Antiviral Research, 10 (1988) 71–81
Elsevier

AVR 00304

Studies on the in vitro biological activities of recombinant bovine tumor necrosis factor (rBoTNF) alpha. I. Synergistic antiviral efficacy of rBoTNF alpha, recombinant bovine interferon gamma (rBoIFN gamma) and their combination

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(Received 4 June 1988; accepted 6 July 1988)

Summary

The recent demonstration of the antiviral activity of recombinant human TNF has launched an interest in the use of TNF alpha for antiviral therapy in veterinary medicine. In the precent report, we demonstrate that pretreatment of bovine cells with rBoTNF alpha reduces the yield of bovine herpesvirus type-1 (BHV-1) from infected cells. Reduction in yield was similar to that observed in the presence of rBoIFN gamma. Similarly, rBoTNF alpha was able to protect bovine cells from virus-induced cytopathology. Enhanced antiviral activity was demonstrated when rBoTNF alpha was administered in combination with rBoIFN gamma. Studies on the induction of 2′,5′-oligoadenylate synthetase (2′-5′A^S) production by cytokine-treated cells showed that although rBoTNF alpha by itself did not induce 2′-5′A^S activity it was capable of enhancing the production of 2′-5′A^S by rBoIFN gamma-treated cells. Combination of these two cytokines was also evident in the inhibition of proliferation of treated cells. In contrast, the cytotoxic effect of rBoTNF alpha towards actinomycin D-treated cells was not affected by the combination of rBoTNF alpha with rBoIFN gamma.

Bovine; Tumor necrosis factor; Interferon

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Introduction

Tumor necrosis factor (TNF) alpha is a macrophage-derived molecule originally identified by its tumoricidal action, reviewed by Flick and Gifford (1985). However, recent evidence indicates that TNF alpha has a number of biological activities in addition to its cytostatic/cytotoxic action against tumor cells (Beutler and Cerami, 1986). Some of these newly described functions make TNF an attractive molecule for its use in veterinary medicine. TNF has been recently shown to be produced in response to viral infections (Aderka et al., 1986) and to possess antiviral activity against a variety of RNA and DNA viruses (Arakawa et al., 1987; Kohase et al., 1986; Mestan et al., 1986; Wong and Goeddel, 1986). Human TNF has been shown to interact synergistically with interferons (IFN) in inhibiting virus replication in a variety of cell lines (Sugarman et al., 1986). Successful cloning of the bovine TNF alpha gene has made this molecule (rBoTNF alpha) available for studies in animal diseases of economic importance.

Bovine herpesvirus type 1 (BHV-1) is responsible for a number of economically significant clinical manifestations (Kahrs, 1977) and it has been shown that of all the recombinant bovine interferons available (rBoIFN), rBoIFN gamma has the least in vitro antiviral activity against BHV-1 (Bielefeldt Ohmann et al., 1987; Czarniecki et al., 1986). It was therefore of interest to investigate the capacity of TNF to act alone and in combination with rBoINF gamma in inducing an effective antiviral stage against BHV-1.

Materials and Methods

Tissue culture media and reagents

Modified Eagle's minimum essential medium (MEM) was supplemented with 50 μg/ml of gentamycin (both from Gibco Laboratories, Grand Island, N.Y.), 2 mg/ml sodium bicarbonate (Fisher Scientific So., Fraiz Lawn, N.J.), and 25 mM HEPES (BDH Chemicals, Toronto, Canada) and 10% fetal bovine serum (FBS) (Gibco).

Recombinant bovine cytokines

Bovine IFN-gamma and TNF-alpha, produced by recombinant DNA technology and purified to homogeneity as determined by high-performance liquid chromatography from Genentech Inc., S. San Francisco, CA. The specific antiviral activity of rBoIFN-gamma was 1×10^7 units/mg protein. No biological standard is yet available for rBoTNF-alpha, therefore, only references to weight-based concentrations are used in the present studies.

Cell lines

Continuous bovine cell lines Georgia Bovine kidney (GBK) and Madin Darby Bovine kidney (MDBK) as well as bovine fibroblast culture established in our laboratory from bovine skin (BSF) were maintained in monolayer cultures on plastic tissue culture flasks (Corning Glassware, Corning, N.Y.) in MEM as described previously (Rouse and Babiuk, 1977).

Virus and virus assays

Stock preparations of the P8-2 strain of bovine herpesvirus type 1 (BHV-1) were prepared in GBK cells as described previously (Rouse and Babiuk, 1977). For the plaque assay, confluent GBK cell cultures in 96-well plates were inoculated with 10-fold dilutions of the virus containing samples and overlaid with a 1:500 dilution of anti BHV-1 antibody and the plaques were counted 2 days later. In order to evaluate the capacity of recombinant bovine cytokines to protect infected cells from viral-induced cytopathology, GBK cells were grown to confluency in 96-well plates. Recombinant bovine cytokines were then added and the cells were inoculated with the virus as indicated. When control cultures (i.e. not treated with cytokines) showed extensive cytopathic effect (36–48 h post inoculation), the monolayers were stained with a 2.0% solution of neutral red. The protection against viral cytopathic effect was measured by the quantity of dye uptake by the cells. The amount of dye taken up by the cells was quantified after elution of the dye with 50% v/v ethanol, 0.1 M sodium phosphate monobasic solution and determination of the light absorbance of 540 nm.

Assays for cellular proliferation

GBK and MDBK cells (5×10^4 cells/ml) were cultured for 60 h in 200 µl of MEM containing the indicated concentrations of single cytokines or their combinations. To assess proliferation, cells were incubated for 12 h with *methyl*-[^{3}H]thymidine before harvesting onto glass fiber paper. The radioactivity incorporated was determined using liquid scintillation counting. Neutral red incorporation by viable cells was also used to evaluate the effect of 48 h treatment of bovine cells with rBoTNF alpha and rBoIFN gamma.

Cytotoxicity assays

A standard ^{51}Cr release assay (Campos et al., 1986) as well as neutral red dye incorporation by viable cells, as described above, were used to evaluate the cytotoxic capacity of rBoTNF alpha.

2,5-Oligoadenylate synthetase induction assay

The level of 2-5A^S activity in GBK cells was determined essentially as described by Revel et al. (1982), using poly (rI):(rC) agarose beads for isolation of the enzyme from the cytosol (Merlin et al., 1981).

Results

Synergistic anti-BHV-1 action of rBoTNF alpha and rBoIFN gamma

Initial experiments were aimed at evaluating the antiviral capacity of rBoTNF alpha against BHV-1. Various concentrations of rBoTNF alpha were added to confluent monolayers of GBK and MDBK cells in 24-well culture plates. After 24 h, the medium was removed and 100 PFU per ml of BHV-1 was added to each. After 2 h, nonadsorbed virus was carefully washed off with warm media and 1 ml

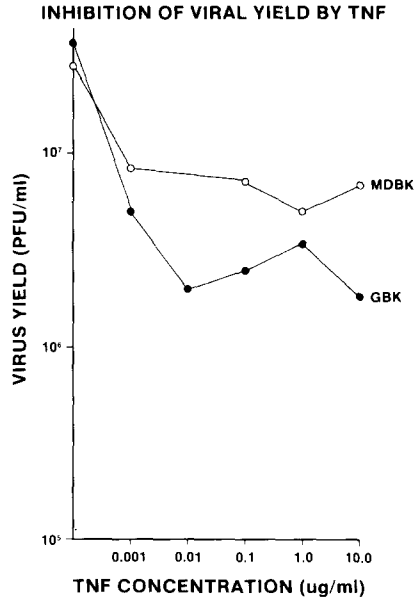

Fig. 1. Anti-BHV-1 action of rBoTNF alpha. The indicated concentrations of rBoTNF were added to confluent monolayers of GBK and MDBK cells in 24-well culture plates. After 24 h 100 PFU/ml of BHV-1 was added to each well. Non-absorbed virus was removed and 1 ml of fresh media was added to each well. The yield of BHV-1 from rBoTNF alpha-treated and non-treated controls was determined using a standard plaque assay.

of fresh media was added to each well. Forty-eight hours after infection the yield of BHV-1 was determined by plaque assay, as described in Materials and Methods. Concentrations of rBoTNF alpha ranging from 10 μg/ml to 1 ng/ml were capable of reducing viral production by both cell types used (Fig. 1). Similar experimental protocols were used to evaluate the antiviral effect of rBoIFN gamma alone and in combination with rBoTNF alpha using 3 different bovine cell types (MDBK cells and 2 lines of bovine skin fibroblasts). In all three cell types, rBoIFN gamma and rBoTNF alpha had comparable antiviral activity at the concentrations tested. However, enhancement of viral inhibition was always obtained when rBoTNF alpha and rBoIFN gamma were combined (Fig. 2).

Enhancement of 2-5A induction on rBoIFN gamma treated cells by rBoTNF alpha

In order to explore whether rBoTNF alpha exhibits its antiviral activity by the same mechanisms as interferons, the ability of rBoTNF alpha to induce 2′–5′A synthetase, a potent inhibitor of viral replication (Merlin et al., 1981), was studied. GBK cells were exposed to rBoTNF alpha either alone or in combination with rBoIFN gamma at various doses and time periods, followed by assay for enzyme activity in the cytosol. No induction of enzyme activity was detectable even after extended exposure of the cells to rBoTNF alpha alone (up to 5 days; data not

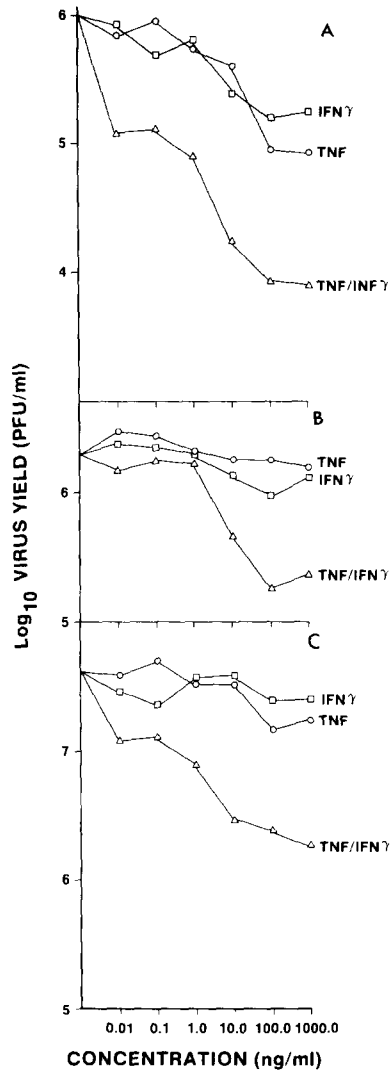

Fig. 2. Inhibition of viral yield by rBoTNF alpha, rBoIFN gamma and their combinations. Various concentrations of rBoTNF alpha, rBoIFN gamma and their combinations at equal concentrations were added to confluent monolayers of bovine skin fibroblasts (A, BSF 70 and B, BSF 74) and to MDBK cells (C) in 24-well culture plates. After 24 h the medium was removed and 100 PFU/ml of BHV-1 was added to each well. Forty-eight hours after incubation, the yield of BHV-1 was determined by plaque assay.

shown). However, rBoTNF alpha had an enhancing effect on the activity induction exerted by rBoIFN gamma alone, when cells were treated simultaneously with the two cytokines (Fig. 3). This effect was dose-dependent with regard to both rBoIFN gamma and rBoTNF alpha. Data are depicted in Fig. 3 for one fixed rBoIFN gamma dose combined with various concentrations of rBoTNF alpha.

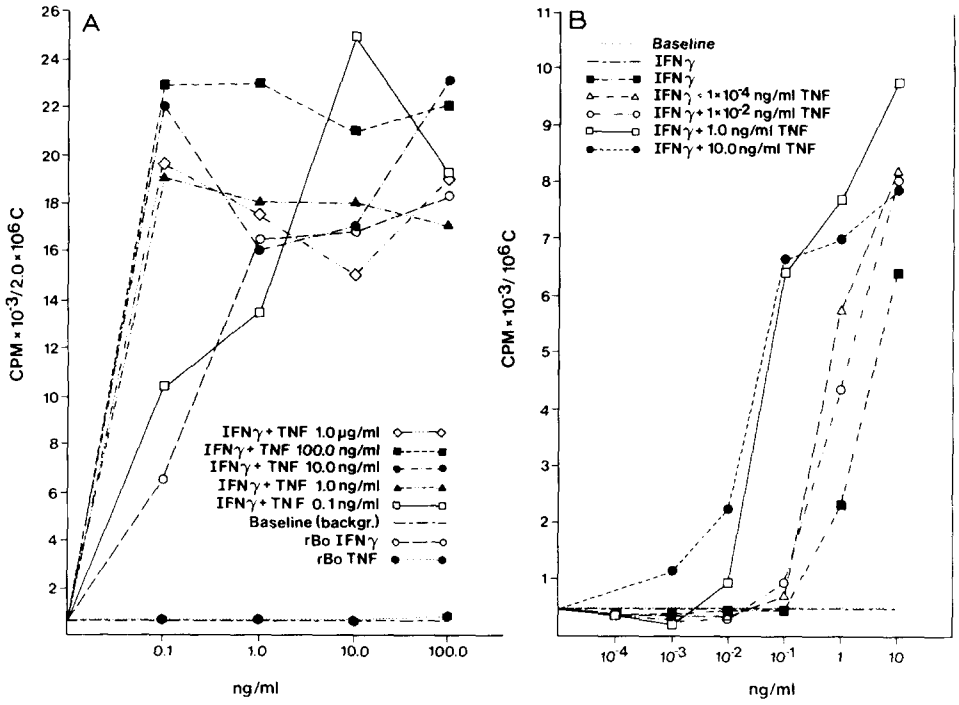

Fig. 3. Enhancement of rBoIFN gamma (at the concentration shown in the X axis of the figure) induced 2′–5′A synthetase activity in GBK cells after simultaneous exposure to rBoTNF alpha for 20 h. The data depicted are representative examples from a larger range of concentration-combinations tested out in three independent experiments, (A) high-dose range; (B) low-dose range.

Cytotoxic and cytostatic actions of rBoTNF alpha to bovine cell lines

It has been suggested that virus-infected cells are selectively killed by TNF and that this activity is accelerated by IFN. It was therefore of interest to study the cytotoxic capacity of rBoTNF alpha and its combination with rBoIFN gamma on bovine cells. Short- (18 h) and long- (48 h) term cytotoxicity assays using [^{51}Cr]release, and neutral red uptake methodologies failed to detect cytotoxic activity of TNF against bovine targets (data not shown). Nevertheless, treatment of bovine cell lines with 1 µg of actinomycin D for 2 h before the assay rendered these bovine cells susceptible to the cytotoxic action of rBoTNF alpha. Briefly, GBK cells were seeded at a concentration of 5×10^4 cells per well in flat bottom microtiter plates. After overnight incubation, 100 µl of media containing 1 µg of actinomycin D per ml was added to each well. After a 2 h incubation period, actinomycin D-containing media were removed and fresh media containing the indicated concentrations of rBoTNF alpha were added to each well. Viability of actinomycin D-treated cells, as evaluated by neutral red uptake, was markedly reduced at concentrations of rBoTNF alpha as low as 0.1 ng/ml (Fig. 4). To evaluate the effect of rBoIFN gamma on rBoTNF alpha-mediated cytotoxicity, actinomycin D-treated GBK and MDBK cells were exposed to different concentrations of these two cy-

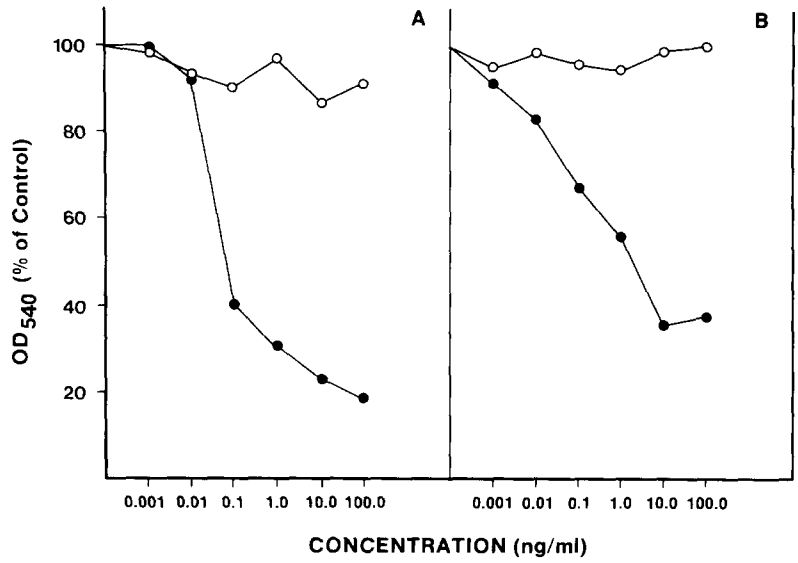

Fig. 4. Cytotoxic activity of rBoTNF alpha against GBK (A) and MDBK (B) cells before (○) and after (●) 2 h treatment with actinomycin D (1 μg/ml). GBK and MDBK cells were seeded in 96-well flat bottom microtiter plates and treated for 18 h with the indicated concentrations of rBoTNF. The OD_{540} of neutral red stained cells was used as the indicator of the number of viable cells at the termination of the assay.

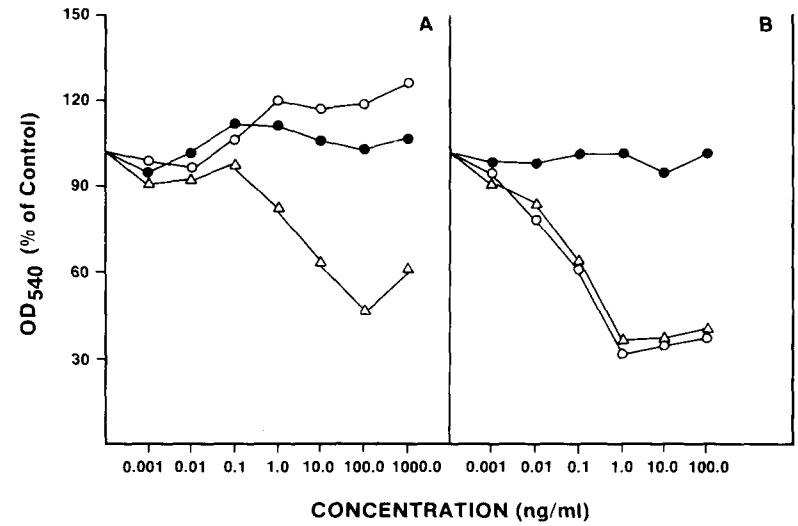

Fig. 5. Antiproliferative activity (A) and cytotoxic activity to actinomycin D-treated MDBK cell (B) of rBoIFN gamma (●), rBoTNF alpha (○) and their combinations (△). MDBK cells were seeded in 96-well flat bottom microtiter plates and treated either 48 h (A) or 18 h (B) with the indicated concentrations of cytokines. Viable cells were stained using neutral red uptake method.

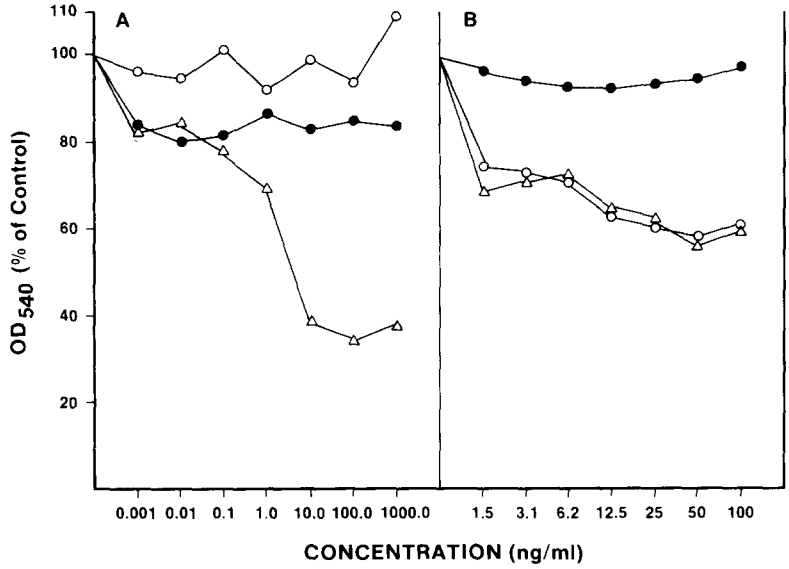

Fig. 6. Antiproliferative activity (A) and cytotoxic activity to actinomycin D-treated GBK cells (B) of rBoIFN (●), rBoTNF alpha (○) and their combinations (△). GBK cells were seeded in 96-well flat bottom microtiter plates and treated either 48 h (A) or 18 h (B) with the indicated concentrations of cytokines. Viable cells were stained using the neutral red uptake method.

tokines alone and in combination. As shown in Figs. 5B and 6B, both cell types were susceptible to the cytotoxic action of rBoTNF alpha. Recombinant BoIFN gamma did not cause cytotoxicity of GBK or MDBK cells, nor did it enhance the cytotoxic activity of rBoTNF alpha.

In order to evaluate the cytostatic action of rBoTNF alpha and its interaction with rBoIFN gamma, proliferation of GBK and MDBK cells was measured using a [^{3}H]thymidine incorporation assay after 4 days of culture in the presence of rBoTNF alpha, rBoIFN gamma, and their combinations. Under these experimental conditions, GBK cells were shown to be more sensitive than MDBK cells to the antiproliferative action of rBoIFN gamma, whereas rBoTNF alpha caused greater cytostasis of MDBK cells. However, in both cell types, the combination of both rBoTNF alpha and rBoIFN gamma markedly enhanced the antiproliferative action of either cytokine alone (data not shown).

To examine the antiproliferative action of these cytokines further, MDBK cells seeded in microtiter plates were treated with various concentrations of rBoTNF alpha, rBoIFN gamma, and rBoTNF alpha/rBoIFN gamma combinations. After 48 h of incubation, cultured cells were stained with neutral red, to quantitate the number of viable cells. The proliferation of MDBK cells after 48 h was not affected by either cytokine at concentrations as high as 1 μg/ml (Fig. 5A). Using the same experimental protocol, GBK cells were shown to be sensitive to the antiproliferative action of rBoIFN gamma (between 15 to 20% inhibition at the tested concentrations). The combination of rBoTNF alpha with rBoIFN gamma resulted in a marked enhancement of the cytostatic action of rBoIFN gamma (Fig. 6A).

Discussion

The ability of interferons to inhibit viral replication has been extensively documented as it has been the focus of numerous publications both in the biomedical and veterinary fields. When the sensitivity of BHV-1 to bovine IFN was evaluated and compared to that of other bovine viruses, BHV-1 was demonstrated to be relatively resistant to the antiviral action of bovine IFN and in particular of rBoIFN gamma (Czarniecki et al., 1986). The antiviral activity of TNF, on the other hand, has only been recently described with human TNF (Arakawa et al., 1987; Kohase et al., 1986; Mestan et al., 1986; Sugarman et al., 1985), where it was shown to potentiate the antiviral actions of IFN (Sugarman et al., 1985). In the present study, we have demonstrated that rBoTNF alpha is capable of reducing the yield of BHV-1 from infected cells to levels similar to those obtained with IFN gamma alone and to enhance the anti-BHV-1 actions of rBoIFN gamma.

A number of mechanisms have been suggested to play a role in the antiviral properties of TNF. It has been postulated that virus-infected cells are selectively killed by TNF alpha and beta (Eifel et al., 1979; Kohase et al., 1986) and that this activity is accelerated by IFN (Bielefeldt Ohmann et al., 1986; Lam Wan et Babink, 1987). Using standard cytotoxic assay, we were not able to detect preferential lysis of virus-infected cells by rBoTNF alpha (results not shown), nor could we enhance the cytotoxic action of rBoTNF to actinomycin D-treated cells with rBoIFN gamma, suggesting that the anti-viral activity to BHV-1 of rBoTNF alpha alone or in combination with rBoIFN gamma, is separable from the cytotoxic action of this cytokine. In certain cell lines rHuTNF has been shown also to be capable of inducing 2–5A synthetase – mRNA in treated cells (Sugarman et al., 1985) and to induce 2–5A synthetase activity in fully confluent monolayers (Mestan et al., 1986). Others have shown that the antiviral action of TNF against encephalomyocarditis virus-infected human fibroblast is mediated by the capacity of TNF to induce IFN beta in treated cells, suggesting an IFN-dependent mechanism for the TNF-mediated antiviral action (Kohase et al., 1986). In contrast, in the present study we were not able to detect 2–5A synthetase activity, a reliable marker for IFN activity (Revel et al., 1982), in rBoTNF alpha-treated cells, suggesting that rBoTNF is capable of inducing an antiviral state to BHV-1 by an IFN-independent mechanism. Although rBoTNF alpha was not capable of inducing 2–5A synthetase activity in treated cells, it was able to enhance the induction of this enzyme by rBoIFN gamma dramatically. The enhancement of 2–5A synthetase activity obtained with the combination of these two cytokines could be responsible for the more effective anti-BHV-1 activity observed in this study when rBoTNF alpha and rBoTNF gamma were used in combination. Additionally, rBoTNF was shown to inhibit proliferation of bovine cell lines and to enhance the action of rBoIFN gamma in this function. Similar antiproliferative properties have been documented in human cell lines using both alpha and beta TNF (Lee et al., 1984; Sugarman et al., 1985). It could be possible that the antiviral action of rBoTNF alpha is associated with the capacity of this cytokine to regulate cell metabolism, and that this capacity could be accentuated by the effects of rBoIFN gamma. However, previous

studies have demonstrated that of all the available recombinant bovine IFNs, rBoIFN gamma has the least in vitro activity against virus infection and the greatest antiproliferative action (Czarniecki et al., 1986), suggesting that inhibition of proliferation alone is not sufficient for induction of an antiviral state.

Although the in vivo relevance of the present observations remains to be tested the fact that rBoTNF enhances the antiviral activity of rBoIFN gamma against a relatively IFN resistant virus suggests a potential use of combinations of these cytokines in BHV-1 infections of cattle.

Acknowledgements

This work was supported by grants from CIBA-GEIGY Ltd., Canada Ltd. and the Natural Sciences and Engineering Research Council of Canada. Dr. Helle Bielefeldt Ohmann received special support by grants SJVF No. 13-3681 and SJVF No. 13-3857 from the Danish Agricultural and Veterinary Research Council during the course of these investigations. We also thank Irene Kosokowsky for her secretarial help with this manuscript.

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