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MODULATION OF TUMOR NECROSIS FACTOR ACTIVITIES BY A POTENTIAL ANTICACHEXIA COMPOUND, HYDRAZINE SULFATE

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Abstract — Experiments were performed to determine the effects of hydrazine sulfate (HS), a potential anti-cachexia agent, on tumor necrosis factor activities (cachectin/TNF) *in vitro*. We present evidence that HS significantly inhibits the lytic activity of TNF on L-929 cells, that HS has no direct effect on TNF itself, and that the minimum amount of time for maximum inhibition of TNF activity to occur after HS treatment is between 1 and 4 h. In addition to HS's effect on the cytolytic activity, we also determined its effect on the antiviral activity of TNF. We found that HS greatly potentiates TNF antiviral activity, while having no significant antiviral activity itself over a range of concentrations, that the potentiation was likely between HS and TNF-induced interferon-B_1, and was maximal following 4 and 8 h of treatment with HS. Although the lytic activity of TNF has not been directly correlated with its cachectic activities, these studies provide evidence for an effect of HS on cachectin and its role in the wasting process. Furthermore, a rationale is provided for use of HS in conjunction with TNF for prevention and/or treatment of viral infection.

Tumor necrosis factor (TNF) has been shown to be produced by monocyte/macrophages in response to endotoxin and other stimuli. It was initially described by its ability to preferentially cause a hemorrhagic necrosis of some tumor but not normal cells (Carswell, Old, Kassel, Green & Williamson, 1975; Old, 1985). In addition to the above, TNF has now been shown to have a plethora of activities which include antiviral activity (Kohase, Henrickson-Stefano, May, Vilcek & Seghal, 1986; Mestan, Digel, Mittnacht, Hillen, Blohm, Moeller, Jacobsen & Kirchener, 1986; Wong & Goeddel, 1986), potentiation of interferon's (IFN) antiviral activity (Wong & Goeddel, 1986; Hughes, Caspar & Coppenhaver, 1988), enhancement of monocyte motility (Mustaha, Schlamsteig, Hughes, Rajarman, Rudloff & Goldman, submitted for publication) and activation of monocyte/macrophage cytotoxic activities (Philip & Epstein, 1986). Recently, TNF has been shown to be identical to cachectin, a presumed mediator of wasting in neoplastic states (Beutler, Greenwald, Hulmes, Chang, Pan, Mathison, Uletvitch & Cerami, 1985a). This wasting process greatly accelerates the morbidity and mortality of affected individuals. The mechanisms by which TNF/cachectin exert their effects are not entirely known, but it has been suggested that they are mediated by the suppression of lipoprotein lipase activity (Beutler, Mahoney, Le Trang, Pekala & Cerami, 1985b).

It has been reported that hydrazine sulfate (HS), a non-competitive inhibitor of phosphoenolpyruvate carboxykinase in gluconeogenesis, can inhibit the wasting process in cancer patients (Gold, 1975, 1987). It was hypothesized that increased levels of lactic acid derived from highly glycolytic neoplastic tissue, amino acids from peripheral protein breakdown, and glycerol from lipid mobilization contributed to the development of a greater than normal gluconeogenic pathway. This in turn resulted in greater amounts of energy being lost from normal host tissue to contribute to the obligatory production of glucose; hence, the wasting process. However, the possibility exists that HS may also be playing a role by inhibiting TNF/cachectin by some as yet undefined mechanism. We therefore attempted to determine the effe ɛxof HS on TNF activity *in vitro*. Here, we report that HS significantly inhibits TNF cytolytic activity and, in addition, potentiates its antiviral activity. Implications for its use are further discussed.

EXPERIMENTAL PROCEDURES

Inhibition of TNF cytolytic activity by hydrazine sulfate

Mouse L-929 cells (American Type Culture Collection) were grown to confluency in 96-well tissue culture plates (Costar, Cambridge, MA) in

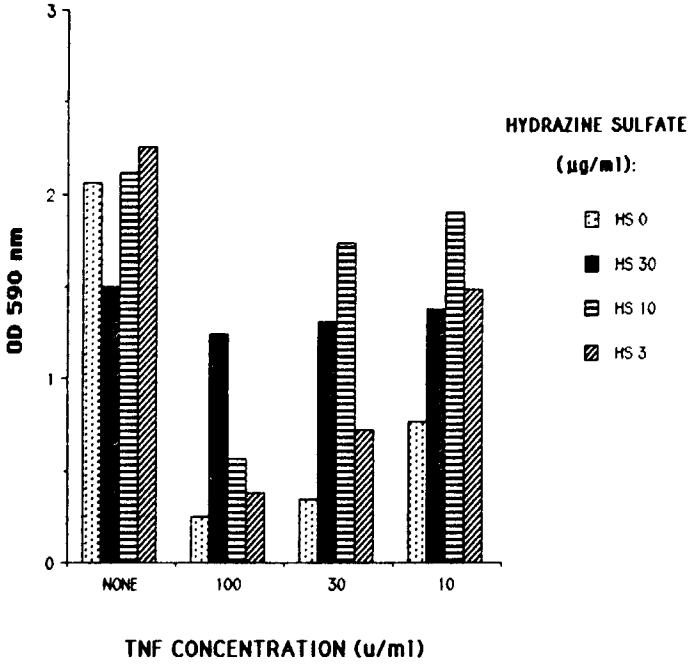

Fig. 1. Inhibition of TNF cytolytic activity by hydrazine sulfate. Mouse L-929 cells were treated with TNF in the absence or presence of hydrazine sulfate at the indicated concentrations of both for 24–36 h. Following incubation, the cells were stained and destained as before and OD_{590} was determined on an automated ELISA reader.

Eagle's minimal essential medium with Earle's salts supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/100 µg/ml) (EMEM 10%) at 4% CO_2, 37°C. Following 24 h incubation or upon confluence, TNF cytolytic activity was measured on L-929 cells as described elsewhere (Flick & Gifford, 1984). Briefly, media was replaced with EMEM 2% containing 5 µg/ml actinomycin D in the absence or presence of various concentrations of recombinant TNF (Suntory, Tokyo, Japan). Twenty-four hours later, cells were stained with 1% crystal violet in 20% methanol, destained with Sorenson's buffer, and optical densities were determined at a wavelength of 590 nm on an automated ELISA reader.

To determine the effects of hydrazine sulfate (Sigma, St Louis, MO) on TNF activity, concentrations ranging from 0 to 300 µg/ml in EMEM 2% were added to the above-described assays at various times before or after TNF addition. Inhibition of TNF activity was reflected as an increased optical density of the HS/TNF-treated cells when compared to those treated with TNF alone.

Potentiation of the antiviral activity of TNF by HS on HEp-2 cells

HEp-2 cells (American Type Culture Collection) were grown in 96-well tissue culture plates in EMEM 10% as before. Upon confluency, various concentrations of TNF in EMEM 2% were placed on the cells. Twenty-four hours later and following removal of TNF, cells were challenged with vesicular stomatitis virus (VSV) at a multiplicity of infection of 0.5. Virus was harvested 24 h later and yields were determined on L-929 cells in a modified plaque reduction assay (Campbell, Grumberger, Kochman & White, 1975).

To determine the effects of HS on TNF antiviral activity, concentrations of HS ranging from 0 to 30 µg/ml were placed on the cells at various times relative to TNF addition. Virus yields were determined as before. In some experiments IFN-B_1 (Triton Biosciences, Palo Alto, CA) was used in place of TNF in a similar fashion.

RESULTS

Inhibition of TNF cytolytic activity by hydrazine sulfate

TNF has been shown to be cytolytic for several transformed cell lines in tissue culture, the standard assay being its cytotoxicity for actinomycin D-treated L-cells (Carswell *et al.*, 1975: Old, 1985).

Table 1. Lack of a direct effect of HS on TNF*

TNF (U/ml)†	Treatment‡	OD 590 nm (± S.E.M.)§
0	—	1.034 ± .38
100	—	0.333 ± .013
100	Dialysis	0.308 ± .030
100	HS (24H) + dialysis	0.281 ± .021

*Representative of two separate experiments.
†Concentration expressed as cytolytic activity determined in a standard assay in actinomycin D-treated L-cells (Flick & Gifford, 1984).
‡TNF was diluted in EMEM 2% with or without HS at a concentration of 1 mg/ml to the indicated concentrations. Twenty-four hours later, both samples were exhaustively dialyzed against phosphate-buffered saline (pH 7.4). Following dialysis, TNF standard and treated samples were tested for cytolytic activity as before.
§Optical density at a wavelength of 590 nm and 1:100 dilution.

Therefore, we tested the ability of HS to block TNF cytolysis in this system. As shown in Fig. 1, L-cells treated with 30, 10 or 3 μg/ml HS for 24 h prior to exposure to 100, 30 or 10 lytic units of TNF and actinomycin D exhibited a significant decrease in cytolysis when compared to control. The possibility existed that HS was acting directly on TNF because of HS's reducing properties. We tested this possibility by treating TNF (300 U/ml) with high concentrations of HS (1 mg/ml). Following treatment, HS was exhaustively dialyzed away. As shown in Table 1, HS had no direct effect on TNF itself. In support of this, we also found that inhibition of TNF cytolysis caused by pre-treatment with HS could not be reversed by extensive washing of the cells. This would indicate that the protective effect is relatively stable (data not shown).

Kinetics of development of the inhibitory effect

We next attempted to determine the kinetics of development of the inhibitory effect of HS on TNF's cytolytic activity in L-cells. L-cells were treated with 100, 30 or 0 μg/ml of HS for 16, 8, 4, 2, 1 or 0 (briefly exposed) h. The cells were then washed three times and TNF was added in the presence of actinomycin D. As seen in Fig. 2, maximum resistance to TNF activity occurred between 1 and 4 h of HS treatment.

Effect of HS on TNF's antiviral activity

In addition to being cytolytic, TNF has been shown to have an antiviral effect on some cells in culture (Kohase *et al.*, 1986; Mestan *et al.*, 1986; Wong & Goeddel, 1986). We therefore determined whether HS had an effect on TNF's antiviral activity. As shown in Table 2, HS at a concentration of 30 μg/ml potentiates the antiviral activity of TNF several hundred fold in some cases. It can also be seen that HS alone had no effect on virus yield. Since TNF is thought to induce its antiviral activity through IFN-β_1 (Van Damme, Deley, Snick, Dinarello & Billiau, 1987), we next determined if HS directly potentiated this IFN. As shown in Table 3, HS directly potentiated IFN-β_1 antiviral activity. Thus, the possibility exists that HS is potentiating TNF antiviral activity through IFN-β_1. At present, however, we cannot determine the relative contributions of any intrinsic TNF antiviral activity or that of IFN-β_1 to the total potentiation of HS/TNF mixture.

Kinetics of development of HS potentiation of TNF antiviral activity

We next determined the kinetics of development of potentiation of TNF antiviral activity by HS. HEp-2 cells were treated with 30 μg/ml of HS for 16, 8, 4, 2, 1 or 0 (briefly exposed) h prior to addition of 1000 units of TNF. Cells were washed free of HS prior to addition of TNF. As shown in Fig. 3, there is a time-dependent effect of HS on the potentiation of TNF antiviral activity. The minimum amount of time for maximum potentiation of TNF by HS appeared to be between 4 and 8 h, which roughly corresponds to TNF's induction IFN-β (Hughes *et al.*, 1988).

DISCUSSION

These experiments were initially aimed at determining the effects of HS, a potential anti-cachexia agent, on the cytolytic activities of TNF. We found that: (1) HS significantly inhibits TNF cytolysis of L-cells in a dose-dependent fashion, (2) the HS-induced protective effect is stable to washing of the cells, (3) HS has no direct effect on TNF itself, and (4) the minimum amount of time of HS treatment required to induce the maximum amount of protection of cells from TNF cytolysis was between 1 and 4 h. In addition to HS's effect on TNF's cytolytic activity, we determined its effects on TNF's antiviral activity. Surprisingly, we found that: (1) HS potentiated TNF's antiviral activity, (2) HS had no significant antiviral activity itself at the concentrations used, (3) this potentiation was possibly between HS and IFN β_1, and, (4) the

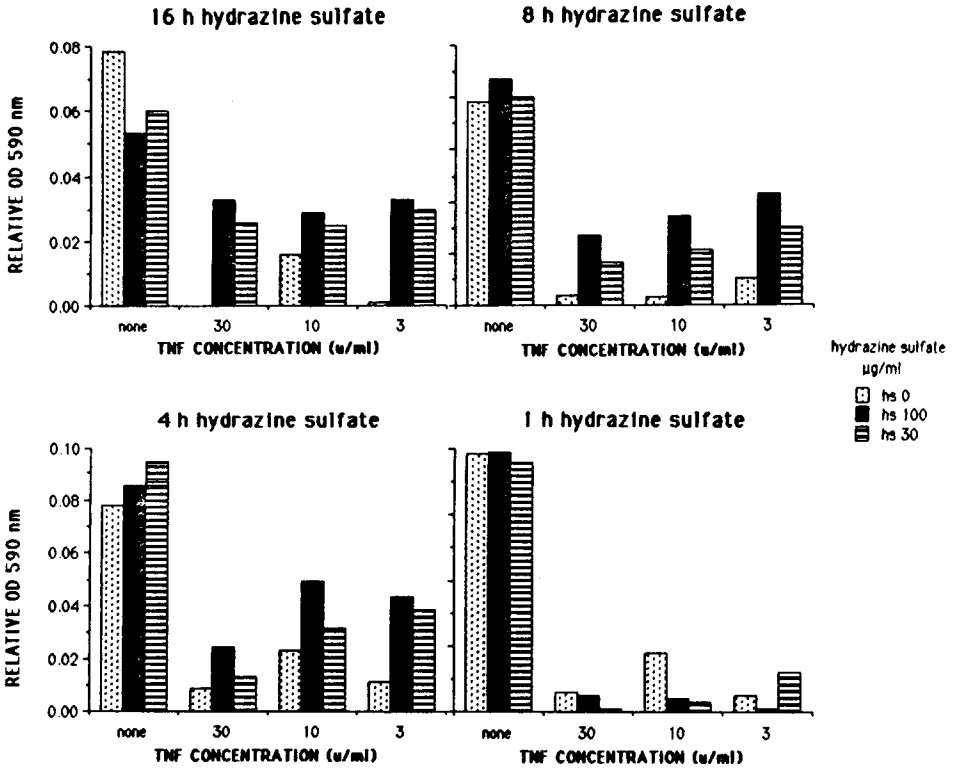

Fig. 2. Kinetics of development of the inhibition of TNF cytolytic activity by hydrazine sulfate on mouse L-929 cells. Mouse L-929 cells were treated with the indicated concentrations of hydrazine sulfate for the indicated periods of time. Cells were then washed and TNF was added at the above concentrations in EMEM 2% with 5 µg/ml actinomycin D. Following 24 h incubation, the cells were stained and destained as before and OD_{590} was determined on an automated ELISA reader.

potentiation was maximal after 4 and 8 h of treatment with HS.

HS has been used in clinical trials with varying degrees of success as an agent to inhibit the wasting syndromes in cancer patients (Gold, 1975, 1987). It was hypothesized that HS, which is a non-competitive inhibitor of phosphenolpyruvate carboxykinase, would interrupt host energy wasting which was a result of rapid tumor glycolysis and host gluconeogenesis. Gold (1987) hypothesized that lactic acid from highly glycolytic neoplastic tissue, amino acids from peripheral protein breakdown and glycerol from lipid mobilization all contributed to the development of greater than normal gluconeogenic pathway. Progressively greater amounts of energy would be lost from normal host tissue to feed this pathway, which would result in the wasting process. In addition to this hypothesized mechanism, however, we feel that an additional mechanism might be an inhibition of TNF, which has been shown to be identical to cachectin — an agent found to contribute to cachexia or wasting (Beutler, 1985a). At present we are not certain as to the mechanism by which HS inhibits TNF cytolytic activity. However, it has been theorized that TNF might induce its cytolytic activity through the generation of free radicals such as superoxide (O_2^-) (Clark, Thumwood, Chaudhri, Cowden & Hunt, 1988). Since HS is a reducing compound, a reduction of 0_2^- to a less toxic compound, i.e. H_2O_2, might explain its activity against TNF cytotoxicity. This possibility is being examined.

In addition to the effects on the cytolytic activity of TNF, we determined the effects of HS on its antiviral activity. Surprisingly, in some instances, the antiviral activity was potentiated several hundred fold. TNF is believed to induce its antiviral activity through the induction of β_1 and/or β_2 (IL-6) (Kohase *et al.*, 1986; Van Damme *et al.*, 1987). Our experiments on determining HS's effects on these IFNs suggest that the potentiation of the antiviral activities could possibly involve TNF-induced IFN β_1.

Table 2. Potentiation of TNF antiviral activity by hydrazine sulfate

TNF U/ml	HS treatment	Virus yield (log 10)	Fold inhibition Exp.†	Obs.‡	Potentiation
0	−	5.5	—	—	—
0	+	5.4	—	1.2	—
10,000	−	4.5	—	10.4	—
10,000	+	2.1	11.6	2538.5	218.8
3000	−	4.6	—	8.5	—
3000	+	1.9	9.7	4400.0	455.5
1000	−	4.6	—	7.8	—
1000	+	2.0	9.0	3000.0	334.8
300	−	4.7	—	6.3	—
300	+	2.3	7.5	1833.0	244.4
100	−	4.6	—	7.9	—
100	+	2.3	9.1	1571.0	173.4

HEp-2 cells were treated with TNF at the indicated concentrations in the absence (−) or presence (+) of 30 μg/ml hydrazine sulfate. Twenty-four hours later, cells were infected with VSV (0.5 m.o.i.). Virus was harvested 24 h later and the yields determined by a modified plaque reduction assay on L-929 cells.

†Observed fold reduction in virus yield $= \frac{\text{virus yield in virus control}}{\text{virus yield in experimental}}$.

Expected fold reduction in virus yield = observed fold reduction in experimental$_1$ + observed fold reduction in experimental$_2$.

‡Fold potentiation $= \frac{\text{observed fold reduction}}{\text{expected fold reduction}}$.

Table 3. HS potentiates the antiviral activity of IFN-β_1

IFN (U/ml)*	HS(μg/ml)	Virus yield (pfu/0.1 ml)	Fold reduction virus yield† Obs.	Exp.	Fold potentiation‡
—	0	40×10^5	—	—	—
—	30	9×10^5	4.4	—	—
—	10	29×10^5	1.4	—	—
—	3	25×10^5	1.6	—	—
B_1 1000	0	53×10^3	75.4	—	—
B_1 1000	30	26×10^2	1538.5	79.8	19.3
B_1 1000	10	52×10^2	769.2	76.8	10
B_1 1000	3	26×10^3	153.8	77	2
B_1 300	3	17×10^5	2.4	—	—
B_1 300	30	16×10^3	250.0	6.8	36.8
B_1 300	10	61×10^3	65.5	3.8	17.2
B_1 300	0	14×10^4	28.6	4	7.2
B_1 100	0	18×10^5	2.2	—	—
B_1 100	30	12×10^4	33.3	6.6	5.1
B_1 100	10	10×10^5	4.0	3.6	1.1
B_1 100	3	4×10^5	10.0	3.8	2.6

*HEp-2 cells (5×10^4 cells/well) in microtiter plates were treated with IFN-B_{ser} (Triton Biosciences) and/or HS at the above indicated concentrations in EMEM 2%. Twenty-four hours later supernatant fluids were decanted and the cells were infected with VSV (multiplicity of infection = 0.5). After 1 h, nonattached virus was decanted and replaced with EMEM 2%. Virus yields were determined as before.

†‡See Table 2 legend for calculation of potentiation values. Representative of three identical experiments.

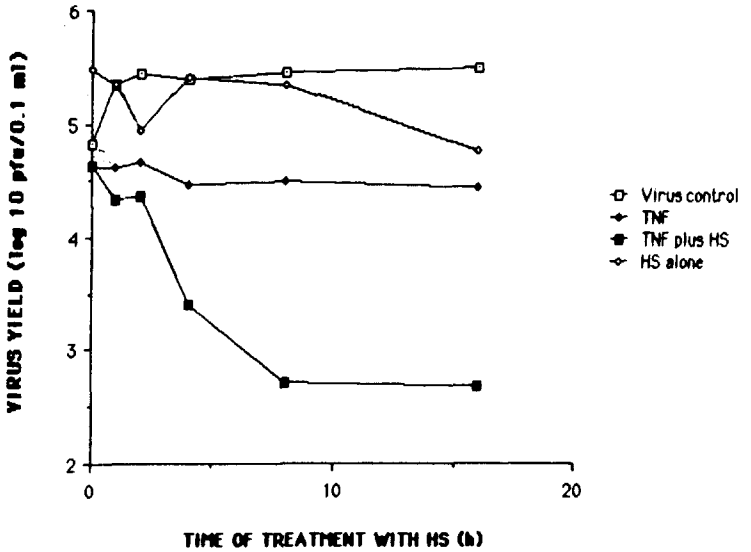

Fig. 3. Kinetics of development of the potentiation of TNF antiviral activity by hydrazine sulfate. HEp-2 cells were treated with hydrazine sulfate at the indicated concentrations and periods of time. Prior to the addition of TNF at the concentrations above, the cells were washed. Twenty-four hours later, VSV was added at a multiplicity of infection of 0.5. Virus yields were harvested 24 h later, and yields were determined by a modified plaque reduction assay on mouse L-929 cells.

Thus, HS may potentiate the antiviral activity of TNF through IFN β_1. We have also tested the possibility on L-cells, which are highly susceptible to TNF's cytolytic activity, that TNF might also induce an antiviral state in the presence of HS. However, no significant decrease in virus yields were found in this system (data not shown).

Although the lytic activity of TNF has not been directly associated with its cachectic activity, these studies are consistent with the hypothesis that HS is affecting TNF in some manner to inhibit the wasting process. In addition, since TNF is shown to have antiviral activity in some cell culture systems (Kohase *et al.*, 1986; Mestan *et al.*, 1986; Wong & Goeddel, 1986) and *in vivo* (Coppenhaver, Sarzotti-Kelsoe, Syiruktasuth, Poast, Singh & Baron, 1988), a rationale for its use in prevention and/or treatment of viral diseases is indicated.

REFERENCES

BEUTLER, B., GREENWALD, D., HULMES, J. D., CHANG, M., PAN, Y. C. E., MATHISON, J., ULEVITCH, R. & CERAMI, A. (1985a). Identity of tumor necrosis factor and the macrophage secreted factor cachectin. *Nature*, **316**, 532–554.

BEUTLER, B., MAHONEY, J., LETRANG, N., PEKALA, P. & CERAMI, A. (1985b). Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endotoxin induced RAW 264.7 cells. *J. exp. Med.*, **161**, 984–995.

CAMPBELL, J. B., GRUMBERGER, J., KOCHMAN, M. A. & WHITE, S. L. (1975). A microplaque reduction assay for human and mouse interferon. *Can. J. Microbiol.*, **21**, 1247–1253.

CARSWELL, E. A., OLD, L. J., KASSEL, R. L., GREEN, S. & WILLIAMSON, B. (1975). An endotoxin induced serum factor that causes necrosis of tumors. *Proc. natn. Acad. Sci. U.S.A.*, **72**, 3666–3670.

CLARK, I. A., THUMWOOD, C. M., CHAUDHRI, G., COWDEN, W. B. & HUNT, N. H. (1988). Tumor necrosis factor and reactive oxygen species: implication for free-radical-induced tissue injury. In *Oxygen Radicals and Tissue Injury Symposium Proceedings* (ed. Halliwell, B.) pp. 122–129. Federation of American Societies for Experimental Biology, Bethesda, M.D.

COPPENHAVER, D. H., SARZOTTI-KELSOE, M. S., SYIRUKTASUTH, P., POAST, J., SINGH, I. P. & BARON, S. (1988). Combined antiviral action of TNF and IFN *in vivo*. *Antivir. Res.*, **9**, 159.

FLICK, D. A. & GIFFORD, G. E. (1984). Comparison of *in vitro* cell cytotoxic assays for tumor necrosis factor. *J. Immun. Meth.*, **68**, 167–175.

GOLD, J. (1975). Use of hydrazine sulfate in terminal and pre-terminal cancer patients: results of investigational new drug (IND) study in 84 evaluable patients. *Oncology*, **32**, 1 – 10.

GOLD, J. (1987). Hydrazine sulfate: a current perspective. *Nutr. Cancer*, **9**, 59 – 66.

HUGHES, T. K., KASPAR, T. A. & COPPENHAVER, D. (1988). Synergy of antiviral actions of TNF and IFN-γ: evidence for a major role of TNF induced IFN-β. *Antivir. Res.*, **10**, 1 – 9.

KOHASE, M. D., HENRICKSON-STEFANO, D., MAY, L. T., VILCEK, J. & SEGHAL, P. B. (1986). Induction of B_2 interferon, by tumor necrosis factor: a homeostatic mechanism in the control of cell proliferation. *Cell*, **45**, 659 – 666.

MESTAN, J., DIGEL, W., MITTNACHT, S., HILLEN, H., BLOHM, D., MOELLER, A., JACOBSEN, H. & KIRCHENER, H. (1986). Antiviral effects of tumor necrosis factor *in vitro*. *Nature*, **323**, 816 – 819.

OLD, L. J. (1985). Tumor necrosis factor (TNF). *Science*, **230**, 630 – 632.

PHILIP, R. & EPSTEIN, L. B. (1986). Tumor necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ interferon and interleukin-1. *Nature*, **323**, 86 – 89.

VAN DAMME, J., DELEY, J. M., SMICK, J. V., DINARELLO, C. A. & BILLIAU, A. (1987). The role of interferon β_1 and the 26 kDa protein (interferon-B_2) as mediators of the antiviral effect of interleukin-1 and tumor necrosis factor. *J. Immun.*, **139**, 1867 – 1872.

WONG, G. H. W. & GOEDDEL, D. V. (1986). Tumor necrosis factors α and β inhibit virus replication and synergize with the interferon. *Nature*, **323**, 819 – 822.