNF-$\alpha$, IFN-$\gamma$, combinations thereof, or left untreated. 24 h later, the cells were infected with HSV-1. Following an additional 24 h incubation period, the samples were harvested and virus titers were determined. Each bar represents the mean infectious titer of samples titrated in triplicate. Error bars indicate standard errors of the mean.

(Jacobsen et al., 1989; Ito and O'Malley, 1987). As noted above, in tests with single cytokines IFN-$\beta$ produced the greatest anti-HSV effect. Thus, its putative induction in HCF by dual cytokine treatment might account for the synergism observed. To investigate this possibility TNF-$\alpha$ and IFN-$\gamma$ individually, and combined, were tested for their capacity to induce IFN-$\beta$ synthesis in HCF. Fig. 4 shows that 40 IU/ml IFN was detected in cell lysates 24 h after dual cytokine treatment. Only 5 IU/ml of IFN was found in the TNF-$\alpha$ treated sample, and $<2.5$ IU/ml of IFN was detected in the control and IFN-$\gamma$ treated samples. Similar results were obtained in two additional independent experiments using HCF cultures from different donors. The antiviral activity in the IFN assay was specifically neutralized by monoclonal antibody to human IFN-$\beta$ but not by antibodies to human IFN-$\alpha$, IFN-$\gamma$, or TNF-$\alpha$. Collectively, these results indicated that TNF-$\alpha$ and IFN-$\gamma$ together were at least 8-fold more efficient at inducing IFN-$\beta$ than either cytokine alone.

*Neutralizing antibodies to human IFN-$\beta$ abrogated TNF-$\alpha$ + IFN-$\gamma$ synergism.* If the induction of IFN-$\beta$ synthesis was an important factor in the synergism exhibited by TNF-$\alpha$ + IFN-$\gamma$, then antibody to IFN-$\beta$ might be expected to

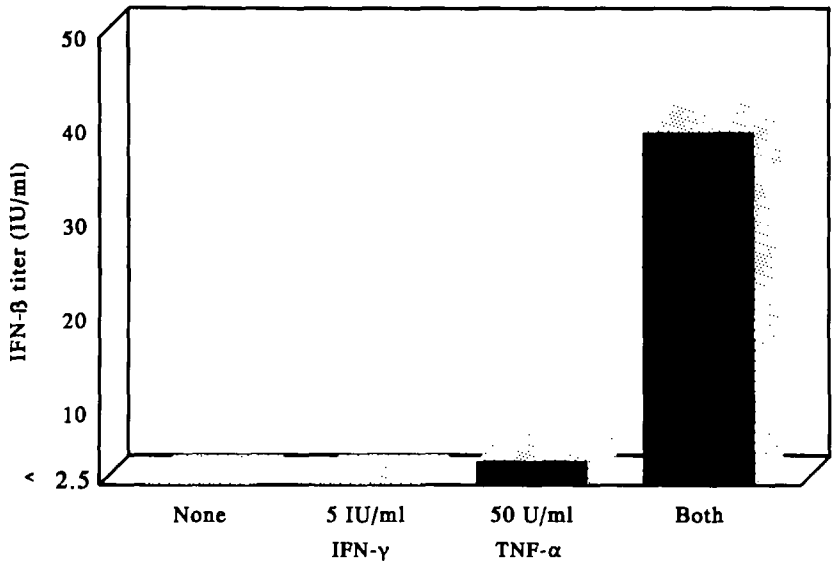


Fig. 4. IFN-$\beta$ production following cytokine treatment. Fibroblasts were exposed to 5 IU/ml of IFN-$\gamma$, 50 U/ml of TNF-$\gamma$, both, or left untreated. 24 h later, the samples were harvested and assayed for interferon content. The IFN-$\beta$ activity was calibrated using a World Health Organization international reference preparation of Human IFN-$\beta$ from NIH, and confirmed by neutralization with anti-human IFN-$\beta$ antibody.

markedly reduce the antiviral effect. To investigate this hypothesis HCF cultures were exposed to dual cytokine treatment in the presence of monoclonal antibody to human IFN-$\beta$. Controls received antiserum to human IFN-$\alpha$ or saline. Fig. 5 shows that the synergistic antiviral effect of TNF-$\alpha$ + IFN-$\gamma$ was significantly reduced by anti-IFN-$\beta$ antibody ($P < 0.005$) but not by antibody to IFN-$\alpha$. These results, which were confirmed in a second experiment, indicated that the IFN-$\beta$ induced by TNF-$\alpha$ + IFN-$\gamma$ played an important role in the mechanism of synergism.

As shown in Fig. 4, HCF treatment with 50 U/ml TNF-$\alpha$ led to the induction of a modest amount of IFN. Anti-IFN-$\beta$ but not anti-IFN-$\alpha$ antibodies abrogated the modest antiviral effect of TNF-$\alpha$ (Fig. 5, panel C). Taken collectively, these two observations suggest the anti-HSV-1 activity of TNF-$\alpha$ was due to IFN-$\beta$ induction rather than to a direct antiviral effect.

*Relative susceptibility of HSV-1 to IFN-$\beta$ vs. TNF-$\alpha$ + IFN-$\gamma$ treatment.* We next investigated whether the amount of IFN-$\beta$ induced in HCF by TNF-$\alpha$ + IFN-$\gamma$ had an antiviral effect comparable to that produced by dual cytokine treatment. The results of two such experiments using cell cultures from two different donors are shown in Fig. 6. It was found that the addition of IFN-$\beta$ at 20–40 IU/ml exerted an inhibitory effect virtually identical to that seen with

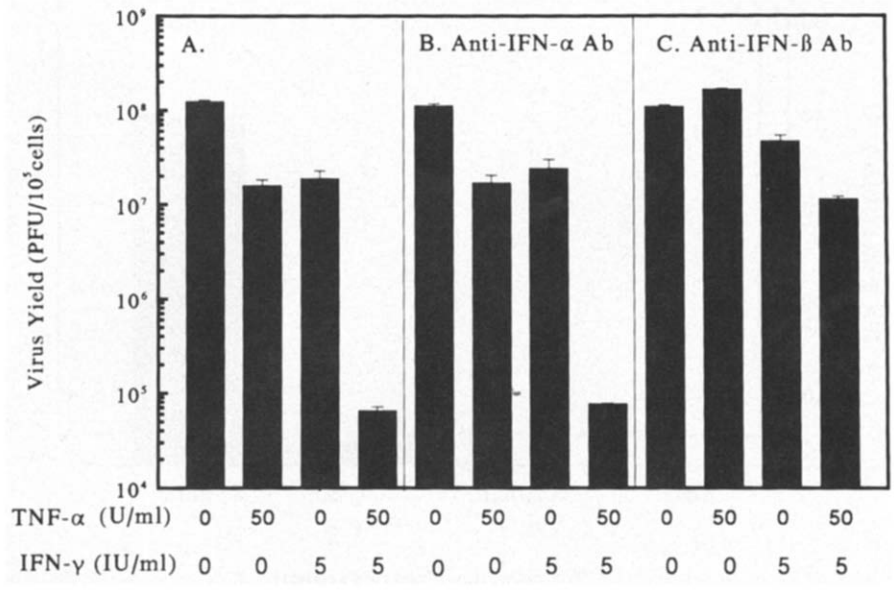


Fig. 5. Effect of anti-IFN-α or anti-IFN-β treatment on the synergistic antiviral effect of TNF-α + IFN-γ. Fibroblasts were exposed to (A) saline, (B) 250 neutralizing units (NU)/ml sheep antiserum to human IFN-α, or (C) 250 NU/ml monoclonal antibody to human IFN-β 10 min prior to cytokine treatment. 24 h after antibody and cytokine treatment, the cells were washed and infected with HSV-1. Following virus infection, the cells were incubated in fresh medium supplemented with 250 NU/ml of the desired antibody or saline. After 24 h of incubation, the samples were harvested and virus titers were determined. Each bar represents the mean infectious titer of samples titrated in triplicate. Error bars indicate standard errors of the mean.

TNF-α + IFN-γ. These data imply that the synergistic antiviral effect produced by TNF-α + IFN-γ can be largely explained by their induction of IFN-β synthesis.

## Discussion

The present study was designed to investigate how effectively TNF-α alone, or in combination with α, β, or γ IFN could inhibit HSV-1 replication in fibroblasts derived from human corneas. The major observation made was that the combination of TNF-α and IFN-γ resulted in a synergistic antiviral effect that was extremely potent at suppressing virus growth. Importantly, this synergism was demonstrable with low concentrations of each cytokine. Dual treatment with 50 units/ml TNF-α and 5 units/ml IFN-γ proved to be 1000- to 2000-fold more suppressive than either cytokine alone. Moreover, this inhibition was achieved against a relatively IFN resistant strain of HSV-1 (Lausch et al., 1991).

TNF-α has been reported to kill HSV-infected cells but not uninfected cells

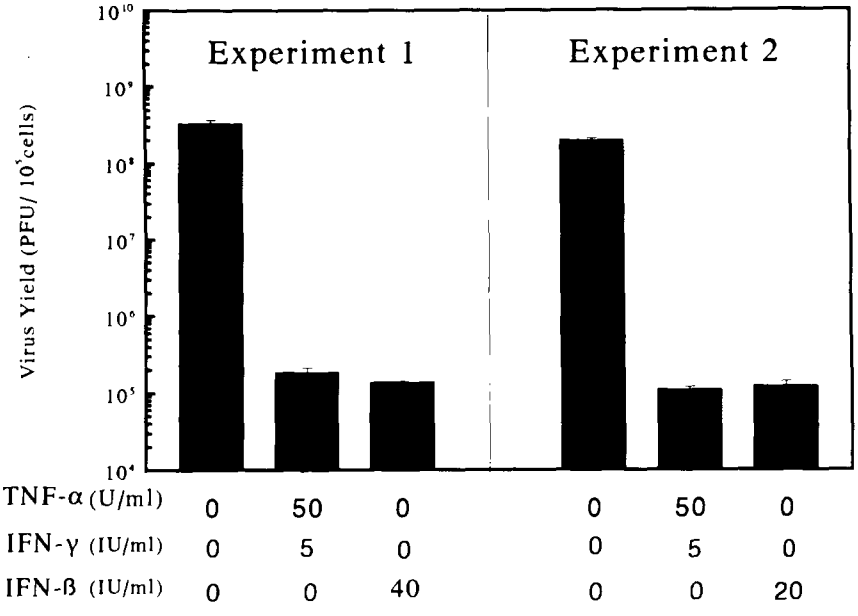


Fig. 6. Relative susceptibility of HSV-1 to IFN-$\beta$ versus TNF-$\alpha$ + IFN-$\gamma$ treatment. Fibroblasts were exposed to 50 U/ml of TNF-$\alpha$ + 5 IU/ml of IFN-$\gamma$, 20–40 IU/ml of IFN-$\beta$, or left untreated. 24 h later, the cells were infected with HSV-1. Following an additional 24 h incubation period, the samples were harvested and virus titers were determined. Each bar represents the mean infectious titer of samples titrated in triplicate. Error bars indicate standard errors of the mean.

(Koff and Fann, 1986) and IFN-$\gamma$ also may exhibit cytotoxic activity (Dijkmans et al., 1989). In addition, TNF-$\alpha$ can synergize with IFNs to lyse tumor cells (Williamson et al., 1983; Stone-Wolff et al., 1984). Thus, it was important to establish that the reduction in virus yield did not reflect cytokine toxicity for HCF. We could find no evidence that the two cytokines, individually or collectively, were harmful to uninfected or HSV-infected HCF at the concentrations we used. To the contrary, the cells remained viable after cytokine exposure as assessed by trypan blue exclusion, and continued to proliferate. Furthermore, if dual cytokine treatment was toxic to the cells, such cytotoxicity would not be expected to be reversed by antibody to IFN-$\beta$.

A number of reports have suggested that at least certain transformed cell lines exposed to TNF-$\alpha$ alone, or in combination with IFN-$\gamma$, will lead to IFN-$\beta$ synthesis (Jacobsen et al., 1989; Ito and O'Malley, 1987). Therefore, we investigated whether induction of this latter cytokine might account for the synergistic effect. Five lines of evidence were generated in support of this hypothesis. First, we found that IFN-$\beta$ was a potent inhibitor of HSV-1 in HCF. Indeed, it was an order of magnitude more effective than TNF-$\alpha$, IFN-$\alpha$ or IFN-$\gamma$. Second, there was no evidence that TNF-$\alpha$ itself exerted a direct antiviral effect in HCF. Rather, TNF-$\alpha$ by itself was found to induce a modest amount of IFN the activity of which was neutralized by antibody to IFN-$\beta$ but

not by antibody to IFN-α. Third, a substantially larger amount of IFN could be detected in the lysates of HCF after combined treatment with TNF-α and IFN-γ. This antiviral activity could be neutralized by antibody to IFN-β but not by antibody specific for IFNs α or γ, or TNF-α. Fourth, antibody to IFN-β was able to abrogate >99% of the synergistic effect. The failure to totally reverse the suppression may mean not all of the IFN-β was accessible to antibody. Finally, the amount of IFN-β induced in HCF by dual cytokine treatment had an inhibitory effect equivalent to that produced by TNF-α and IFN-γ synergism. Collectively, these data support the view that the synergistic antiviral effect produced by TNF-α and IFN-γ in HCF can be entirely explained by the induction of IFN-β.

Hughes et al. conducted similar studies using a 20-fold higher dose of TNF-α and a highly IFN sensitive challenge virus, vesicular stomatitis virus. They found that the TNF-α+IFN-γ synergism observed in HEp-2 cells was significantly abrogated by anti-IFN-β treatment. However, they failed to detect any IFN-β activity in the supernatant after TNF-α and IFN-γ treatment. In contrast to our results, anti-IFN-β treatment did not block the antiviral activity of TNF-α. Apparently, the mechanism by which TNF-α prevents virus growth will vary depending upon the target cells and challenge virus used (Ito et al., 1991; Schijns et al., 1991).

In a recent study Feduchi and Carrasco reported that TNF-α could synergize with IFN-γ to inhibit HSV-1 protein synthesis. Antibodies to IFN-β were reported to inhibit this synergism. These authors concluded that TNF-α induced IFN-β and both cytokines must synergize with IFN-γ to inhibit HSV-1. In their study, however, the amount of IFN-β induced was not determined, and reconstitution experiments were not done.

While earlier studies linked IFN-β with the TNF-α and IFN-γ antiviral effect, none established that it was solely responsible for the synergism. In the present study, we were able to quantitate IFN-β production after TNF-α+IFN-γ treatment, and duplicate the synergism phenomenon by reconstitution with the amount of IFN-β we detected. With this additional information it is possible to conclude that the potent anti-herpes effect of TNF-α+IFN-γ in HCF is attributed largely, and perhaps entirely, to the induction of IFN-β.

Since TNF-β binds to the same cell receptors as TNF-α (Aggarwal et al., 1985b) it was of interest to determine if the former also possessed antiviral activity in HCF. We found that TNF-β was 2–5-fold less inhibitory than TNF-α when tested at 50 U/ml. TNF-β also synergized less effectively with IFN-γ than did TNF-α. Wong and Goeddel had found that TNF-β was as inhibitory as TNF-α in inhibiting HSV-2 replication, and synergized with IFN-γ as effectively as TNF-α. However, their studies were carried out in human renal carcinoma cells and A549 cells using nanogram concentrations of the cytokines, and this may account for the different results.

How might TNF-α synergize with IFN-γ to induce abundant IFN-β synthesis? The regulatory region for the IFN-β gene is known to be highly complex, containing both positive and negative regulatory domains (Williams,

1991). These domains interact with various proteins, some of which can be induced by TNF-$\alpha$ and IFN-$\gamma$ (Fujita et al., 1989; Meichle et al., 1990; Osborn et al., 1989). Presumably the two cytokines act in synergy to remove repressors and induce transcription factors which promote IFN-$\beta$ expression. Present evidence indicates that IFN-$\beta$ blocks HSV replication by inhibiting the synthesis of immediate early genes (Mittnacht et al., 1988; Klotzbücher et al., 1990).

Preliminary studies in our laboratory have established that low dose TNF-$\alpha$ and IFN-$\gamma$ also collaborate in a synergistic manner to suppress HSV-1 replication in mouse corneal fibroblasts (Chen and Lausch, unpublished observations). Previous work had shown that passive transfer of neutralizing antibodies to IFN-$\alpha/\beta$ enabled normally avirulent HSV strains to induce herpetic stromal keratitis (Su et al., 1990; Lausch et al., 1991). In addition, sensitized T cells, the anticipated producers of IFN-$\gamma$, are functionally demonstrable in mice (Lausch et al., 1990) at the time virus is being cleared from ocular tissue, ie about day 5–7 post-infection (Lausch et al., 1989). A variety of cell types including resident Langerhans cells may release TNF-$\alpha$ in the cornea (Asbell and Kamenar, 1987; Larrick et al., 1989). Thus, one can envision that the interaction of TNF-$\alpha$ and IFN-$\gamma$ in vivo could play a critical role in promoting virus clearance from the eye. Future experiments will be directed toward testing this premise in the murine-HSV ocular infection model.

## Acknowledgements

The authors thank Nikki Nixon for typing this manuscript. This work was supported by Public Health Service Grant EY05099 from the National Eye Institute.

## References

Aderka, D., Novick, D., Hahn, T., Fischer, D.G. and Wallach, D. (1985) Increase of vulnerability to lymphotoxin in cells infected by vesicular stomatitis virus and its further augmentation by interferon. Cell Immunol. 92, 218–225.

Aggarwal, B.B., Kohr, W.J., Hass, P.E., Moffat, B., Spencer, S.A., Henzel, W.J., Bringman, T.S., Nedwin, G.E., Goeddel, D.V. and Harkins, R.N. (1985a) Human tumor necrosis factor. J. Biol. Chem. 260, 2345–2354.

Aggarwal, B.B., Eessalu, T.E. and Hass, P.E. (1985b) Characterization of receptors for human tumor necrosis factor and their regulation by $\gamma$-interferon. Nature 318, 665–667.

Asbell, P.A. and Kamenar, T. (1987) The response of Langerhans cells in the cornea to herpetic keratitis. Curr. Eye Res. 6, 179–182.

Beutler, B. and Cerami, A. (1989) The biology of cachectin/TNF – A primary mediatory of the host response. Annu. Rev. Immunol. 7, 625–655.

Cubitt, C.L., Lausch, R.N. and Oakes, J.E. (1993) IL-8 gene expression in cultures of human corneal epithelial cells and keratocytes. Invest. Ophthalmol. Vis. Sci., in press.

Dijkmans, R., Decock, B., Heremans, H., Damme, J.V. and Billiau, A. (1989) Interferon-$\gamma$ is cytotoxic for normal mouse fibroblasts: Enhancement by tumor necrosis factor and interleukin 1. Lymphokine Res. 8, 25–34.

Feduchi, E. and Carrasco, L. (1991) Mechanism of inhibition of HSV-1 replication by tumor necrosis factor and interferon $\gamma$. Virology 180, 822–825.

Fujita, T., Reis, L.F.L., Watanabe, N., Kimura, Y., Taniguchi, T. and Vilcek, J. (1989) Induction of the transcription factor IRF-1 and interferon-$\beta$ mRNAs by cytokines and activators of second-messenger pathways. Proc. Natl. Acad. Sci. USA 86, 9936–9940.

Havell, E.A. and Vilcek, J. (1972) Production of high-titered interferon in cultures of human diploid cells. Antimicrob. Agents Chemother. 2, 476–484.

Hendricks, R.L. (1991) Role of lymphokines in HSV-1 corneal stromal disease. Invest. Ophthalmol. Vis. Sci. 32, 854.

Hendricks, R.L., Weber, P.C., Taylor, J.L., Koumbis, A., Tumpey, T.M. and Glorioso, J.C. (1991) Endogenously produced interferon $\alpha$ protects mice from herpes simplex virus type 1 corneal disease. J. Gen. Virol. 72, 1601–1610.

Hooks, J.J., Chan, C.C. and Detrick, B. (1988) Identification of the lymphokines, interferon-gamma and interleukin-2, in inflammatory eye diseases. Invest. Ophthalmol. Vis. Sci. 29, 1444–1451.

Hughes, T.K., Kaspar, T.A. and Coppenhaver, D.H. (1988) Synergy of antiviral actions of TNF and IFN-$\gamma$: evidence for a major role of TNF-induced IFN-$\beta$. Antiviral Res. 10, 1–9.

Ito, M. and O'Malley, J.A. (1987) Antiviral effects of recombinant human tumor necrosis factor. Lymphokine Res. 6, 309–318.

Ito, M., Nakano, T., Kamiya, T., Kitamura, K., Ihara, T., Kamiya, H. and Sakurai, M. (1991) Effects of tumor necrosis factor alpha on replication of varicella-zoster virus. Antiviral Res. 15, 183–192.

Jacobsen, H., Mestan, J., Mittnacht, S. and Dieffenbach, C.W. (1989) Beta interferon subtype 1 induction by tumor necrosis factor. Mol. Cell. Biol. 9, 3037–3042.

Kaufman, H.E. (1981) Local therapy of herpes simplex virus ocular infections. In: A.J. Nahmias, W.R. Dowdle and R.F. Schinazi (Eds), The Human Herpesviruses: An Interdisciplinary Perspective, pp. 466–477. Elsevier, New York.

Klotzbücher, A., Mittnacht, S., Kirchner, H. and Jacobsen, H. (1990) Different effects of IFN-$\gamma$ and IFN-$\alpha/\beta$ on "Immediate Early" gene expression of HSV-1. Virology 179, 487–491.

Koff, W.C. and Fann, A.V. (1986) Human tumor necrosis factor-alpha kills herpes-infected but not normal cells. Lymphokine Res. 5, 215–221.

Larrick, J.W., Morhenn, V., Chiang, Y.L. and Shi, T. (1989) Activated Langerhans cells release tumor necrosis factor. J. Leukocyte Biol. 45, 429–433.

Larrick, J.W. and Wright, S.C. (1992) Cytokine modulation of viral infections. In: S.L. Kunkle and D.G. Remick (Eds), Cytokines in Health and Disease, pp. 181–195. Marcel Dekker, New York.

Lausch, R.N., Oakes, J.E., Metcalf, J.F., Scimeca, J.M., Smith, L.A. and Robertson, S.M. (1989) Quantitation of purified monoclonal antibody needed to prevent HSV-1 induced stromal keratitis in mice. Curr. Eye Res. 8, 499–506.

Lausch, R.N., Staats, H., Metcalf, J.F. and Oakes, J.E. (1990) Effective antibody therapy in herpes simplex virus ocular infection. Characterization of recipient immune response. Intervirology 31, 159–165.

Lausch, R.N., Su, Y.H., Ritchie, M. and Oakes, J.E. (1991) Evidence endogenous interferon production contributed to the lack of ocular virulence of an HSV intertypic recombinant. Curr. Eye Res. 10, 39–45.

Meichle, A., Schutze, S., Hensel, G., Brunsing, D. and Kronke, M. (1990) Protein kinase c-independent activation of nuclear factor B by tumor necrosis factor. J. Biol. Chem. 265, 8339–8343.

Mestan, J., Digel, W., Mittnacht, S., Hillen, H., Blohm, D., Moller, A., Jacobsen, H. and Kirchner, H. (1986) Antiviral effects of recombinant tumor necrosis factor in vitro. Nature 323, 816–819.

Mittnacht, S., Straub, P., Kirchner, H. and Jacobsen, H. (1988) Interferon treatment inhibits onset of herpes simplex virus immediate-early transcription. Virology 164, 201–210.

Osborn, L., Kunkel, S. and Nabel, G. (1989) Tumor necrosis factor $\alpha$ and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor B. Proc. Natl. Acad. Sci. USA 86, 2336–2340.

Pinto, A.J., Morahan, P.S., Brinton, M., Stewart, D. and Gavin, E. (1990) Comparative therapeutic

efficacy of recombinant interferon-α, -β and -γ against alphatogavirus, bunyavirus, flavivirus, and herpesvirus infections. J. Interferon Res. 10, 293–298.

Rossol-Voth, R., Rossol, S., Schütt, K.H., Corridori, S., de Cian, W. and Falke, D. (1991) In vivo protective effect of tumor necrosis factor α against experimental infection with herpes simplex virus type 1. J. Gen. Virol. 72, 143–147.

Sanitato, J.J., Varnell, E.D., Kaufman, H.E. and Raju, V.K. (1984) Difference in natural and recombinant interferon for herpes keratitis in two animal models. Invest. Ophthalmol. Vis. Sci. 25, 874–876.

Schijns, V.E.C.J., Van der Neut, R., Haagmans, B.L., Schellekens, H. and Harzinek, M.C. (1991) Tumor necrosis factor-α, interferon-γ and interferon-β exert antiviral activity in nervous tissue cells of rat. J. Gen. Virol. 72, 809–815.

Stanton, G.J., Jordan, C., Hart, A., Heard, H., Langford, M.P. and Baron, S. (1987) Nondetectable levels of interferon gamma is a critical host defense during the first day of herpes simplex virus infection. Microbiol. Pathogenesis 3, 179–183.

Stone-Wolff, D.S., Yip, Y.K., Kelkar, H.C., Le, J., Henriksen-Destefano, D., Rubin, B.Y., Rinderknecht, D.E., Aggarwal, B.B. and Vilcek, J. (1984) Interrelationships of human interferon-gamma with lymphotoxin and monocyte cytotoxin. J. Exp. Med. 159, 828–843.

Su, Y.-H., Oakes, J.E., and Lausch, R.N. (1990) Ocular avirulence of a herpes simplex virus type 1 strain is associated with heightened sensitivity of alpha/beta interferon. J. Virol. 64, 2187–2192.

Trinchieri, G. (1992) Lymphocytes and NK cells as producers of TNF. In: B. Beutler (Eds), Tumor Necrosis Factors: The Molecules and their Emerging Role in Medicine, pp. 515–530. Raven Press, New York.

Weiss, R.C. (1989) Synergistic antiviral activities of acyclovir and recombinant human leukocyte (alpha) interferon on feline herpes replication. Am. J. Vet. Res. 50, 1672–1677.

Williams, B.R.G. (1991) Transcriptional regulation of interferon-stimulated genes. Eur. J. Biochem. 200, 1–11.

Williamson, B.D., Carswell, E.A., Rubin, B.Y., Prendergast, J.S. and Old, L.J. (1983) Human tumor necrosis factor produced by human B-cell lines: synergistic cytotoxic interaction with human interferon. Proc. Natl. Acad. Sci. USA 80, 5397–5401.

Wong, G.H.W. and Goeddel, D.V. (1986) Tumor necrosis factors α and β inhibit virus replication and synergize with interferons. Nature 323, 819–822.